The glucocorticoid receptor (GR) HBD must be bound to the protein chaperone hsp90 in order to acquire the high affinity steroid binding conformation. Despite this crucial role of hsp90, its binding site in GR remains poorly defined. Large portions of the GR HBD have been implicated and no similarity has been established between steroid receptor HBDs and the catalytic domains of the protein kinases (e.g. pp60c-src, Raf) that also form stable heterocomplexes with hsp90. Thus, it has been thought that some general property of the proteins, such as exposure of hydrophobic residues in partially denatured regions, determines the assembly of stable hsp90 heterocomplexes. In this work, we have studied fusion proteins containing glutathione S-transferase (GST) and very short amino-terminal truncations just before and at the beginning of the rat GR HBD that are otherwise intact to the carboxyl terminus. Overexpression in COS cells of the chimeras GST537C and GST547C was found to yield receptors that were bound to hsp90 and had wild-type steroid binding affinity. However, removal of 7 more amino acids to form GST554C resulted in a fusion protein that did not bind either hsp90 or steroid. Additional mutations revealed that the role of these 7 amino acids was neither to provide a spacer between protein domains nor to expose a protein surface by introducing a bend in the conserved a-helix. Instead, these observations support a model in which the sequence of the 7 amino acids directly or indirectly affects hsp90 binding to the GR HBD. Thus, a region of GR that has not been thought to be relevant for hsp90 binding is now seen to be of critical importance, and these data argue strongly against the commonly accepted model of receptor-hsp90 heterocomplex assembly in which the chaperone initially interacts nonspecifically with hydrophobic regions of the partially denatured HBD and subsequently assists its folding to the steroid binding conformation.

The hormone-binding domain (HBD) of the glucocorticoid receptor (GR) is comprised of the carboxyl-terminal one-third of the protein (for review, see Refs. 1 and 2). However, the HBD cannot bind steroid unless the receptor is bound to the chaperone hsp90 (3, 4). hsp90 is a component of a multiprotein chaperone system (including hsp70, p60, and p23) that directs the ATP-dependent assembly of hsp90 into complexes with a variety of transcription factors and protein kinases (for review, see Refs. 5 and 6). It is not known what properties permit these proteins to form relatively stable complexes with hsp90, whereas most proteins do not. It is clear, though, that hsp90 binds directly to the GR HBD (7, 8). When the GR HBD is fused to another transcription factor, the fusion protein is bound to hsp90 and binds steroid (9). Furthermore, the activity of the fused transcription factor is usually controlled by steroid binding (for review, see Ref. 10).

Several approaches have been taken to determine the region of the GR HBD involved in steroid binding and hsp90 binding. When GR was translated in reticulocyte lysates, only the full-length translation product was recovered in association with hsp90 (11), suggesting that the HBD is completely formed before the original hsp90 binding occurs. When GR mutants lacking increasing portions of the carboxyl terminus were translated in reticulocyte lysate, Dalman et al. (12) identified a minimal region from 616—671 (rat GR) that was required for a high yield of high affinity hsp90 binding. With the same approach, Howard et al. (13) found that the region 568—616 was sufficient to yield some hsp90 binding. Taken together, these reports suggested a minimal hsp90-binding site of about 100 amino acids (568—671). However, Cadepond et al. (14) divided the human GR HBD into three subregions of roughly equal length and showed that the fusion of each segment to GR with carboxyl-terminal truncation at amino acids 550 or 568 was sufficient to confer hsp90 binding. One of these regions sufficient for conserving hsp90 binding was the carboxyl-terminal one-third of the HBD (697—777 human or 715—795 rat), which lies completely outside of the minimal hsp90-binding region determined from carboxyl-terminal truncations. In a similar study, Schowalter et al. (15) showed that three separate regions of the progesterone receptor HBD could confer hsp90 binding when fused to a mutant PR lacking the HBD. As with the GR (14), deletion of any of these regions did not abolish hsp90 binding (15).

Thus, for the last several years, it has been the conclusion

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1 The abbreviations used are: HBD, hormone-binding domain; hsp, heat shock protein; GR, glucocorticoid receptor, PR, progesterone receptor; ER, estrogen receptor; TA, triamcinolone acetonide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione S-transferase; DHFR, dihydrofolate reductase; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid.

2 The amino acid numbering in this paper is for rat GR.
that several regions throughout the GR and PR HBDS are involved in hsp90 complex formation. No critical sequences or motifs required for hsp90 binding have been identified, and no similarity has been established between steroid receptor HBDS and the catalytic domains of protein kinases, such as p60src and Raf, that are also assembled into hsp90-containing heterocomplexes by the same hsp90-based chaperone machinery (16, 17). It is important to bear in mind that the previously identified hsp90 binding sequences may represent only a final product and bear little relationship to the region of the HBD, or even the whole receptor, required for the initial interaction with the multiprotein chaperone machinery. In any case, it is thought that some general property, such as exposure of hydrophobic residues in partially denatured regions (18–20), must account for the ability of the steroid receptor HBDS to form relatively stable complexes with hsp90.

Unfortunately, none of the above approaches could provide convincing evidence regarding the site of hsp90-GR interaction involved in ligand binding because steroid binding was not concomitantly determined. Using a series of such fusion proteins containing dihydrofolate reductase and HBD fragments with deletions at the amino- and carboxyl-terminal ends, Xu et al. (21) found that some sequence, which did not have to be of the GR, fused to the amino terminus of the HBD was absolutely necessary for the production of stable protein, even when the resulting protein could not bind steroid. Furthermore, the boundaries of the steroid-binding domain were localized to 550–795 of the rat GR (21). These results raised the question of whether the loss of steroid binding upon deletion of amino acids beyond 550 was due to the absence of sequences necessary for interaction with steroid or to the lack of residues required for hsp90 binding.

In this work, we decided to examine the steroid-binding and hsp90-binding properties in parallel for a series of fusion proteins containing very short amino-terminal truncations of, or deletions at, the amino and carboxyl termini. We show that the chimeric proteins lose all hsp90 binding activity and all steroid binding activity with the regions corresponding to the amino acid residues 520 to 795 of GR were amplified by PCR using pSVLGR (22) as the template. The PCR primers were as follows: 5′ primer is 5′-GCCAGATTACATGTCCTCATACTAGG-3′; 3′ primer is 5′-TATAGCTGGGATCCACACCGGAA-3′. Amino acids 520 to 795 of GR were amplified by PCR using pSVLR (22) as the template. The PCR primers were as follows: 5′ primer is 5′-GTATAGCATGGCAGCAAGCAGCTG-3′; 3′ primer is 5′-GGGAATTACATTTTCTTAAAGCACC-3′. Both PCR products were digested with EcoRI plus 4.7-kilobase vector described above to generate pMTGST520C.

pMTGST547C, pMTGST552C, and pMTGST554C were derived by exchange of amino acids 520 to 781 (SphI to EcoRI) in pMTGST520C with amino acids 547, 552, or 554 to 781 (SphI to EcoRI) in plasmid pdrf547C, pdrf552C, or pdrf554C, respectively. The 5′ primer that was used to prepare pdrf547C was originally (21) incorrectly reported and is 5′-GTATAGCATGGCACGCAAGCAGCTG-3′. All the constructs were confirmed by dideoxy sequencing using Sequenase Version 2.0 kit (Amersham).

pMTGST547CAC8C, pMTGST554CAC5C, and pMTGST554C/ APA5C were prepared by first digesting pMTGST547C and pMTGST554C with BglII/BstXI. The excised sequences of GST547C and GST554C were ligated into the BstII/BamHI-restricted Blue-Script vector using K+ expression vector (Stratagene). The primers 5′ACA5a-b (5′-CGACGTCTGCGCCGCAGCACGATCAG-3′) and 5′-CTGTCGACGGGCCGCTGCGCGATAGCAG-3′, respectively) and primers 5′ACA5a-b (5′-CGACGTCTGCGCCGCAGCACGATCAG-3′) and 5′-CAGCGGCGCCGCGATAGCAG-3′, respectively) were inserted between the GST and receptor sequences of BSGST547C and BSGST554C vectors after linearization with SphI. Sequencing was performed to identify correct insertion, orientation, and number of the alanine linkers. The sequences GST547C/ACA8 and GST554CAC5C and GST554C/ACA5 were then excised from the Blue-script vector using EcoRI and were used to replace the corresponding regions in pMTGST547C and pMTGST554C, respectively.

The pMTGST545C/ACA5 vector was used to convert the linker ACAAAAAA to APAAAAAC. Site-directed mutagenesis was performed according to the specification of the GeneEditor kit (Promega) using oligo ACA5/554PRO (5′-TGATAGCATGGACGCAAGCAGCTG-3′). A unique PvuII restriction site was created at the site of the Pro mutation, and PvuII digestion was used to confirm mutant.

pSPGST537C and pSPGST547C were prepared from the pMTGST537C and pMTGST547C vectors, respectively, by BglII/SacI double digestion. The excised GST/GR sequences were then ligated with the pSP73 vector (Promega), after linearization by BglII/SacI double digestion, by using T4 ligase. pSPGST547C/ACA8, pSPGST554C, and pSPGST554C/ACA5 constructs for TNT studies: GST547C/ACA8, GST554C, and GST554C/ACA5 were removed from pMTGST547C/ACA8, pMTGST554C, and pMTGST554C/ACA5, respectively, by SacI/BglII double digestion and ligated into a SacI/BglII linearized pSP73 vector. 

Cell Growth and Transfection—Monolayer cultures of COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 5% heat inactivated fetal bovine serum. All the fusion proteins were expressed in transiently transfected COS-7 cells as described previously (23).

Assay of Steroid Binding—Cytosol of transiently transfected COS-7 cells containing the steroid-free receptors was obtained by the lysis of cells at 80°C and centrifugation at 15,000 × g as described previously (24). [3H]Dexamethasone or [3H]17α-Hydroxyprogesterone binding assays and competition binding assays all contained 20 μM sodium molybdate. Briefly, 30% cytosol was incubated at 0°C for 2.5 h with 50 nM [3H]steroid, mixed with dextran-coated charcoal, and, after centrifugation, the supernatant was counted in Hydrofluor Scintichek and analyzed at 0°C for 18 h with various concentrations of [3H]Dexamethasone ≥ 100-fold excess of non-radioactive Dex. Unbound [3H]Dexamethasone was removed with dextran-coated charcoal and the samples processed as above.

Reconstitution of GST/GR-hsp90 Heterocomplexes—Aliquots (200 μl) of undiluted cytosol from transiently transfected COS-7 cells were mixed with mammalian hsp90, 15% with anti-GR antibody or preimmune IgG and subsequently incubated with 8-μl pellets of protein A-Sepharose. Immunoadsorbed GST fusion proteins were stripped of associated hsp90 by incubating the immune pellets an additional 2 h at 4°C with 0.5 mM NaCl, followed by one wash with 1 ml of TEG buffer (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol) and a second wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4). Immune pellets containing...
Binding of hsp90 to the Glucocorticoid Receptor

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Cell-free Transcription and Translation—Cell-free transcription and translation of pSPGST357C and pSPGST547C was performed using a TNT kit (Promega) according to the procedure of the manufacturer. Aliquots of translation mixture containing 35S)methionine-labeled fusion protein were immunoadsorbed with the 3G3 monoclonal IgM against hsp90 as described by Dalman et al. (11). The radiolabeled fusion protein co-adsorbed with hsp90 was detected by autoradiography. The immunoblot was cut just above the full-length fusion protein translation product and probed with AC88 for hsp90. The blot was incubated a second time with 125I-labeled counterantibody and developed by autoradiography. The fusion protein and hsp90 bands were excised and counted for 35S and 125I radioactivity, respectively. Nonradioactive translation mixture was used for quantitation of 35H)dexamethasone binding as described above.

Steroid Binding of GST/GR HBD Fusion Proteins—For assay of GST fusion proteins and associated hsp90, immune pellets were boiled in SDS sample buffer with 10% β-mercaptoethanol, and proteins were resolved on 7% SDS-polyacrylamide gels. Proteins were then transferred to Immobilon-P membranes and probed with 0.2% anti-GST or 0.01% α51 for fusion proteins and 1 μg/ml AC88 for hsp90. The immunoblots were then incubated a second time with 125I-conjugated goat anti-mouse IgG to visualize the immunoreactive bands.

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GST/GR HBD fusion proteins were immunoadsorbed from 200-μl aliquots of cytosol from transiently transfected COS-7 cells with anti-GST antibody. After washing the immune pellets, receptor-associated hsp90 was stripped from four samples with 0.5M NaCl and stripped pellets were incubated for 20 min at 30 °C with rabbit reticulocyte lysate as described under “Methods.” After the incubation, the immune pellets were washed twice and proteins were resolved by SDS-PAGE andWestern blotting. A portion of each immune pellet was incubated with [3H]TA to determine steroid binding activity, except for condition 5 where 20 nM [3H]TA was present during the 20-min heterocomplex reconstitution at 30 °C. For each construct listed below, conditions are: lane 1, native GST-fusion-monkey hsp90 heterocomplex in unstripped immune pellet; lane 2, stripped GST-fusion pellet incubated with buffer; lanes 3–5, stripped nonimmune (lane 3) and immune (lanes 4 and 5) pellets incubated with reticulocyte lysate in the absence (lanes 4 and 5) or presence (lane 5) of [3H]TA. A, GST537C; B, GST547C; C, GST552C; D, GST554C.

On the basis of a general model based on the x-ray structures of retinoic acid receptor and 9-cis-retinoic acid receptor (RXR) HBDs (26), the three-dimensional structure of amino acids 547–553 of the rat GR is thought to be that of an α-helix. However, the presence of two prolines in this region suggests that a kink may be present near the beginning of this helix (helix 1). Such a kink could move the rest of the receptor (or GR in the fusion protein) out of the way of a surface required for hsp90 binding to GR. To test this hypothesis, a proline was introduced into GST534C/ACA5C to cause a break near the beginning of the introduced α-helix (Fig. 3A). This new chimera (GST554C/ACA5C) contains the linker APAAAAAC. As indicated in Fig. 3A, the presence of this kinked linker still did not cause any increase in steroid binding over that seen for GST554C. Again, this was not due to the absence of expressed proteins in the transfected COS cells (Fig. 3B).

Cell-free hsp90 Binding to Stripped Chimeric Receptors—Rabbit reticulocyte lysate contains the multicomponent chaperone system that forms stable GR-hsp90 heterocomplexes and reactivates steroid binding activity (4, 6). As shown in Fig. 2A, when the monkey hsp90 was stripped from the GST537C fusion protein, steroid binding activity was lost (lane 2). Incubation of the stripped immune pellet with reticulocyte lysate resulted in reassociation of a portion of the GST537C with rabbit hsp90 and there was ~30% reactivation of steroid binding activity (lane 4). The reticulocyte lysate system is dynamic in that receptor-hsp90 heterocomplexes are constantly being assembled and disassembled (27). We have recently shown that incubation of GR with reticulocyte lysate in the presence of [3H]TA allows detection of complexes that would otherwise have been disassembled and thus not be in a steroid binding state by the end of the incubation (28). Thus, in lane 5 of Fig. 2A, when [3H]TA was present during the incubation to bind the fusion protein as soon as complexes with rabbit hsp90 were formed, about 52 ± 9% (±S.D., n = 4) of the GST537C was restored to the steroid binding state. Surprisingly, the reactivation of steroid binding activity for stripped GST547C in the reticulocyte lysate system was much lower (8.9 ± 0.5%, n = 2) (Fig. 2B, lanes 4 and 5). Little or no hsp90 heterocomplexes were formed with GST552C or GST554C in either COS cells or the reticulocyte lysate reconstitution system (Fig. 2, C and D, respectively).

Stability of GST/GR HBD-hsp90 Heterocomplexes—The decrease in recovery of reconstituted fusion protein-hsp90 heterocomplexes with truncation of the amino terminus of the HBD from 537 to 547 could reflect a progressive decrease in the cell-free stability of the heterocomplexes that were formed, rather than a progressive impairment of heterocomplex formation. In the experiment of Fig. 4, cytosols were prepared in molybdate-free buffer from cells transfected with either pMTGST547C or pMTGST537C. Aliquots of each cytosol were incubated for various times at 25 °C and steroid binding activity was assayed. It can be seen that GST547C (open circles) loses its ability to bind steroid at the same rate as GST537C (closed circles).

The inset of Fig. 4 shows Western blots prepared from cytosol samples that were incubated for 60 min at 25 °C and immunoabsorbed with anti-GST. The bands in the immunoblots were
with hsp90. Replicate 100-µl aliquots of cytosol from COS-7 cells expressing GST547C (open circles) or GST537C (closed circles) were incubated at 25 °C, and at the indicated times, the aliquot was divided and incubated overnight at 0 °C with 50 nM [3H]TA in the presence or absence of 50 µM competing dexamethasone. Unbound ligand was adsorbed with charcoal and bound radioactivity was assayed. Specific binding is plotted as a fraction of the zero time control. The data represent the average of three separate experiments ± S.E. Inset, zero time samples and samples incubated for 60 min at 25 °C were immunoadsorbed with anti-GST; immune pellets were washed, and proteins were resolved by SDS-PAGE and Western blotting with primary antibody followed by 125I-labeled counterantibody. The radioactive bands were excised and counted to determine an arbitrary amount of hsp90 corrected for relative amounts of fusion protein at each time, and from that value, the percent of heterocomplex disassociation was excised and the radioactivity from the 125I-labeled counterantibody was counted. Sixty-nine percent of the hsp90 disassociated from GST537C heterocomplexes and 74% from GST547C heterocomplexes during the 60-min incubation. Thus, we conclude that the two fusion proteins formed hsp90 complexes of similar stability.

**Assembly of hsp90 Heterocomplexes with Fusion Proteins**—The ability of hsp90 to associate with wild-type receptors is similar for receptors synthesized in intact cells and for in vitro translated receptors (11). Thus, when immunoadsorbed, stripped, wild-type GR from whole cell cytosol is incubated with reticulocyte lysate, we reactivate 70–100% of the steroid binding activity of the immunoadsorbed native GR-hsp90 heterocomplex (29). When the wild-type GR is translated in reticulocyte lysates, all of the full-length [35S]methionine-labeled translation product is in a heterocomplex with hsp90 (11). The very low efficiency of hsp90 binding to GST547C in the reticulocyte lysate (lanes 4 and 5 versus lane 1 in Fig. 2B) thus raises the question of whether the reticulocyte lysate is deficient in some component that is required for the reconstitution of at least the GST547C. This question was examined by determining the ability to observe steroid binding in the various in vitro translated chimeric receptors. At the end of the reaction, molybdate was added to stabilize heterocomplexes and hsp90 was immunoadsorbed with the 3G3 monoclonal (IgM) antibody. In the experiment shown in Fig. 5, pSPGST537C and pSPGST547C were each co-immunoadsorbed with hsp90 in roughly the same proportion as hsp90 itself. The data from three separate translations are presented in the bar graph of Fig. 5C. In that immunoadsorption of ~70% of the hsp90 yielded co-immunoadsorption of roughly the same percentage of fusion protein, we conclude that all of the GST537C and GST547C translated in reticulocyte lysate was bound to hsp90. Thus, heterocomplex assembly during in vitro translation (Fig. 5) was more efficient than reconstitution of heterocomplexes with hsp90 by reticulocyte lysate (Fig. 2, A and B). This issue was further examined by determining whether the 8-amino acid α-helical linker of ACAAAAAC, which was unable to increase the steroid binding activity of the GST554C receptors expressed in intact cells, might have a different effect with in vitro translated receptors. The presence of the α-helical linker in GST554C/ACA5C did not cause any increase in the steroid binding activity of GST554C, despite the fact that comparable amounts of GST554C/ACA5C and GST547C receptor protein were made (data not shown).

**DISCUSSION**

Using GST/GR HBD fusion proteins, we have identified a region at the amino terminus of the GR HBD that is required for assembly of a stable heterocomplex with hsp90. Deletion of 10 amino acids from GST537C to form GST547C results in a reduction of both hsp90 binding and steroid binding activity (perhaps reflecting a reduced amount of fusion protein), but only minor effects on affinity, while deletion of the next 7 amino acids to form GST554C results in complete loss of stable HBD-hsp90 heterocomplex formation and steroid binding activity.

Cadepet al. (14) and Schowalter et al. (15) have shown that segments of the GR or PR HBD lying carboxyl-terminal to helix 1 are sufficient for conferring stable hsp90 binding onto truncated receptors lacking the HBD. These observations led to the notion that several regions of the HBD are involved in binding hsp90 through some undetermined quality of the HBD tertiary structure (15). However, the approach used in those studies may have led to the formation of chimeras containing HBD segments that are incompletely folded or partially denatured. Thus, much like the carboxyl-terminal deletion approach...
Fig. 6. Sequence alignment of human steroid receptors in the amino-terminal region of the HBD required for assembly of GST/GR HBD-hsp90 complexes. The sequence of the rat GR (rGR) (22) is above the comparable alignments for human GR (hGR) (34), PR (hPR) (35), androgen receptor (hAR) (36), mineralocorticoid receptor (hMR) (37), and ER (hER) (38). The invariant residues are shaded and conserved hydrophilic residues are boxed. Helix 1 of the HBD is indicated by the large box and the autonomous transactivation domain, AF2-a, is indicated by the bold bracket.

of Dalman et al. (12) and Howard et al. (13), these experiments may have led to hsp90 heterocomplex formation in a manner that is quite different from the manner in which assembly occurs when the entire HBD assumes its normal, steroid-binding tertiary structure.

Using the approach of truncating from the amino-terminal side and leaving the rest of the HBD intact, we have shown that GST554C does not form a heterocomplex with hsp90 or have steroid binding activity. As shown in Fig. 5, all of the GST54C that is translated in reticulocyte lysate was bound to hsp90; thus, the loss of ability of the fusion protein to form a heterocomplex with hsp90 occurred between amino acids 547 and 554 and was almost complete with removal of just 5 amino acids to give GST552C (Fig. 2C). The loss of steroid and hsp90 binding activity appears to be sequence specific as replacement of the wild-type sequence of TPTTVSL with CAAAAAC did not regenerate any of the steroid binding activity lost with GST554C (Fig. 3A).

Several features of this 7-amino acid sequence that could influence hsp90 binding have been considered. As shown in Fig. 6, amino acids 547–554 of rat GR reside within what has been proposed to be helix 1 of the HBD of all steroid receptors (26) and overlap the amino-terminal one-third of an autonomous transactivation domain, AF2-a (30–32) at the carboxyl terminus of the a domain (33). However, no conserved sequence is evident in an alignment with the other steroid-binding receptors (Fig. 6). The sequence of 547–553 of rat GR does contain four of the seven hydrophobic amino acids common to all steroid receptors (boxed in Fig. 6). The L550A/V551A mutations in GST556C/ACA5C and GST/APA5C would not be expected to dramatically alter the hydrophobic environment. The mutation of Leu-553 to the weakly polar cysteine is not likely to be of major importance as this residue is unchanged in GST556C, which retained only marginal binding activity (Fig. 2C). The invariant Leu-554, plus the other hydrophobic residues of 554–562, are not sufficient in that GST556C has lost essentially all binding activity (Figs. 2D and 3A). A minimal physical separation between the GST and GR HBD domains, to avoid possible stereric interference in hsp90 binding, appears to have been eliminated by the inactivity of the GST556C/ACA5C with an α-helical linker (Fig. 3A). To the extent that this region in the wild-type GR is predicted to be α-helical (26), the presence of an α-helix should not be detrimental. The presence of a polyalanine linker per se was also found not to present problems (Fig. 3A).

Finally, the importance of a possible kink in the predicted helix 1 can be discounted by the inability of a kinked α-helix to restore binding activity in the context of the proline containing linker of GST554C/APA5C (Fig. 3A). Thus, we conclude that features of the amino acid sequence TPTTLVSL of 547–553, such as tertiary structure or contributions to a larger surface for protein-protein interactions, are involved in hsp90 binding to the GR.

The marked decrease in reticulocyte lysate mediated reassociation of hsp90 with stripped GST54C, compared with GST53C, suggests that the 10 amino acids of 537–546 also have an important role under some conditions. In particular, the sequence of 537–546 is not required for hsp90 association in intact cells but is necessary for high levels of hsp90 reconstitution with stripped GR in the reticulocyte lysate. The molecular basis for this behavior is not known, but it may simply reflect variations in the efficiency of hsp90 association in the two systems. Alternatively, these results may reflect different initial, or intermediary, attachment points of hsp90 with newly translated GR (i.e. amino acids 547–554) versus pre-folded and then stripped GR (i.e. amino acids 537–547). In this case, the data would argue for hsp90 association with GR being a dynamic process with at least one intermediate being formed prior to the final product.

In conclusion, a combined assaying of steroid binding and hsp90 binding to GR chimeras has directly determined that the sequence upstream of, and including the amino-terminal region of, the GR HBD is required for hsp90 binding. This observation stands in opposition to the accepted model of steroid receptor-hsp90 heterocomplex assembly based on experiments with fragments of steroid receptor HBDs (14, 15). Although binding of the HBD to hsp90 may ultimately require a general property such as exposure of hydrophobic residues in partially denatured regions as determined in in vitro reactions with other chaperones (18–20), the requirement of a specific sequence for hsp90 binding to the intact HBD implies an initial event that enables interaction of the chaperone with more carboxyl-terminal hydrophobic residues not otherwise exposed. A structure defined by the 7-amino acid required sequence may be involved in an initial opening or unfolding of the steroid binding pocket by the multiprotein hsp90-based chaperone machinery (39–41), an unfolding that exposes hydrophobic residues in the hsp90 contact region, permitting stable HBD-hsp90 heterocomplex assembly.

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