Valine 571 Functions as a Regional Organizer in Programming the Glucocorticoid Receptor for Differential Binding of Glucocorticoids and Mineralocorticoids*

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The glucocorticoid receptor (GR) interacts specifically with glucocorticoids, whereas its closest relative, the mineralocorticoid receptor (MR), interacts with both glucocorticoids and mineralocorticoids, such as aldosterone. To investigate the mechanism underlying the glucocorticoid/mineralocorticoid specificity of the GR, we used a yeast model system to screen for GR ligand-binding domain mutants, substituted with MR residues in the segment 565–574, that can be efficiently activated by aldosterone. In all such increased activity mutants, valine 571 was replaced by methionine, even though most mutants also contained substitutions of other residues with their MR counterparts. Further analysis in yeast and COS-7 cells has revealed that the identity of residue 571 determines the behavior of other MR substituted residues in the 565–574 segment. Generally, MR substitutions in this region are only consistent with aldosterone binding if residue 571 is also replaced with methionine (MR conformation). If residue 571 is valine (GR conformation), most other MR substitution mutants drastically reduce interaction with both mineralocorticoid and glucocorticoid hormones. Based on these functional data, we hypothesize that residue 571 functions as a regional organizer involved in discriminating between glucocorticoid and mineralocorticoid hormones. We have used a molecular model of the GR ligand-binding domain in an attempt to interpret our functional data in structural terms.

The steroid hormone receptors belong to the nuclear receptor family (1) of ligand-induced transcription factors. They are structurally and functionally related, and their structure can be divided into three main domains. A central domain binds to hormone response elements in the DNA, an N-terminal region mediates transactivation, and a C-terminal domain binds the steroid (2). The unliganded steroid receptors are complexed with chaperone proteins, including a dimer of hsp90 (3). The steroid hormone is an integral part of the transcriptionally active receptor complex and is almost completely buried within the folds of the ligand-binding domain (LBD). Binding of the steroid actively modulates the structure of the receptor into a DNA-binding transcriptionally active complex (4–6).

The mineralocorticoid receptor (MR) and GR are two closely related members of this family with structural and functional similarities. The DNA- and ligand-binding domains are relatively well conserved (94 and 57% sequence identity, respectively), whereas the N-terminal domains are less well conserved (7, 8). The receptors bind to the same hormone responsive elements in target genes (7, 9, 10) and have overlapping steroid-binding specificity. MR binds mineralocorticoids and most glucocorticoids with high affinity, whereas GR only binds glucocorticoids with high affinity (7, 10, 12). There is also an overlap in function between the steroids, so that even though mineralocorticoids regulate renal sodium retention and are the main regulating components of salt balance, blood pressure, and intravascular volume, glucocorticoids also have a pronounced effect on these physiological processes, involving both MR- and GR-dependent mechanisms (13). Glucocorticoids also specifically regulate a range of other processes, including the development of specific tissues, glucose metabolism, and the immune response (13).

The ligand-binding domain of GR consists of about 250 amino acids, which is thought to fold into a hydrophobic pocket that binds the steroid. The crystal structure is not yet resolved, but it is likely to be quite similar to the structures of the ligand-binding domains of PR, ER, TR, retinoic acid receptor, and retinoid X receptor, which are all folded into three-layered antiparallel α-helical sandwich structures (5, 6, 14–16). The difference between the crystal structures of retinoic acid receptor and retinoid X receptor LBD, as well as the structure of ER bound to various ligands, has clearly shown that the ligand actively induces a conformational change in the structure of the domain, particularly with regard to the positioning of helix 12. Mutational analysis has been used to analyze the importance of different amino acids in the binding of steroids, and most mutations lead to receptors with decreased affinity (17–23), but there are also studies in which mutant receptors with increased affinity or changes in specificity have been created (22, 24–27). A model of MR LBD based on functional mutational
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A miniprep of DNA (Wizard miniprep, Promega, Madison, WI) was made from a pool of bacterial colonies and used to transform yeast (Saccharomyces cerevisiae strain FY 23 [MAT α, ura 3–52, trp 1-3-63, leu 2-Δ1]); generously provided by Dr. Fred Winston) by the method of Gietz and Schiestl (32). Screening was performed by replica plating approximately 1000 yeast transformants onto X-gal plates containing galactose and a battery of different hormones. Interesting yeast transformants from this screening were rescreened by spotting a suspension of each yeast transformant containing about 5 × 10⁵ cells on X-gal plates. The activity of the different mutants relative to wild type was determined by the degree of blueness the colonies achieved.

Directed Mutagenesis—Directed mutagenesis was used to reconstruct the mutants that were selected for further studies after the yeast screen and to construct additional mutants that were characterized in the β-galactosidase assay. Kunkel mutagenesis was performed using oligonucleotides encoding the different amino acid substitutions and dideoxy sequencing was performed to confirm the mutations.

β-Galactosidase Assay—Yeast cells were grown in galactose-containing medium for 18–24 h to a density of A₆₅₀ of 0.2–0.8. They were then diluted to A₆₅₀ of 0.1, and 10-ml aliquots were incubated in a shaker at 30 °C with various concentrations of different hormones for 6 h. The cells were then harvested and assayed for β-galactosidase activity and protein concentration (29). β-Galactosidase activity was expressed as nanomoles of o-nitrophenyl-β-D-galactoside substrate converted/min/mg of protein.

Sequencing of Yeast Mutants—Preparation of DNA from yeast was made according to the method described by Kaiser and Auer (33) with the modification that electrocompetent bacteria were plated out and allowed to form single colonies before growth in culture. DNA was then prepared with Wizard minipreps, and Sanger dideoxy sequencing was performed using Sequenase (Amersham Pharmacia Biotech).

Mammalian Cell Culture and Transfection—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml), at 37 °C in a humidified atmosphere with 5% CO₂. When making dose-response curves, 6-cm plates containing cells at 60–80% confluency, plated out the day before transfection, were transfected with 0.1 μg of expression vector and 4 μg of p19TK luc, using DOTAP (Roche Molecular Biochemicals). 4.5 h after transfection, hormones were added to the cells, and 20–24 h later, a luciferase assay was performed. For ligand binding assays and competitive binding assays, 10-cm plates containing cells at 60–80% confluency, plated out 1–3 days before transfection, were transfected with 15 μg of expression vector using DOTAP. Cells were incubated 48 h after transfection before assays on cytosolic cell extracts were performed.

Luciferase Assay—Transfected cells from 6-cm plates were scraped into 1 ml of phosphate-buffered saline, centrifuged for 1 min in a microcentrifuge, and resuspended in 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 in a total volume of 500 μl containing 0.2% bovine serum albumin, 20 μM tetradsodium pyrophosphate, 10 mM magnesium acetate, 20 mM dithiothreitol, 2 mM EDTA, 0.2 mM ATP, 90 mM Tris acetate, pH 7.8, and 0.2 mg of luciferin/ml. The reaction was started by the addition of luciferin and enzyme activity was measured directly. The results are expressed as light units measured. All assays were performed in triplicate using three separate plates of transfected cells.

Ligand Binding and Competitive Binding Assays—Cells were washed with and scraped into phosphate-buffered saline and spun in a microcentrifuge. They were then resuspended in EPGMo buffer (1 mM

| Mutants superactive on X-gal plates containing aldosterone | No. of clones |
|----------------------------------------------------------|-------------|
| V571M                                                   | 5           |
| V571M/A573E                                             | 4           |
| V571M/A573Q                                             | 2           |
| V571M/A574V                                             | 3           |
| R569K/V571M                                             | 3           |
| V571M/A573E/A574V                                       | 4           |
| G567A/V571M/A574V                                       | 1           |

Limited information is available regarding which amino acids in the LBD of hGR discriminate between glucocorticoids and mineralocorticoids. The differences in structure between the mineralocorticoids deoxycorticosterone, aldosterone, 18-OH-deoxycorticosterone, and 18-OH-corticosterone and the glucocorticoids cortisol and corticosterone are at the 11β and 18 positions of the steroid. Thus, the regions of the LBD interacting with these positions would be expected to be important in the selective recognition of glucocorticoids and mineralocorticoids. One segment possibly involved is that centered around Gly-567 in hGR, because it has been suggested that the corresponding residue in human PR (G722), is important in accommodating the bulky 11β-side chain of RU 486 (21). Mutation of the Gly-722 in human PR to cysteine, which has a larger side chain, abolished binding of RU 486, but not binding of an agonist. Warriar et al. (25) mutated Gly-567, Met-565, and Ala-573 in hGR LBD to the corresponding amino acids in the human MR sequence (Ala, Arg, and Gln, respectively). They found that mutants M565R and A573Q bound aldosterone and dexamethasone better than wild type, whereas binding of these steroids to the mutant G567A was almost completely abolished (25).

In this paper, we have taken a broader approach to determining the role of the amino acid segment Met-565–Ala-574 of the hGR LBD in discriminating between glucocorticoid and mineralocorticoid binding. Following in vitro mutagenesis of this segment, screening for mutants with increased activity with aldosterone was carried out in a yeast system. By further characterization of the mutants in COS-7 cells, we could show that residues in this region play qualitatively different roles in the discrimination between glucocorticoids and mineralocorticoids.

EXPERIMENTAL PROCEDURES

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

Plasmids—The single copy number yeast vector prS315-NX expressing hGR, the yeast reporter vector pLOZ-TAT, the mammalian vector pCMV-hGR expressing hGR, and the mammalian reporter vector p19-luc-TK are described elsewhere (26, 29).

Random Mutagenesis and Screening—Mutagenesis was performed according to the refined method of Kunkel (30, 31). Mutant forms of prS315NX were constructed by oligonucleotide-directed mutagenesis to change the six amino acids in the segment 565–574 of hGR LBD that differ between GR and MR (Met-565, Gly-567, Arg-569, Val-571, Ala-573, and Ala-574) to the corresponding ones in MR (Arg, Ala, Lys, Met, Gln, and Val, respectively). By using a degenerate oligonucleotide, mutant receptors with mutation of one to six amino acids in all possible combinations were created. Only one base in each codon had to be changed into the equivalent MR amino acid, except at position 573, where two bases had to be altered. The oligonucleotide (see Fig. 1) was synthesized by adding equal amounts of the GR and MR base for each codon. At position 573, where two bases had to be altered, two more alternative amino acids, Pro and Glu, in addition to Gln, could be created. The resulting combination of different mutant plasmids was transformed into Escherichia coli by electroporation.

The single copy number yeast vector pRS315-NX expressing hGR, and the mammalian reporter vector p19-luc-TK, the yeast reporter vector pLGZ-TAT, the mammalian vector pCMV-hGR expressing hGR, and the yeast reporter vector p19-luc-TK are described elsewhere (26, 29).

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EDTA, 20 mM potassium phosphate, pH 7.8, 10% glycerol, 20 mM sodium molybdate, and 1 mM dithiothreitol) and homogenized with a glass homogenizer, and the lysate was spun for 30 min at 100,000 g at 4 °C. For ligand binding assays, different concentrations of [3H]TA (0.1–2.5 nM) were added. For competitive binding assays, 2.5 nM [3H]TA and increasing concentrations of cold aldosterone were added. The extracts were incubated at 4 °C overnight. Bound and free [3H]TA were then separated by gel filtration on a nick column (Amersham Pharmacia Biotech), and the amount of [3H]TA bound was measured in a scintillation counter. Free [3H]TA was calculated as total minus bound [3H]TA. The level of unspecific binding was negligible as controlled by adding a 200-fold excess of cold TA to the different concentrations of [3H]TA.

Statistical Analysis and Curve Fitting—Analysis of variance was carried out using the Newman-Keuls test using the program STATGRAPHICS for Windows (StatSoft, Inc., Tulsa, OK). Curve fitting was carried out using the program Kaleidagraph. The following functions were used for curve fitting.

Dose-response transactivation curves were calculated as follows,

$$R = \frac{(R_{\text{max}} \cdot H)}{(EC_{50} + H)} + b$$  \hspace{1cm} (Eq. 1)

where $r$ = response, $H$ = hormone concentration, and $b$ = background. Competitive binding was calculated as follows,

$$B = \frac{(B_{\text{max}} \cdot B)}{B + EC_{50} \cdot \left(1 + \frac{H}{k_i}\right)} + b$$  \hspace{1cm} (Eq. 2)

where $B$ = concentration bound radiolabeled hormone, $H$ = concentration of free competing hormone, and $b$ = background. $k_i$ values were calculated using the Cheng-Prusoff equation,

$$k_i = \frac{IC_{50}}{1 + \left(\frac{L}{EC_{50}}\right)}$$  \hspace{1cm} (Eq. 3)

where $L$ = concentration tritiated steroid in competitive binding assays.

RESULTS

Screening for Mutants in Yeast—To study the role of amino acids 565–574 in hGR LBD in discriminating between glucocorticoid and mineralocorticoid binding, we performed in vitro mutagenesis of this segment. The six amino acids that differ between the two receptors in this segment were randomly mutated using a degenerate oligonucleotide that gave equal probability of having an MR or GR amino acid at each position (see Fig. 1). A pool of mutant plasmids was thus created, containing receptors with one to six mutations in different combinations. The mutant plasmids were transformed into...
yeast together with a GRE-Lac Z reporter and approximately 1000 yeast transformants were replica-plated to X-gal plates containing aldosterone, cortisol, progesterone, TA, or deoxycorticosterone. The primary aim was to find mutants with increased activity on aldosterone, but other hormones were also included in the screening to allow detection of mutants with altered specificity.

45 colonies that were more blue than wild type on all or some hormones were re-screened by spotting an equal number of cells from each clone onto X-gal plates containing different hormones, and of those, 30 still remained more active on all or some hormones. 22 of the most blue superactive clones on aldosterone plates were sequenced, and all 22 clones were found to contain the mutation V571M, either in isolation or in combination with other mutations (Table I). We also sequenced some randomly selected inactive mutations (white colonies) and found the single mutation M565R and the double mutations G567A/R569K and G567A/A574V, none of which contained the V571M mutation. Due to the design of the degenerate oligonucleotide (see under “Experimental Procedures” and Fig. 1), two additional amino acids at position 573 were also possible, in addition to the GR- and MR-specific alanine and glutamine (glutamate and proline). Mutants containing these amino acid substitutions were not considered for further studies.

For further characterization in yeast we chose the superactive mutants [V571M, R569K/V571M, V571M/A573Q, and GM6 (cf. Fig. 1)] detected in the plate screening. To minimize the risk that the mutants found in the screen contained nonprogrammed mutations elsewhere in the receptor, we recrreated the mutations by directed mutagenesis. In addition, in order to analyze the influence of the single mutations in the receptors containing multiple mutations, we also created all six single mutations separately (including V571M, which was already found in the screen) and one receptor containing all six mutations in combination (GM6<sub>RAKMQV</sub> (cf. Fig. 1).)

The phenotypes of the selected mutants were tested in a β-galactosidase assay with the hormones TA, aldosterone, and corticosterone (Fig. 2). The mutations could be grouped into three groups with regard to their activity on aldosterone compared with wild type; those with lower activity (group 1), those with similar activity (group 2), and those with higher activity (group 3). Group 1 consists of the single mutations G567A, R569K, and A574V (Fig. 2A), which had less activity than wild type on all three hormones, and M565R, which had about equal activity with TA, but clearly less activity on corticosterone and aldosterone.

Group 2 consists of the double mutant R569K/V571M, which in this assay had less activity with TA, but about the same activity with corticosterone and aldosterone (Fig. 2B). Finally, group 3 consists of the mutants having higher activity with aldosterone: V571M, A573Q, the double mutant V571M/A573Q, the triple mutant GM3<sub>MV</sub>, and the mutant with all six amino acids changed GM6<sub>RAKMQV</sub> (Fig. 2C). All mutants in this group were more active with all hormones, with the exception of V571M, which had the same activity with TA as wild type GR.

Characterization of Mutants with Regard to Transactivation in COS-7 Cells—To confirm the phenotypes of the mutants in COS-7 cells, we transfected wild type and mutant cDNA into a mammalian CMV-based expression vector and co-transfected these into the COS-7 cells together with a luciferase reporter vector consisting of two GREs and the tk-promoter upstream of the luciferase gene. Because our goal was to identify mutants with increased function on aldosterone, mutants with the same or lower activity compared with wild type could be eliminated from further studies.

In order to identify mutants with lower activity than wild type GR in COS-7 cells, we made a first screen at one concentration of TA, aldosterone and corticosterone. In this assay M565R, G567A, R569K, and A574V (group 1) all had less activity on aldosterone (Fig. 3), thus reflecting their behavior in yeast. G567A, R569K, and A574V had less activity than wild type with all hormones, whereas M565R had less activity with aldosterone and corticosterone and similar activity with TA. The mutant R569K/V571M was also excluded from further studies due to its similarity in activity to wild type in yeast (Fig. 2B).

The phenotypes of the remaining mutants (group 3 that were superactive in yeast), were first characterized with regard to their sensitivity to aldosterone by adding increasing amounts of aldosterone to plates containing transfected cells, thus cre-
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Summary of transcriptional activation and binding affinity for triamcinolone acetonide and aldosterone for wild type and mutant GR

| Mutant          | EC50 aldosterone | IC50 aldosterone | kD aldosterone | EC50 TA | Kd TA |
|-----------------|------------------|------------------|----------------|---------|-------|
| Wild type       | 491 ± 215        | 224 ± 40         | 32 ± 5.8       | 0.33 ± 0.17 | 0.42 ± 0.07 |
| V571M           | 143 ± 103        | 91 ± 27          | 13 ± 3.8       | 0.18 ± 0.087 | 0.42 ± 0.14 |
| A573Q           | 554 ± 251        | ND               | ND             | ND      | ND    |
| V571M/A573Q     | 42 ± 18          | 91 ± 28          | 5.7 ± 1.7      | 0.07 ± 0.03  | 0.17 ± 0.03 |
| GM3AMV          | 51 ± 26          | ND               | ND             | 0.25 ± 0.11 | ND    |
| GM6RAKMQV       | 63 ± 17          | ND               | ND             | 0.12 ± 0.08  | ND    |

a Significantly different from wild type GR (p < 0.05).
b ND, not determined.

ting dose-response curves (Fig. 4). Four of the five mutants were more sensitive to aldosterone than wild type in this experiment (Fig. 4A), with EC50 values ranging from 3.4 to 12 times less than that of the wild type receptor (Table II). The decreases in EC50 values were statistically significant compared with wild type GR for all four mutants. However, there was no statistically significant difference between the EC50 of the V571M mutant (3.4 times less than wild type GR) compared with the other three mutants V571M/A573Q, GM3AMV and GM6RAKMQV (7–12 times less than that of wild type GR). We have not been able to show any significant alteration in the sensitivity of the single point mutant GR A573Q (Fig. 4B), compared with wild-type GR and thus this mutant was not studied further with TA or aldosterone.

To investigate whether the increased sensitivity of the mutants was specific for the mineralocorticoid aldosterone, we also performed dose-response curves with the potent glucocorticoid TA. The IC50 values were significantly more sensitive to TA than wild type GR (p < 0.05), having EC50 values of 4.7 and 2.7 times less than that of wild type receptor, whereas the IC50 for the V571M and GM3AMV mutants were not significantly different from that of wild type (Table II).

We thus conclude that the single mutation V571M increases the sensitivity to aldosterone without affecting the sensitivity to TA and that this mutation in combination with other amino acid substitutions in the segment Met-565–Ala-574 of GR LBD can result in increased sensitivity to aldosterone alone or to both TA and aldosterone.

Analysis of Hormone Binding with Wild Type and mutant Receptors—Because the first step in activation of hormone receptors is binding of the hormone, we wanted to investigate whether the changes in sensitivity of the mutant receptors in the transactivation assays could be explained by changes in hormone binding. For the binding studies, we selected V571M, which had increased sensitivity to aldosterone, and the double mutant V571M/A573Q, which had increased sensitivity to both aldosterone and TA in the transactivation assay.

The equilibrium constants for TA were calculated by incubating cytosolic extracts of COS-7 cells containing mutant or wild type receptor with increasing amounts of [3H]TA. After separation of bound and free steroid by gel chromatography, Scatchard analysis was performed to calculate the Kd. The mutant V571M had affinity for TA similar to that of wild type GR (Kd = 0.42 nM), whereas the mutant V571M/A573Q had significantly higher affinity (Kd = 0.17 nM; p < 0.05) (Table II).

To estimate the binding affinity for aldosterone, competitive binding experiments were performed by adding increasing amounts of unlabeled aldosterone to 2.5 nM [3H]TA in cytosolic extracts of COS-7 cells, containing wild type or mutant receptor. The IC50 values for both mutants were about 2.5 times less than that of wild type (p < 0.05) (Fig. 6 and Table II), and the k values calculated according to the Cheng-Prusoff equation (34) were 2.5 times less that of wild type GR (p < 0.05) for the V571M mutant and 5.6 times less than wild type (p < 0.05) for the V571M/A573Q mutant (Table II). The changes in transactivation sensitivity thus correlate well with altered ligand-binding affinity for both aldosterone and TA.

Transactivation Analysis of GR Mutants M565R and A573Q—As shown in Fig. 4B, we were unable to show a significant increase in aldosterone sensitivity for the A573Q mutant. This is of some interest because previous studies by Warriar et al. (25) showed that mutations A573Q and M565R had a higher relative affinity for aldosterone in steroid-binding competition assay with [3H]dexamethasone. Thus, these mutants might have been expected to be more sensitive to aldosterone. We therefore made a complete aldosterone dose-response curve also with mutant M565R, which showed that M565R is much less sensitive to aldosterone (Fig. 7A). Thus, any increase in affinity of these mutants for aldosterone does not appear to lead to increased transactivation sensitivity. In the same study, Warriar et al. reported that A573Q and M565R had an increased sensitivity to dexamethasone. We have attempted to repeat this experiment, and for A573Q we saw a similar increase in sensitivity of about 10-fold (Fig. 7B). The EC50 values we measured for A573Q and wild type receptor were 0.097 ± 0.041 nM (n = 4) and 0.767 ± 0.563 nM (n = 4) respectively. However, in our hands, the sensitivity of the M565R mutant to dexamethasone (EC50 = 0.859 ± 0.739 nM (n = 4)) did not differ significantly from that of wild type GR (Fig. 7B), in contrast to the previous report (25) in which the sensitivity of the M565R mutant was increased about 100-fold.

DISCUSSION

The aim of this paper was to understand the mechanisms underlying the glucocorticoid/mineralocorticoid binding specificity of GR. The GR interacts specifically with glucocorticoids, whereas its closest relative, the MR, interacts with both glucocorticoids and mineralocorticoids such as aldosterone.

The main difference in structure between glucocorticoids and mineralocorticoids are at the 11β and 18 position of the steroid,
so the region of GR-LBD interacting with these positions would be expected to be involved in the differential binding of glucocorticoids and mineralocorticoids. One possible important segment is that centered around Gly-567, because the corresponding residue in human PR (Gly-722) seems to be involved in accommodating the bulky 11β-side chain of Ru486 (21).

Furthermore, Warriar et al. (25) performed mutations in this region and found some mutants with increased mineralocorticoid specificity. These findings led us to randomly mutate the six amino acids in the segment 565–574 of hGR, which differ between the two receptors, to the corresponding amino acids in the MR sequence. A yeast system was used to screen for mutants with increased activity with aldosterone, and interesting mutants were also further characterized in COS-7 cells.

Interestingly, all multiple mutations selected for their higher activity with aldosterone in the yeast screen contained the mutation V571M (Table I), which was also found as a single point mutation, indicating the key importance of this mutation for the increased aldosterone specificity. None of the other single GR to MR mutations alone displayed increased aldosterone sensitivity (Figs. 2A and Fig. 3), even though they could be expected to make the receptor more “MR-like.” In fact, all but A573Q had decreased sensitivity. One of the more important findings was that the V571M mutation had a positive, dominant effect in combination with other MR substitutions that by themselves are almost inactive, such as in GM3AMV, resulting in a receptor more sensitive to aldosterone (Fig. 4A). We therefore hypothesize that residue 571 functions as a regional organizer, such that the identity of the residue 571 seems to determine the phenotype associated with other MR derived residues in the 565–574 segment. Generally, MR substitutions in this region are only consistent with aldosterone binding if residue 571 is valine (GR conformation) most other MR substitute mutants drastically reduce interaction with both mineralocorticoid and glucocorticoid hormones. The function of va-
line 571 in wild type GR might be to reduce the interaction with mineralocorticoids.

The observation that the affinity of the V571M mutant for the powerful glucocorticoid TA is unaltered (Table II) indicates that the increased affinity for aldosterone is mineralocorticoid-specific. In our yeast system, however, the V571M mutant was also more active with corticosterone (Fig. 2C), which binds with nearly equal affinity to both MR and GR but which has lower affinity for the glucocorticoid receptor than TA. This mutation may thus result in increased binding of pure mineralocorticoids, such as aldosterone, but also of steroids such as corticosterone (which are good mineralocorticoids but relatively weak glucocorticoids compared with TA). The mutations V571M/A573Q and GM6RAKMQV were more sensitive to both TA (Fig. 5 and Table II) and aldosterone, but the increase in sensitivity to TA was less pronounced. Because TA, apart from being a potent glucocorticoid, also acts as a mineralocorticoid, although with somewhat weaker potency than aldosterone, these mutants still display a more mineralocorticoid-specific characteristic.

Binding studies were not performed with all mutants, but

![Fig. 8. Model for the change in specificity seen in mutant forms of GR. A homology model of hGR ligand-binding domain was built from the ERα x-ray crystallographic structure. Initial sequence alignment between the receptors was obtained using the PileUp program from the GCG package. This sequence alignment was then refined during semiautomated homology modeling by performing iterative runs of the program modeler as supplied with QUANTA 96, taking available scientific data into account (e.g., point mutations and covalent affinity labeling experiments). Dexamethasone was then docked in the ligand-binding cavity, and the protein-ligand interaction energy was minimized using the program CHARMM. The complex was then subjected to molecular dynamics using the Verlet and Shake algorithms, followed by reminimization. Other ligands were then fitted in the same orientation as dexamethasone and calculated protein-ligand interactions were correlated with experimental affinity binding data. The final model was produced to obtain the best correlation between experimental and calculated binding affinities (Footnote 2). A, model for the increased affinity of GR mutant V571M for aldosterone. In the model, mutation of Val-571 in helix 3 to Met results in steric interaction with Met-752 and Leu-753 in helix 12, resulting in reorientation of helix 12. The 11,18-hemiacetal group of aldosterone is directed toward helix 12. B, model for the increased affinity of GR mutant A573Q for dexamethasone. The mutated residue participates in a network of hydrogen bonds with the 3-keto group of the steroid, as shown in the figure.](image-url)
V571M and V571M/A573Q showed a good correlation between altered binding affinity and altered transactivation capacity (Table II), even if the EC_{50} value for the double-mutant to aldosterone is somewhat more decreased than the \( k_a \) value (Table II). We suggest that the shift in dose response curves for all mutants is mainly due to altered binding affinity on the first hand, but we cannot exclude that slight changes in transactivation potency have occurred in addition to the increased affinity.

The affinity of wild type GR for aldosterone in this study (\( k_a \), 32 nM; Table II) was very similar to that previously reported (11, 35). The affinity of MR for aldosterone is in the range \( K_d \) 0.52–1.3 nM (7, 11, 28, 36), so the mutants in this study had affinities for aldosterone intermediate between that of GR and MR (Table II). The region 565–574 can thus not be the sole determinant of mineralocorticoid/glucocorticoid specificity, but is probably an important part in an interplay between different regions of the receptor that determine the binding specificity.

Mutational studies of the same region performed by Warriar et al. (25) revealed that M565R and A573Q seemed to have higher affinity for aldosterone. In our transactivation assays in COS-7 cells, we could not see any increase in sensitivity to aldosterone with these mutants (Figs. 4B and 7A), but in similarity to their results, the A573Q mutant had higher sensitivity to dexamethasone (Fig. 7B). There might thus be a difference between our experimental systems, or alternatively, the increased affinity for aldosterone that they reported (25) is not reflected as an increase in transactivation sensitivity (Table II). In our study, A573Q seemed to be more sensitive to aldosterone in yeast (Fig. 2C) but not in COS-7 cells (Fig. 4B). A possible explanation is that the difference in phenotype in yeast and COS-7 cells for this mutant is due to interaction of the receptor with cell-specific factors that differ in yeast and COS-7 cells or due to other differences intrinsic to the two cell systems.

We have attempted to explain the functional changes obtained with the mutants by using a homology model of the GR LBD based on the crystal structure of human ER LBD.\(^2\) In the model, Val-571 does not seem to contact the ligand directly (distance for side chain to steroid is > 8 A). The increased affinity for aldosterone and the positive, dominant effect of V571M may therefore be explained by conformational changes induced by exchanging methionine for valine. The longer side chain of methionine 571 appears to come too close to amino acids Met-752 and Leu-753 in helix 12, and as a consequence of repulsive interactions, the quite flexible helix 12 could be displaced, leading to a somewhat rearranged binding pocket (Fig. 8A). This could create a receptor with increased affinity for aldosterone, which might explain why the triple mutant GM334445 is more sensitive to aldosterone despite the inactivity of the G567A and A574V single mutations alone. According to the model, alanine substituted for the smaller glycine in G567A would make an unfavorable contact with the A-ring, causing the ligand to move, and thereby disrupt the important interaction between Ala-570 and the 3-keto group of the steroid. What happens in the case of the A574V cannot easily be explained by the model, but Ala-574 does not seem to directly contact the ligand. The rearranged binding pocket created by V571M might be able to compensate for the loss of interaction between Ala-570 and the 3-keto group and thus allow aldosterone to bind better even in the presence of the bigger side chain at positions G567A and A574V, each of which disrupts binding as a single mutation.

Naturally, we cannot be completely sure that our structural model for the GR-LBD is correct, but its prediction that V571M does not contact the ligand directly is consistent with our functional data. Furthermore, the flexibility in the positioning of helix 12 suggested in our model has been seen previously in the crystal structure of human ER LBD, in which the antagonist raloxifene causes a different positioning of the helix than the agonist estradiol (5).

The increased affinity of the A573Q mutant for dexamethasone could be explained by creation of an extra hydrogen bond within the receptor that stabilizes the interaction with the A-ring. Gln-570 and Arg-611 form a hydrogen-bonding network with the 3-keto group of the steroid, including a bridging water molecule as has been described in both ER and PR (5, 14). The mutation A573Q results in an additional hydrogen-bonding of the 573 glutamine with Gln-570 and the backbone carboxyl of Glu-542 (Fig. 8B).

Finally, we have shown in this paper, as well as in a previous paper (26), that the yeast-system is a valuable tool for isolation of interesting change-of-function mutations that can be used to test the structural models of receptors that are now becoming available. We have identified the single mutant V571M, which binds aldosterone with higher affinity than wild type GR, and multiple mutations containing V571M that were more sensitive to aldosterone and in which V571M seemed to have a positive dominant effect. We thus conclude that the V571M mutant is the key to the increased affinity for aldosterone in the Met-565–Ala-574 region of hGR LBD and that valine at this position is likely to play an important role in determining the more limited steroid-binding profile of the GR.

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REFERENCES

1. Evans, R. M. (1988) Scand. J. 20, 889–919
2. Carlstedt-Duke, J., Strunsmuir, P. E., Wrangle, O., Bergman, T., Gustafsson, J.-Å., and Jornvall, H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4437–4440
3. Pratt, W. B., Hutchison, K. A., and Scherrer, L. C. (1992) Trends Endocrinol. Metab. 3, 263–268
4. Parker, M. G., and White, R. (1996) Nat. Struct. Biol. 3, 113–115
5. Brzozowski, A. M., Pice, A. C., Dauter, Z., Hubbard, R. K., Bonn, T., Engstrom, O., Ohman, L. E., Gustafsson, J.-Å., and Carlquist, M. (1997) Nature 390, 753–758
6. Wagner, R. L., Aprillieti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fetterick, R. J. (1995) Nature 378, 690–697
7. Arriza, J. L., Weinberger, C., Cerelli, G., Glasser, T. M., Handelin, B., Housman, D. E., and Evans, R. M. (1987) Science 237, 268–275
8. 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
9. Pearce, D., and Yamamoto, K. R. (1993) Science 259, 1161–1165
10. Rupprecht, R., Arriza, J. L., Spengler, D., Reul, J. M., Evans, R. M., Holsboer, F., and Damm, K. (1993) Mol. Endocrinol. 7, 597–603
11. Rupprecht, R., Reul, J. M., van Steensel, B., Spengler, D., Soder, M., Berning, B., Holsboer, F., and Damm, K. (1993) Eur. J. Pharmacol. 247, 145–154
12. Arriza, J. L., Simerly, R. B., Swanson, L. W., and Evans, R. M. (1988) Neuron 1, 897–900
13. Miller, W. L., and Tyrrell, J. B. (1995) in Endocytosis and Metabolism (Felg, P., Baxter, J. D., and Frohman, L. D., eds) pp. 555–711, 3rd Ed., McGraw-Hill Inc., New York.
14. Williams, S. P., and Sigler, P. B. (1998) Nature 393, 392–396
15. Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995) Nature 378, 681–689
16. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) Nature 375, 377–382
17. Danielsen, M., Northrup, J. P., Jonklaas, J., and Ringold, G. M. (1987) Mol. Endocrinol. 1, 816–822
18. Chen, D., and Stallcup, M. R. (1994) J. Biol. Chem. 269, 7914–7918
19. Danielsen, M., Northrup, J. P., and Ringold, G. M. (1986) EMBO J. 5, 2513–2522
20. Byravan, S., Milhon, J., Rahhindran, S. K., Olinger, B., Garabedian, M. J., Danielsen, M., and Stallcup, M. R. (1991) Mol. Endocrinol. 5, 752–758
21. Bennhams, O. B., Garcia, T., Learsge, T., Vergez, A., Goallo, D., Biggcn, C, Chambon, P., and Gronemeyer, H. (1992) Science 255, 206–209
22. Chen, D., Kohli, K., Zhang, X., Daniels, M., and Stallcup, M. R. (1994) Mol. Endocrinol. 8, 422–430
23. Milhen, J., Kohli, K., and Stallcup, M. R. (1994) J. Steroid Biochem. Mol. Biol. 51, 11–19
24. Chakraborti, P. K., Garabedian, M. J., Yamamoto, K. R., and Simons, S. S., Jr. (1991) J. Biol. Chem. 266, 22075–22078

\(^2\) M. Gillner, U. Lind, J. Carlstedt-Duke, J. D. Baxter, and P. Greenidge, manuscript in preparation.
25. Warrier, N., Yu, C., and Govindan, M. V. (1994) J. Biol. Chem. 269, 29010–29015
26. Lind, U., Carlstedt-Duke, J., Gustafsson, J.-Å., and Wright, A. P. (1996) Mol. Endocrinol. 10, 1358–1370
27. Zhang, S., Liang, X., and Danielsen, M. (1996) Mol. Endocrinol. 10, 24–34
28. Fagart, J., Wurtz, J. M., Souque, A., Hellallevy, C., Moras, D., and Rafestin-Oblin, M. E. (1998) EMBO J. 17, 3317–3325
29. Wright, A. P., Carlstedt-Duke, J., and Gustafsson, J.-Å. (1990) J. Biol. Chem. 265, 14763–14769
30. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
31. McClary, J. A., Witney, F., and Geisselsoder, J. (1989) BioTechniques 7, 282–289
32. Gietz, R. D., and Schiestl, R. H. (1991) Yeast 7, 253–263
33. Kaiser, P., and Auer, B. (1993) BioTechniques 14, 552
34. Cheng, Y., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099–3108
35. Veldhuis, H. D., Van Koppen, C., Van Ittersum, M., and De Kloet, E. R. (1982) Endocrinology 110, 2044–2051
36. Binart, N., Lombes, M., Rafestin-Oblin, M. E., and Baulieu, E. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10681–10685