Mutations at Positions 547–553 of Rat Glucocorticoid Receptors Reveal That hsp90 Binding Requires the Presence, but Not Defined Composition, of a Seven-amino Acid Sequence at the Amino Terminus of the Ligand Binding Domain*

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Glucocorticoid receptors (GRs) must heterocomplex with hsp90 to have an open steroid binding cleft that can be accessed by steroid. We reported that a seven-amino acid sequence (547–553 of rat GR) overlapping the amino-terminal end of the ligand binding domain is required for hsp90 binding to GR. We have now conducted saturation mutagenesis of this sequence, which appears to be part of the surface where the ligand binding cleft merges with the surface of the ligand binding domain. No single point mutation causes significant changes in any of a variety of biochemical and biological properties in addition to hsp90 binding. A triple mutation (P548A/T549A/V551A) increases by >100-fold the steroid concentration required for half-maximal induction without affecting the level of maximal induction or coactivator response. Interestingly, this triple mutant displays reduced binding of steroid and hsp90 in whole cells, but it possesses wild type affinity for steroid and normal hsp90 binding capacity under cell-free conditions. This phenotype of a dramatic shift in the dose response for transactivation would be expected from an increase in the rate of disassembly of the triple mutant GR-hsp90 heterocomplex in the cell. Mutation of the entire seven-amino acid region to CAAAAAC maintains the presence of a critical α-helical structure and heterocomplex formation with hsp90 but eliminates steroid binding and transcriptional activation, thus disconnecting hsp90 binding from opening of the ligand binding cleft and steroid binding.

Glucocorticoid receptors (GRs) are recovered from hormone-free cells as large multiprotein heterocomplexes containing a dimer of hsp90 (for a review, see Ref. 1). One GR function that is absolutely hsp90-dependent is steroid binding activity (2, 3). Steroid binding is the first in a series of steps by which GR translates the intracellular concentration of steroid hormone into altered levels of selected, biologically active proteins. Accordingly, hsp90 was shown to be essential for the steroid-induced activity of the GR in yeast (4). When the GR is stripped of its associated hsp90, it immediately loses its ability to bind steroid, and steroid binding activity is regenerated when GR-hsp90 heterocomplexes are reformed by the hsp90/hsp70-based chaperone machinery (5, 6). Steroid ligands bind deep in a hydrophobic cleft that appears to be collapsed in the absence of ligand, such that the receptor must change its conformation to allow entry of the ligand (7). Hsp90 binds to the ligand binding domain (LBD) of the receptors (1), and the hsp90/hsp70-based chaperone machinery carries out an ATP-dependent opening of the binding cleft in the GR LBD such that it can be accessed by steroid. In addition to opening the steroid binding cleft, hsp90 promotes conformational changes that increase the sensitivity of the GR LBD to attack by thiol derivatizing agents and trypsin (8–10).

It has proven difficult to define the site of hsp90 binding within the LBD. It was originally found that the entire LBD of the glucocorticoid or progesterone receptor had to be deleted before hsp90 binding was eliminated (11–13), and several subregions of the LBD could confer hsp90 binding onto a receptor fragment (14, 15). Because no consensus protein binding site was identified for hsp90, it has been suggested that a general property, such as exposure of hydrophobic residues in a partially denatured protein, may determine hsp90 binding (16–18). However, there is no indication that steroid receptors that have been stripped of hsp90 by mild salt treatment are in any way denatured prior to their reactivation by the hsp90/hsp70-based chaperone machinery. The deletion approach to studying the site of hsp90 binding also destroyed steroid binding activity, thus abrogating any relationship between hsp90 binding and the steroid binding activity resulting from the normal action of the hsp90/hsp70-based chaperone machinery on the receptor.

We took a different approach. We previously found that no stable proteins are produced when plasmids containing just the GR LBD sequence are transiently transfected into cells. However, fusing the GR LBD to dihydrofolate reductase creates a stable steroid binding unit that allowed the N-terminal end of the LBD to be localized to the segment of amino acids 550–795 of the rat GR (19). Definition of an N terminus permits the study of hsp90 binding to an intact, native LBD possessing a normal steroid binding site when it is bound to hsp90. Thus, any requirements for hsp90 binding can be correlated with the appropriate hsp90-mediated conformational change leading to steroid binding. Using the GR LBD fusion proteins, we then
demonstrated that a seven-amino acid segment (positions 547–553) lying in the predicted helix 1 of the rat GR LBD (20) is required for both LBD-hsp90 heterocomplex assembly and steroid binding activity (21).

Additional support for the notion that the seven-amino acid segment is critical for hsp90 regulation of receptor function in vivo comes from a recent study by Mackem et al. (22). They found that when a GR fragment ending with this segment is fused to the complementary retinoic acid receptor LBD fragment extending to its C terminus, the chimera is complexed with hsp90 and undergoes cytoplasmic/nuclear translocation in response to retinoic acid. Thus, this N-terminal region of the GR LBD appears to confer those properties associated with bound-hsp90 to the retinoic acid receptor, which normally does not bind hsp90 (1), and is the likely site of interaction between the chaperone machinery and the GR when the LBD is in its native conformation. By examination of the predicted GR structure, which is based on the x-ray structure of the closely related human progesterone receptor LBD (23) and the similar human estrogen receptor alpha LBD (24), this seven-amino acid segment would form part of the rim of the ligand binding cleft of the receptor (7, 25). The presence of hydrophobic clefts are a universal feature of all properly folded proteins, and regions where clefts merge with the surface of a protein may be the general topological feature that is recognized by the hsp90/hsp70-based chaperone machinery. Another example of such cleft recognition may be reflected in the ability of the hsp90/hsp70-based machinery to facilitate the entry of heme into its hydrophobic binding cleft in aponeuronal nitric-oxide synthase (26).

We have taken a mutational approach to further explore the role of the seven-amino acid segment of the GR LBD in hsp90 action. The segment contains all but the C-terminal leucine of an LXXLL motif that has been found important for a number of protein interactions of nuclear receptors, including the GR (27–29). Thus, we initially suspected that this LXXLL sequence might be involved in hsp90 binding to GR. Surprisingly, mutation of the first two leucines in the LXXLL motif decreased steroid binding capacity and transcriptional activity without altering receptor levels, cell-free steroid binding affinity, or hsp90 binding (25). The biological inactivity was found to be due to an increased rate of steroid dissociation from the activated/transformed mutant receptor. Therefore, the LXXLL motif was not required for the hsp90/hsp70-based chaperone machinery to assemble an LBD-hsp90 complex and open the steroid binding cleft, but it apparently aided in keeping the cleft closed once steroid had bound and the receptor was transformed, a step that eliminates its persistent interaction with hsp90.

In this work, we have made single point and multiple mutations of the five remaining amino acids in the seven-amino acid segment. We have examined the steroid binding and hsp90 binding activities as well as the transcriptional activity of each mutant. As a further indicator of normal receptor structure and function, we assayed the response to added coactivator. These studies were conducted with both full-length GR and chimeric constructs of either GAL4-DBD or GST with GR-LBD to determine the possible role of other GR sequences. The results indicate that hsp90 binding to GRs can be dissociated from steroid binding and that the presence, but not the sequence, of the seven amino acids (Thr–547 to Leu–553) is required for the formation of stable GR-hsp90 heterocomplexes.

MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 0 °C.

**Chemicals—**Dexamethasone (Dex) was obtained from Sigma. Droxetanone (Dex-Ox) (30) and Dexamethasone (Dex-Mex) (31) were prepared as described. [1,2,4,6-3H]Dex (91 Ci/mmol); [6,7-3H]triamcinolone acetonide (TA; 38 Ci/mmol); and [3H]triamcinolone acetonide (TA; 38 Ci/mmol), and 125I-conjugated goat anti-mouse and anti-rabbit IgGs were from PerkinElmer Life Sciences. Nonimmune IgG and the monoclonal anti-GST antibody (clone GST-2) were from Sigma. The AC88 monomonal IgG against hsp90 and the N2T73-4 anti-72/73 kDa hsp monoclonal IgG (anti-hsp70) were from StressGen (Victoria, Canada), mouse monoclonal IgG against GAL4 DBD was obtained from CLONTECH (Palo Alto, CA), and the BuGR2 monoclonal IgG against the GR (Millipore, MA). Hydrofluor scintillation mixture was from National Diagnostics.

**Polyclonal and Mutant Anti-GR Antibodies—**Polyclonal anti-72/73-kDa hsp monoclonal IgG and the monoclonal anti-GST antibody (clone GST-2) were from Sigma. The AC88 monomonal IgG against hsp90 and the N2T73-4 anti-72/73 kDa hsp monoclonal IgG (anti-hsp70) were from StressGen (Victoria, Canada), mouse monoclonal IgG against GAL4 DBD was obtained from CLONTECH (Palo Alto, CA), and the BuGR2 monoclonal IgG against the GR (Millipore, MA). Hydrofluor scintillation mixture was from National Diagnostics.

**Complementary Retinoic Acid Receptor LBD Fragment Extend-**

**Cell Culture and GR Transfection Assay—**Monolayer cultures of COS-7 and CV-1 cells were grown at 37 °C in Dulbecco’s modified...
Eagle’s medium (Invitrogen) supplemented with 5% and 10%, respectively, fetal calf serum (Biofluids Inc., Rockville, MD) in a humidified incubator (5% CO2). Triplicate samples of CV-1 cells in 60-mm dishes were transiently transfected with 100 ng of wild type or mutant GR plasmids, GreTkLUC (1 μg), Renilla (200 ng), and enough phBSK+-DNA (1.5 μg) to bring the total to 3 μg. For the reporter used for Gal/GalR-525C constructs was FRLuc. The cells were induced 24 h after transfection and harvested 2 days after transfection for analysis of the luciferase activity (34).

Steroid Binding Assay—Transient transfection of COS-7 cells with 5 μg/100-mm dish of GR plasmid DNA was performed as described (35). Cytosols of transfected cells containing the steroid-free receptors were obtained by the lysis of cells on dry ice and centrifugation at 15,000 × g (36). Thirty percent cytosol with 20 mM sodium molybdate was added to varying concentrations of [3H]Dex with or without a 100-fold excess of nonradioactive Dex and incubated at 0 °C for 18 h. Unbound [3H]Dex was removed by dextran-coated charcoal.

Whole cell steroid binding was performed by incubating suspensions of COS-7 cells (1.5 × 106) transiently transfected with 1.2 μg/100-mm dish of GR plasmid DNA, with increasing concentrations of [3H]Dex (1.5–50 nM) in 200 μl of serum-free medium in the presence or absence of a 100-fold molar excess of unlabeled Dex (each with 1.2% ethanol) for 30–45 min at 37 °C. The binding was terminated by the addition of 2 ml of phosphate-buffered saline, followed by centrifugation for 1 min at room temperature. Cells were washed three times with phosphate-buffered saline at room temperature. In both cases, the total binding was determined by liquid scintillation counting. The specific binding was calculated by subtracting the background dpm (100-fold Dex) from the total [3H]Dex binding. The binding capacity and affinity were determined by Scatchard plot analysis by plotting the ratio of bound steroid/free steroid, versus bound steroid.

The amount of GR protein was determined by Western blotting with a1-anti-GR antibody (1:40,000) or with anti-GLD BBDB antibody (1: 10,000) for GAL/GR chimeras and visualization by enhanced chemiluminescence as described (10).

Reconstitution of GR-hsp90 Heterocomplexes—Aliquots (100 μl) of cytosol from transfected COS-7 cells were immunoadsorbed with 7 μl of FGR ascites or nonimmune IgG precipitated with 14-ml pellets of protein A-Sepharose suspended in 200 μl of TEG buffer (10 mM Tris, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol). Prior to incubation with the mixture of five purified proteins, immunoadsorbed receptors were stripped of associated hsp90 by incubating the immunopellet for 2 h at 4 °C with 350 μl of 0.5 M NaCl in TEG buffer. The pellets were then washed once with 1 ml of TEG buffer followed by a second wash with 1 ml of TEG buffer (10 mM Tris, pH 7.35). For assembly of GR-hsp90 heterocomplexes (5), immunopellets containing GR stripped of chaperones were incubated with the five-protein assembly system (20 μg of purified hsp90, 15 μg of purified hsp70, 0.6 μg of purified human Hop, 6 μg of purified p23, 0.4 μg of purified YDJ-1) adjusted to 50 mM potassium phosphate, 2 mM magnesium acetate, and 100 units/ml creatine phosphokinase. The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG buffer with 100 units/ml creatine phosphokinase). The amount of GR protein was determined by Western blotting with a1-anti-GR antibody (1:40,000) or with anti-GLD BBDB antibody (1: 10,000) for GAL/GR chimeras and visualization by enhanced chemiluminescence as described (10).

RESULTS

Biological Properties of Single Point Mutations of the Full-length GR—Of the seven amino acids in the sequence of Thr-547 to Leu-553 that were implicated in hsp90 binding to GRs (Fig. 1) (21), Leu-550 and Leu-553 were previously found not to be involved (25). To examine the role of the remaining five residues (i.e., Thr-547, Pro-548, Thr-549, Val-551, and Ser-552), single point mutations were prepared in the context of the full-length GR (Fig. 1). We decided to use a variety of GR transactivation properties (total level of induced gene product, fold induction, the position of the dose-response curve (or EC50), and the partial agonist activity of anti-glucocorticoids) to screen for possible effects of these mutations on hsp90 binding to GRs for two reasons. First, the ability to detect steroid binding to GRs with affinities that are >10-fold less than the wild type GR is severely limited by the background, nonspecific binding. Second, and more importantly, if defects in hsp90 binding alter any aspects of GR function other than steroid binding, assays of the final biological activity of GRs should be able to detect them.

Representative data from the whole cell bioassays are given for the full-length wild type (wt) GR and three point mutants (P548A, V551A, and S552A) in Fig. 2A. No significant differences in the total levels of induced luciferase activity or the fold induction (equal to 52 ± 22-fold (S.E., n = 6) for wild type GR) with saturating concentrations of the agonist Dex are seen for any of the single mutants. When the data are replotted as a percentage of maximal induction by Dex (Fig. 2B), there are
again only minor differences between the wild type and mutant GRs in the EC$_{50}$ or the position of the dose-response curve for Dex induction of the luciferase reporter gene. A summary of these data for all of the single point mutants, after normalization to the properties of the wild type GR, is presented in Table I. This table also gives the values for the partial agonist activity of each mutant GR with the antiglucocorticoids Dex-Mes and Dex-Ox, each of which is relatively constant. Thus, none of the single point mutations has an appreciable effect on any of the properties examined.

Properties of Triple Mutants of the Full-length GR—Because protein-protein interactions usually involve multiple residues and can be relatively insensitive to single point mutations, we examined two triple mutations. The first changed all of the hydrophilic residues that might be expected to be on the surface of the LBD and in contact with water in aqueous solutions (i.e. Thr-547, Thr-549, and Ser-552). As shown in Fig. 3A and Table I, the properties of this triple mutant are only marginally different from those of the wild type receptor. A closer examination of the data for the single mutants suggested that the greatest effects were seen upon mutation of Pro-548, Thr-549, and Val-551. Therefore, the triple mutant of these residues was next prepared. Again, there is no notable effect on the maximal level of transactivation or -fold induction. However, a dramatic shift in the position of the dose-response curve is apparent (Fig. 3B). When the data are plotted as a percentage of maximal induction, it can readily be determined that the EC$_{50}$ of the 548/549/551 triple mutant is shifted to the right by a factor of more than 300 (Table I). At the same time, the partial agonist activity of Dex-Mes is almost eliminated with the 548/549/551 triple mutant (Table I). Western blots show that equivalent levels of wild type and triple mutant GRs are expressed (Fig. 3C). Therefore, it is likely that the transactivation capacities of both the wild type GR and the 548/549/551 triple mutant (Fig. 3B) are similar.

In an effort to understand why the 548/549/551 triple mutant is an effective inducer of GR-responsive genes only at very high concentrations of steroid, we determined the binding affinity of the mutant receptor by Scatchard analysis. In cell-free systems at 0 °C, the affinity of the triple mutant for [3H]Dex is nearly identical to that of the wild type GR (Fig. 4A). However, the binding capacity of the 548/549/551 triple mutant is routinely one-half to one-third that of the wild type receptor, although equal amounts of protein are expressed (see Fig. 3C). Therefore, not all of the triple mutant receptors isolated from transiently transfected COS-7 cells are able to bind steroid under cell-free conditions. Interestingly, in intact cells at 37 °C, transiently transfected triple mutant GR displays much less binding than equivalent amounts of wild type GR and only negligible binding above that seen for the low levels of endogenous GR (Fig. 4B). Obviously, there is binding to the triple mutant in intact cells, but the affinity must be more than 10-fold lower than that of the wild type GR and thus difficult to observe in this assay.

Effects of Various Mutations Are Seen in the Absence of Amino-terminal and DNA Binding Domains—Recent evidence suggests that the amino- and carboxyl-terminal domains of GRs combine in interacting with some transcriptional cofactors (34, 42), just as has been observed for other steroid/nuclear receptors (43–47). We therefore asked whether the effects described above for the various mutations can be observed in the absence of the amino-terminal and DNA binding domains of the GR. To address this question, we prepared chimeras of the GAL4 DNA binding domain with the GR LBD (19) and 25 amino acids of the hinge region (Fig. 1). We find that all of the properties of each single mutant and the 547/549/552 triple mutant are similar in the context of the GAL/GR-525C chimera to what they are in the context of the full-length GR (Table I; other data not shown but included for review). Similarly, the 548/549/551 triple mutant displays the same properties in the context of the GAL/GR-525C chimera as it does in the full-length GR (Table I). The dose-response curve is shifted to the right by 2 orders of magnitude, and the partial agonist activity of Dex-Mes is eliminated.
Coactivator Activity with the Full-length GR and GAL/GR525C Chimera Containing the 548/549/551 Triple Mutations—One possible explanation for the dramatically higher EC50 of the 548/549/551 triple mutant (Fig. 3B and Table I) and the undetectable binding affinity at 37 °C (Fig. 4B) is that the mutations have rendered the structure of the LBD much more labile at 37 °C. If this is the case, then the ability of p160 coactivators, such as TIF2, to augment GR transactivation

**Table I**

Activities of full-length GRs and chimeric GRs (GAL/GR525C)

| Mutation          | Total activity | Induction fold | EC50  | Partial agonist activity |       |       |
|-------------------|----------------|----------------|-------|--------------------------|-------|-------|
|                   |                |                |       |                          |       |       |
| **Full-length GR**|                |                |       |                          |       |       |
| Wild type         | 1.11 ± 0.29    | 1.40 ± 0.08    | 2.68 ± 0.20 | 0.33 ± 0.05 | ND    |       |
| T547A             | 1.21 ± 0.03    | 1.01 ± 0.23    | 1.23 ± 0.18 | 0.88 ± 0.13 | 0.96 ± 0.13 |
| P548A             | 1.14 ± 0.04    | 1.22 ± 0.01    | 2.46 ± 0.70 | 1.17 ± 0.04 | 0.79 ± 0.13 |
| T549A             | 1.07 ± 0.03    | 1.02 ± 0.03    | 3.91 ± 0.28 | 0.60 ± 0.01 | 0.58 ± 0.03 |
| V551A             | 1.15 ± 0.03    | 1.15 ± 0.11    | 2.61 ± 0.14 | 0.39 ± 0.02 | 0.63 ± 0.03 |
| S552A             | 1.16 ± 0.03    | 1.20 ± 0.03    | 0.75 ± 0.00 | 1.32 ± 0.04 | 1.02 ± 0.04 |
| 547/549/552       | 1.07 ± 0.06    | 1.05 ± 0.06    | 2.28 ± 0.00 | 0.93 ± 0.08 | 0.86 ± 0.10 |
| 548/549/551       | 0.95 ± 0.08    | 1.17 ± 0.25    | 336 ± 75   | 0.03 ± 0.03 |       |
| **GAL/GR525C**    |                |                |       |                          |       |       |
| Wild type         | 1.11 ± 0.29    | 1.40 ± 0.08    | 2.68 ± 0.20 | 0.33 ± 0.05 | ND    |       |
| 547/549/552       | 0.57 ± 0.14    | 0.62 ± 0.03    | 151 ± 47   | 0.00 ± 0.00 | ND    |       |
| 548/549/551       | 1.11 ± 0.29    | 1.40 ± 0.08    | 2.68 ± 0.20 | 0.33 ± 0.05 | ND    |       |

**Fig. 3.** **Induction properties of the wild type GR and GRs containing three point mutations.** Triplicate samples of CV-1 cells were transiently transfected as in Fig. 2 with full-length GRs without and with the triple mutations to alanine at positions 547/549/552 (A) and 548/549/551 (B). In both cases, the data are expressed as the total relative amounts of luciferase activity ± S.D. and then plotted against the corresponding concentration of Dex. Similar results were obtained in at least one additional experiment. C, Western blot of overexpressed GRs. Overexpressed wild type and mutant (548/549/551) GRs from transiently transfected COS-7 cells were analyzed on SDS-polyacrylamide gels and visualized by enhanced chemiluminescence after Western blotting with αP1 anti-GR antibody as described under “Materials and Methods.” For comparison, equivalent amounts of mock-transfected cells (mock) and cytosol from HTC cells, which contain endogenous rat GRs (10) (HTC cell), were also analyzed. The positions of molecular weight markers, with the indicated size, are displayed on the left edge of the gel.
would also be expected to change because they act by binding to a hydrophobic pocket formed by helices 3, 4, 5, and 12 of the receptor LBD (48, 49). When this is examined in the context of the full-length GR, the ability of TIF2 to increase the total levels of transactivation by saturating concentrations of Dex is almost the same for the triple mutant as for the wild type GR (Fig. 5A). Furthermore, we have previously reported that overexpressed TIF2 causes a shift in the dose-response curve to lower concentrations of Dex and an increase in the partial agonist activity of antiglucocorticoids (50, 51). When the data of Fig. 5A are replotted as percentage of maximal induction, it is clear that the ability of added TIF2 to modulate both the dose-response curve of GR-agonist complexes (Fig. 5B) and the partial agonist activity of GR-antagonist complexes (data not shown) is retained by the triple mutant.

Similarly, the ability of TIF2 to augment transactivation and to shift the position of the dose-response curve to lower concentrations of Dex is undiminished by the presence of the 548/549/551 triple mutation in the context of the GAL/GR525C chimera (Fig. 5, C and D). Likewise, added TIF2 increases the partial agonist activity of Dex-Mes in both the triple mutant and wild type GR sequence in the context of GAL/GR525C (data not shown).

Because TIF2 is able to modify all of the examined properties of the wild type and 548/549/551 triple mutant to the same extent in both the full-length and chimeric GR constructs (Fig. 5 and data not shown), we conclude that these three mutations have not produced widespread changes in the LBD conformation that can account for the greatly increased EC50 and reduced partial agonist activity of antiglucocorticoids. These data also rule out a possible dramatic decrease in the binding of coactivators to the triple mutant as the cause for the marked alterations of both the EC50 and the partial agonist activity of antiglucocorticoids (50). Furthermore, we can conclude that all of the properties of the single mutants, and especially of the 548/549/551 triple mutant, are due to factors that are independent of the amino-terminal and DNA binding domains of GR. Finally, it should be noted that changes in the position of the dose-response curve and the amount of partial agonist activity are independent of the absolute levels of induced transactivation for a given receptor (Fig. 5 and Table I).

**Hsp90 Binding to Full-length Mutant GRs—** We next asked whether the altered properties of the 548/549/551 triple mutation are due to changes in the binding of hsp90 to full-length receptors. Cytosols from cells overexpressing wild type GR or the triple mutant receptor were immunoadsorbed with either nonimmune or anti-GR antibody, and the protein A-Sepharose pellets were washed to remove loosely bound proteins. The protein of the immune pellets was both assayed for steroid binding activity and resolved on SDS-polyacrylamide gels, followed by Western blotting, to visualize the retained GR, hsp90, and hsp70. As in the experiment of Fig. 4A, the triple mutant has about one-third of the steroid binding activity of the wild type GR for the same amount of receptor protein (Fig. 6A). However, the amount of hsp90 and hsp70 bound to the triple mutant is about twice as much as would be expected from the decreased steroid binding capacity. This suggests that not all of the receptor mutants that are in heterocomplexes with hsp90 are able to bind steroid.

We next asked whether the modest reduction in hsp90 bind-
ing and the extensive decrease in steroid binding activity are inherent properties of the triple mutant GR. To determine this, the GR-containing immune pellets were stripped of hsp90 by salt, and the stripped receptors were incubated with the purified five-protein (hsp90, hsp70, Hop, YDJ-1, p23) assembly system to form GR/Hsp90 heterocomplexes and to regenerate steroid binding activity (5). As shown in Fig. 6B, equal amounts of wild type and mutant GR yield nearly identical amounts of both receptor-bound hsp90 and steroid binding activity after incubation with the hsp90/hsp70-based assembly machinery (lane 3 versus lane 6). This suggests that, under the forcing conditions of the reconstitution system (see “Discussion”), there is no difference in the capacity of wild type and 548/549/551 triple mutant receptors to bind hsp90 and, consequently, steroid hormone.

Hsp90 Binding to GAL/GR Chimeras—At this point, it appears that no single mutation, or even triple mutation, of the sequence of amino acids 547–553 of rat GRs is able to eliminate hsp90 binding, because all mutants retain steroid binding activity at 0 °C (Table I) (25). Furthermore, all of the mutants examined also bind hsp90 (Fig. 6) (25). However, deletion of the seven amino acids eliminates hsp90 binding (21). These obser-
Cell cytosols were prepared from transiently transfected COS-7 cells overexpressing wild type or 547/549/551 triple mutant GR. The GRs were immunoadsorbed from aliquots (50 μl for counts or 100 μl for blots) of cytosol with nonimmune (NI) or FiGR (I) antibody in TEGM buffer. Immune pellets were washed and assayed for GR, hsp90, and hsp70 by Western blotting (middle panel). The amount of hsp90 specifically bound to wild type (WT) and triple mutant (Mut) GRs was quantified by densitometry and plotted as the percentage of hsp90 that co-immunoprecipitated with wild type GRs (lower panel). Samples were also incubated overnight with [3H]triamcinolone acetonide to determine steroid binding activity (top panel). Conditions were as follows: wild type GR, nonimmune (lane 1) and immune (lane 2); triple mutant GR, nonimmune (lane 3) and immune (lane 4). The steroid binding activities and hsp90/GR ratios represent the means ± S.E. from at least three experiments. B, reconstituted GR:hsp90 heterocomplex. Wild type and 545/549/551 triple mutant GRs were immunoadsorbed from aliquots of COS-7 cell cytosol and stripped of native chaperones with NaCl. The stripped GR immune pellets were then incubated with the purified five-protein system to reconstitute receptor:hsp90 heterocomplexes. Immune pellets were washed and assayed for GR, hsp90, and hsp70 by Western blotting (middle panel). The amount of hsp90 specifically bound to wild type and triple mutant GRs was quantified by densitometry and plotted as the percentage of hsp90 that co-immunoprecipitated with wild type GRs (lower panel). Samples were also incubated overnight with [3H]triamcinolone acetonide to determine steroid binding activity (top panel). Conditions were as follows: stripped wild type GR, immune (lane 1); reconstituted wild type GR, nonimmune (lane 2) and immune (lane 3); stripped triple mutant GR, immune (lane 4); reconstituted triple mutant GR, nonimmune (lane 5) and immune (lane 6). The steroid binding activities and hsp90/GR ratios represent the means ± S.E. from at least three experiments.

The present results show that single mutations of each of the seven amino acids in the sequence of 547–553, has on the binding of hsp90 in the context of GST/547C–551 triple mutant show that the presence or absence of a proline, which is present in the native sequence (Fig. 1) and would be expected to permit a bend in the protein structure, is not a determining factor. We therefore conclude that the binding of hsp90 depends more on the presence of the seven amino acids at positions 547–553 than their particular sequence or chemical properties.

**DISCUSSION**

hsp90 binding to the GR is critical for its steroid binding activity, but the location of the hsp90 binding site in the GR sequence has resisted elucidation. Here we pursue our recent observations that hsp90 binding to rat GRs requires seven amino acids in the sequence of 547–553 (21) in a manner that is unaffected by mutation of Leu-550 and Leu-553 to Ser (25). The present results show that single mutations of each of the other amino acids in this seven-amino acid region have relatively minor effects on a variety of transcriptional properties.
The forms of GR in which the mutations listed in the leftmost column are incorporated are as follows: GR, full-length GR; GAL, GAL/552C, GST/570C, GST/547C; and dhfr, dhfr/547C. The symbols used in scoring the activities listed at the top of the chart indicate whether the activity is (+) or not (−) observed. ND, not determined. For qualitative measurements (EC_{50}, and partial agonist activity), a larger number of plus symbols (+) indicates a lower EC_{50} or more partial agonist activity.

| GR mutations | Form of GR | hsp90 binding | Steroid binding | Transcriptional activity | EC_{50} | Partial agonist activity of DM |
|--------------|------------|---------------|-----------------|--------------------------|--------|-------------------------------|
| Wild type^{a,b,c,d} | GR, GAL, GST/520C, GST/547C, dhfr | + | + | + | + | + + + + + + |
| Single mutations in 547–553 | GR, GAL | + | + | ND | + | + | + | + |
| T547A/T549A/S552A | GR, GAL | + | + | ND | + | + | + | + |
| F548A/T549A/V551A | GR, GAL | + | + | − | + | + | + | + |
| L550S/L553S | GR, GST/552C | + | + | − | + | + | + | + |
| ^{547}CAAAAAAC^{555} (CA5C)^{a,c} | GAL, GST/547C | + | − | ND | ND | ND | ND | ND |
| ^{547}PAAAAAAC^{553} (PA5C)^{a,c} | GST/547C | + | − | ND | ND | ND | ND | ND |
| Δ547–551 | GST/547C, dhfr | − | − | ND | ND | ND | ND | ND |
| Δ547–553 | GST/547C, dhfr | − | − | ND | ND | ND | ND | ND |

^{a} This report.
^{b} Ref. 25.
^{c} Ref. 21.
^{d} Ref. 19.

(Table I). Furthermore, good binding of hsp90 is seen both in the presence and absence of the first 546 amino acids of the rat GR (Figs. 6 and 7) (21). Therefore, these sequences are not needed for hsp90 binding to the GR. However, the next seven amino acids are required in a manner that is unrelated to their actual sequence. As is summarized in Table II, more extensive modifications of the native seven amino acid sequence progressively affect more properties of the GR. The first properties to be changed are the steroid binding at 37 °C, the EC_{50}, and the partial agonist activity of antisteroids in the 548/549/551 triple mutant. The last property to be affected is hsp90 binding. Even after all residues are mutated, as in the CA5C and PA5C constructs, and all steroid binding activity is lost, hsp90 binding remains. It is only after deletion of some or all of these seven amino acids (in GST/552C and GST/554C) that hsp90 binding is lost.

The 548/549/551 triple mutant is of special interest in that its dose-response curve for transactivation is shifted ~2 orders of magnitude to the right with respect to wild type GRs (Fig. 3B, Table I). A similar right shift in the dose-response curve is seen with the wild type GR when hsp90 expression in yeast is reduced more than 20-fold (4). In the cell, the efficiency of the interaction of the 548/549/551 triple mutant with hsp90 may be reduced, much as the efficiency of the wild type GR is reduced when the level of hsp90 is very low. To understand the right shift in the dose-response curve, it is necessary to consider the dynamic nature of receptor interactions with hsp90 in the cell.

Although we isolate receptor-hsp90 heterocomplexes from cytosol at 0 °C under conditions where the heterocomplexes are biochemically stable (e.g. Fig. 6A), in intact cells these heterocomplexes are in a steady-state cycle of assembly and disassembly (52). In cytosol prepared from transfected COS cells with a normal level of hsp90, expressed wild type GRs are entirely in the 9 S, hsp90-bound form (53). However, when cells are exposed to geldanamycin, which inhibits hsp90 heterocomplex assembly, the whole cell steroid binding activity of progesterone (54) or glucocorticoid (55) receptors declines rapidly (with a t_{1/2} of 5–10 min) as the receptor-hsp90 heterocomplexes disassemble. When cells are ruptured and cytosols are prepared at 0 °C for assay of steroid binding capacity and receptor-bound hsp90, the data reflect a “stop-action” picture of the steady state condition of receptors that are in an assembly/disassembly cycle in the hormone-free cell and have been trapped by the lowered temperature. The data of Fig. 6A show that the state of the 548/549/551 triple mutant is different from the wild type GR.

Lowering the temperature to 0 °C freezes the whole cell population of mutant receptors in a state where about one-third of the 548/549/551 triple mutant is bound to hsp90 and binds steroid with a normal steroid binding affinity (Figs. 4A and 6A). Another third of the mutant receptors is bound to hsp90 but does not possess steroid binding activity. The fact that the CA5C and PA5C mutant GRs are bound to hsp90 but do not bind steroid (Fig. 7) provides an example where mutation of the seven-amino acid region yields a LBD that is only in the hsp90-binding/non-steroid-binding state. The remaining third of the 548/549/551 triple mutant GR is not bound to hsp90 and does not bind steroid (Fig. 6A). However, when the 548/549/551 triple mutant is stripped of hsp90 and incubated with the five-protein heterocomplex assembly system, it behaves like the wild type GR, both with respect to assembly of GR-hsp90 heterocomplexes and generation of steroid binding activity (Fig. 6B). Thus, all forms of the triple mutant receptor can, under the appropriate circumstances, be converted to the hsp90-bound form with an open steroid binding cleft.

The conditions of the five-protein system are “forced” toward GR-hsp90 heterocomplex assembly in that both p23 and 20 mM molybdate are present to stabilize heterocomplexes once they are formed. The situation in the cell is more dynamic in that rapid disassembly is occurring simultaneously with heterocomplex assembly. The process of heterocomplex disassembly in the cell has not been defined at all. But the 548/549/551 triple mutant GR appears to be more susceptible to disassembly, such that at any time, only about two-thirds of the receptors are bound by hsp90 and, of these complexes, only one-half are in a steroid binding state.

Assembly of receptor heterocomplexes with hsp90 protects the receptors from ubiquitination and proteasomal degradation in the cell (for a review, see Ref. 56). The 548/549/551 triple mutation that alters the dynamics of heterocomplex assembly/disassembly still yields protection against this degradation, with the level of the mutant receptor being roughly the same as the wild type (Fig. 3C). Thus, at high enough concentrations of steroid to elicit a maximum response, the 548/549/551 triple mutant produces nearly the same transcriptional activation as the wild type GR (Fig. 3). However, the drug concentration required to activate the triple mutant is now more than 2 orders of magnitude higher than that for the wild type GR (Fig. 3, Table I).

This requirement for higher concentrations of steroid exists in the intact cell despite the fact that the 548/549/551 triple mutant receptor binds steroid in cytosol at 0 °C with the same...
affinity as the wild type GR (Fig. 4A). This is exactly the phenotype one would expect, however, if the sole difference between the mutant and the wild type receptors is a more rapid disassembly of the receptor-hsp90 complex on the part of the 548/549/551 triple mutant receptor in intact cells. At any instant, only a few of the triple mutant receptors in the cell would have an open steroid binding cleft and be accessible to steroid. Because of rapid disassembly of the receptor-hsp90 heterocomplex, the proportion of time that the cleft is open for the triple mutant receptor in the cell is very short, and high concentrations of steroid must be present to ensure entry before hsp90 dissociates and the ligand binding cleft closes. After steroid binding, the 548/549/551 triple mutant GR undergoes steroid-dependent activation, interacts with co-factors (Fig. 5), and induces gene expression (Fig. 3) in apparently normal fashion. At present, we do not know how a mutation, such as P548A/V551A, can have so little effect on the total activity of an antagonist and such a dramatic effect on the partial agonist activity of an antagonist. However, such information would be very useful for use in antisteroid therapies. Factors that modulate the partial agonist activity of antisteroids have the potential of reducing the number of side effects by selectively repressing the expression of a limited number of responsive genes while allowing the majority of genes to still be induced.

Until now, we have considered the GR as being only in the hsp90-bound state with an open steroid binding cleft or in an hsp90-free state with a closed cleft and no steroid binding activity. The 548/549/551 triple mutant gives us an appreciation of how the dynamics of the GR-hsp90 heterocomplex assembly/disassembly process can dramatically affect the dose-response curve for transactivation in the cell without affecting the receptor’s intrinsic affinity for steroid, as determined in cytosols at 0°C. The alanine replacement of the segment required for hsp90 binding, as in the CA5C and PA5C constructs, maintains both the α-helical structure of this region and assembly of heterocomplexes with hsp90 (Fig. 7). However, assembly of CA5C or PA5C heterocomplexes with hsp90 does not result in any stable opening of the steroid binding cleft, as indicated by the facts that GRs containing these mutations display no steroid binding activity in cytosol (Fig. 7) or transactivation activity in intact cells, even at 100 μM DEX (Table II). Thus, the CA5C and PA5C mutants provide a starting point for future work to determine by replacement mutation what is required to link hsp90 heterocomplex assembly with steroid binding cleft opening.

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