To elucidate which amino acids in the glucocorticoid receptor ligand-binding domain might be involved in determining steroid binding specificity by interaction with the D-ring of glucocorticoids, we have performed site-directed mutagenesis of the four amino acids Met-560, Met-639, Gln-642, and Thr-739 based on their proximity to the steroid in a model structure. Mutations of these residues affected steroid binding affinity, specificity, and/or steroid-dependent transactivation. The results indicate that these residues are located in close proximity to the ligand and appear to play a role in steroid recognition and/or transactivating sensitivity, possibly by changes in the steroid-dependent conformational change of this region, resulting in the formation of the AF-2 site. Mutation of Gln-642 resulted in a marked decrease in affinity for steroids containing a 17α-OH group. This effect was alleviated by the presence of a 16α-CH3 group to a varying degree. Thr-739 appears to form a hydrogen bond with the 21-OH group of the steroid, as well as possibly forming hydrophobic interactions with the steroid. Met-560 and Met-639 appear to form hydrophobic interactions with the D-ring of the steroid, although the nature of these interactions cannot be characterized in more detail at this point.

The glucocorticoid receptor (GR) belongs to the superfamily of hormone-dependent nuclear receptors and consists of three structural and functional main domains: the N-terminal domain, which harbors the major transactivating function (AF-1); the central domain, which binds to DNA in glucocorticoid regulated genes; and the C-terminal domain, which binds the ligand (1–4).

The ligand-binding domain (LBD) comprises approximately 250 amino acids and is in its unliganded state associated with a complex containing heat shock proteins and immunophilins. Upon ligand binding this complex dissociates and a cascade of events are triggered leading to induction or repression of target genes. Within the ligand-binding domain there are also a hormone-dependent nuclear localization signal (6) and hormone-dependent transactivation functions (AF-2) (7–10).

The crystal structure of the GR LBD is not yet available, but the crystal structures of the LBDs of other members of the nuclear receptor superfamily including the peroxisome proliferator activated receptor, retinoic acid receptor, retinoid X receptor, thyroid hormone receptor, progesterone receptor (PR), estrogen receptor α (ERα), and estrogen receptor β (ERβ) have been solved (11–17). Their structures contain 12 α-helices that are folded in a very similar way into a three-layered antiparallel α-helical sandwich that creates a hydrophobic pocket for the ligand. Upon ligand binding a conformational change occurs, mainly involving helix 12, which folds up against the protein body and creates a lid for the ligand binding pocket. This also leads to formation of the AF-2 interface, which has been shown to interact with transcriptional coactivators (18–21).

The mechanisms that determine the binding affinity and specificity of steroid hormone receptors for different ligands is not well understood, but the liganded crystal structures of ERα, ERβ, and PR have given some information (15–17). A number of van der Waals’ interactions and a few hydrogen bonds between receptor and steroid were identified. The A-ring of the steroid, which has quite a similar structure in all classes of steroids, also seems to be anchored in a very similar manner via a hydrogen bond network with water and two amino acids of the receptor. In all three co-crystallized receptors an arginine, which is conserved throughout the nuclear receptor family, makes a hydrogen bond to the 3-OH substituent of estradiol (ERα), the 3-keto group of progesterone (PR), and the corresponding hydroxyl position in genistein and raloxifene (ERβ). The other amino acid involved is a glutamine in PR and a glutamate in ERα and ERβ. Because all steroid receptors binding ligands with a 3-keto containing A-ring (PR, androgen receptor, MR, and GR) have a glutamine in the corresponding position, A-ring binding selectivity is probably determined by the presence of a glutamate or a glutamine at this position.

The D-ring, which is anchored at the opposite end of the ligand binding pocket, shows a greater variability of its substituents between steroids, and the amino acids of different receptors interacting with the D-ring also seem to be more variable. Of six amino acids identified to interact with the D-ring of estradiol in ERα, four amino acids at the corresponding positions in PR interacted with the D-ring of progesterone, none of which were conserved between the receptors (15, 16,
Steroid-interacting Amino Acids of Glucocorticoid Receptor

22). The D-ring interactions are thus likely to be involved in binding specificity.

The key feature at the D-ring of most glucocorticoids is a 17β side chain containing a 20-carbonyl and a 21-OH group likely to be engaged in hydrogen bonding. Many glucocorticoids also contain additional substituitions at carbon 16 and 17, for example methyl, hydroxyl or 16α,17α-acetone groups, that could be involved in specific interactions.

To identify possible interactions between steroids and receptors whose structures are not available, homology models based on resolved crystal structures for other receptors can be built.

We have developed a homology model of the GR LBD based on the ER LBD crystal structure (16), in which experimental binding affinity data of several ligands were correlated to calculated binding affinity data to create an optimal model.

To investigate the interactions between glucocorticoid receptor and the D-ring substituents of various glucocorticoids, we have in this paper performed site-directed mutagenesis of four amino acids (Met-560, Met-639, Gln-642, and Thr-739) likely to interact with substituents on the D-ring of the ligand as deduced from the homology model. The homology model was subsequently revised to accommodate the functional data in an optimal manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]TA was obtained from NEN Life Science Products, unlabeled steroids from Sigma, and cell culture medium, fetal bovine serum, and penicillin-streptomycin from Life Technologies, Inc.

**Plasmids**—The vector pCMV-hGR expressing hGR is described elsewhere (23), and the reporter vector p19-luc-TK, containing two glucocorticoid response elements upstream of a truncated thymidine kinase promoter linked to the luciferase gene, was a kind gift from Paul T. van der Saag (Hubebrcht Laboratory, Netherlands Institute for Developmental Biology) and is a modified version of pG29LtkCAT (24).

**Site-directed Mutagenesis**—Site-directed mutagenesis of pCMV-hGR according to the refined method of Kunkel (25,26) was used to construct the mutants. The mutant plasmids were transformed into Escherichia coli by electroporation, minipreps of DNA (Wizard miniprep, Promega, Madison, WI) were made, andideoxy sequencing was performed to confirm the mutations.

**Mammalian Cell Culture and Transfection**—COS-7 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, penicillin-streptomycin (100 μg/ml), and 1% Triton X-100. Luciferase activity was measured in 30 μl of culture medium, supplemented with 100 μl of lysis buffer (25 mM Tris acetate, pH 7.8, 2 mM dithiothreitol, 1.5 mM EDTA, 10% glycerol, and 1% Triton X-100). Luciferase activity was measured in 30 μl of extract in a Bioorbit 1253 luminometer using the Genglow kit (Bioorbit). The results are expressed as light units measured. All assays were performed in triplicate using three separate plates of transfected cells.

**Statistical Analysis**—Analysis of variance was carried out using the Newman-Keuls test using the program STATISTICA for Windows (StatSoft, Inc., Tulsa, OK). Statistical analysis of binding data and transactivation data was carried out for each individual series of experiments corresponding to one particular site of mutation.

**GR Homology Models**—Initial multiple sequence alignments of the ligand binding nuclear receptor sequences were obtained using the Pileup program from the GCG program package (27) (available from Oxford Molecular, Oxford,OX4 4GA, UK). For semi-automated homology modeling, Modeler (28), as supplied with Quanta96 (Modeler, Santa, and CHARMm; Molecular Simulations, Inc., San Diego, CA) was run using the no optimization option with the human ER-a LBD/estriadiol complex x-ray crystallographic structure (16) (Protein Data Bank accession number 1ERE) as the template and the human glucocorticoid receptor primary sequence (Swiss-Prot accession number P04150) as target (29).

The initial model was constructed using molecular dynamics/mechanics. Hydrogen atoms were added to the homology model using the HBUILD routine in CHARMM (30). Sodium and chloride counterions were placed in the maximum likelihood of the protein electrostatic potential near charged amino acid residues so as to achieve net neutrality of the system. The C and N termini were made neutral.

The three-dimensional molecular model of QUANTA 96 was used to build the various glucocorticoids. The constructed glucocorticoids were minimized in vacuo using Gasteiger-Huckel charges and a dielectric constant of 78. Partial atomic charges for the resulting structures were calculated by fitting the water-accessible surfaces of the molecules to their 6–31G* electrostatic potentials according to Singh and Kollman (30), as implemented in Gaussian 94 (Gaussian, Inc., Carnegie, PA). The 6–31G* ESP charges were used for the ensuing protein-ligand interaction studies.

The fit of dexamethasone in the binding site with the lowest ligand-protein interaction energy after minimization of various explored alternative starting orientations was chosen as an initial conformation for subsequent molecular dynamics. The minimization was carried out within CHARMM and started with 200 initial cycles of steepest descent and continued by the adopted-basis-Raphson algorithm until the root mean square energy gradient was less than 0.01 kcal/A. The all-atom force field and parameters as implemented in QUANTA 96 were used. The nonbonded interactions were cut-off beyond 10 Å. The van der Waals and electrostatics functions were applied between 11 and 14 Å. The default heuristic nonbonded list update method and a distance-dependent dielectric function (scaled with 1/r) were used. The protein-ligand interaction energy actions were then required calculated for each resulting minimized conformation. The system was subjected to molecular dynamics using the Verlet and Shake algorithms (41,42) using the same conditions, with 2 ps of dynamics, after minimization was followed by 11 Å solvent cap of transferable intermolecular potential 3 waters (43) centered on the ligand for the dynamics simulation (31). The initial dynamics simulation was for 10 ps using a step size of 0.01 followed by 60 ps with a stop size of 0.02. The solvent cap was then removed, and the remaining dexamethasone-GR complex structure resulting from the final trajectory after 70 ps of dynamics was energy-minimized using the same constraints as described above and thereafter used for energy-minimization with other ligands instead of dexamethasone.

Following the functional analysis of the effects of the mutations, a revised model of GR LBD based on the initial ER-derived model was constructed. Torsion angles of amino acid side chains were assigned using SCWRL 2.1 (University of California, San Francisco, CA) (32) holding conserved amino acid residues fixed (f=option) and using the dexamethasone-GR ligand extracted from the 1ERE crystallographic structure as a steric constraint (f=option). The backbone of the 1ERE template structure and the preliminary GR homology model were left squares fit using Sybyl 6.6 (Tripos Associates, St. Louis, MO), and the crystallographically determined water molecules and the estradiol ligand were copied from the 1ERE structure to the initial ER basic GR homology model (atoms hydrogen, oxygen, and sulfur atoms) were added using Sybyl. The N- and C-terminal residues and charged amino acid side chains not involved in salt bridges were neutralized (with the exception of Arg-611) by adding or subtracting hydrogen atoms using MacroModel 7.0 (Schrodinger, Inc, Jersey City, NJ) (33). The estradiol ligand was then "mutated" to triamcinolone acetonide (TA), and the structure of the ligand was minimized.
Steroid-interacting Amino Acids of Glucocorticoid Receptor

Fig. 1. Structures of the steroids used in this study. 

RESULTS

In an initial homology model of hGR LBD the four amino acids Met-560, Met-639, Gln-642, and Thr-739 were located at the surface of the steroid-binding pocket and have the opportunity to interact with the 20-carboxyl, the 17-OH, the 16-oxygen, and the 21-OH group of the D-ring of the steroid, respectively (for steroid structures see Fig. 1). To elucidate the role of these amino acids in ligand binding, site directed mutagenesis was performed, and the mutants were characterized with regard to binding and transactivation. Generally, amino acid substitutions were chosen to be as conservative as possible, but such that they would lead to disruption of the potential specific interactions with the ligand (e.g. hydrogen bond or electrostatic interaction) as deduced from the model. In some cases, alanine mutants were also created to mimic removal of the particular amino acid side chain. The assumption was made that if an amino acid interacts with a specific group on the steroid, mutation of this amino acid would decrease the affinity of the receptor only for steroids containing this group. The affinity for ligands containing different functional groups was estimated by competitive binding assays.

Mutation of Gln-642—In the initial model, the amide nitrogen of Gln-642 appeared to make a hydrogen bond (distance 3 Å) to the 16-oxygen of triamcinolone acetone, desonide, and triamcinolone. To investigate the role of Gln-642 in steroid binding we first created mutants Q642A and Q642V. As seen in Table I (Gln-642, Series 1), Q642A had an affinity for TA similar to that of wild type, whereas Q642V had a slightly but significantly reduced affinity. A more substantial loss in affinity might have been expected if the interaction with the 16-oxygen atom was important. However, ether oxygen atoms are not very strong hydrogen bond acceptors, and van der Waals’ interactions of the receptor with the hydrophobic acetone moiety of TA may largely compensate for the loss of a hydrogen-bonding interaction.

To examine whether the specificity of the mutant receptors for other steroids was affected, the affinity for a range of steroids containing different functional groups was determined in binding competition assays (Fig. 2). Interestingly, both Q642A and Q642V had a clearly reduced affinity for the steroids cortisol, 9α-fluorocortisol, prednisolone, triamcinolone, and dexamethasone, containing a 17α-OH group as a common feature (Fig. 2, A–E), whereas the affinity for desonide, corticosterone, and deoxycorticosterone, lacking the 17α-OH group, was unaltered (Fig. 2, F–H), or even somewhat enhanced for the Q642V mutant (deoxycorticosterone and corticosterone). These interesting specificity changes suggested to us that there might be a direct interaction between the Gln-642 side chain and the 17α-OH group and that the shorter and more hydrophobic alanine and valine were not able to make this interaction.

Therefore, to further examine the role of Q642 in steroid binding, we created two additional mutants, Q642E and Q642N, having side chains more similar in size and composition to glutamine. Like the alanine and valine mutants, Q642N had almost similar affinity for TA as wild type, whereas the Q642E mutant displayed a significantly reduced affinity (Table I, Gln-642, Series 2).

Similar to the alanine and valine mutants, both Q642E and Q642N had a severe reduction in affinity for the steroids containing a 17α-OH group (Fig. 3, A–D), with the exception of dexamethasone, where a milder reduction was seen (Fig. 3E). Both mutants, however, also had a slightly reduced affinity for steroids lacking a 17α-OH group (Fig. 3, F–H). In the case of Q642E the loss in affinity for desonide, corticosterone, and deoxycorticosterone was similar to the loss in affinity for TA, because the binding curves were overlapping. A summary of the steroid binding specificity of Gln-642 mutants as measured by competition assays, and analysis by log-logit plots is shown in Table II. Statistical analysis of the data was not performed because of the relatively low number of analyses for each specific set of criteria. However, clear trends can be identified in the data presented.

Transactivation studies, following transient expression of Gln-642 mutants, were carried out using various concentrations of TA. Interestingly, there was a varied degree of coupling between changes in affinity and changes in transactivation sensitivity for the four mutants studied. As seen in Fig. 4A and Table I, no difference in transactivation sensitivity was detected with mutant Q642V, even though the affinity of this mutant for TA was slightly reduced (p < 0.05). In contrast, the Q642A mutant showed an increase in transactivation sensitivity with an EC50 of around four times less than that of wild type despite similar binding affinity. This might indicate an active role for Gln-642 in the steroid-dependent conformational change in its immediate environment and the formation of the transactivating surface. As expected, Q642E, which had reduced binding affinity, was clearly less sensitive in the transactivation assay (Fig. 4B and Table I) having an EC50 of more than 50 times that of wild type. No significant difference was seen in the transactivating sensitivity of the mutant Q642N compared with wild type (Fig. 4B and Table I).

Mutation of Thr-739—The hydroxyl group of Thr-739 appeared to make a hydrogen bond (distance < 3 Å) to the 21-OH group of the steroid in the initial model. The potential function of this residue was tested by mutation to alanine and valine.
There was no significant change in binding affinity for [3H]TA with either of the mutants (Table I). Binding specificity of these mutants for corticosterone, deoxycorticosterone, and 11β-OH progesterone was analyzed by competition assay to test the possible interaction of Thr-739 with the 21-OH group of the steroid (Table III). The steroids selected also resulted in an

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**TABLE I**

| Amino Acid | Series 1 | Series 2 |
|------------|----------|----------|
|            | K<sub>d</sub> (nM) | K<sub>d</sub> relative to wild type | EC<sub>50</sub> (nM) | EC<sub>50</sub> relative to wild type |
| Gln-642, | Wild type GR | 0.562 ± 0.074 (n = 5) | 1.11 ± 0.085 | 0.206 ± 0.074 (n = 5) |
|           | Q642A     | 0.734 ± 0.079 (n = 5) | 1.36 ± 0.157 | 0.48 ± 0.032 (n = 5)<sup>a</sup> |
|           | Q642V     | 0.890 ± 0.165 (n = 5)<sup>a</sup> | 1.69 ± 0.060 (n = 5) | 0.861 ± 0.284 |
| Gln-642, | Wild type GR | 0.366 ± 0.128 (n = 4) | 1.26 ± 0.12 (n = 3) | 0.267 ± 0.12 (n = 3) |
|           | Q642E     | 1.48 ± 0.751 (n = 3)<sup>a</sup> | 3.73 ± 0.416 | 13.8 ± 3.0 (n = 3)<sup>a</sup> |
|           | Q642N     | 0.534 ± 0.146 (n = 4) | 1.54 ± 0.427 | 0.91 ± 0.38 (n = 3) |
| Thr-739, | Wild type GR | 0.473 ± 0.222 (n = 9) | 1.72 ± 0.144 (n = 4) | 15.9 ± 12.8 |
|           | T739A     | 0.578 ± 0.227 (n = 8) | 1.38 ± 0.32 | 1.65 ± 0.276 (n = 4)<sup>a</sup> |
|           | Met-560   | 0.727 ± 0.178 (n = 4) | 1.33 ± 0.19 | 0.394 ± 0.238 (n = 4) |
|           | M560L     | 0.506 ± 0.220 (n = 4) | 0.425 ± 0.317 (n = 3) | 31.25 ± 0.071 |
|           | M560T     | 0.851 ± 0.576 (n = 4) | 1.53 ± 0.49 | 0.225 ± 0.141 (n = 2) |
|           | Met-639   | 0.794 ± 0.353 (n = 4) | 1.58 ± 0.14 | 8.2 ± 1.046 (n = 2)<sup>a</sup> |
|           | Wild type GR | 0.601 ± 0.125 (n = 3) | 0.437 ± 0.208 (n = 2) | 28.7 ± 14.0 |
|           | M639V     | 1.36 ± 0.286 (n = 3)<sup>a</sup> | 2.27 ± 0.31 | 11.1 ± 0.141 (n = 2)<sup>a</sup> |

<sup>a</sup> Significantly different from wild type GR (p < 0.05).
Steroid-interacting Amino Acids of Glucocorticoid Receptor

Relative binding affinity was determined by competition binding assay. IC\(_{50}\) values (nM) were determined by log-logit plots of the competition binding data. Binding affinity relative to wild type GR is also given.

## Table II

| Steroid          | Wild type GR | Q642A | Q642V | Q642E | Q642N |
|------------------|--------------|-------|-------|-------|-------|
| Cortisol         | 51.0 ± 21.5  | >300  | >300  | >300  | >300  |
| 9α-F cortisol    | 10.2 ± 7.1   | >100  | >100  | >100  | >100  |
| Prednisolone     | 17.6 ± 5.8   | >100  | >100  | >100  | >100  |
| Triamcinolone    | 21.4 ± 3.9   | >100  | >100  | >100  | >100  |
| Dexamethasone    | 8.45 ± 2.9   | >100  | >100  | >100  | >100  |
| Desonide         | 11.1 ± 5.0   | >12   | 8     | 11    | 2     |
| Corticosterone   | 40.7 ± 15.4  | 55 ± 8| 21    | 18    | 12    |
| Deoxycorticosterone | 63.2 ± 29   | 76 ± 35| 8    | 11    | 12    |

## Table III

| Steroid          | Wild type GR | T739A | T739V |
|------------------|--------------|-------|-------|
| Corticosterone   | 17.3 ± 15.7  | 50.9 ± 44.7 | 23.4 ± 13.9 |
| Deoxycorticosterone | 20.4 ± 8.7  | 93.9 ± 7.1  | 12.0 ± 8.0  |
| 11β-OH progesterone | 35.6 ± 27.5  | 41 ± 28.1  | 16.3 ± 15.0  |

Fig. 4. Dose response of Gln-642 mutants in transactivation induced by TA. COS-7 cells were transfected with wild type or mutant GR together with a luciferase reporter system and incubated with a range of concentrations of triamcinolone acetonide. Luciferase activity is expressed relative to the maximum level of activity induced. For average IC\(_{50}\) values see Table I. Wt, wild type.

Fig. 5. Steroid binding specificity of Thr-739 wild type and mutant GR. Relative binding affinity was determined by competition binding assay. IC\(_{50}\) values (nM) were determined by log-logit plots of the competition binding data. Binding affinity relative to wild type GR is given.

**A** Relative luciferase activity vs. [TA] (nM).

**B** Relative luciferase activity vs. [TA] (nM).

**C** Relative luciferase activity vs. [TA] (nM).

analysis of the function of the 11β-OH group. The alanine mutant had a reduction in relative affinity for corticosterone and deoxycorticosterone (Fig. 5, A and B) but not for 11β-OH progesterone (Fig. 5C), supporting the hypothesis of an interaction between Thr-739 and the 21-OH group. The T739V mutant, on the other hand, did not display the same specificity change. There was no relative change in affinity for corticosterone, whereas there was a small relatively increased affinity for the more hydrophobic ligands deoxycorticosterone and 11β-OH progesterone (Table III and Fig. 5).

In the transactivation assay, T739A had an EC\(_{50}\) similar to that of wild type, whereas T739A was significantly less sensitive to TA having an EC\(_{50}\) 16 times higher than that of wild type (Table I). This is in clear contrast to the lack of significant change in affinity to TA for the T739A mutant. Thus, Thr-739 appears to play an active role in the signal transduction from hormone to the transactivating surface of the receptor.

**Mutation of Met-560**—In the initial model, the sulfur of Met-560 made a putative favorable electrostatic interaction (distance, <3 Å) with the 20-carbonyl oxygen of the steroid. Such nonbonded sulfur-nucleophile close contacts have been reported earlier in the crystallographic literature (35–37). Two mutants were created; the relatively conservative M560L and the less conservative, more polar M560T. As seen in Table I, two such nonbonded sulfur-nucleophile close contacts have been reported earlier in the crystallographic literature (35–37). Two mutants were created; the relatively conservative M560L and the less conservative, more polar M560T. As seen in Table I, the relative sensitivity for TA is also significantly affected for either mutant. In the transactivation assay with TA, however, M560T was clearly less sensitive than wild type (Table I) despite similar binding affinity, perhaps indicating that M560T affects the AF-2 domain. M560L had a sensitivity for TA similar to that of wild type in the transactivation assay (Table I). This is in clear contrast to the lack of significant change in affinity to TA for the T739A mutant. Thus, Thr-739 appears to play an active role in the signal transduction from hormone to the transactivating surface of the receptor.

Another possible interaction of Met-560 with the 17β side chain of the steroid D-ring was investigated by competition assay with corticosterone and 11β-OH progesterone (Fig. 6). M560L had the same relative affinity for both ligands as wild type GR, whereas M560T had greatly reduced affinity for both ligands. Thus, there is no correlation with the presence of a 21-OH group in the steroid or not. Instead, the results indicate a more general hydrophobic interaction between Met-560 and...
the ligand, which is apparently lost by replacing methionine with the smaller and more hydrophilic threonine. Replacing methionine with the slightly more hydrophobic leucine appears to maintain this interaction.

Mutation of Met-639—in the initial model, Met-639 potentially interacts with the 17α-OH group of the steroid (distance, 4–5 Å). We mutated this amino acid to the smaller and slightly more hydrophilic valine residue. M639V had significantly lower affinity for [3H]TA and was much less sensitive in the transactivation assay with TA (Table I). To test the role of the 17α-OH group, binding competition assays with cortisol and corticosterone were carried out (Fig. 7). M639V had a decreased affinity for both steroids to a similar degree, independent of the presence of 17α-OH. Thus, Met-639 clearly plays an active role in steroid binding, although the nature of the interaction remains unclear.

Mutation of Asn-564—in our model only Thr-739 seemed to make an interaction with the 21-OH group. However, in a homology model of the closely related MR, in addition to the corresponding amino acid to Thr-739 (MR Thr-945), the amino acid corresponding to Asn-564 (MR Asn-770) could interact with the 21-OH group, which was also supported by functional analysis (38). In our initial model Asn-564 was quite far from the steroid with the closest distance being 4–6 Å to the 11β-OH group, depending on which steroid is docked. To test whether there was any interaction with the 21-OH of the steroid in GR as described for MR or alternatively with the 11β-OH, we mutated Asn-564 to alanine and valine. Both mutations decreased binding of [3H]TA to a level where binding affinity was hard to determine (data not shown). Transactivation assays with 11β-OH progesterone and deoxycorticosterone were performed to test the possible interaction with either the 21-OH or 11β-OH groups, respectively. No activity could however be detected with either mutant (N564A and N564V), after the addition of up to 1 μM 11β-OH progesterone or 10 μM deoxycorticosterone, in contrast to wild type GR that showed an 11–12-fold induction (data not shown).

Thus, mutation of Asn-564 destroys some important interaction or destroys the ligand-binding site in GR, whereas mutation of Asn-770 in MR to alanine reduced binding only of 21-OH containing steroids (38). In our GR model Asn-564 could make a hydrogen bond to Glu-748 (helix 12), which might stabilize the structure of the LBD.

DISCUSSION

The recently resolved crystal structures of the ligand binding domains of the ERα, ERβ, and PR showed that whereas the A-ring seems to be anchored in a similar manner, the interactions of the steroid with the D-ring seem to be more varied (15, 16, 22). This correlates well with the fact that many steroid specificity determinants are found in the D-ring. The crystal structure of the hGR LBD is not yet resolved, and to identify possible interactions between steroid and receptor we created a homology model of GR LBD derived from the estrogen receptor crystal structure. To investigate possible interactions with the D-ring of glucocorticoids, we have performed site directed mutagenesis of Met-560, Met-639, Gln-642, and Thr-739, which in the initial model made hydrogen bonds or electrostatic interactions with substituents of the D-ring of the steroid. In addition Asn-564 was mutated, although not interacting with the ligand in our initial model (4–6 Å from 11β-OH), because the corresponding amino acid in a model of MR interacted with the 21-OH group (38). The equivalent residues in the known crystal structures were shown to interact with the steroid in one or more cases (Table IV).

In our model the orientation of the steroid in GR is the same as the orientation described in the three published structures and in contrast to the model postulated by Wurtz et al. (39). This orientation entails the anchoring of the A-ring of the steroid by hydrogen bonding between the the 3-keto group and Gln-570 and Arg-611, which results in the positioning of Gln-642 in proximity of the D-ring of the steroid. Mutation of Gln-642 and the resulting change in steroid specificity show no correlation to structures in the A-ring (A1; compare prednisolone and cortisol, Table II) or the B-ring (9α-F; compare 9α-F cortisol and cortisol, Table II). The affinity for both corticosterone and deoxycorticosterone, which differ only with regard to the 11β-OH group in the C-ring, was slightly increased for Q642A and Q642V and slightly decreased for Q642E and Q642N. Thus, Gln-642 does not seem to correlate to structures in the C-ring either. However, there are clear changes in steroid specificity for the mutants studied, related to specific structures in the D-ring of the steroid (Tables II and III and Figs. 2–6). Thus, the similarity of the orientation of the steroid in GR LBD in comparison with ER and PR can be confirmed functionally. All the sites of mutations studied affected binding specificity and/or affinity (Tables I–III), thereby indicating that these residues are probably located in close proximity to the ligand. In two cases (T739A and M560T), there was a significant decrease in transactivating sensitivity induced by TA without any significant change in binding affinity (Table I). The mutations Q642E and M639V significantly reduced both relative binding affinity and transactivating sensitivity for TA. Of particular interest are the mutations Q642A, which demonstrated a significant 4-fold increased transactivating sensitivity toward TA without any change in affinity, and Q642V, which demonstrated a slightly decreased affinity for TA (p < 0.05) without any significant change in transactivating sensi-
Steroid-interacting Amino Acids of Glucocorticoid Receptor

TABLE IV
Comparative residues in steroid receptors equivalent to GR sites of mutation in this study

| GR residue | Helix position | Putative interaction in initial GR model | Equivalent residue in steroid receptor |
|------------|----------------|----------------------------------------|---------------------------------------|
| GR         | Ta            | ERα                | ERβ                | PR                  | MR | AR |
| Met-560    | 3             | 20-O               | Met-343            | Met-295             | Leu-715 | Leu-766 | Leu-701 |
| Asn-564    | 3             | (11β-OH)           | Treminderino-299   | Treminderino-299   | Asn-719 | Asn-770 | Asn-705 |
| Met-639    | 7             | 17α-OH             | Thr-347            | Thr-299             | Asn-719 | Asn-770 | Asn-705 |
| Gln-642    | 7             | 16-O               | Ile-424            | Ile-373            | Phe-794 | Met-845 | Met-780 |
| Thr-739    | 11            | 21-OH              | Met-528            | Met-479            | Thr-894 | Thr-945 | Leu-880 |

*a* Interacts with 17β-estradiol in the crystal structure of ERα LBD (16, 22).  
*b* Interacts with genistein in the crystal structure of ERβ LBD (17).  
*c* Interacts with progesterone in the crystal structure of PR LBD (15).  
*d* Specifically interacts with the antagonist raloxifene in ERα/ERβ (17).  
*e* Functional indication of interaction with 21-hydroxyl group in MR model (30).  

...tivity (Table I). Thus, the residues at these positions appear to be playing roles in both steroid recognition and binding as well as in the continued steroid-dependent induction of transactivating activity.

Mutation of Gln-642 resulted in very clear changes in binding specificity (Figs. 2 and 3 and Table II). All four mutants at this position showed a clear decrease in affinity for steroids containing a 17α-OH group (cortisol, 9α-F cortisol, prednisolone, triamcinolone, and dexamethasone; Fig. 1). The direct correlation with the 17α-OH group is most clearly seen by comparing the IC₅₀ values for cortisol and corticosterone (Figs. 2, A and G, and 3, A and G, and Table II). The presence of a 16α-CH₃ group greatly reduced the effect of mutation with regard to the negative correlation with the 17α-OH group as seen when comparing IC₅₀ values for dexamethasone and prednisolone (Figs. 2, C and E, and 3, C and E, and Table II) especially for the mutants Q642E and Q642N. Also, the presence of a 16α-OH group had a weakly protective effect (compare prednisolone and triamcinolone, Fig. 3, C and D, or 9α-F cortisol and triamcinolone, Fig. 3, B and D), with regard to the binding specificity for Q642E and Q642N. The presence of 16α,17α-acetonide resulted in relatively minor effects of Gln-642 mutation on steroid binding (compare desonide and prednisolone, Figs. 2, C and F, and 3, C and F, and Table II, and TA and triamcinolone, Table I and II.). In both these cases, the mutant Q642E had a reduced affinity for 16α,17α-acetonides, although this effect was much less dramatic than the negative correlation with the 17α-OH group. Finally, mutation of Gln-642 resulted in various effects on the relative affinity for corticosterone and deoxycorticosterone (Figs. 2, G and H, and 3, G and H, and Table II). The mutant Q642V, with a more hydrophobic substituent at this position, resulted in increased relative affinity for these two steroids. The mutant Q642N, with a shorter side chain at this position, resulted in a relatively decreased affinity for corticosterone and deoxycorticosterone.

Removal of the side chain (Q642A) or the introduction of a more polar, charged side chain of the same length (Q642E) had a neutral effect with regard to the relative affinity for these two steroids. However, Q642E had a generally reduced affinity for all steroids including TA (Table I).

Thus, the interaction of Gln-642 with the D-ring of the steroid is complex. In the initial model, the N-terminal group of this side chain is 3 Å from the 16-O atom, 5–6 Å from the 17-O atom in TA, and 4 Å from the 16α-CH₃ group in dexamethasone. Although there is a very strong correlation between the effect of mutation at this position and the presence of a 17α-OH group, there does not appear to be any possibility for direct interaction between these two groups. The introduction of a more hydrophobic group (Val) results in an energetically favorable interaction with steroids that are relatively hydrophobic in the 16 and 17 positions (corticosterone and deoxycorticosterone) and an energetically unfavorable interaction with steroids with polar substituents at these positions. Thus, the spatial distribution of polar groups within this region of the steroid-binding surface centered around position 642 as well as hydrophobic interactions appear to play important roles in steroid recognition and binding.

In the initial GR LBD model, Thr-739 was hypothesized to form a hydrogen bond with the 21-OH of glucocorticoids. The distance between the oxygen in Thr-739 and the 21-OH group was <3 Å. Mutation to the smaller alanine resulted in reduced affinity for corticosterone and deoxycorticosterone (Fig. 5 and Table III), both of which have a 21-OH group. However, T739A bound 11β-OH progesterone with unchanged affinity. Thus, there is clear functional evidence for a hydrogen bond as indicated in the model. Mutation of the threonine to the more hydrophobic valine also resulted in a change in specificity (Table III). In this case, T739V had increased affinity for the more hydrophobic steroids deoxycorticosterone and 11β-OH progesterone but unchanged affinity for corticosterone. In contrast to T739A, there was no correlation to a specific hydroxyl group within the steroid but rather a correlation to the number of hydroxyl groups (one instead of two). The increased hydrophobic interaction with valine could compensate for the loss of the hydrogen bond to 21-OH with threonine. The distance between the C₅ of Thr-739 and C-21 of the steroid is 4–5 Å. Thus there are possibilities for hydrophobic interactions between this residue and the side chain of the steroid, in addition to the hydrogen bond with 21-OH. The γ methyl of the corresponding residue in MR, Thr-945, was suggested to make van der Waals’ interactions with the 20 and 21 positions of the steroid. Similar to our findings in this study, T945A mutation of MR resulted in reduced affinity for 21-OH containing steroids (38). Threonine is conserved in this position in GR, MR, and PR whose cognate steroids all contain the 17β side chain (C₂₁ steroids). In contrast, the corresponding residues in ER and androgen receptor are methionine and leucine, respectively (Table IV). Thus, Thr-739 appears to play an important role in differentiating between the different structures of the 17β side chain of the steroid.

The result of the mutagenesis of Met-560 and Met-639 indicates that general hydrophobic interactions between these amino acids and the steroid might be more important than the specific interactions suggested by the model. It is known that hydrophobic interactions are important for high receptor binding affinity of steroids, whereas hydrogen bonds might provide ligand-binding specificity (40). Mutagenesis of Met-560 to leucine did not affect binding affinity for any of the tested ligands (Table I and Fig. 6), whereas mutation to threonine affected binding of corticosterone and 11β-OH progesterone (Fig. 6). Met-560 might thus make hydrophobic interactions with the steroid, which are maintained by the relatively hydro-
phobic leucine but destroyed in the presence of the smaller and more polar threonine. Met-560 was in close proximity of the 20 and 21 positions in the initial model. The corresponding residues in ER and PR (Table IV) made hydrophobic contacts with the D-ring of the steroid as inferred from the corresponding crystal structure. That the affinity for TA was not significantly affected for M560T (Table I) might result from the fact that TA, because of its acetonide groups, makes other contacts or additional contacts within the ligand-binding pocket compared with corticosterone and 11β-hydroxyprogesterone. Despite almost similar binding affinity for TA, the sensitivity of M560T for TA in the transactivation assay was significantly reduced (Table I), suggesting that M560T affects the AF-2 site.

Mutation of Met-639 to the smaller valine resulted in reduced affinity for all ligands (Table I and Fig. 7), as well as reduced transactivating properties of Met-639 was located closest to the 17α-OH group in cortisol in the initial GR LBD model (distance 4–5 Å). However, our results indicate no correlation to the 17α-OH but instead indicate a possible role of hydrophobic interactions. In the case of dexamethasone and TA, there is a possibility that Met-639 makes hydrophobic interactions with the 16α-CH₃ or the acetonide-CH₃, respectively (distance from C, 3–4 Å).

Following the results of the functional analysis of the mutations, a revised model of GR LBD bound to TA was constructed using Sybyl and MacroModel (Fig. 8). In this revised model, the side chains of Gln-642, Thr-739, and Asn-564 are in closer proximity to the steroid (2.0, 2.0, and 3.2 Å, respectively). Gln-642 is hydrogen bonded to the 16α-oxygen of the acetonide group, and Thr-739 is hydrogen bonded to the 21-hydroxyl group, both of which agree with the functional data obtained in this study. Compared with the initial model obtained by molecular dynamics, Asn-564 is located closer to the 11β-hydroxyl group and could now form a weak hydrogen bond. Because no binding or transactivation was obtained with the Asn-564 mutants tested in this study, this aspect of the model cannot be further evaluated at this stage. Finally, in the revised model, Met-560 and Met-639 are located further away from the steroid compared with the initial model. Also, this agrees with the functional data obtained because less specific effects were seen with mutations of these two residues, indicative of hydrophobic interactions in the first hand. In the revised model, Met-560 is located 3.9 Å from the 20-carbonyl and 3–4 Å from the 16 and 17 substituents. Met-639 is distant from the 17α-hydroxyl group (5.5 Å) but only 3.6 Å from one of the acetonide methyl groups.

In conclusion, there is an active interaction between a number of residues and the D-ring of the steroid. In the case of GR, Gln-642, Thr-739, Met-560, and Met-639 all appear to play an active role in the recognition of this part of the steroid and thereby steroid binding specificity. Mutation of a number of these residues affected TA-dependent transactivation rather than binding affinity. This would indicate that there is a marked degree of plasticity in this region of the receptor and that these residues play an active role in the steroid-dependent conformational change of the protein, resulting in the formation of the AF-2 site. The residues corresponding to the sites of mutation in this study have all been shown to play an active role in interaction with the steroid ligand in the crystal structures published. However, there is a receptor-specific combination of different residues at these positions that interact with and recognize the specific steroid ligand (Table IV). In addition, there is a difference between the role of some of these residues in ER in the interaction with agonist as compared with antagonist. The role of these residues in GR will be more clear when the crystal structure of GR LBD has been solved.

REFERENCES

1. Evans, R. M. (1988) Science 240, 889–895
2. Mangeldal, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Marks, M., and Chambon, P. (1995) Cell 83, 835–839
3. Beato, M., Herrlich, P., and Schütz, G. (1995) Cell 83, 851–857
4. Carlstedt-Duke, J., Strömstedt, P.-E., Wrang, O., Bergman, T., Gustafsson, J.-Å., and Jornvall, H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4437–4440
5. Pratt, W. B., Hutchison, K. A., and Scherer, L. C. (1992) Trends Endocrinol. Metab. 3, 326–333
6. Picard, D., and Yamamoto, K. R. (1987) EMBO J. 6, 3333–3340
7. Hollenberg, S. M., and Evans, R. M. (1988) Cell 55, 899–906
8. Daniels, M., Northrop, J. P., Jonklaas, J., and Ringold, G. M. (1987) Mol. Endocrinol. 1, 816–822
9. Godowski, P. J., Picard, D., and Yamamoto, K. R. (1988) Science 241, 812–816
10. Hollenberg, S. M., Giguere, V., Segui, P., and Evans, R. M. (1987) Cell 49, 39–46
11. Wolfe, R. T., Wisse, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurakawa, R., Rosenfeld, M. G., Williams, T. M., Glass, C. K., and Milburn, M. V. (1988) Nature 335, 137–143
12. Renaud, J.-P., Rochel, N., Ruff, M., Herrlich, P., Chambon, P., and Moras, D. (1995) Nature 378, 681–689
13. Bourguet, W., Ruff, M., Chambon, P., Grumey, H., and Moras, D. (1995) Nature 375, 377–382
14. Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fleiterick, R. J. (1995) Nature 378, 690–697
15. Williams, S. P., and Sigler, P. B. (1998) Nature 399, 392–396
16. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bohn, T., Engström, O., Ohman, L., Greene, G. L., Gustafsson, J.-Å., and Carlquist, M. (1997) Nature 389, 753–758
17. Pike, A. C. W., Brzozowski, A. M., Hubbard, R. E., Bohn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J.-Å., and Carlquist, M. (1999) Nature 395, 137–143
18. Renda, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1997) Nature 378, 681–689
Steroid-interacting Amino Acids of Glucocorticoid Receptor

10. Singh, U. C., and Kollman, P. A. (1984) J. Computat. Chem. 5, 129–145
11. Brooks, C. L., III, Brunger, A., and Karplus, M. (1985) Biopolymers 24, 843–865
12. Bower, M. J., Cohen, F. E., and Dunbrack, R. L., Jr. (1997) J. Mol. Biol. 267, 1258–1282
13. Mohamadi, F., Richards, N. G. J., Guida, W. C., Liskamp, R., Caufield, C., Chang, G., Hendrickson, T., and Still, W. C. (1990) J. Comput. Chem. 11, 450–467
14. McDonald, D. Q., and Still, W. C. (1992) Tet. Lett. 33, 7743–7746
15. Burling, F. T., and Goldstein, B. M. (1992) J. Am. Chem. Soc. 114, 2313–2320
16. Rosenfield, E. E., Parthasarathy, R., and Dunitz, J. D. (1977) J. Am. Chem. Soc. 99, 4860–4862
17. Burling, F. T., and Goldstein, B. M. (1993) Acta Crystallogr. Sect. B Struct. Sci. 49, 736–748
18. Fagart, J., Wurtz, J. M., Souque, A., Hellallevy, C., Moras, D., and Rafestinoblin, M. E. (1998) EMBO J. 17, 3317–3325
19. Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Chambon, P., Moras, D., and Gronemeyer, H. (1996) Nat. Struct. Biol. 3, 87–94
20. Wolff, M. E., Baxter, J. D., Kollman, P. A., Lee, D. L., Kuntz, I. D., Bloom, E., Matulich, D. T., and Morris, J. (1978) Biochemistry 17, 3201–3208
21. Verlet, L. (1967) Physiol. Rev. 59, 88–105
22. Ryckaert, J.-P., Cicotti, G., and Berendsen, H. J. C. (1977) J. Comput. Phys. 23, 327–341
23. Jorgensen, W. L., Chandrasekhar, J., and Madura, J. D. (1983) J. Chem. Phys. 79, 926–935

EMBO J. 18, 4608–4618

18. Feng, W., Ribeiro, R. C., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998) Science 280, 1747–1749
19. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallep, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Genes Dev. 12, 3343–3356
20. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) Cell 95, 927–937
21. Mak, H. Y., Hoare, S., Henttu, P. M., and Parker, M. G. (1999) Mol. Cell. Biol. 19, 3895–3903
22. Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5998–6003
23. Lind, U., Carlstedt-Duke, J., Gustafsson, J.-Å., and Wright, A. P. (1996) Mol. Endocrinol. 10, 1358–1370
24. Schule, R., Muller, M., Kaltschmidt, C., and Renkawitz, R. (1988) Science 242, 1418–1420
25. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
26. McClary, J. A., Witney, F., and Geisselsoder, J. (1989) BioTechniques 7, 282–289
27. Womble, D. D. (2000) Methods Mol. Biol. 132, 3–22
28. Sali, A. (1995) Curr. Opin. Biotechnol. 6, 437–451
29. Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985) Nature 318, 635–641
Functional Probing of the Human Glucocorticoid Receptor Steroid-interacting Surface by Site-directed Mutagenesis: Gln-642 PLAYS AN IMPORTANT ROLE IN STEROID RECOGNITION AND BINDING

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