Hsp90 association with glucocorticoid receptors (GRs) is required for steroid binding. We recently reported that seven amino acids (547–553) overlapping the amino-terminal end of the rat GR ligand-binding domain are necessary for hsp90 binding, and consequently steroid binding. The role of a LXXLL motif at the COOH terminus of this sequence has now been analyzed by determining the properties of Leu to Ser mutations in full-length GR and glutathione S-transferase chimeras. Surprisingly, these mutations decreased steroid binding capacity without altering receptor levels, steroid binding affinity, or hsp90 binding. Single mutations in the context of the full-length receptor did not affect the transcriptional activity but the double mutant (L5508/L553S) was virtually inactive. This biological inactivity was found to be due to an increased rate of steroid dissociation from the activated mutant complex. These results, coupled with those from trypsin digestion studies, suggest a model in which the GR ligand-binding domain is viewed as having a “hinged pocket,” with the hinge being in the region of the trypsin digestion site at Arg. The pocket would normally be kept shut via the intramolecular interactions of the LXXLL motif at amino acids 550–554 acting as a hydrophobic clasp.

Steroid hormone receptors are members of a superfamily of proteins with a common zinc finger motif for DNA binding. While the initially discovered members of this superfamily bind steroid and thyroid hormones, the majority of the family members still have no defined ligand (1, 2). Those receptors that bind hormone are ligand activated transcription factors and are thus of interest both as models for the control of gene transcription and as mediators of hormone action. Steroid binding to a receptor requires both a properly folded protein and an intact ligand-binding domain (LBD). In general, the carboxyl-terminal — 250 amino acids of receptors encode the LBD, although the precise boundaries have rarely been defined. For rat glucocorticoid receptors (GR), the last 246 amino acids (positions 550–795) encompass the LBD (3). Some receptors, such as the estrogen receptor, contain additional residues after the LBD, commonly called the F domain. However, these added amino acids do not appear to be required for ligand binding (4). It was initially thought that any mutation in the LBD was detrimental for steroid binding. Subsequent research, although, has revealed that many mutations are without effect (5) and some increase the affinity and/or selectivity of receptors (6, 7).

Defining the determinants of protein folding that are required for steroid binding has proven to be a difficult task. As with many protein domains, LBDs are transferable and retain their activity when attached to other proteins (8, 9). This led to the view that receptor LBDs were independent entities containing all of the information necessary for ligand binding. It was, therefore, surprising to find that the GR LBD was not stable as an isolated unit but required other, upstream sequences, which did not have to be those of GR, in order to obtain a stable protein (3).

Another crucial component for hormone binding to GRs is hsp90. In fact, the GR cannot bind hormone unless the receptor is already complexed with hsp90 (10, 11). Hsp90 is an ubiquitous cellular protein that is part of a multiprotein complex with chaperone activity (12). When hsp90 dissociates from GRs, steroid binding is lost but can be regenerated by incubating immobilized GRs with reticulocyte lysate or with a five-protein, minimal chaperone system consisting of hsp90, hsp70, Hop, hsp40, and p23 (11–13). Several sequences of the GR have been implicated in the binding of hsp90, but most are near the middle of the GR LBD (amino acids 568–671 of the rat GR (14–16)). We recently reported (3) that a more amino-terminal sequence of 547–553, which lies at the border of the GR LBD, was essential for the binding of hsp90 (17). This seven-amino sequence appeared to be specifically required, as a polyalanine spacer was incapable of maintaining hsp90 binding, and consequently steroid binding activity.

No common sequence required for hsp90 binding has yet been discerned among the numerous transcription factors and proteins kinases with which hsp90 forms complexes (reviewed in Refs. 12 and 18). Indeed, three different regions of both the human GR (15) and the chicken progesterone receptor (PR) (19) were sufficient to confer hsp90 binding. It is generally thought that, like other chaperones, hsp90 initially binds to exposed hydrophobic residues of partially denatured proteins and that this binding assists the protein in folding to its final, native conformation (20–23). However, for those proteins that undergo ATP-dependent assembly into stable heterocomplexes with hsp90, such as steroid receptors, this simple model is not sufficient. In addition to creating steroid-binding sites, hsp90 complexation with GRs also opens up the LBD to permit both
the attack of thiol residues by a thiol-derivatizing agent (24) and the proteolytic cleavage at basic amino acids by trypsin (25, 26). These data, coupled with those of GR mutants (17), support the notion (27) that the hsp90-based chaperone machinery directs the partial unfolding of the LBD, thus opening the hydrophobic steroid binding cavity for access by steroid.

The purpose of this study was to determine the role of amino acids 547–553 of rat GR in the binding of hsp90 and steroid. We noted that this seven-amino acid sequence contains an LXXLL motif, which has been found to mediate some protein-protein interactions (28–30). Several point mutants of this motif were, therefore, prepared to assess its function in the context of glutathione S-transferase (GST) chimeras and full-length GRs. Both the kinetics and thermal stabilities of steroid and hsp90 binding were monitored along with the susceptible sites for the receptors to protease digestion. These studies demonstrated that the seven amino acids 547–553 are important for two separable activities, hsp90 binding and steroid binding. A molecular model for some of the determinants of these two activities is presented that is based on the x-ray structure of the progesterone LBD (31).

MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 0 °C. Chemicals—[1,2,4,6-3H]Dexamethasone (Dex; 81 Ci/mmol) and the Thermo Sequenase radiolabeled terminator cycle sequencing kit were obtained from Amersham Pharmacia Biotech. [6,7-3H]Triamcinolone acetonide (TA; 33 Ci/mmol) and [131I]labeled goat anti-mouse and anti-rabbit IgGs were from NEN Life Sciences Products. Nonimmune IgG and the monoclonal anti-GST antibody (clone GST-2) were from Sigma. The AC8 monoclonal IgG against hsp90 was from Stressgen (Victoria, British Columbia), the BuGR2 monoclonal IgG against the steroid and hsp90 binding were monitored along with the susceptibility sites for the receptors to protease digestion. These studies demonstrated that the seven amino acids 547–553 are important for two separable activities, hsp90 binding and steroid binding. A molecular model for some of the determinants of these two activities is presented that is based on the x-ray structure of the progesterone LBD (31).

Construction of Plasmids—The pSVLGR vector was a gift from Dr. Keith Yamamoto (34). Constructs obtained by the GeneEditor in vitro Site-Directed Mutagenesis System and the Dual-Luciferase Reporter Assay System were from Promega. LipofectAMINE Reagent was obtained Life Technologies, Inc. Enzyme preparations were purchased from the supplier (35). Cytosols of transfected cells and the steroid-free receptors were obtained by the lysis of cells at −80 °C and centrifugation at 15,000 × g as described previously (36). [3H]Dex binding assays all contained 20 nm sodium molybdate. Experiments for Scatchard analysis were conducted at 0 °C for 18 h with 30% cytosol and 0.625–50 nM [3H]Dex. [3H]Dex was removed by dextran-coated charcoal, and the supernatant was counted in Hydrofluor. The affinity (Kd) was determined by plotting the ratio of bound steroid/free steroid versus bound steroid. For the temperature sensitivity assays, the 30% cytosol extracts were incubated with 50 nm [3H]Dex for 100-fold excess of nonradioactive Dex. Unbound [3H]Dex was removed with dextran-coated charcoal, and the supernatant was counted in Hydrofluor. The affinity (Kd) was determined by plotting the ratio of bound steroid/free steroid versus bound steroid. For the temperature sensitivity assays, the 30% cytosol extracts were incubated with 50 nm [3H]Dex for 100-fold excess of nonradioactive Dex.

Overexpressed receptor proteins were visualized following Western blot analysis (see below) using enhanced chemiluminescence. Gel shift bands were quantitated on a Macintosh Power G3 computer using the public domain program NIH Image 1.6 (developed at the U.S. National Institutes of Health and available on the Internet). An equal region of the film without any bands was used to calculate the background.

The intensity of the specific protein band was then determined by subtracting the background value from that of the receptor protein. To normalize the steroid binding per unit receptor protein, the specific disintegrations/min of “Hysteroid bound to each receptor were divided by the above calculated amount of receptor protein.

For Biological Activity—Forty ng of wild type and mutant pSVLGR constructs, 1 μg of a GRElLuc reporter, 200 ng of a Renilla pRLlLuc reporter (Promega), and enough pBSK+ vector to bring total DNA concentration to 3 μg/60-mm dish, were transiently transfected into COS-7 cells. As recommended by the supplier, 10 μl of LipofectAMINE was added 2 μg of DNA, serum-free Dulbecco’s modified Eagle’s medium and added to the cells for 3 h. At the end of the transfection period, cells were washed, replenished with complete media, and incubated overnight at 37 °C. The cells were then treated with EtOH + Dex or Dex 21-mesy late (Dex-Mes) for an additional 24 h. At the end of the incubation period, the cells were harvested in 1× Passive Lysis Buffer (0.5 ml/dish, Promega). Luciferase activity was assayed using luciferin reagents in the Promega Dual Luciferase Reporter Assay kit.

For Protein A-Sepharose. Immuno-
creatinine phosphokinase) were added to all assays to yield a final assay volume of 56 μl. The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 5 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG buffer with 20 mM sodium molybdate), and GST fusions and hsp90 were assayed in each sample by Western blotting.

A portion of each immune pellet was assayed for steroid binding by incubation overnight in 100 μl of TEGM buffer plus 4 μg dithiothreitol and 50 nM [3H]triamcinolone acetonide (TA). Samples were then washed twice with 1 ml of TEGM and counted by liquid scintillation spectrometry as described previously (13). The steroid binding is expressed as counts/minute of [3H]TA bound/anti-GST immune pellet prepared from 100 μl of cytosol.

**Assay of Steroid Dissociation**—Prior to immunoadsorption, the BuGR2 antibody was prebound to protein A-Sepharose pellets by incubating 65 μl of a 20% slurry of Protein A-Sepharose for 2 h at 4 °C with 45 μl of antibody at a concentration of 100 μg/ml and 200 μl of 10 mM Hepes, pH 7.4, followed by centrifugation and washing with Hepes buffer. The transfected wild type GR and L550S/L553S mutant GR that had been prebound with [3H]Dex × 500-fold excess [3H]Dex were allowed to dissociate for various lengths of time and then adjusted to contain 20 mM molydate to block further dissociation of hsp90. The receptors were then immunoadsorbed from 600 μl of diluted cytosol by rotation for 2 h at 4 °C with 15 μl of protein A-Sepharose prebound with BuGR2 antibody, followed by washing the immunopellets three times with 1 ml of TEGM.

**Gel Electrophoresis and Western Blotting**—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 0.01% aP1 for fusion proteins, 1 μg/ml BUGR-2 for full-length GR, and 1 μg/ml AC88 for hsp90. The immunoblots were then incubated a second time with the appropriate [3H]-conjugated counter antibody to visualize the immunoreactive bands. For quantitative Western blotting, the GST/GR HBD fusion proteins, or hsp90, were excised from the immunoblots and counted for associated [3H]-conjugated goat anti-rabbit (for GR) or anti-mouse (for hsp90) radioactivity. The determined ratio of hsp90 per unit of GR associated radioactivity. The determined ratio of hsp90 per unit of GR

**Assessment of Steroid Binding**—In order to dissociate the 65 kDa species (38). This construct would further reveal any contributions of the hinge region in hsp90 binding to GR. In order to examine the biological consequences of these mutations, full-length GR mutants were prepared.

**Steroid Binding Activity and Expression of Mutant Proteins**—Western blots indicated that each mutant was expressed at about same level as the wild type construct (Fig. 2, insets). Surprisingly, though, the level of [3H]steroid binding was not similarly constant. The single mutations decreased the amount of binding of the chimeras by about 75% while the double mutant displayed almost no binding (Fig. 2, A and B). In all cases, the effect of each mutation was muted in the context of the full-length receptor (Fig. 2C). However, Scatchard analysis revealed that each mutation did not alter the affinity of those receptors that did bind steroid (Table 1). This decrease in binding with maintenance of wild type affinity could be explained by a heterogeneity among the receptors, some of which were unable to bind steroid. Alternatively, the kinetics of binding could be very different among the mutant GRs. However, [3H]Dex binding to each of the GST520C chimeras was found to be able to look at the effect of these mutations on the tertiary structure of GR, the GST520C chimera was also selected, as proteolysis of this ligand-free chimera does afford the 16-kDa species (38). This construct would further reveal any contributions of the hinge region in hsp90 binding to GR. In order to examine the biological consequences of these mutations, full-length GR mutants were prepared.

**Effect of Mutations on Interactions with Hsp90**—Western blots indicated that each mutant was expressed at the same level as the wild type construct (Fig. 2, insets). Surprisingly, though, the level of [3H]steroid binding was not similarly constant. The single mutations decreased the amount of binding of the chimeras by about 75% while the double mutant displayed almost no binding (Fig. 2, A and B). In all cases, the effect of each mutation was muted in the context of the full-length receptor (Fig. 2C). However, Scatchard analysis revealed that each mutation did not alter the affinity of those receptors that did bind steroid (Table 1). This decrease in binding with maintenance of wild type affinity could be explained by a heterogeneity among the receptors, some of which were unable to bind steroid. Alternatively, the kinetics of binding could be very different among the mutant GRs. However, [3H]Dex binding to each of the GST520C chimeras was found to be able to look at the effect of these mutations on the tertiary structure of GR, the GST520C chimera was also selected, as proteolysis of this ligand-free chimera does afford the 16-kDa species (38). This construct would further reveal any contributions of the hinge region in hsp90 binding to GR. In order to examine the biological consequences of these mutations, full-length GR mutants were prepared.

**Effect of Mutations on Interactions with Hsp90**—We had previously observed that GST547C, overexpressed in COS-7 cells, was associated with hsp90 (17). One model for the decreased steroid binding capacity without altered affinity of the L550S and/or L553S mutants is that hsp90 binding to GR has become more labile and only a fraction of the receptors remain associated with hsp90. Thus, under cell-free conditions at 0 °C that do not permit hsp90 reassociation with GR (11), only those receptors that retained hsp90 would bind with an affinity that might be indistinguishable from wild type receptors. This hy-
Steroid and Hsp90 Binding to Glucocorticoid Receptors

Fig. 2. Steroid binding activity of wild type and mutant receptors. Duplicate samples of cell-free extracts from COS-7 cells that had been transiently transfected with plasmids encoding (A) GST547C, (B) GST520C, or (C) full-length receptors (pSVLGR) ± point mutations were analyzed for total steroid binding activity as described under “Materials and Methods.” At the same time, the total receptor protein in each sample was determined by Western blotted following separation on 8% SDS-polyacrylamide gels (insets). The GST chimeras were detected by anti-GST (shown) or anti-GR (aP1 or hGR; data not shown) antibodies. The anti-GR antibody aP1 was used with the full-length receptors. The intensity of the Western blotted receptor bands was determined by NIH Image, as described under “Materials and Methods,” and the total binding per unit of Western blotted protein was expressed relative to the wild type construct. The data plotted are the average ± S.D. of three or four (GST547C) independent experiments. The average dpm/10 µl of 100% cytosol was 53,051 (GST547C), 48,353 (GST520C), and 23,859 (wt GR).

TABLE I

| Constructs | Mutations present in receptor |
|------------|------------------------------|
| Wild type  | L550S | L553S | L550S/L553S |
| GST547C   | 5.2 ± 2.1 | 7.5 ± 4.0 | ND* | ND |
| GST520C   | 4.2 ± 0.7 | 3.2 ± 0.3 | 3.8 ± 0.1 | 3.9 ± 2.1 |
| Full-length | 2.0 ± 0.7 | 1.7 ± 0.4 | 2.0 ± 0.5 | 2.1 ± 0.5 |

* ND, not determined.

pothesis was addressed by heating ligand-free GRs at 20 °C to accelerate the dissociation of hsp90 and the loss of steroid binding. For GST547C ± L550S chimeras, the loss of steroid binding activity was found to be identical (t1/2 = 60 min; data not shown). These results indicated that the decreased binding of GST547C/L550S was not a consequence of weaker hsp90 binding that reduced the fraction of hsp90-bound GR and, thus, the steroid binding capacity of the total GR.

Another possibility was that the mutations altered the folding of the GR proteins such that there was a partitioning of the receptors between GR that could and could not bind hsp90. Thus, those molecules that reached a conformation capable of binding hsp90 might then bind steroid with the same affinity as wild type receptors. To assess this possibility, we quantitated the steroid and hsp90 binding of the GST520C mutants. These chimeras were used because they seemed to have the same tertiary structure as the wild type receptors, as determined by protease digestion. A 16-kDa tryptic fragment, corresponding to the sequence 652–795 (25, 38), is obtained after trypsin digestion of steroid-free, full-length receptors and chimeras containing sequences upstream of amino acid 547 (such as 537C, 520C, and 494C) but not from constructs starting at 547C (3, 38).

Interestingly, GST520C and all of the mutant chimeras (L550S, L553S, and L550S/L553S) contained hsp90 (Fig. 3, B–E, lane 2). Even the L550S/L553S double mutant with little residual binding contained large amounts of hsp90 (Fig. 3E, lane 2). In all cases, the amount of associated hsp90 was dramatically reduced in the stripped pellets (lane 3 of Figs. 3, B–E) and restored after incubation with reticulocyte lysate (lane 4 of Fig. 3, B–E). Most surprising, however, was that the ratio of hsp90 to GR was relatively constant for all constructs (Fig. 3F).

Thus, the quantity of hsp90 associated with the wild type 520C and each mutant was about the same even though there were major differences in total steroid binding activity. This demonstrates that, while hsp90 has been found to be necessary for steroid binding (10, 11), hsp90 binding to GR is not sufficient for steroid binding. These data also establish that the decreased binding of the L550S, L553S, and L550S/L553S mutations of GST520C occur without any major disruption of hsp90 binding to GR. Therefore, the amino acid sequence of 547–553 is involved in the expression of two independent activities of GR, steroid binding and hsp90 binding.

Sequences between Amino Acids 520 and 546 Are Not Required for Hsp90 Binding—We have previously demonstrated that GST547C contains sufficient amino acid residues for the association of receptors with hsp90 in intact cells. However, the possibility remained that residues between 520 and 546 could contribute to hsp90 binding and were responsible for the unraveled binding of hsp90 to the GST520C mutants in Fig. 3. The same experiments were therefore conducted in the context of the GST547C chimera to see if a similar insensitivity of hsp90 binding existed in the presence of mutations that reduced steroid binding. As shown in Fig. 4, the ratio of hsp90 to GR was not appreciably different for GST520C and GST547C. Most significantly, this ratio was virtually identical between GST520C and GST547C/L550S, which retained only 25% of the steroid binding activity of the wild type GST547C (see Fig. 2A).

Therefore, as for the GST520C chimeras, mutations in the LXXL sequence of 547–553 can dramatically reduce steroid binding activity with little or no effect on hsp90 binding. Furthermore, the sequence of 547–553 is sufficient to convey steroid and hsp90 binding to the rest of the GR LBD (554–795) as...
residues between 520 and 546 do not make significant contributions.

Whole Cell Biological Activity of Full-length GR Mutants—

Dex inducibility of a transiently transfected reporter construct by full-length receptors containing a single mutation was uniformly higher (1.3–2.6-fold) than that by wild type GR (Fig. 5). In contrast, the activity of the double mutant was decreased 5-fold (0.19 ± 0.06-fold; S.D., n = 3). These changes in biological activity could not be accounted for by the observed alter-
Steroid and Hsp90 Binding to Glucocorticoid Receptors

FIG. 6. Stability of steroid binding activity in ligand-free wild type versus mutant GR. Duplicate aliquots of cytosols from COS-7 cells that had been transiently transfected with plasmids encoding wild type GR (pSVLGR) or L550S/L553S mutations were incubated at 20 °C for the indicated time. The samples were cooled to 0 °C and treated with [3H]Dex ± 500-fold excess [1H]Dex to quantitate the remaining steroid binding activity, which was plotted as percent of the initial binding activity. Similar results were obtained in a second experiment.

FIG. 7. Dissociation of steroid and hsp90 from full-length, wild type GR (pSVLGR) and L550S/L553S mutant. Cytosols from transfected COS-7 cells were prebound at 0 °C with [3H]Dex and then allowed to dissociate at 20 °C. At the indicated times, the samples were placed in ice to stop the dissociation. All samples were treated with 20 mM molybdate at the end of the assay (120 min) to block further dissociation of hsp90. Receptor with its associated hsp90 was then immunoadsorbed using either a nonimmune antibody or anti-GR antibody (BUGR2). The graph displays the bound [3H]Dex at each time as percent of initial binding for full-length, wild type GR (pSVLGR, ●), and the L550S/L553S double mutant (□) in addition to the hsp90/GR ratio for full-length wild type GR (○) and the L550S/L553S double mutant (○). The autoradiogram (inset) shows the Western blots of immunoadsorbed GR and coadsorbed hsp90. These bands were incubated with 125I-labeled secondary antibody, excised, and counted to generate the hsp90/GR ratios in the graph. Similar results were obtained in a second experiment.

FIG. 8. Dissociation of prebound steroid from full-length L550S/L553S GR in the presence of molybdate and/or Dex. Aliquots of cytosols from COS-7 cells transfected with full-length GR with the L550S/L553S mutation were prebound with [3H]Dex ± 500-fold [1H]Dex with or without 20 mM sodium molybdate, as indicated. Solutions containing molybdate were then split so that half received a 500-fold excess of [1H]Dex to prevent rebinding of any dissociating [3H]Dex. Duplicate samples of all treatments were then heated at 20 °C for the indicated times, at which point the remaining specifically bound [3H]Dex was determined and the averages ± range were plotted as percent of the initial binding. Similar results were obtained in a second experiment.

Full-length GR (Figs. 2C versus 5) could be due to the different temperatures of the two assays (0 versus 37 °C). In fact, when steroid-free receptors were heated at 20 °C, the full-length double mutant (WT/L550S/L553S) lost steroid binding activity much more rapidly than did the wild type GR (Fig. 6). This could result from either a more rapid dissociation of hsp90 from the double mutant or weakened intramolecular interactions in the double mutant, thus permitting a more facile opening of the LBD. When receptors pre-bound with steroid were heated, conditions that cause the activation/transformation of receptor-steroid complexes (39), the loss of steroid was again more rapid from the double mutant (Fig. 7). However, this more rapid loss of steroid from the activated mutant complexes occurred without any preferential loss of hsp90 (circle data points). Thus, as seen above in Figs. 3 and 4, steroid binding capacity can be altered without affecting the binding of hsp90.

The more rapid rate of dissociation of steroid from the double mutant (Fig. 7), and the lack of biological activity (Fig. 5), could be due either to a mutation-induced blockage of activation, thus preventing the activation/transformation of the mutant receptor-steroid complex to the more slowly dissociating form (40–42), or to more rapid dissociation of steroid from the activated form of mutant GR. To distinguish between these two possibilities, we made use of the fact that bound at 0 °C with [3H]Dex blocks the conversion of the rapidly dissociating, unactivated receptor-steroid complex to the more stable, activated complex for both GR (41) and estrogen receptors (43). Therefore, the rates of steroid dissociation at 20 °C from unactivated (in the presence of molybdate) and activated (in the absence of molybdate) double mutant GR-[3H]Dex complexes were determined. As shown in Fig. 8, the receptor-steroid complex was quite stable in the presence of molybdate. The slight increase in steroid binding...
with time at 20 °C (triangles) was most likely due to the fact that the initial incubation period of 2 h was not sufficient to bind all of the receptors. However, in the presence of excess non-radioactive steroid to prevent the rebinding of [3H]Dex, the rate of dissociation of steroid in the absence of molybdate was much faster (t1/2 ~ 20 min) than in the presence of molybdate (t1/2 ~ 80 min). Thus, a temperature-induced modification of the full-length L550S/L553S mutant GR-steroid complex has occurred which causes a more rapid dissociation of steroid. Furthermore, this temperature-induced change is inhibited by sodium molybdate. We interpret this as indicating that the L550S/L553S mutation of the full-length GR does not block activation but rather accelerates the dissociation of bound steroid once the activated complex has been formed. This rapid loss of steroid from activated complexes would further explain why the full-length double mutant displays good steroid binding ability at 0 °C (Fig. 2C) but is biologically inactive (Fig. 5).

**Trypsin Digestion of Steroid-free Mutant Receptors to Yield the 16-kDa Fragment**—The atypical accelerated dissociation of steroid from activated versus unactivated mutant complexes in Fig. 8 is most readily explained as resulting from changes in GR tertiary structure due to mutations of the LXXLL motif. We, therefore, looked for additional evidence of conformational differences in the mutant GRs. We previously documented that steroid-free GR is uniquely vulnerable to trypsin digestion to yield a 16-kDa fragment (25) corresponding to the sequence of steroid-free GST520C and full-length GR. Cell-free extracts of COS-7 cells that had been transiently transfected with plasmids encoding (A) GST520C or (B) full-length receptors ± point mutations were treated, in the absence of added steroid, with the indicated concentrations of trypsin for 1 h at 0 °C. Tryptic fragments, especially the 16-kDa species (indicated by arrow), were separated by SDS-polyacrylamide gel electrophoresis and visualized by Western blotting with anti-GR antibody (aP1) followed by enhanced chemiluminescence. The positions of the molecular weight markers was determined by staining with Ponceau S and marking with fluorescent paint. Comparable results were obtained in 1 and 2 additional experiments for A and B, respectively.

**DISCUSSION**

The seven-amino acid sequence of 547–553 of rGR is established here to participate in two distinct activities. This sequence was previously reported to be essential for hsp90 binding to GR (17). The absence of steroid binding in constructs lacking this sequence was thought to result from the loss of hsp90 binding. However, mutations within this sequence now illustrate that, while hsp90 binding to GR is required for steroid binding (10, 11), mutations of Leu550 and Leu553 to serine dramatically reduced steroid binding (Fig. 2) and biological activity (Fig. 5) without altering the binding of hsp90 (Figs. 3, 4, and 7). Therefore, the sequence of 547–553 contributes to the expression of two independent and separable GR activities, steroid binding and hsp90 binding. The steroid binding activity depends on a LXXLL motif at amino acids 550–554.

LXXLL motifs have recently been described to mediate the protein-protein interactions of transcriptional cofactors with steroid/nuclear receptors (28–30). The presence of such a motif at the COOH terminus of amino acids 547–553 of rGR suggested that it might similarly be crucial for the protein-protein interactions required in hsp90 binding and steroid binding. Convincing evidence for the role of the LXXLL sequence in steroid binding per se came from the rates of steroid dissociation in the presence and absence of molybdate from full-length GR complexes containing the L550S/L553S double mutation (Fig. 8). The rate of steroid dissociation from the wild type GR decreases after being exposed to activating conditions, such as elevated temperatures (40–43). In contrast, the L550S/L553S mutations resulted in an accelerated dissociation of bound steroid after activation. Furthermore, discrepant rates of steroid dissociation from GR complexes were observed under conditions causing no difference in the amount of hsp90 retained by the wild type or mutant GR (Fig. 7).

An examination of the predicted GR structure, based on the x-ray structure of the closely related human PR LBD (31), suggests a molecular model for the role of the LXXLL motif in steroid binding. This sequence is thought to be α-helical and part of helix 1 of the LBD (31, 45). Therefore, as adjacent amino acids of an α-helix are 3.5 residues apart, the mutations of Leu550 and/or Leu553 would affect amino acid substituents that all lie on one side of the α-helix. Furthermore, Leu550 and Leu553 of rGR are well located for intramolecular van der Waals interactions that would stabilize the tertiary structure of the GR LBD (Fig. 10A). Leu550 (yellow stick model) would form hydrophobic bonds with residues both in the bend between helices 3 and 4 (Pro600 of rGR) and in helix 9 (Tyr711 and Glu714 of rGR) while Leu553 (red) would contact Pro600 (see figure legend for details). Leu554 (blue) would interact with Trp595, Ile599, Prp600, Leu682, Thr686, Tyr711 in helix 3, between helices 3 and 4, and in helix 8 of rGR. It should be noted that almost all of these residues, including Leu550, Leu553, and Leu554, are completely conserved between rGR and the human PR (31). We therefore propose that the LXXLL sequence of...
550–554 is a hydrophobic patch that makes important contributions to the stability of the GR LBD tertiary structure and, consequently, steroid binding activity.

The importance of this LXXLL sequence can be further appreciated from a different view of the LBD, in which the amino-terminal half of the domain (547–651) forms one side of the steroid binding pocket (Fig. 10B). The two sides of the pocket are “hinged” in the region of Arg651, which appears not to exist as an α-helical segment (44), and closed at the other end, in part, by the hydrophobic interactions of Leu550, Leu553, and Leu554.

Additional considerations of the hinged pocket model offer explanations as to why the magnitude of reduced binding capacity at 0 °C following various mutations of the LXXLL motif was not uniform in all receptor constructs examined. A single mutation in the GST chimeras of the GR LBD caused a greater loss of steroid binding activity than did the presence of both mutations in the full-length receptor (Fig. 2). Thus, GR sequences upstream of amino acid 520 were able to counteract some of the disruptive effects of the mutations of Leu550 and Leu553. This does not appear to result from a stabilization of hsp90 binding as the rate of loss of steroid binding activity upon heating steroid-free receptors at 20 °C was actually faster for the full-length L550S/L553S double mutant ($t_{1/2}$ = 20 min,
structural consequences that would affect trypsin digestion at P600L, which modifies the proline contacted by Leu550, Leu553, tryptic fragment, due to inadequate intramolecular contacts of rapid dissociation at elevated temperatures following activation of the dissociation of the two peptides. This would facilitate the elevated temperatures that cause activation, any destabilization of the complexes resulting from the dissociation of non-receptor proteins like hsp90 may be partially compensated by the steroid-induced compaction of the LBD (45, 53). However, the double mutation causes more extensive disruptions of the hydrophobic clasp around Leu550. The loss of these important interactions allows the hinged pocket to open more easily at 37 °C, with the subsequent dissociation of steroid and the production of a transcriptionally inactive form of GR.

The hinged pocket model of the GR LBD also offers an attractive hypothesis for how mutations around Leu550 have structural consequences that would affect trypsin digestion at Arg651 to produce the 16-kDa tryptic fragment, which correlates to amino acids 652–795 (38) (Fig. 9). Trypsin digestion can be an especially revealing probe of GR structure (25, 26) and, in some cases, is a more sensitive probe than steroid binding (3). The GR LBD contains many basic residues but is cleaved by trypsin at only very few sites (25, 26, 38), presumably due to most of the residues being hidden by the folded tertiary structure and associated proteins. Trypsin digestion of steroid-free GR does not open up the LBD after cutting at Arg651 in the hinge because the two halves of the LBD (518–651 and 652–789) remain non-covalently associated (26, 38). However, mutation of the hydrophobic clasp at the other end of the hinged pocket (Leu550 and Leu553) would be expected to reduce the binding of the two tryptic fragments, thereby allowing the dissociation of the two peptides. This would facilitate further trypsin digestion of the LBD, and the 16-kDa fragment, at previously inaccessible residues, just as was observed (Fig. 9). We feel that the observed differences in trypsin digestion reflect a functionally relevant loosening of the receptor tertiary structure as opposed to a nonspecific denaturation because 1) all of the mutants retain nearly equivalent levels of hsp90 binding and 2) the steroid binding at 0 °C of each of the full-length mutant GRs is similar while the biological activities are quite dissimilar.

Four mutant GRs have been described with properties similar to those defined in this study. Mouse GR with the Leu to Arg mutation at positions equivalent to 553 and 554 of rGR has been reported for LBDs with the amino-terminal regions of androgen (46–48), estrogen (49, 50), and progesterone (51) receptors and implicated for the amino terminus of GR by the presence of apparently normal hsp90 binding may be a relatively common phenotype.

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