p50\textsuperscript{cdc37} Binds Directly to the Catalytic Domain of Raf as Well as to a Site on hsp90 That Is Topologically Adjacent to the Tetra tricopeptide Repeat Binding Site*

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Adam M. Silverstein\textsuperscript{§}, Nicholas Grammatikakis\textsuperscript{§}, Brent H. Cochran\textsuperscript{§}, Michael Chinkers\textsuperscript{§}, and William B. Pratt\textsuperscript{¶}

From the \textsuperscript{§}Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109, the \textsuperscript{¶}Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111, and the \textsuperscript{¶}Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201

A variety of transcription factors and protein kinases have been recovered from cytosols in native heterocomplexes with the abundant, ubiquitous, and essential protein chaperone hsp90\textsuperscript{1} (for review, see Refs. 1 and 2). Several other proteins, all of unknown function, have been recovered in steroid receptor-hsp90 and protein kinase-hsp90 heterocomplexes. Steroid receptor-hsp90 heterocomplexes contain one of several high molecular weight immunophilins or the protein serine/threonine phosphatase PP5 (1). The protein kinase heterocomplexes contain a 50-kDa phosphoprotein that was originally identified as a component of the p60\textsuperscript{v-raf}-hsp90 heterocomplex (for review, see Refs. 3 and 4).

We and others have recently cloned p50 and identified it as the vertebrate homolog of the yeast cell cycle control protein Cdc37 (5–7). Genetic evidence suggests that Cdc37 is necessary for Src function (8) and for signaling via the sevenless receptor, a protein tyrosine kinase of Drosophila (9). The cyclin-dependent protein kinase Cdk4 is also recovered in heterocomplexes with hsp90 and p50\textsuperscript{cdc37} (6, 10), and we (10) and Stepanova et al. (6) have shown that p50\textsuperscript{cdc37} binds directly to Cdk4 as well as to hsp90.

Three high molecular weight immunophilins, FKBP52 (formerly called p59 or hsp56) (11–14), FKBP51 (15–17), and CyP-40 (18, 19), exist in steroid receptor-hsp90 heterocomplexes. The immunophilins exist in independent receptor heterocomplexes. Each of the three immunophilins contains three tetrar icopeptide repeats (TPRs), which are degenerative sequences of 34 amino acids (20) that are required for binding to hsp90 (21–23). It has been shown that CyP-40 and FKBP52 compete with each other for binding to hsp90 (21, 24), and that these immunophilins exist in independent receptor-hsp90-FKBP52 and receptor-hsp90-CyP-40 heterocomplexes (24, 25).

A variety of transcription factors and protein kinases have been recovered from cytosols in native heterocomplexes with the abundant, ubiquitous, and essential protein chaperone hsp90\textsuperscript{1} (for review, see Refs. 1 and 2). Several other proteins, all of unknown function, have been recovered in steroid receptor-hsp90 and protein kinase-hsp90 heterocomplexes. Steroid receptor-hsp90 heterocomplexes contain one of several high molecular weight immunophilins or the protein serine/threonine phosphatase PP5 (1). The protein kinase heterocomplexes contain a 50-kDa phosphoprotein that was originally identified as a component of the p60\textsuperscript{v-raf}-hsp90 heterocomplex (for review, see Refs. 3 and 4).
Using FLAG-tagged p50cdc37 and PP5, we show that p50cdc37 exists in separate hsp90 heterocomplexes from the TPR proteins. In addition to binding to hsp90, p50cdc37 binds directly to Raf. It is known that, during the process of Rafhsp90 heterocomplex assembly, Raf is transiently associated with p60 (also called Hop) (33), which binds to hsp90 via its TPRs (34). p60/Hop is required for assembly of hsp90 heterocomplexes (35), and we show here that p60/Hop competes for the binding of both TPR domain proteins and p50cdc37 to hsp90. Our observations are consistent with a model in which dissociation of p60/Hop from the newly formed Rafhsp90 complex results in an open region on the surface of the hsp90 dimer that can be occupied by either p50cdc37 or a TPR protein. With continued exchange binding of p50cdc37 and TPR domain proteins to Raf-associated hsp90, Rafhsp90/p50cdc37 complexes are rapidly selected because p50cdc37 also binds directly to Raf.

EXPERIMENTAL PROCEDURES

Materials

Unlabeled rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). [35S]-L-Conjugated goat anti-mouse and anti-rabbit IgGs were from NEN Life Science Products. Goat anti-mouse IgG-herosarized peroxidase conjugate, monoclonal nonimmune IgG and IgM, purified rabbit anti-myc monoclonal anti-glutathione S-transferase (GST) clone GST-2 ascites, and purified glutathione S-transferase from Sigma. The AC88 monoclonal IgG against hsp90 was from StressGen (Victoria, British Columbia, Canada). The 3G3 monoclonal IgM against hsp90, and the anti-cycliphilin 40 (COOH-terminal peptide) antibody were from Affinity Bioreagents (Golden, CO). The anti-FLAG M2 monoclonal IgG, M2-agarose, and the FLAG peptide were from IBI (New Haven, CT). The C-12 rabbit anti-Raf-1 IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Raf antiserum prepared against the carboxy-terminal 12 amino acids of human Raf-1 (34) was kindly provided by Dr. Richard Jove (Moffitt Cancer Center, Tampa, FL). The DS14F5 monoclonal antibody against p60/Hop (36) and ErbB2 was provided by Dr. Jack-Michel Renoir (University of Paris, France). The UPJ56 rabbit antiserum against hsp56 (38) was a kind gift from Dr. Karen Grillo (38) was a kind gift from Dr. Karen Leach (The Upjohn Co., Kalamazoo, MI). The rabbit antiserum against hsp56 (38) was a kind gift from Dr. Karen Leach (The Upjohn Co., Kalamazoo, MI). The rabbit antiserum against hsp56 (38) was a kind gift from Dr. Karen Leach (The Upjohn Co., Kalamazoo, MI). The rabbit antiserum against hsp56 (38) was a kind gift from Dr. Karen Leach (The Upjohn Co., Kalamazoo, MI).

Binding of Proteins to Purified hsp90—Rabbit hsp90 was purified from brain cytosol as described by Hutchison et al. (40). Aliquots (30 μl) of purified rabbit hsp90 (1 mg/ml) were immobilized to 12-μl 20 mM Hepes, 1 mM dithiothreitol, 150 mM NaCl were added, maintaining the same final incubation volume of 100 μl, 1 mM EDTA, 10% (w/v) glycerol, 20 mM sodium molybdate, pH 7.6, and 10% (w/v) glycerol and proteins were resolved by SDS-PAGE and Western blotting.

Expression of p60 and GST-FKB52 Fusion Protein—Bacterially expressed p60/Hop was prepared as described previously (35). For bacterial lysates containing GST-FKB52, the expression plasmid containing the cDNA for the 59-kDa rabbit immunophilin subcloned into the SmaI site of pGEX-2T prepared by Le Bihan, et al. (37) was used to transform E. coli strain BL21(DE3). Purification of rabbit FKB52 was performed by binding the GST-FKB52 to GSH-agarose and incubation at 4 °C with threom, which cleaves at a site between the GST domain and the FKB52 domain.

Preparation of a Recombinant Baculovirus Expressing FLAG-tagged p50cdc37—The cDNA for p50cdc37, isolated from a human lymphocyte cDNA library through hybridization with the previously described chick cd37 DNA homolog (5, 10),1 served as template to amplify by polymerase chain reaction the open reading frame, starting from codon 2 and including 285 base pairs of 3′-untranslated sequence. The amplified human p50cdc37 cDNA was subcloned into the NcoI site of pFastBAC1-FLAG, a modified version of the baculoviral pFastBAC1 vector (Life Technologies, Inc.), in frame with a FLAG epitope sequence. The resulting construct was verified by DNA sequencing and subsequently used to generate FLAG-p50cdc37 encoding recombinant baculoviruses and high titer stocks, using the BAC-TO-BAC baculovirus expression system from Life Technologies, Inc.

1N. Grammatikakis, unpublished results.
Fig. 1. The PP5 TPR domain does not compete for binding of p50<sup>cdc37</sup> to hsp90. Protein A-Sepharose pellets linked to 3G3 antibody alone or 3G3 prebound with purified hsp90 were incubated on ice with 30 μl of the rabbit brain hydroxylapatite pool containing p60/Hop, FKBP52, p50<sup>cdc37</sup> and CyP-40 (but not hsp90) in the presence or absence of 30 μg of purified FLAG-tagged TPR domain of PP5. After washing, pellet-associated proteins were resolved by SDS-PAGE and Western blotting. Lane 1, 3G3 pellet without hsp90 incubated with hydroxylapatite pool; lane 2, 3G3 pellet with bound hsp90 incubated with hydroxylapatite pool; 3G3 pellet with bound hsp90 incubated with hydroxylapatite pool and purified PP5 TPR.

Purification of FLAG-p50<sup>cdc37</sup> from S9 Cells—S9 cells (1.8 × 10<sup>7</sup>) were cultured into T-162 cm tissue culture flasks and infected with a baculovirus expressing FLAG-p50<sup>cdc37</sup> at a multiplicity of infection of 3, then incubated for 2 days at 27 °C. Cytosol was prepared from infected cells and diluted 1:1 with TEG, the nonionic detergent Nonidet P-40 was added to 0.02%, and the diluted cytosol was rotated for 1 h at 4 °C and centrifuged at 100,000 × g. FLAG-tagged p50<sup>cdc37</sup> was then purified using M2-agarose beads (IBI) according to manufacturer’s instructions.

Preparation of an Antibody against p50<sup>cdc37</sup>—Human p50<sup>cdc37</sup> (amino acids 2-378) expressed as GST fusion protein was purified by GSH-Sepharose chromatography and used to generate p50<sup>cdc37</sup>-specific antisera in rabbits. Although the rabbit anti-p50<sup>cdc37</sup> antiserum exhibits a wide reactivity for p50<sup>cdc37</sup> across species, it does not recognize the endogenous p50<sup>cdc37</sup> expressed in insect S9 host cells.

RESULTS

Competition for Binding of p50<sup>cdc37</sup> to hsp90—In a previous study (29), we showed that a fragment containing the TPR domains of CyP-40 competed for the binding of FKBP52 and CyP-40 to hsp90. However, the binding of p60/Hop and p50<sup>cdc37</sup> was not inhibited by the highest achievable level of the CyP-40 TPR fragment. Subsequently, we found that the fragment of PP5 containing its four TPRs bound much more tightly to hsp90 than p50<sup>cdc37</sup> alone or 3G3 prebound with purified hsp90 (lane 2). The PP5 TPR fragment (amino acids 2-378) expressed as GST fusion protein was purified by GSH-Sepharose chromatography and used to generate p50<sup>cdc37</sup>-specific antisera in rabbits. Although the rabbit anti-p50<sup>cdc37</sup> antiserum exhibits a wide reactivity for p50<sup>cdc37</sup> across species, it does not recognize the endogenous p50<sup>cdc37</sup> expressed in insect S9 host cells.

Fig. 2. p50<sup>cdc37</sup> and TPR proteins compete for the binding of each other to hsp90. A, bacteriologically expressed p60/Hop competes for the binding of p50<sup>cdc37</sup> to hsp90. Pellets with 3G3 antibody alone or 3G3 prebound with hsp90 were incubated on ice in the presence of lysate from control bacteria or bacteria expressing p60/Hop, then incubated with the rabbit brain hydroxylapatite pool. Lane 1, pellet without hsp90; lane 2, p60/Hop-bound pellet; lane 3, p60/Hop-bound pellet preincubated with control bacterial lysate; lane 4, p60/Hop-bound pellet preincubated with lysate from bacteria expressing p60/Hop. B, S9-expressed p50<sup>cdc37</sup> competes for binding of p60/Hop to hsp90. Pellets were preincubated in the presence of lysate from S9 cells transfected with wild-type baculovirus or S9 cells expressing FLAG-p50<sup>cdc37</sup>, then incubated with the rabbit brain hydroxylapatite pool. p50<sup>cdc37</sup> was detected with the anti-FLAG antibody. Lane 1, pellet without hsp90; lane 2, p60/Hop-bound pellet; lane 3, p60/Hop-bound pellet plus control S9 lysate; lane 4, p60/Hop-bound pellet plus lysate from S9 cells expressing p50<sup>cdc37</sup>. C, S9-expressed PP5 competes for binding of p50<sup>cdc37</sup> to hsp90. Pellets were treated as in B. Lane 1, pellet without hsp90; lane 2, hsp90-bound pellet; lane 3, hsp90-bound pellet plus control S9 lysate; lane 4, hsp90-bound pellet plus lysate from S9 cells expressing PP5.

was assayed. It is clear from lane 4 that p60/Hop competes for the binding of p50<sup>cdc37</sup> to hsp90. In Fig. 2B, p60/Hop-bound immune pellets were preincubated with lysate from S9 cells expressing FLAG-p50<sup>cdc37</sup> and then incubated with the hydroxylapatite pool. In the presence of the S9-expressed p50<sup>cdc37</sup> (lane 4), the binding of p60/Hop to hsp90 was competed. As shown in Fig. 2C, S9-expressed FLAG-PP5 also competes for the binding of p50<sup>cdc37</sup> to hsp90.

The cloning and sequencing of p50<sup>cdc37</sup> showed that it does not possess a TPR domain (6, 7), yet intact TPR domain proteins compete for its binding to hsp90. As shown in the experiment of Fig. 1, we have occasionally observed an increase in the amount of p50<sup>cdc37</sup> binding to hsp90 when the PP5 TPR fragment is present. Such an increase in p50<sup>cdc37</sup> binding would occur if binding of TPR proteins to the TPR acceptor site on hsp90 prevented access of p50<sup>cdc37</sup> to its binding site, but the small PP5 TPR fragment did not.

p50<sup>cdc37</sup> Does Not Exist in Native hsp90 Heterocomplexes with TPR Proteins—These competition data suggest that the binding site for p50<sup>cdc37</sup> may be close enough to the TPR binding site on the surface of hsp90 such that the binding of a protein to one site blocks access of the other protein to its binding site. If that is true, p50<sup>cdc37</sup> should not exist in a native hsp90-TPR protein complex unless there is a binding site for each of the proteins on each half of the hsp90 dimer. In which case, immunoabsorption of an hsp90-bound TPR protein should yield not only co-immunoabsorption of some p50<sup>cdc37</sup> but also of other TPR-containing proteins. In the experiment of Fig. 1, either hsp90 or p60/Hop was immunoabsorbed from rabbit reticulocyte lysate and the washed immune pellets were assayed for coadsorbed proteins. Immunoabsorption of hsp90 (lane 2) yielded coadsorption of the four TPR proteins (p60/Hop, PP5, FKBP52, and CyP-40) as well as the non-TPR-containing p50<sup>cdc37</sup>. Immunoabsorption of p60/Hop (lane 4) yielded coadsorption of a substantial amount of hsp90 but no coadsorption of p50<sup>cdc37</sup> or of other TPR proteins.
and stripped with salt and heating (31). The antiserum against p50 was preadsorbed with nonimmune IgG or 3G3 antibody against hsp90. Other 100-μl aliquots of reticulocyte lysate were incubated for 30 min at 30 °C with 10 μl of Sf9 cytosol overexpressing FLAG-p50cdc37 or FLAG-PP5, and then immunoadsorbed with nonimmune IgG or the M2 monoclonal IgG against the FLAG epitope. Lane 1, immunoadsorption with nonimmune antibody, lane 2, immunoadsorption with the antibody indicated at the top of each pair of lanes. Note that the AC58 antibody used to blot hsp90 reacts with rabbit but not insect (Sf9) hsp90 (31). Because PP5 migrates close to FKBP52 on SDS-PAGE, any FKBP52 that might be present in the immune adsorbate would be obscured by the large amount of FLAG-PP5; thus, FKBP52 was not assayed.

Binding of p50cdc37 to hsp90 and Raf

**Fig. 3.** Native hsp90 p60/Hop heterocomplexes do not contain p50cdc37. Aliquots (150 μl) of rabbit reticulocyte lysate were immunoadsorbed with the 3G3 antibody against hsp90 or the F5 antibody against p60/Hop. Lane 1, nonimmune IgM; lane 2, 3G3 anti-hsp90; lane 3, nonimmune IgG; lane 4, F5 anti-p60/Hop.

It is possible that p60/Hop is unique among TPR proteins in that it is present in hsp90 heterocomplexes free of p50cdc37. We were unable to test this possibility by immunoadsorption of hsp90 heterocomplexes with antibodies directed against p50cdc37 or the immunophilins because of their substantial cross-reactivity. The antiserum against p50cdc37 (α-p50), for example, reacts on immunoblots with both PP5 and CyP-40 (data not shown). Given the cross-reactivity of the antisera, we used a monoclonal antibody against the FLAG epitope to immunoadsorb Sf9-expressed FLAG-p50cdc37 and FLAG-PP5 and assayed for immunoadsorbed proteins. In the experiments of Fig. 4, a small amount of Sf9 cytosol with the expressed FLAG-tagged protein was first incubated with rabbit reticulocyte lysate to ensure complete equilibration of the FLAG-p50cdc37 and FLAG-PP5 with rabbit hsp90. The FLAG-tagged proteins were then immunoadsorbed with the M2 monoclonal anti-FLAG IgG, and coadsorbed proteins were assayed. It is clear that immunoadsorption of FLAG-p50cdc37 yields coadsorption of hsp90, but there is no coadsorption of the rabbit TPR domain proteins PP5 or FKBP52. Similarly, immunoadsorption of FLAG-PP5 yielded coadsorption of hsp90, but there is no coadsorption of p50cdc37. Taken together, these immunoadsorption observations and the competition data of the previous section lead us to conclude that p50cdc37 can bind to hsp90 when the TPR acceptor site is occupied by the TPR domain fragment of PP5 but not when the site is occupied by an intact TPR domain protein.

**Fig. 4.** p50cdc37, PP5 and FKBP52 exist in independent heterocomplexes with hsp90. Aliquots (100 μl) of rabbit reticulocyte lysate were immunoadsorbed with nonimmune IgM or 3G3 antibody against hsp90. Other 100-μl aliquots of reticulocyte lysate were incubated for 30 min at 30 °C with 10 μl of Sf9 cytosol overexpressing FLAG-p50cdc37 or FLAG-PP5, and then immunoadsorbed with nonimmune IgG or the M2 monoclonal IgG against the FLAG epitope. Lane 1, immunoadsorption with nonimmune antibody, lane 2, immunoadsorption with the antibody indicated at the top of each pair of lanes. Note that the AC58 antibody used to blot hsp90 reacts with rabbit but not insect (Sf9) hsp90 (31). Because PP5 migrates close to FKBP52 on SDS-PAGE, any FKBP52 that might be present in the immune adsorbate would be obscured by the large amount of FLAG-PP5; thus, FKBP52 was not assayed.

**DISCUSSION**

Previous studies have shown that FKBP52 and CyP-40 compete with each other for binding to hsp90 (21, 24) and that these two immunophilins and the TPR-containing protein phosphatase, PP5, exist in separate heterocomplexes with hsp90 (28). In this work, we provide evidence that p50cdc37 cannot bind to hsp90 when the TPR acceptor site on hsp90 is occupied by one of the TPR domain proteins, such as p60/Hop or PP5. However, p50cdc37 does bind to hsp90 when the small TPR domain fragment of PP5 occupies the TPR acceptor site and prevents binding of the TPR domain proteins. These com-
have been identified in transcription factors that are recovered from cytosols in stable complexes with hsp90. The TPR protein can be p60/Hop, PP5, or any of the immunophilins that have been identified in transcription factors-hsp90 heterocomplexes. To date, p50<sup>cdc37</sup> has been recovered only with protein kinase-cdc37 heterocomplexes. Molybdate (MoO<sub>4</sub><sup>-</sup>), which stabilizes hsp90 in its ATP-dependent conformation (50), interacts with the nucleotide binding site.

petition data suggest that the p50<sup>cdc37</sup> binds to a site on the surface of hsp90 that is close to the TPR binding site and that binding of a protein to one site may block binding of a protein to the other site.

It could be argued that binding of a protein, such as p60/Hop, PP5, or an immunophilin, to the TPR binding site on hsp90 influenced the conformation of hsp90 such that the affinity of a p50<sup>cdc37</sup> binding site located at some distance from the TPR binding site was reduced. However, the fact that binding of the PTP TPR fragment to hsp90, if not augmenting, at least does not reduce the binding of p50<sup>cdc37</sup> argues against such an allosteric effect. Thus, we propose that p50<sup>cdc37</sup> binds to a site on hsp90 that is topologically adjacent to the TPR binding site, and at any instant in time, an hsp90 heterocomplex contains either p50<sup>cdc37</sup> or one of the TPR domain proteins.

Although hsp90 is present in cytosols as a dimer, it is likely that only one molecule of p50<sup>cdc37</sup> or TPR domain protein can be bound by the dimer. In the event that independent binding sites were available on each dimer, we should have recovered mixed complexes in which immunoadsorption of one TPR protein from cytosol yields coimmunoadsorption of other TPR proteins and p50<sup>cdc37</sup>. A stoichiometry in which one of these proteins is bound per hsp90 dimer is consistent with careful cross-linking studies of Gehring and his co-workers (42–44), who established a stoichiometry for untransformed steroid receptor heterocomplexes. Molybdate (MoO<sub>4</sub><sup>-</sup>), which stabilizes hsp90 in its ATP-dependent conformation (50), interacts with the nucleotide binding site.

The evidence of this study suggests that the p50<sup>cdc37</sup> component of protein kinase-hsp90 heterocomplexes binds, in vitro, to a site that is topologically adjacent to the TPR binding site on hsp90 but that p50<sup>cdc37</sup> and a TPR domain protein may not be able to bind to the same hsp90 dimer. The dashed borders of the TPR domain protein and p50<sup>cdc37</sup> in Fig. 6 indicate the overlapping space occupied by both proteins that accounts for their mutual competition for binding to hsp90. Because p50<sup>cdc37</sup> binds directly to Raf (Fig. 5) and to Cdk4 (6, 10), it has also been positioned such that it could contact the chaperoned protein as well as hsp90.

In the dynamic state when Raf-hsp90 complexes are being assembled, dissociation of the p60/Hop component of the assembly machinery would expose on hsp90 both the binding site for TPR domains and the adjacent binding site for p50<sup>cdc37</sup>. As both the TPR domain proteins and p50<sup>cdc37</sup> bind in a readily reversible manner to their respective sites on hsp90, simultaneous binding of p50<sup>cdc37</sup> directly to Raf should rapidly select for Raf-hsp90-p50<sup>cdc37</sup> complexes, which is the composition of native Raf-hsp90 heterocomplexes isolated from cytosols (31). Thus, the combination of exclusive binding of p50<sup>cdc37</sup> versus a TPR domain protein to hsp90 plus direct binding of p50<sup>cdc37</sup> to Raf allow the protein kinase to determine the dominant heterocomplex composition.

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