p50^{cdc37} Is a Nonexclusive Hsp90 Cohort Which Participates Intimately in Hsp90-Mediated Folding of Immature Kinase Molecules†

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Received February 10, 2000; Revised Manuscript Received April 18, 2000

ABSTRACT: Hsp90 and p50^{cdc37} provide a poorly understood biochemical function essential to certain protein kinases, and recent models describe p50^{cdc37} as an exclusive hsp90 cohort which links hsp90 machinery to client kinases. We describe here the recovery of p50^{cdc37} in immunoadsorptions directed against the hsp90 cohorts FKBP52, cyp40, p60HOP, hsp70, and p23. Additionally, monoclonal antibodies against FKBP52 coadsorb maturation intermediates of the hsp90-dependent kinases p56^{lck} and HRI, and the presence of these maturation intermediates significantly increases the representation of p50^{cdc37} and hsp90 on FKPB52 machinery. Although the native heterocomplex between hsp90 and p50^{cdc37} is salt-labile, their dynamic interactions with kinase substrates produce kinase−chaperone heterocomplexes which are highly salt-resistant. The hsp90 inhibitor geldanamycin does not directly disrupt the native association of hsp90 with p50^{cdc37} per se, but does result in the formation of salt-labile hsp90−kinase heterocomplexes which lack the p50^{cdc37} cohort. We conclude that p50^{cdc37} does not simply serve as a passive structural bridge between hsp90 and its kinase substrates; instead, p50^{cdc37} is a nonexclusive hsp90 cohort which responds to hsp90's nucleotide-regulated conformational switching during the generation of high-affinity interactions within the hsp90−kinase−p50^{cdc37} heterocomplex.

The 90-kDa heat-inducible protein (hsp90)[1] provides one or more functions essential to certain members of the protein kinase and steroid hormone receptor superfamilies [reviewed in refs (*1*, *2*)]. Although the biochemical mechanisms underlying hsp90 function remain poorly understood, this phosphoprotein exhibits several properties which suggest that it acts as a molecular chaperone to support kinase folding in vivo [(*3*) and references cited therein]. Consistent with this postulated chaperone function, hsp90 is physically associated with immature kinase molecules and is essential for their proper maturation (*3*−*8*). Additionally, hsp90 provides prolonged reiterative support necessary to maintain some kinases (*3*−*7*) and certain hormone-receptor transcription factors [reviewed in ref (*2*)] in responsive conformations while they await specific regulatory stimuli. These properties suggest that hsp90 functions as a "signal transduction chaperone," acting at interfaces between overlapping pathways of protein folding and regulation (*9*−*11*). This unique positioning of hsp90 in cellular physiology provides a novel target for chemotherapeutic attacks by hsp90 inhibitors such as geldanamycin, herbimycinA, and radicicol (*12*−*14*).

In addition to its client proteins, hsp90 associates with various partner proteins, or "cohorts" [reviewed in ref (*2*)]. Hsp90's cohorts do not represent partially folded substrates per se; instead, individual cohorts appear to provide various ancillary activities that contribute to hsp90 function. For instance, the 60-kDa mammalian hsp90−hsp70 organizing protein [p60HOP (*15*), known as Sti1 in yeast (*16*)] simultaneously binds hsp90 and hsp70 and may coordinate their nucleotide-regulated chaperone cycles (*17*−*21*). Alternatively, the acidic 23-kDa hsp90 cohort [p23 (*22*−*25*), known as Sba1 in yeast (*26*)] associates with hsp90 in a nucleotide-responsive fashion (*18*, *27*, *28*) and appears to stabilize the interaction of hsp90 with steroid hormone receptors (*29*). Additionally, protein phosphatase 5 (PP5) occurs in native hsp90 heterocomplexes (*30*); however, its potential role (*31*, *32*) in these complexes is unknown. The family of hsp90 cohorts also includes various high M_r immunophilins [reviewed in ref (*2*)] and the immunophilin-

† This work was supported by grants from the Oklahoma Center for the Advancement of Science and Technology (HN6-018 to S.D.H.), the National Institute of General Medicine, NIH (GM51608 to R.L.M.), and the National Institute of Environmental Health Sciences, NIH (ES04299 to R.L.M.), and by the Oklahoma Agricultural Experiment Station (Project 1975).

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[1] Abbreviations: p56^{lck}, the 56-kDa lymphoid cell kinase found in association with the CD4 receptor; p60^{src}, nonreceptor tyrosine kinases produced by either cellular or viral homologues of the *src* gene; HRI, the heme-regulated serine kinase responsible for phosphorylating the α subunit of eukaryotic initiation factor 2; hsp90, heat shock protein 90, denoting members of the family of 90-kDa heat-inducible chaperone proteins; hsp70, heat shock protein 70, denoting members of the family of 70-kDa heat-inducible chaperone proteins; p60HOP, the 60-kDa mammalian hsp90−hsp70 organizing protein; FKBP52, the 52-kDa FK506-binding protein; p50^{cdc37}, the 50-kDa product of the *cdc37* gene; cyp40, cyclophilin 40; PP5, protein phosphatase 5; TPR, tetratricopeptide repeat motif governing protein−protein interactions; p23, the 23-kDa acidic hsp90 cohort; XAP2/ARA9, the 37-kDa hepatitis B virus X-associated protein, aka the aryl hydrocarbon receptor associated protein; RRL, rabbit reticulocyte lysate, typically supplemented with buffers, salts, and ATP-regenerating system necessary to support protein synthesis and folding.

10.1021/bi000315r CCC: $19.00
Published on Web 06/02/2000

like protein XAP2, also known as AIP or ARA9 (*33*−*35*); these immunophilins and immunophilin-like proteins are not functionally equivalent (*36*−*39*), and it has been proposed that they mediate intracellular trafficking of protein heterocomplexes (*40*).

Many of hsp90's cohorts contain tetratricopeptide repeat (TPR) motifs [reviewed in ref (*41*)] which mediate their interactions with hsp90 (*30*, *39*, *40*, *42*−*45*). This family of hsp90 cohorts includes p60HOP, PP5, XAP2/ARA9, and the high M_r immunophilins FKBP52, FKBP51, and cyp40 (*2*, *33*, *34*, *45*). However, the TPR-containing cohorts do not appear to be capable of simultaneous binding to hsp90: antibodies directed against p60HOP, PP5, FKBP52, or cyp40 do not coadsorb detectable amounts of the alternative TPR cohorts (*30*, *40*, *46*−*48*). Consistent with this postulated exclusivity, competition assays indicate that the full immunophilin coterie does not associate with hsp90 in the presence of a molar excess of an individual recombinant TPR cohort or its TPR domain; instead, the competing cohort (or its TPR domain) is the primary hsp90 associate recovered (*30*, *40*, *44*, *46*, *48*). These results suggest that hsp90's TPR cohorts compete for a common TPR-acceptor site on hsp90, thus generating discrete hsp90 machineries [reviewed in ref (*2*)].

The 50-kDa product of the mammalian *cdc37* gene [p50^{cdc37} (*6*, *49*, *50*)] has been postulated to represent a similarly exclusive hsp90 cohort (*48*). p50^{cdc37} is physically associated with native hsp90 machinery (*48*, *49*, *51*−*54*), but does not contain a discernible TPR domain (*50*). Although p50^{cdc37} appears to utilize a distinct sequence motif to associate with hsp90, p50^{cdc37} has not been detected in hsp90 machinery immunoadsorbed by antibodies directed against the TPR cohorts p60HOP or PP5 (*40*, *48*). Consistent with these results, p50^{cdc37} is not detected in hsp90 heterocomplexes formed in the presence of competing amounts of recombinant p60HOP or PP5 (*40*, *48*). Similarly, competing recombinant p50^{cdc37} compromises the recovery of hsp90 heterocomplexes containing p60HOP or PP5 (*48*). However, a truncated TPR domain of cyp40 or PP5 does not similarly compromise the association of p50^{cdc37} with hsp90 (*40*, *48*). These results suggest that hsp90 has topologically adjacent acceptor sites for p50^{cdc37} and TPR cohorts, and that the close proximity of these sites results in steric hindrance which prohibits the binding of more than one cohort molecule (*48*). Consistent with this model, hsp90, but not the immunophilin FKBP52, can be detected in reconstituted heterocomplexes formed with recombinant p50^{cdc37} (*48*). Nonetheless, the composition and character of native hsp90−p50^{cdc37} heterocomplexes remain somewhat uncertain regarding the potential presence of immunophilins: it has not been determined whether hsp90's immunophilin cohorts and p50^{cdc37} compete for binding to hsp90. Furthermore, attempts to compare hsp90 machinery containing immunophilins with that containing p50^{cdc37} have been complicated by the potential for direct recognition of p50^{cdc37} by anti-immunophilin antibodies (*40*).

Although the role of p50^{cdc37} in hsp90 machinery is unknown, the recombinant *cdc37* gene product maintains β-galactosidase in a refolding-competent form in vitro and enhances the in vitro activity of casein kinase II, suggesting a potential chaperone-like function in vivo (*55*). Consistent with this in vitro chaperone activity, preparations of purified p50^{cdc37} show latent kinase binding activity (*6*, *48*, *52*). Genetic approaches have revealed a functional relationship between *hsp90* and *cdc37*: overexpression of *cdc37* alleviates the kinase-deficient phenotype of *hsp90*-deficient yeast (*55*), and p50^{cdc37} increases the association of hsp90 with its substrate kinases in various in vivo and in vitro assays (*6*, *52*). Consistent with this functional relationship, the hsp90-dependent viral kinase p60^{src} is compromised in the absence of full *cdc37* function (*56*), and coexpression of p50^{cdc37} with the hsp90-dependent kinases Raf-1 or Cdk4 enhances their activities (*6*, *52*). Additionally, p50^{cdc37} is readily recovered in heterocomplexes with hsp90-associated kinases (*5*, *6*, *48*, *51*, *52*, *57*−*61*). However, p50^{cdc37} has not been detected in substrate heterocomplexes containing hsp90-dependent steroid hormone receptors (*54*, *59*), and overexpression of *cdc37* does not correct an hsp90-deficient yeast phenotype with regard to receptor function (*55*). Conversely, *cdc37* deficiency in yeast has been reported to compromise steroid hormone receptor function (*62*). Nonetheless, current models propose that p50^{cdc37} is an exclusive hsp90 cohort responsible for recruiting hsp90 chaperone machinery to client kinases (*6*, *52*).

As part of our ongoing studies of the hsp90-dependent kinases p56^{lck} (*3*, *4*, *63*, *64*) and HRI (*7*, *65*−*67*), we characterized the interaction of p50^{cdc37} with these two kinases. Additionally, we characterized native p50^{cdc37} heterocomplexes with regard to hsp90, several of its cohorts, and hsp90 antagonists. We report here that p50^{cdc37} and immunophilins coexist in native hsp90 heterocomplexes and that binding of incompletely folded kinase molecules to FKBP52 machinery greatly increases the representation of p50^{cdc37} and hsp90 on this machinery. Additionally, we find that hsp90 and p50^{cdc37} display geldanamycin-sensitive salt-stable interactions with kinase folding intermediates that are distinct from their salt-labile interactions with each other. We conclude that p50^{cdc37} does not simply serve as a passive structural bridge between hsp90 and its kinase substrates; instead, p50^{cdc37} is a nonexclusive hsp90 cohort which responds to hsp90's nucleotide-regulated conformational switching during the generation of high-affinity interactions within the hsp90−kinase−p50^{cdc37} heterocomplex.

EXPERIMENTAL PROCEDURES

Identification of cdc37 cDNA and Production of Recombinant Protein. Two *cdc37* cDNA plasmid constructs present in the EST database were identified by alignment to the published *cdc37* sequence (*6*, *50*) and were obtained from Genome System Inc. The first plasmid (GenBank gi610344, Accession No. AA172101) contained a partial cDNA encoding the human *cdc37* gene product from amino acids 8 to 378. This gene segment was subcloned into the *E. coli* expression vector pET-30a(+) for expression of the His-tagged recombinant fusion protein. The second cDNA identified (GenBank gi946705, Accession No. R87892) was sequenced in its entirety and was found to contain the full human *cdc37* coding region; this EST plasmid has been identified previously (*57*) and its entire sequence archived as GenBank Accession No. U63131. Standard molecular cloning techniques were used to construct plasmids derived from pSP64T (*68*) in which an SP6 RNA polymerase promoter drove expression of the *cdc37* gene. Plasmids for the expression of fusion genes encoding kinase proteins

(p56^{lck} or HRI) with N-terminal poly-His epitope tags were similarly constructed.

Immunological Reagents. Agarose resins for immobilization of immunocomplexes were produced as previously described (*66*). Recombinant *cdc37* gene product was expressed in *E. coli*, purified to 90% homogeneity by nickel affinity chromatography, and used for immunization of mice to produce polyclonal ascites antibodies. Antibodies thus produced recognized a single protein (M_r = 50) present in unfractionated rabbit reticulocyte lysates, in immunocomplexes containing protein kinases or hsp90, and in immunocomplexes isolated with a commercial monoclonal antibody against p50^{cdc37} [C1 as described in (*49*), aka MA3-029 from Affinity BioReagents]. Other antibodies utilized include the following: an irrelevant (nonimmune control) mouse IgG from the MOPC-21 hybridoma (Sigma M7894); EC1 mouse monoclonal antibody recognizing FKPB52 [(*69*); from Dr. Lee Faber]; polyclonal rabbit antibody raised against a peptide representing the C-terminus of cyp40 [Affinity BioReagents PA3-022]; F5 (aka DS14F5) mouse monoclonal antibody recognizing p60HOP [(*15*); from Dr. David Smith]; BB70 mouse monoclonal antibody recognizing hsp70 [(*15*); from Dr. David Toft]; 3M/1B5p50 mouse IgM antibody recognizing p50^{cdc37} [(*54*); from Dr. Gary Perdew]; JJ3 and JJ5 mouse monoclonal antibodies recognizing p23 [(*23*); from Dr. David Toft]; antigen-affinity-purified mouse antibodies recognizing pentaHis epitope tags (Quiagen 34660); polyclonal rabbit antibody recognizing the N-terminus of hsp90alpha [(*70*); from Affinity BioReagents PA3-013]; and polyclonal mouse antibodies raised against recombinant p56^{lck} as described in (*8*).

Rabbit Reticulocyte Lysates. Native rabbit reticulocyte lysate reaction mixtures (RRL) were assembled to contain 50% (v/v) rabbit reticulocyte lysate, 10 mM creatine phosphate, 20 units/mL creatine phosphokinase, 30 μM of each amino acid, 10 mM TrisHCl (pH 7.7), 0.2 mM GTP, 1 mM $Mg(OAc)_2$, and 76 mM KCl. Reactions were incubated for 10 min at 37 °C prior to immunoadsorption. Salt-stripped RRL were prepared as previously described (*27*), with variations as indicated in the figure legends. p50^{cdc37}, p56^{lck}, and HRI were translated in nuclease-treated RRL reactions via coupled transcription/translation as previously described (*3*, *7*, *63*).

Immunoadsorptions. Antibodies against individual hsp90 cohorts or against poly-His epitope tags were immobilized on agarose resins (15 μL), and immunoresins were washed 3 times with 10 mM PIPES, 50 mM NaCl (pH 7.0). Washed immunoresins were incubated with RRL (30−50 μL) which had been chilled on ice and clarified by centrifugation at 10000*g* for 5 min. After adsorption for 1−2 h on ice, immunopellets were washed 5 times with buffer containing 10 mM PIPES, pH 7.0, 0.05% Tween-20, and the indicated concentrations of NaCl and/or sodium molybdate. Adsorbed materials were separated by SDS−PAGE, transferred to PVDF, and analyzed by Western blotting or autoradiography, as appropriate. Band intensities were quantified by densitometry using Bio-Rad's Multi-Analysis software in conjunction with their GS700 Imaging Densitometer. Images of Western blots and autoradiograms were prepared with Adobe Photoshop and Deneba Canvas and were subjected to the minimal manipulations necessary to duplicate the appearance of the original data.

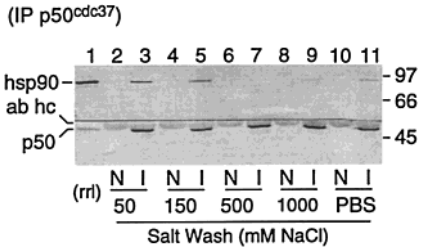

FIGURE 1: Salt-lability of the p50^{cdc37}−hsp90 heterocomplex. Rabbit reticulocyte lysate reaction mixtures containing an ATP regeneration system were immunoadsorbed with nonimmune or anti-p50^{cdc37} antibodies. Immunopellets were washed with buffer containing 50−1000 mM NaCl as indicated, or with phosphate-buffered saline, after which bound materials were eluted by boiling in SDS−PAGE sample buffer and analyzed by Western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (hsp90), p50^{cdc37} (p50), and the heavy chain of the adsorbing antibody (ab hc) are indicated. Migrations of molecular mass markers (kDa) are indicated along the right side of the panel. Lane 1, unfractionated lysate reaction loaded as a standard for the detection of hsp90 and p50^{cdc37}; lanes 2, 4, 6, 8, and 10, adsorptions with nonimmune (control) antibody; lanes 3, 5, 7, 9, 11, adsorptions with anti-p50^{cdc37}; lanes 2, 3, immunopellets were washed with 50 mM NaCl; lanes 4, 5, immunopellets were washed with 150 mM NaCl; lanes 6, 7, immunopellets were washed with 500 mM NaCl; lanes 8, 9, immunopellets were washed with 1.0 M NaCl; lanes 10+11, immunopellets were washed with phosphate-buffered saline.

Assay for Dynamic Associations. p50^{cdc37} was translated in hemin-replete RRL reactions containing [^{35}S]Met to generate a radiolabeled p50^{cdc37} population, while His-tagged HRI template was translated in separate hemin-replete reactions to generate radiolabeled HRI molecules with a unique epitope tag. After synthesis, reinitiation of protein synthesis in each reaction was arrested by addition of 2 mM aurintricarboxylic acid, and lysates were incubated with excess unlabeled Met (25 μM) to terminate radiolabeling. Equal volumes of the separate hemin-replete reactions were mixed with each other and incubated for 20 min at 30 °C to allow formation of heterocomplexes between [^{35}S]p50^{cdc37} and [^{35}S]H7−HRI. Subsequently, a fraction of the mixture was transferred to 4 volumes of RRL chase reaction lacking radiolabeled p50^{cdc37}. H7−HRI heterocomplexes were immunoadsorbed with anti-pentaHis antibodies at various times after assembly of the chase reaction and analyzed by SDS−PAGE, Western blotting, and autoradiography.

RESULTS

p50^{cdc37} Associates with Hsp90 via Salt-Labile Interactions. To determine the nature of the native (substrate-free) interaction between p50^{cdc37} and hsp90, we characterized the resistance of the native hsp90−p50^{cdc37} heterocomplex to various concentrations of salt. This characterization was prompted by previous observations that moderate-to-high concentrations of salt disrupt the native interaction of hsp90 with its TPR-containing cohorts (*15*, *17*, *71*), while p50^{cdc37} had been described as binding tightly to hsp90 (*40*, *49*). To determine the mode by which p50^{cdc37} associated with hsp90, we immunoadsorbed p50^{cdc37} from ATP-replete RRL and assayed for the coadsorption of hsp90 (Figure 1). These immunoadsorptions recovered p50^{cdc37} in an immune-specific fashion. Additionally, hsp90 was coadsorbed in an immune-specific fashion, thus confirming the previously described native heterocomplex between p50^{cdc37} and hsp90. This

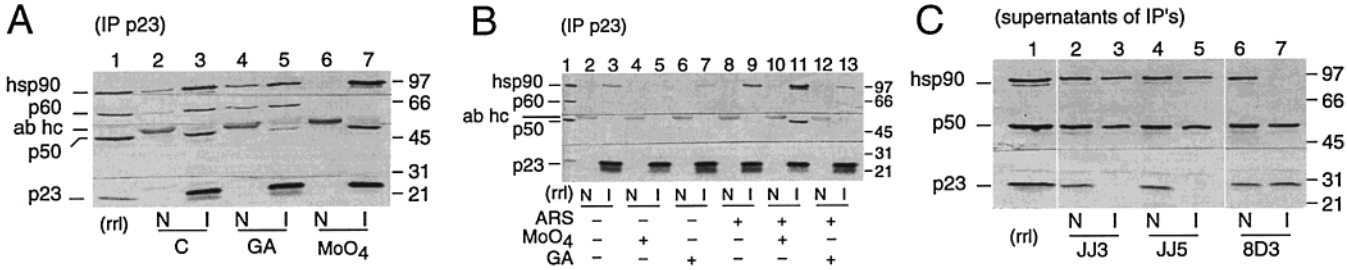

FIGURE 2: Characterization of p23 chaperone machinery. Panel A: p23 machinery from native lysates. Native rabbit reticulocyte lysate reaction mixtures containing an ATP regeneration system and drug additions as indicated were immunoadsorbed with either nonimmune antibody or anti-p23 antibody. Immunopellets were washed with buffer containing either 20 mM NaCl or 20 mM sodium molybdate. Washed immunocomplexes were eluted by boiling in SDS−PAGE sample buffer and analyzed by Western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (hsp90), p60HOP (p60), p50^{cdc37} (p50), p23 (p23), or the heavy chain of the adsorbing antibody (ab hc) are indicated. Migrations of molecular mass markers (kDa) are indicated along the right side of the panel. Lane 1, lysate reaction loaded as a standard for detection of chaperone proteins; lanes 2, 4, 6, adsorptions with nonimmune (control) antibody; lanes 3, 5, 7, adsorptions with JJ3 anti-p23 monoclonal antibody; lanes 2, 3, adsorptions from lysates with no drug additions; lanes 4, 5, adsorptions from lysates containing geldanamycin; lanes 6, 7, adsorptions from lysates containing molybdate. Panel B: reassociation of p23 and hsp90 in salt-strippped lysate. Rabbit reticulocyte lysates were treated with 0.5 M KCl, dialyzed to restore quasi-physiological salt conditions, and were, or were not, supplemented with ATP and an ATP regenerating system (ARS+ATP). Lysates were incubated for 5 min, after which molybdate or geldanamycin was added as indicated and the reactions were further incubated for 10 min. Reactions were then immunoadsorbed and analyzed as for panel A. Lane 1, lysate reaction loaded as a standard for detection of chaperone proteins; lanes 2−7, lysate reactions without ARS+ATP supplementation; lanes 8−13, lysate reactions supplemented with ARS+ATP; even-numbered lanes, adsorptions with nonimmune (control) antibody; lanes 3, 5, 7, 9, 11, 13, adsorptions with JJ3 anti-p23 antibody; lanes 4, 5, 10, 11, lysate reactions supplemented with molybdate; lanes 6, 7, 12, 13, lysate reactions supplemented with geldanamycin. Panel C: depletion assay of chaperone machinery in unstripped lysates. Aliquots (4 μL) of ATP-replete RRL were immunoadsorbed using either nonimmune antibodies, anti-p23 antibodies (JJ3 or JJ5), or anti-hsp90 antibodies (8D3). Unbound fractions were collected and analyzed by Western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (hsp90), p50^{cdc37} (p50), and p23 (p23) are indicated. Migrations of molecular mass markers (kDa) are indicated along the right side of the panel. Lane 1, an aliquot representing the amount of lysate input; lanes 2, 4, 6, unadsorbed fractions from lysates adsorbed with nonimmune antibody; lane 3, unadsorbed fraction from lysate adsorbed with JJ3 anti-p23 antibody; lane 5, unadsorbed fraction from lysate adsorbed with JJ5 anti-23 antibody; lane 7, unadsorbed fraction from lysate adsorbed with 8D3 anti-hsp90 antibody.

heterocomplex was readily detected when immunocomplexes were washed with buffer containing 50 mM NaCl (lanes 2 and 3). When the anti-p50^{cdc37} immunopellet was washed with buffer containing 150 mM NaCl, 30% as much hsp90 was coadsorbed (lanes 4 and 5). When anti-p50^{cdc37} immunocomplexes were washed with concentrations of 500 mM NaCl or higher, no coadsorbing hsp90 could be detected (lanes 6−9). Additionally, the presence of 20 mM sodium phosphate in 150 mM NaCl wash buffers further compromised hsp90 coadsorption (lanes 10 and 11), a result similar to previous reports that phosphate buffers compromised the recovery of cyp40 in progesterone receptor heterocomplexes (*72*). These results indicated that in ATP-replete RRL, p50^{cdc37} and hsp90 existed in native heterocomplexes that were predominately salt-labile.

p50^{cdc37} Is a Component of Hsp90 Heterocomplexes Containing p23. Previous characterizations of native hsp90 heterocomplexes and the effects of hsp90 antagonists thereon (*24*, *27*, *28*, *72*) provided a useful conceptual framework within which to evaluate hsp90 machinery containing p50^{cdc37}. Hsp90 had been described to occur in several distinct native heterocomplexes, each containing unique compositions of cohorts. One such heterocomplex contains hsp90, p60HOP, and hsp70; this complex is proposed to be recruited to steroid hormone receptors during intermediate stages of chaperone-mediated refolding. The formation of the native complex formed between hsp90 and p60HOP is favored in the presence of ADP or the hsp90 antagonist geldanamycin. In contrast, late stages of receptor refolding are postulated to recruit a preassociated complex containing hsp90, immunophilins, and an acidic cohort termed p23. The formation of the native heterocomplex containing hsp90 and p23 requires ATP or nonhydrolyzable ATP analogues, and this heterocomplex is stabilized by molybdate. To analyze p50^{cdc37}−hsp90 machinery within this previously established framework, we immunoadsorbed p23 or p50^{cdc37} from ATP-replete RRL containing or lacking hsp90 antagonists and Western-blotted the washed immunopellets for hsp90 and select hsp90 cohorts.

We began by characterizing chaperone machinery associated with p23 and the effects of hsp90 antagonists thereon. Although p23 had been described as a component of native hsp90 heterocomplexes [e.g. (*23*)] and as a component of heterocomplexes with kinase molecules (*61*, *67*), previous characterizations of native p23 heterocomplexes had not detected the presence of p50^{cdc37} (*40*). For our characterizations, ATP-replete RRL were immunoadsorbed with monoclonal antibody against p23, and the washed immunocomplexes were analyzed for the presence of p23, hsp90, p60HOP, and p50^{cdc37} (Figure 2, panel A). Immunoadsorption of p23 from ATP-replete RRL resulted in the immune-specific coadsorption of hsp90 (Figure 2A, lanes 2 and 3), a result consistent with previous reports. Additionally, p23 immunoadsorptions also coadsorbed p60HOP at levels greater than those observed for nonspecific background binding (Figure 2A, lanes 2 and 3). Although the presence of p60HOP on p23 heterocomplexes seemed somewhat inconsistent with predominant models describing hsp90 machineries, similar results had been described previously (*27*). When anti-p23 immunopellets were examined for the presence of p50^{cdc37}, p50^{cdc37} was observed to be coadsorbed with anti-p23 in an immune-specific fashion (Figure 2A, lanes 2 and 3). These results indicated that p23 occurred in one or more preexisting heterocomplexes with p50^{cdc37}. When levels of hsp90, p50^{cdc37}, and p23 in unfractionated RRL were compared to the levels of these proteins recovered in anti-

p23 immunoadsorptions, p50^{cdc37} appeared to be well-represented on p23 chaperone machinery.

We wished to test the hypothesis that p50^{cdc37} recognized a specific conformation of hsp90 or a specific subpopulation of hsp90 machinery; however, we initially characterized p23 heterocomplexes since previous studies (*24*, *27*, *28*, *72*) provided a benchmark for our own investigations. As seen in Figure 2A (lanes 4 and 5), the hsp90 antagonist geldanamycin increased the nonspecific binding of hsp90 and p60HOP to control immunoresins. In the presence of this background binding, geldanamycin did not noticeably alter the levels of p60HOP detected in p23 immunocomplexes (Figure 2A, lanes 4 and 5). Addition of geldanamycin reduced the recovery of hsp90 in p23 heterocomplexes by 25%; the magnitude of this reduction was much less than that expected from previously published studies [(*27*); but see below]. In contrast to geldanamycin, molybdate enhanced the coadsorption of hsp90 with anti-p23 immunoresins by 35% while quantitatively eliminating p60HOP associations (Figure 2A, lanes 6 and 7). When the effects of these hsp90 antagonists were examined with regard to the presence of p50^{cdc37} on p23 machinery, geldanamycin reduced the coadsorption of p50^{cdc37} by anti-p23 antibodies by 70% (Figure 2A, lanes 4 and 5). In contrast, molybdate enhanced the coadsorption of p50^{cdc37} by 40% (Figure 2A, lanes 6 and 7). Thus, p50^{cdc37} was associated with native p23 machinery, and molybdate enhanced the levels of both p50^{cdc37} and hsp90 present in this machinery.

The effects of hsp90 antagonists were further examined in RRL that had been treated with 0.5 M KCl to disrupt native interactions between hsp90 and its cohorts. This approach was utilized because geldanamycin only partially antagonized hsp90's association with p23 in our ATP-replete RRL (Figure 2A, lanes 4 and 5), whereas previous investigations utilizing salt-stripped lysates had demonstrated quantitative disruptions (*27*). Additionally, the use of native RRL did not allow us to examine the potential for ATP to mediate interactions between hsp90 and its cohorts. To more fully investigate the effects of nucleotide and hsp90 antagonists, RRL were treated with 0.5 M KCl to disrupt native chaperone heterocomplexes and these lysates were subsequently dialyzed to regenerate quasi-physiological ionic conditions. These lysates then were, or were not, supplemented with ATP and an ATP regenerating system (ARS), geldanamycin, and/or molybdate prior to immunoadsorption of chaperone machinery and characterization of chaperone composition.

Consistent with previous work utilizing salt-stripped RRL (*27*), ATP facilitated the reassociation of hsp90 with p23 in a fashion that was enhanced by molybdate and nearly quantitatively inhibited by geldanamycin (Figure 2B). The nearly quantitative conversion of hsp90 to a conformation that was incapable of interacting with p23 also resulted in the failure to detect p50^{cdc37} in p23 immunocomplexes (Figure 2B, lanes 12+13). Because hsp90 is the only known chaperone target of geldanamycin, this result indicated that p50^{cdc37} did not directly form a stable complex with p23, but instead associated with p23 indirectly by virtue of its interaction with hsp90.

To determine if all molecules of p50^{cdc37} were associated with p23 machinery in RRL, we established low-salt immunoadsorption conditions that quantitatively depleted p23 and asked whether p50^{cdc37} was concomitantly depleted

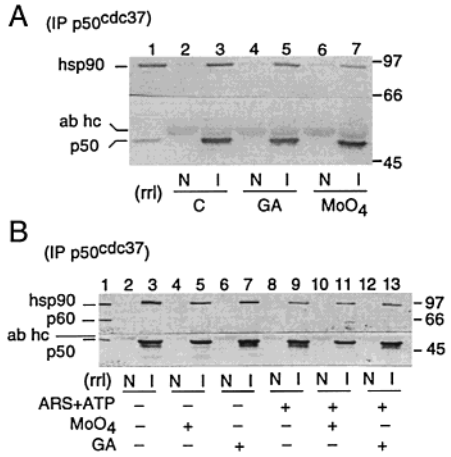

FIGURE 3: Characterization of p50^{cdc37} chaperone machinery. Panel A: p50^{cdc37} chaperone machinery in native rabbit reticulocyte lysates (RRL) was immunoadsorbed from native lysates and assessed as described for Figure 2A. Lane 1, lysate loaded as a standard for detection of chaperones; lanes 2, 3, adsorptions from untreated lysate; lanes 4, 5, adsorptions from lysates treated with geldanamycin; lanes 6, 7, adsorptions from lysates treated with 20 mM sodium molybdate; lanes 2, 4, 6, immunoadsorption with nonimmune (control) antibodies; lanes 3, 5, 7, immunoadsorptions with polyclonal anti-p50^{cdc37}. Panel B: reassociation of p50^{cdc37} and hsp90 in salt-stripped lysate was assessed as described for Figure 2B. Lane 1, lysate loaded as a standard for detection of chaperones; lanes 2−7, no ATP/ATP regeneration system; lanes 9−13, ATP+ATP regeneration system; lanes 2, 3, 8, 9, adsorptions from lysates lacking drug additions; lanes 4, 5, 10, 11, adsorptions from lysates treated with 20 mM sodium molybdate; lanes 6, 7, 12, 13, adsorptions from lysates treated with geldanamycin; even-numbered lanes, immunoadsorption with nonimmune antibodies; lanes 3, 5, 7, 9, 11, 13, immunoadsorptions with polyclonal anti-p50^{cdc37}.

(Figure 2C). We observed that quantitative depletion of p23 from RRL did not similarly deplete p50^{cdc37}; the majority of the p50^{cdc37} population remained in the immunoadsorption supernatant (Figure 2C, lanes 2−5). Similarly, quantitative depletion of hsp90 from RRL did not deplete p50^{cdc37} (Figure 2C, lanes 6+7); similar results were reported during the preparation of this manuscript (*73*). Thus, p50^{cdc37} was not quantitatively associated with p23 or hsp90 in a stable complex under our immunoadsorption conditions.

To determine if geldanamycin and molybdate had direct effects on the native association of p50^{cdc37} with hsp90, anti-p50^{cdc37} antibodies were used to immunoadsorb p50^{cdc37} heterocomplexes from RRL which had been treated with these compounds; these characterizations were performed in native RRL and in salt-stripped RRL. Treatment of unstripped ATP-replete RRL with geldanamycin or molybdate induced only minor variations in the subsequent recovery of hsp90 in anti-p50^{cdc37} adsorptions (Figure 3A). This result indicated that in unfractionated ATP-replete RRL, neither geldanamycin nor molybdate quantitatively induced hsp90 conformations which were incapable of interacting with p50^{cdc37}. To more fully test the hypothesis that p50^{cdc37} might recognize a specific conformation of hsp90, we also examined the association of p50^{cdc37} with hsp90 in salt-stripped RRL. When p50^{cdc37} was immunoadsorbed from salt-stripped RRL, the coadsorption of hsp90 was observed independent of the presence of an ATP-ARS (Figure 3B). Although the association of these proteins was slightly compromised by the presence of an ATP-ARS, this effect seemed to result from nonspecific salt and dilution effects (not shown).

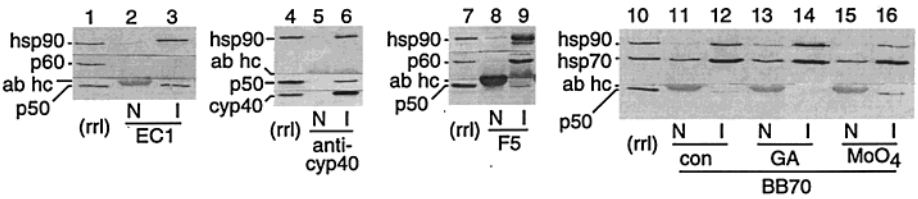

FIGURE 4: Characterization of chaperone machinery associated with TPR-containing cohorts or with hsp70. Rabbit reticulocyte lysate reaction mixtures containing an ATP regeneration system were immunoadsorbed with antibodies against the indicated hsp90 cohorts. Adsorbed materials were eluted by boiling in SDS−PAGE sample buffer and analyzed by Western blotting with anti-chaperone antibodies. Immunoreactive bands representing hsp90 (hsp90), p60HOP (p60), cyp40 (cyp40), hsp70 (hsp70), p50^{cdc37} (p50), p23 (p23), or the heavy chain of the adsorbing antibody (ab hc) are indicated. Lanes 1, 4, 7, 10, lysate reaction loaded as a standard for detection of chaperone proteins; lanes 2, 5, 8, 11, 13, 15, adsorptions utilizing nonimmune (control) antibody; lane 3, adsorption with EC1 anti FKBP52; lane 6, adsorption with polyclonal anti-cyp40; lane 9, adsorption with F5 monoclonal anti-p60HOP; lanes 12, 14, 16, adsorption with BB70 monoclonal anti-hsp70; lanes 1−9, adsorptions from lysate reactions lacking drug additions; lanes 13, 14, adsorptions from reactions supplemented with geldanamycin; lanes 15, 16, adsorptions from reactions supplemented with molybdate.

Additionally, neither geldanamycin nor molybdate abrogated the ability of p50^{cdc37} to interact with hsp90 (Figure 3B). These results indicated that p50^{cdc37} interacted with each of the drug-enforced conformations of hsp90, and that this interaction neither required nor was contradicted by the interaction of p23 with hsp90; this conclusion was consistent with previous work demonstrating that geldanamycin does not disrupt the native hsp90−p50^{cdc37} heterocomplex in vivo (*52*).

p50^{cdc37} Is a Component of Native Heterocomplexes Associated with TPR-Containing Cohorts. Previous studies had demonstrated that polyclonal antisera against specific immunophilins immunoadsorb p50^{cdc37} from cell lysates; this result had been ascribed to direct recognition of p50^{cdc37} by the immunophilin antibodies (*40*). To reexamine the hypothesis that p50^{cdc37} and immunophilins are mutually exclusive hsp90 cohorts, we utilized the EC1 monoclonal antibody against FKBP52 to immunoadsorb FKBP52 and its associated proteins from unstripped ATP-replete RRL. Associated proteins were detected by Western blot analysis of the washed immunopellets; due to comigration of antibody heavy chains with the immunoadsorption target FKBP52, we did not attempt to assay for its recovery. When EC1 immunopellets were examined for the presence of hsp90, hsp90 was readily detected to be present in the anti-FKBP52 immunocomplexes in an immune-specific fashion (Figure 4, lanes 2 and 3); this result described the previously documented native heterocomplex formed between these proteins. When FKBP52 immunopellets were examined for the presence of p50^{cdc37}, p50^{cdc37} was found to be coadsorbed in an immune-specific fashion (Figure 4, lanes 2 and 3). When levels of hsp90 and p50^{cdc37} in unfractionated RRL (lane 1) were compared to the levels of these proteins recovered in anti-FKBP52 immunoadsorptions (lanes 2+3), p50^{cdc37} appeared to be a significant component of FKBP52 chaperone machinery.

We extended our analyses by immunoadsorbing cyp40 and its associated proteins from unstripped ATP-replete RRL using polyclonal antibodies raised against the C-terminus of cyp40. Although antibodies raised against this immunogen had been described as cross-reacting with p50^{cdc37} (*40*), we observed no evidence for cross-reactivity when we assayed purified p50^{cdc37} (an amount equivalent to that present in 30 μL of RRL) by Western blotting with 0.1% anti-cyp40 antibodies (not shown). When we used these anti-cyp40 antibodies to immunoadsorb cyp40 and its associated proteins from ATP-replete unstripped RRL, hsp90 was reproducibly coadsorbed in an immune-specific fashion (Figure 4, lanes 5 and 6); this coadsorption was consistent with previously documented associations between these proteins. When these anti-cyp40 immunopellets were assayed for the presence of p50^{cdc37}, p50^{cdc37} was readily detected to be present in an immune-specific fashion (Figure 4, lanes 5 and 6). When levels of hsp90, cyp40, and p50^{cdc37} in unfractionated RRL (lane 4) were compared to the levels of these proteins recovered in anti-cyp40 immunoadsorptions (lanes 5 and 6), p50^{cdc37} appeared to be well represented on cyp40 chaperone machinery.

Our observation that p50^{cdc37} did not seem to be mutually exclusive versus the TPR immunophilins FKBP52 and cyp40 prompted us to extend our characterizations to a third TPR protein, p60HOP. When F5 monoclonal anti-p60HOP antibodies were used to immunoadsorb p60HOP and its associated proteins from unstripped ATP-replete RRL, hsp90 was readily detected upon Western blot analysis of the immunoadsorption pellet (Figure 4, lanes 8 and 9). The occurrence of hsp90 in this pellet was immune-specific: only trace amounts of hsp90 were detected in parallel adsorptions with nonimmune antibody (cf. lanes 8 and 9). These results described the previously documented heterocomplex formed between these proteins. When anti-p60HOP immunopellets were assessed for the presence of p50^{cdc37}, p50^{cdc37} was detected in the F5 immunoadsorption pellet, and this occurrence was immune-specific (Figure 4, lanes 8 and 9). When the Western blot signals from hsp90, p60HOP, and p50^{cdc37} in unfractionated RRL (lane 7) were compared to the signals recovered in anti-p60HOP immunoadsorptions (lanes 8 and 9), p50^{cdc37} appeared to be a very minor constituent of p60HOP machinery. Our detection of p50^{cdc37} in F5 immunopellets was inconsistent with similar studies which did not detect p50^{cdc37} associated with p60HOP heterocomplexes (*40*). However, the low levels of p50^{cdc37} detected in lane 8 of Figure 4 would not have been detectable by previously available monoclonal anti-p50^{cdc37} IgM (*54*); this IgM had very low sensitivity relative to the polyclonal anti-p50^{cdc37} antibodies used in the current study (not shown).

Because p60HOP simultaneously binds hsp70 and hsp90 (*15*), we extended our coadsorption studies to characterize hsp70 chaperone machinery (Figure 4, lanes 11−12). As is typically observed, some hsp70 was recovered from immunoresins in a nonspecific fashion (lanes 11, 13, 15). Nonetheless, immunoadsorption with monoclonal BB70 anti-hsp70 enhanced hsp70 levels in immunopellets and specifically coadsorbed hsp90 from unstripped ATP-replete RRL (lanes

12, 14, 16); this result was consistent with previously documented interactions between hsp90 and hsp70 via p60HOP. In contrast to hsp90, p50^{cdc37} was barely detectable in anti-hsp70 adsorptions of untreated RRL (Figure 4, lanes 11−14). However, pretreatment of the RRL reactions with molybdate resulted in a more detectable, yet specific, coadsorption of p50^{cdc37} concomitant with decreased levels of hsp90 recovery (Figure 4, lanes 15 and 16). These results confirmed that p50^{cdc37} was not exclusive to a unique hsp90 machine, but rather suggested that p50^{cdc37} could be a very minor component of hsp90−p60HOP−hsp70 heterocomplexes.

p50^{cdc37} Is Tightly Bound to Heterocomplexes Containing Immature Client Kinases. Although p50^{cdc37} had been previously documented to occur in complexes with several kinases, the nature and role of this association had not been well characterized. Thus, we characterized the association of p50^{cdc37} with two well-characterized hsp90-dependent kinases: the T-cell *src*-family tyrosine kinase p56^{lck} and the heme-regulated serine kinase HRI. For wild-type p56^{lck}, a heterocomplex formed with hsp90 represents as an obligate intermediate occurring during kinase biogenesis: p56^{lck} molecules flow from an hsp90-bound state to an hsp90-free state during posttranslational maturation (*3*, *4*, *64*). To explore the role of p50^{cdc37} in this process, RRL translation reactions were programmed for in vitro synthesis of p56^{lck}. These reactions were assembled to produce a pulse of p56^{lck} synthesis, after which reinitiation of synthesis was inhibited. Either this synchronized p56^{lck} population was immediately immunoadsorbed with rabbit anti-p56^{lck} antibodies, or, alternatively, synchronized populations were further incubated for 60 min at 37 °C, after which reactions were similarly immunoadsorbed. Immunocomplexes were washed with either low-salt or high-salt buffers, and the washed immunocomplexes were analyzed by Western blotting to detect the coadsorption of hsp90 and p50^{cdc37}.

Consistent with our previous studies, hsp90 was specifically coadsorbed with p56^{lck} (Figure 5, lanes 1−4), and the association of hsp90 with these p56^{lck} molecules was stable to washing in 0.5 M NaCl (lanes 2 and 4). Also consistent with our previous observations, this association was specific to newly synthesized molecules of p56^{lck}: the salt-stable association of hsp90 with p56^{lck} was not detected if p56^{lck} molecules were allowed to mature prior to immunoadsorption (lanes 6 and 8). This result did not reflect a change in the mode by which hsp90 interacted with p56^{lck}: even when immunocomplexes were washed in the absence of salt, hsp90 was not coadsorbed with mature p56^{lck} molecules (lanes 5 and 7). These results confirmed that hsp90 formed salt-resistant complexes with newly synthesized p56^{lck} molecules, but did not associate with mature p56^{lck}.

To determine if p50^{cdc37} behaved like hsp90, p56^{lck} immunocomplexes were assessed for the presence of p50^{cdc37}. Immunoadsorption of nascent p56^{lck} from unmatured RRL reactions resulted in the specific coadsorption of p50^{cdc37} (Figure 5, lanes 1−4). When the heterocomplex between p50^{cdc37} and immature p56^{lck} molecules was examined for its resistance to washing in the presence of 0.5 M NaCl, the association of p50^{cdc37} with p56^{lck} was found to be highly resistant to high ionic strength buffers, despite the absence of any stabilizing agents such as molybdate (lanes 2 and 4). Upon maturational incubation of p56^{lck}, p50^{cdc37} was no longer detected in association with the synchronized p56^{lck} population regardless of the ionic strength of the wash buffer (lanes 5−8). These results indicated that p50^{cdc37}, like hsp90, formed salt-resistant complexes with newly synthesized p56^{lck} molecules, but did not associate with mature p56^{lck} regardless of ionic conditions. More importantly, these results indicated that hsp90 and p50^{cdc37} each associated with p56^{lck} in a manner distinct from their association with each other.

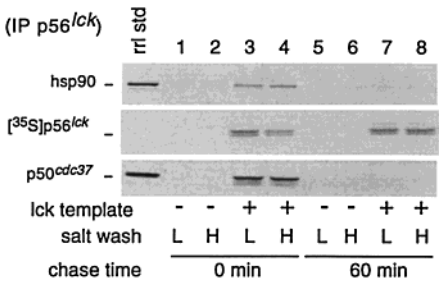

FIGURE 5: Salt-stable association of hsp90 and p50^{cdc37} with p56^{lck} maturation intermediates. RRL translation reactions were, or were not, programmed for in vitro synthesis of p56^{lck}. After brief synthesis (25 min), reinitiation of protein synthesis was inhibited, and reactions either were immediately immunoadsorbed with anti-p56^{lck} antibodies or were incubated further ("chased") prior to anti-56^{lck} immunoadsorption. Immunopellets were washed with buffers containing or lacking 0.5 M NaCl and were eluted by boiling in SDS−PAGE sample buffer. [^{35}S]p56^{lck} ([^{35}S]p56^{lck}) was detected by autoradiography; hsp90 (hsp90) and p50^{cdc37} (p50) were identified by Western blotting. Migrations of molecular mass markers (kDa) are indicated along the right side of the panel. First lane (rrl std), naive lysate loaded as a standard for detection of chaperones; lanes 1−4, adsorption prior to maturational incubation of lysate reactions; lanes 5−8, adsorption after 60-min maturational incubation of lysate reactions; lanes 1, 2, 5, 6, lysate reactions without p56^{lck} template; lanes 3, 4, 7, 8, lysate reactions programmed for p56^{lck} synthesis; odd-numbered lanes, adsorptions washed with buffer lacking 0.5 mM NaCl; even-numbered lanes, adsorptions washed with buffer containing 0.5 M NaCl.

We also characterized the interaction of p50^{cdc37} with an alternative hsp90-dependent kinase having a distinct primary structure and unique regulatory elements, the heme-regulated inhibitor of proteins synthesis (HRI). The physical and functional interaction between hsp90 and HRI has been well characterized (*7*, *65*−*67*). Like p56^{lck}, HRI is only found in salt-stable heterocomplexes with hsp90 during intermediate stages of kinase maturation. In contrast to p56^{lck}, however, HRI does not show an obligate flow toward an hsp90-independent state; instead, HRI structure evolves in the presence of hemin to form kinase molecules that are competent to respond to hemin deficiency, but are nonetheless inactive (*7*). These inactive HRI molecules continue to interact with hsp90 machinery and require continued hsp90 function to maintain their ability to respond to hemin deficiency.

The unique biochemistry of HRI provided a novel opportunity to determine whether the continued association of HRI with hsp90 machinery reflected a static heterocomplex or a dynamic reiterative association. To differentiate these possibilities, p50^{cdc37} and an epitope-tagged HRI (H7−HRI, with an N-terminal poly-His tag) were translated in separate hemin-replete RRL reactions to generate novel radiolabeled protein populations. After synthesis, radiolabeling was terminated, and the individual reactions were mixed with each other. These mixed reactions were incubated briefly to allow formation of heterocomplexes between [^{35}S]p50^{cdc37}

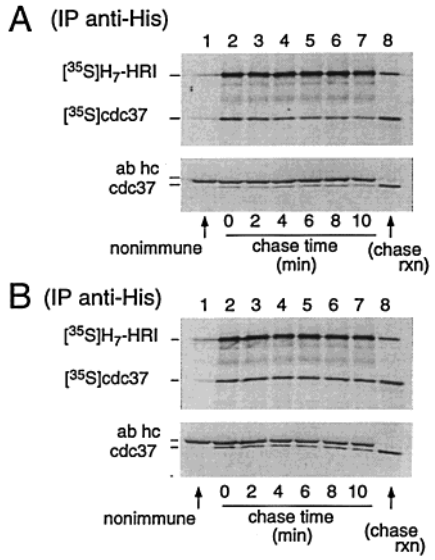

FIGURE 6: Dynamic association of p50^{cdc37} with HRI. p50^{cdc37} and an epitope-tagged HRI were synthesized in separate RRL reactions with concomitant radiolabeling with [^{35}S]Met. After synthesis, radiolabelings were terminated, and the individual reactions were mixed with each other. These mixed reactions were incubated for 20 min to allow formation of heterocomplexes, and a portion of the mixture was transferred ("chased") to a third hemin-replete reaction lacking radiolabel. At the indicated times after assembly, aliquots of the chase reaction were collected for immunoadsorption either with control nonimmune antibody (lanes 1) or with antibodies recognizing the epitope tag on [^{35}S]H7−HRI (lanes 2−7). An aliquot of the unfractionated chase reaction was also analyzed (lane 8). Panel A: analysis in RRL lacking sodium molybdate. Radiolabeled HRI ([^{35}S]H7−HRI) and radiolabeled p50^{cdc37} ([^{35}S]p50^{cdc37}) were detected by autoradiography in the upper panel; net immunoreactive p50^{cdc37} (cdc37) and the immunoadsorbing antibody heavy chain (ab hc) were detected by Western blotting in the lower panel. Panel B: the effect of including 20 mM sodium molybdate to the chase reaction was analyzed in parallel with, and as described for, panel A.

and [^{35}S]H7−HRI, and a portion of the mixture was transferred to a hemin-replete chase reaction lacking radiolabeled p50^{cdc37}. At various times after assembly of the chase reaction, aliquots were collected for immunoadsorption and analysis of H7−HRI heterocomplexes.

As seen in the upper panel of Figure 6A, immunoadsorption of [^{35}S]H7−HRI from the chase reaction immediately after its assembly coadsorbed [^{35}S]p50cdc37 in an immune-specific fashion (lanes 1 and 2). Thus, [^{35}S]p50^{cdc37} could associate with untransformed [^{35}S]H7−HRI during hsp90-mediated kinase maintenance in hemin-replete RRL. During subsequent incubations, however, the amount of [^{35}S]p50^{cdc37} associated with [^{35}S]H7−HRI steadily declined (lanes 2−7). To determine if this decline represented a net decline in the amount of p50^{cdc37} associated with HRI, these adsorptions were Western-blotted with anti-p50^{cdc37} antibodies to quantify the net levels of p50^{cdc37} associated with [^{35}S]H7−HRI (Figure 6A, lower panel). These blots indicated that the net levels of p50^{cdc37} associated with HRI did not decline. Thus, the decline in amounts of [^{35}S]p50^{cdc37} associated with HRI represented a replacement of radiolabeled [^{35}S]p50^{cdc37} with unlabeled endogenous p50^{cdc37}. This result indicated that the p50^{cdc37}−HRI complex was a dynamic entity with which p50^{cdc37} associated in a reiterative fashion. This interpretation was consistent with previous work demonstrating the dynamic reiterative nature of hsp90's association with the progesterone receptor (*74*), and the half-life of p50^{cdc37}−HRI heterocomplexes (5 min) was very similar to that reported for progesterone receptor−hsp90 heterocomplexes.

We wished to determine if molybdate would arrest the dynamic reiterative cycling of p50^{cdc37} with inactive HRI molecules. This characterization was prompted by our previous observations that molybdate inhibits hsp90-mediated folding processes by enforcing high-affinity associations between hsp90 and its substrates, resulting in an arrest of the normal obligate flow of p56^{lck} from hsp90-bound to hsp90-free forms (*64*). To determine if molybdate similarly freezes the dynamic reiterative interaction of p50^{cdc37} with HRI, this interaction was analyzed in reactions containing 20 mM molybdate (Figure 6B). In the presence of molybdate, levels of HRI-associated [^{35}S]p50^{cdc37} did not decline during chase incubations (Figure 6B, lanes 3−7), indicating that radiolabeled p50^{cdc37} was not replaced by endogenous unlabeled p50^{cdc37} in the presence of molybdate. This result indicated that the hsp90 antagonist molybdate arrested the dynamic reiterative processing of inactive kinase folding intermediates by hsp90−p50^{cdc37} machinery, a result that was consistent with molybdate's previously documented effects on other hsp90 folding processes (64).

In the preceding experiments, p50^{cdc37} associated with p56^{lck} in a fashion that was indistinguishable from hsp90's association with this kinase. However, we have previously observed that inhibition of hsp90-mediated kinase folding by geldanamycin (*3*) is accompanied by alterations in hsp90's interactions with its clients: in the presence of geldanamycin, hsp90 binds to p56^{lck} in a salt-labile fashion rather than forming the normal salt-resistant heterocomplex (*64*). To determine if p50^{cdc37} was present in the salt-labile hsp90−kinase heterocomplex formed in the presence of geldanamycin, p56^{lck} was translated in the presence or absence of this drug, kinase heterocomplexes were isolated by immunoadsorption with anti-p56^{lck} antibodies, and immunocomplexes were washed with low or high ionic strength buffers (Figure 7A). From untreated RRL, hsp90 and p50^{cdc37} were specifically recovered in anti-p56^{lck} immunoadsorptions (Figure 7A, lanes 1, 2, 5, 6), and these interactions were resistant to washing with high-salt buffers (lanes 5 and 6). In contrast, when kinase heterocomplexes were isolated from RRL reactions containing geldanamycin and subjected to washing with low ionic strength buffers, p50^{cdc37} was absent from kinase heterocomplexes despite the continued presence of hsp90 (lanes 3 and 4). When p56^{lck} heterocomplexes from geldanamycin-treated RRL were washed with high-salt buffer, neither hsp90 nor p50^{cdc37} could be detected in immunopellets (lanes 7+8). Thus, the hsp90 antagonist geldanamycin inhibited the formation of salt-resistant substrate heterocomplexes containing p50^{cdc37}. Instead, p56^{lck} was trapped in a salt-labile hsp90 chaperone complex reminiscent of previously characterized (*27*, *72*) "intermediate" heterocomplexes formed between steroid hormone receptors and hsp90 in the presence of geldanamycin.

To determine if geldanamycin similarly altered the nature of HRI's interactions with hsp90 and p50^{cdc37}, HRI was synthesized in RRL reactions containing or lacking geldanamycin (Figure 7B). As was observed for p56^{lck}, geldanamycin prevented the formation of salt-resistant HRI−hsp90 heterocomplexes containing p50^{cdc37} (lanes 7 and 8); instead, a salt-labile HRI complex containing hsp90 but lacking p50^{cdc37}

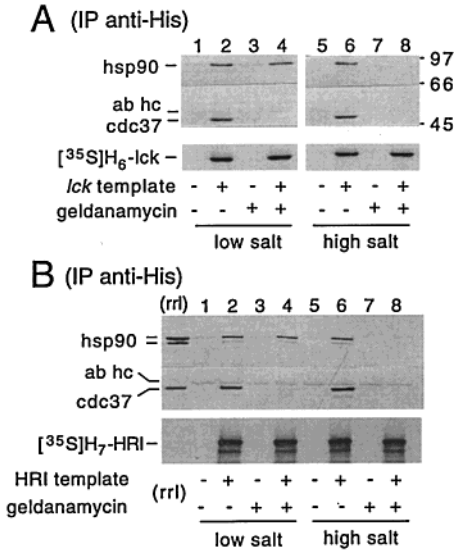

FIGURE 7: Kinase−chaperone heterocomplexes formed in the presence of geldanamycin. Protein synthesis reactions containing (lanes 3, 4, 7, 8) or lacking (lanes 1, 2, 5, 6) geldanamycin were (even-numbered lanes), or were not (odd-numbered lanes), programmed for synthesis of epitope-tagged p56^{lck}. After brief synthesis, reactions were immunoadsorbed with antibodies recognizing the epitope tag, and immunocomplexes were washed with buffers lacking (lanes 1−4) or containing (lanes 5−8) 0.5 M NaCl. Immunocomplexes were analyzed by Western blotting to detect hsp90 (hsp90) and p50^{cdc37} (cdc37); heavy chains of immunadsorbing antibody are also indicated (ab hc). Radiolabeled p56^{lck} ([^{35}S]H6−p56^{lck}) was detected by autoradiography. Migrations of size standards are indicated along the right edge of the panel. Panel B: HRI heterocomplexes were analyzed as described for panel A.

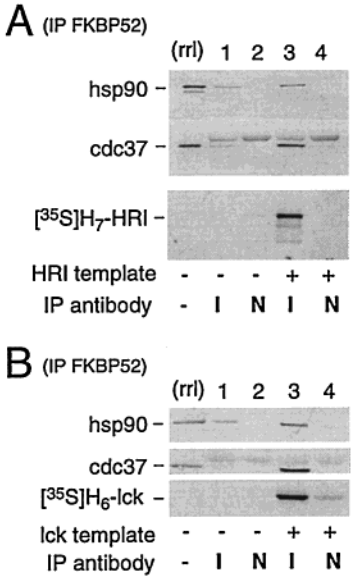

FIGURE 8: Association of p50^{cdc37} and hsp90 with FKPB52 in the presence of kinase substrates. Hemin-replete RRL reactions were (lanes 3+4), or were not (lanes 1+2), programmed for synthesis of HRI. Subsequently, reactions were immunoadsorbed either with nonimmune antibody (lanes 2, 4) or with EC1 monoclonal antibody against FKBP52 (lanes 1+3). Immunopellets were washed with buffer containing 150 mM NaCl, and adsorbed materials were eluted by boiling in SDS−PAGE sample buffer. The first lane (rrl) contains RRL loaded as a standard for detection of chaperones. Hsp90 (hsp90) and p50^{cdc37} (cdc37) were detected by Western blotting; radiolabeled HRI ([^{35}S]H7−HRI) was detected by autoradiography. Panel B: FKPB52 heterocomplexes formed in the presence or absence of p56^{lck} ([^{35}S]H6−p56^{lck}) were analyzed as described for panel A.

was formed (lanes 3 and 4). This result indicated that HRI, like p56^{lck}, had two potential modes for interaction with hsp90, and that the salt-resistant mode correlated with the presence of p50^{cdc37}.

Hsp90, p50^{cdc37}, and FKBP52 Are Simultaneously Present in Complexes with Immature Kinases. As shown in Figure 4, p50^{cdc37} was present in native hsp90 heterocomplexes containing the high M_r TPR immunophilins. To determine if p50^{cdc37} and immunophilins might be present simultaneously in heterocomplexes with immature kinase molecules, HRI or p56^{lck} was translated in hemin-replete RRL reactions. Subsequently, these reactions were immunoadsorbed with EC1 monoclonal antibody against FKBP52 and washed with buffer containing 150 mM NaCl, and the levels of kinase, p50^{cdc37}, and hsp90 present in the immunocomplex were assessed.

Anti-FKBP52 adsorptions specifically coadsorbed nascent molecules of HRI or p56^{lck} (Figure 8A+B, lanes 3+4). This result indicated that chaperone machinery containing FKBP52 recognized and bound immature kinase molecules, a finding consistent with our previous characterization of HRI heterocomplexes (*66*, *67*). The presence of either HRI or p56^{lck} on the FKBP52 heterocomplex significantly increased levels of the representation of hsp90 therein (Figure 8A+B, lanes 1 versus lane 3), a finding reminiscent of the client-enhanced association of XAP2/ARA9 with hsp90−AhR heterocomplexes (*39*). Furthermore, expression of HRI or p56^{lck} increased the levels of p50^{cdc37} associated with FKBP52 relative to the p50^{cdc37} levels observed in native FKPB52 heterocomplexes (Figure 8A,B, lanes 1 versus 3). These results demonstrated the simultaneous occurrence of hsp90, p50^{cdc37}, and FKBP52 in one or more chaperone superassemblies associated with nascent kinase molecules. Similarly, expression of HRI significantly enhanced the association of p50^{cdc37} with cyp40 chaperone machinery (not shown); however, studies examining the effects of p56^{lck} on the association of p50^{cdc37} with cyp40 machinery have been inconclusive to date (Hartson et al., unpublished).

DISCUSSION

Our results indicate that p50^{cdc37} and hsp90's TPR-containing cohorts are not invariantly mutually exclusive components of hsp90 chaperone machinery: p50^{cdc37} is specifically recovered in immunoadsorptions directed against FKBP52, cyp40, and p60HOP (or hsp70) (Figure 4). Although it has previously been suggested that similar coadsorptions may have resulted from direct recognition of p50^{cdc37} by polyclonal antibodies directed against FKBP52 or cyp40 (*40*), we do not favor such immuno-cross-reactivity as an explanation for findings presented in our current work. We minimized the potential for immuno-cross-reactivity by utilizing the EC1 monoclonal antibody to capture FKBP52 heterocomplexes. Additionally, we characterized the polyclonal anti-cyp40 antibody preparation used in our current study and observed no evidence that this antibody preparation recognized p50^{cdc37}. Furthermore, two alternative monoclonal antibodies which immunoadsorb hsp90 machinery via associated cohorts, namely, the F5 monoclonal anti-p60HOP antibody and the BB70 monoclonal anti-hsp70 antibody, also coadsorb p50^{cdc37}. Due to the improbability that all four of these antibody preparations cross-react with p50^{cdc37}, we

conclude that the recovery of p50^{cdc37} in immunoadsorptions of hsp90's TPR cohorts does not reflect an immunological artifact, but rather documents authentic chaperone heterocomplexes containing hsp90, p50^{cdc37}, and the immunoadsorption target. The existence and functional relevance of these machineries are further supported by our finding that kinase molecules are recruited to FKBP52 machinery, and this recruitment greatly increases the representation of both p50^{cdc37} and hsp90 on FKBP52 heterocomplexes (Figure 8). Thus, we conclude that hsp90, p50^{cdc37}, p23, and individual members of hsp90's family of TPR-containing cohorts, especially the immunophilins, have the potential to form a novel four-component chaperone machine which binds kinase folding intermediates.

This conclusion is consistent with previous studies which have detected physical (*59*, *61*, *66*, *67*, *75*, *76*) and functional (*77*) interactions between kinases and immunophilins, but is inconsistent with other work in which FKBP52 was not detected as a component of reconstituted hsp90 heterocomplexes associated with recombinant p50^{cdc37} (*48*). We believe that our ready detection of p50^{cdc37} in hsp90−immunophilin heterocomplexes reflects the enhanced sensitivity of the polyclonal anti-p50^{cdc37} antibodies used in the current study; these antibodies are 1−2 orders of magnitude more sensitive that the 3M/1B5p50 (*54*) monoclonal anti-p50^{cdc37} antibody previously available (not shown).

Although the potential for competition between p50^{cdc37} and immunophilins for binding to hsp90 has not been examined, previous work has demonstrated such competition between p50^{cdc37} and the nonimmunophilin cohort p60HOP (*40*, *48*). Thus, our detection of low levels of p50^{cdc37} in p60HOP immunocomplexes implies an apparent contradiction. This apparent contradiction can be resolved by insights provided through a recent study that examined the nature of hsp90's cohort acceptor sites: isothermal titration calorimetry profiles suggest that hsp90 binds purified Sti 1 (yeast equivalent to p60HOP) or Cpr6 (yeast equivalent to cyp40) with stoichiometries of one molecule of hsp90 per molecule of cohort, suggesting two TPR acceptor sites per hsp90 dimer (*78*). This two-site model predicts that heterogeneous cohort compositions might exist in situ, but that the hsp90 dimer would nonetheless bind only the predominant cohort during competition assays. Furthermore, the two-site model predicts that hsp90 cohorts with a strong potential to dimerize (such as Sti 1/p60HOP) might strongly inhibit the formation of mixed-cohort machineries, while cohorts with less dimerization potential (such as Cpr6/cyp40) might be more permissive in this regard (*78*). Consistent with this speculation, we note that only very low levels of p50^{cdc37} are recovered in p60HOP immunopellets relative to those recovered in FKBP52 or cyp40 immunopellets (Figure 4), despite a lack of any evidence indicating that p50^{cdc37} prefers a specific hsp90 conformation (Figure 3). However, this explanation must be regarded as speculative given our incomplete understanding of hsp90 machinery: the postulation that hsp90 dimers contain two sites for binding of TPR proteins is not universally accepted (*73*), and the purified yeast *cdc37* gene product migrates as a dimer on native PAGE gels (*55*).

Although our understanding of hsp90−p50^{cdc37} machinery is incomplete, we propose that p50^{cdc37} chaperone heterocomplexes should be considered to be analogous to the "late" hsp90 machineries previously described for the progesterone receptor ordered-assembly pathway (*61*, *74*). This conceptual pathway attempts to describe hsp90-mediated folding by dividing the process into discrete stages; at each stage, the receptor−chaperone heterocomplex displays a unique composition of hsp90 cohorts. Based on previously described behaviors of hsp90−chaperone heterocomplexes, we propose three criteria by which p50^{cdc37} should be considered to act as a "late" hsp90 machinery. (1) p50^{cdc37} is well represented in heterocomplexes containing p23 (Figure 2) or immunophilins (Figure 4), but appears to be a very minor constituent of p60HOP machinery (Figure 4). (2) p50^{cdc37} interacts with its kinase substrates in a salt-stable heterocomplex (Figure 5), whereas "intermediate" hsp90 heterocomplexes described to date are invariantly salt-labile [e.g., (*17*)]. (3) The hsp90 antagonist geldanamycin prevents recruitment of p50^{cdc37} to kinase substrates (Figure 7), a characteristic of "late" hsp90 machinery exemplified by hsp90−immunophilin−p23 heterocomplexes (*27*, *28*, *72*). However, although p50^{cdc37} would appear to fit these three criteria for a late complex, we note p50^{cdc37} does not seem to share with p23 the ability to stringently recognize a specific a nucleotide-regulated conformation of hsp90 (*27*, *28*); p50^{cdc37} associates with hsp90 in an ATP-independent fashion, and this interaction is not abolished by geldanamycin (Figure 3).

Despite its putative identity as a "late" kinase−chaperone heterocomplex, it is important to acknowledge that the p50^{cdc37}−kinase interaction does not represent a static, obligatorily terminal heterocomplex. Instead, p50^{cdc37} associates with immature kinase substrates in a dynamic reiterative fashion (Figure 6), as has been described previously for the interaction of hsp90 with the progesterone receptor (*74*). Furthermore, it is important to recognize that p50^{cdc37} interacts only with immature or inactive forms of the kinases p56^{lck} (Figure 5) and HRI (Shao et al., submitted), and similarly recognizes conformational intermediates of viral p60^{src} [reviewed in ref (*51*)], Cdk4 (*6*), Cdk9 (*5*), Cdc28 (*79*), and Cak1 (*79*). These observations suggest that for these kinases, p50^{cdc37} functions strictly as a molecular chaperone and an hsp90 cohort rather than acting as an obligate regulatory subunit or as an adaptor protein. However, kinase maturation mediated by hsp90−p50^{cdc37}−immunophilin heterocomplexes in vivo might be coupled to other cellular processes such as intracellular trafficking (*40*).

Although the biochemical function of p50^{cdc37} is poorly understood, our data indicate that p50^{cdc37} does not serve as a passive structural bridge between hsp90 and client kinases, nor does hsp90 serve as a passive structural bridge between p50^{cdc37} and client kinases. This conclusion derives from the observation that hsp90 and p50^{cdc37} interact with each other in a fashion which is distinct from their interactions with substrate: the hsp90−p50^{cdc37} interaction is salt-labile (Figure 1), while heterocomplexes with kinase folding intermediates are salt-resistant [Figure 5; see also (*51*, *58*)]. Thus, high-affinity heterocomplexes between hsp90, p50^{cdc37}, and kinase folding intermediates appear to result from fundamental alterations in protein−protein interactions rather than from the simple assembly of passive protein components. However, our data do not allow us to differentiate mechanisms which may underlie these fundamental alterations. As one possibility, binding of kinase client to p50^{cdc37} could directly enhance p50^{cdc37}'s affinity of hsp90 [i.e., a "linear" (*39*)

organization of individual components within the heterocomplex]. Alternatively, the stability of the client−chaperone heterocomplex could be enhanced by the simultaneous interaction of each chaperone with both the client and its co-chaperone partners. Additionally, contributions from both mechanisms could enhance the stability of the multicomponent client−chaperone heterocomplex. Similar uncertainties regarding the association of hsp90 and XAP2/ARA9 with the aryl hydrocarbon receptor have been discussed recently [e.g., (*39*)].

Geldanamycin inhibits the formation of high-affinity interactions within hsp90−kinase heterocomplexes; instead, geldanamycin-poisoned hsp90 associates with kinase heterocomplexes via weak ionic interactions [Figure 7, see also (*64*)]. The labile nature of this interaction probably explains the absence of hsp90 from substrate heterocomplexes typically isolated from geldanamycin-treated cells (*64*). We have previously speculated that this low-affinity interaction reflects electrostatic binding of hsp90 to kinase-bound cohort proteins (e.g., p60HOP/hsp70) rather than to the kinase per se (*64*). Because geldanamycin acts at hsp90's nucleotide binding pocket (*80*−*82*), the effects of this drug on the hsp90−kinase heterocomplex indicate that nucleotide-mediated switching of hsp90's conformation is required to establish high-affinity interactions therein (*64*).

However, the p50^{cdc37} component is absent from kinase heterocomplexes formed in the presence of geldanamycin, regardless of the ionic strength of immunoadsorption wash buffers (Figure 7). The inability of p50^{cdc37} to bind to kinase folding intermediates in the presence of geldanamycin does not reflect sequestration or masking of kinase molecules by geldanamycin-poisoned chaperone complexes (Shao et al., submitted). Thus, we conclude that hsp90 regulates the kinase binding activity of p50^{cdc37}.

Similarly, it has been suggested that the failure of p50^{cdc37} to associate with Raf-1 in geldanamycin-treated cells reflects a quantitative sequestration of p50^{cdc37} into inactive hsp90−p50^{cdc37} heterocomplexes (*52*); geldanamycin also inhibits formation of the Cdk4−p50cdc37 heterocomplex (*6*). However, we note that there appears to be an abundance of hsp90-free p50^{cdc37} in RRL (Figure 2). Additionally, geldanamycin does not appear to enforce an interaction between hsp90 and p50^{cdc37}, since this drug does not enrich for such heterocomplexes (Figure 3). Furthermore, geldanamycin treatment of RRL does not induce detectable alterations in the proteolytic fingerprint of the p50^{cdc37} population that predominates therein (Hartson et al., unpublished). Thus, we speculate that the default conformation which p50^{cdc37} assumes in RRL is not competent to bind kinases, and that nucleotide-regulated conformational switching of hsp90 is necessary to unleash p50^{cdc37}'s ability to form high-affinity interactions with kinase folding intermediates. This interpretation would suggest that the modest-to-robust kinase binding activity of purified p50^{cdc37} (*6*, *48*, *52*) represents a property acquired by disregulation of this chaperone during its purification.

However, p50^{cdc37} does not appear to be a strictly subordinate partner to hsp90; p50^{cdc37} potentiates the physical and functional interaction of hsp90 with kinases (*6*, *52*, *55*), suggesting that p50^{cdc37} modulates the kinase binding activity of hsp90. Thus, hsp90 and p50^{cdc37} may represent dynamic partners, each regulating the kinase binding potential of the other. This speculation is consistent with the observed correlation between the salt-labile nature of the hsp90−kinase heterocomplex formed in the presence of geldanamycin and the absence of p50^{cdc37} therein (Figure 7).

The biochemical mechanisms by which hsp90, immunophilins, and p50^{cdc37} fulfill their physiological roles in vivo remain poorly understood. HRI is an endogenous reticulocyte kinase, suggesting that the HRI−immunophilin heterocomplexes which occur in reticulocyte lysates may also occur in the intact reticulocyte. Alternatively, expression of p56^{lck} is normally limited to lymphoid cell populations. Thus, it remains to be determined if p56^{lck} similarly associates with FKBP52 in T cells. Given emerging evidence that individual immunophilins play nonequivalent roles in vivo (*36*−*39*), these considerations are important caveats with regard to the interpretation of reticulocyte lysate modeling. Nonetheless, data presented here demonstrate that p50^{cdc37} is a nonexclusive hsp90 cohort which responds to hsp90's nucleotide-regulated conformational switching during the generation of high-affinity interactions within the hsp90−kinase−p50^{cdc37} heterocomplex.

ACKNOWLEDGMENT

We thank Dr. Douglas Melton (Harvard University), Dr. Paul Burn (Monsanto), Dr. Roger M. Pearlmutter (Howard Hughes Medical Institute, University of Washington), and Dr. Jane-Jane Chen (MIT) for generously providing plasmid DNAs and Dr. David O. Toft (Mayo Clinic, Rochester, MN), Dr. David F. Smith (Mayo Clinic, Scottsdale, AZ), Dr. Lee E. Faber (Medical College of Ohio), and Dr. Gary H. Perdew (Pennsylvania State University) for generously providing antibodies. We also thank Dr. Sheri Uma (Oklahoma State University) for insightful discussions and for critical reading of the manuscript. Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH. Oligonucleotide synthesis and DNA sequencing were performed by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Polyclonal mouse ascites antibodies against p56^{lck} and p50^{cdc37} were prepared by the Oklahoma State University Hybridoma Center for the Agricultural and Biological Sciences. Rabbit antibodies were produced by the Oklahoma State University Lab Animal Resources Unit.

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BI000315R