Cysteines 849 and 942 of Human Mineralocorticoid Receptor Are Crucial for Steroid Binding†

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ABSTRACT: To assess the role of each of the four cysteine residues in the hormone binding domain (HBD) of the human mineralocorticoid receptor (hMR), we have separately substituted C808, C849, C910, and C942 into serine. These mutations were created in the G595-K984 hMR receptor fragment which encompasses the DNA binding domain, the hinge region, and the hormone binding domain. Each mutant was further analyzed by steroid binding assays and transactivation assays using wild-type and mutant proteins expressed in vitro in the reticulocyte lysate. While the C910S mutant exhibited similar wild-type G595-K984 receptor binding properties for aldosterone, the C808S mutant affinity was 3.5-fold higher. In contrast, the C849S mutant showed a drastic drop in affinity for aldosterone and the mutant C942S was unable to bind the steroid. Affinities for the antagonist progesterone were also determined. C808S, C849S, and C910S were found to bind progesterone better than aldosterone (about a 2-fold increase in their affinities). Mutant C942S failed to bind any steroid tested (aldosterone, progesterone, cortisol, and the synthetic antagonist RU26752). No change in the specificity toward various agonists and antagonists could be observed with the mutants when compared to the wild-type G595-K984. When transactivation assays were performed, the properties of mutants C808S and C910S were similar to those of the wild-type G595-K984, while mutant C849S showed reduced sensitivity and C942S was devoid of any transcriptional activity. Our data indicate that C849 and C942 are critical for the ligand binding process of hMR. Moreover, C942 might be involved in a direct contact with the 20-keto group of the steroid.

The steroid mineralocorticoid receptor (MR) mediates the actions of aldosterone in target cells where it acts as a transcriptional factor (*1*). In the absence of ligand, MR occurs in a physiologically inactive form in a heteromeric complex with heat shock proteins (hsps) (*2*, *3*). Upon ligand binding, a conformational change occurs (*4*) which triggers the dissociation of hsps, and then the transformed receptor binds tightly to specific DNA recognition sequences of target gene promoters, called hormone response elements.

As a member of the nuclear receptor superfamily, the MR consists of three functionaly linked distinct domains (*5*): an N-terminal domain which encompasses the constitutive transcriptional activation function AF1, a DNA binding domain (DBD) located in the central region of the protein and a C-terminal hormone binding domain (HBD).[1] The HBD contains all amino acid residues necessary for high-affinity ligand binding, and it also includes sequences involved in hsp interaction, receptor dimerization, and nuclear translocation. In addition, this domain contributes to the hormonally inducible transactivation function by means of the ligand-dependent AF2-activating function, apparently by protein−protein contacts with basal transcription factors and/or transcriptional activators (*6*).

The 250 amino acid HBD is largely hydrophobic and assumed to be arranged in 12 α-helices which, after ligand binding, provide the hydrophobic ligand core according to the model described by Wurtz et al. for nuclear receptors (*7*). Four cysteine residues are scattered along the HBD sequence of the hMR: C808, C849, C910, and C942. Two of them, C849 and C942, are conserved in some of the other members of the family (Figure 1). Note that another position, I871 of the hMR, is occupied by a cysteine residue in all of the other steroid receptors. Although at a molecular level the role of SH groups in the binding of steroids is not precisely established, it has long been known from affinity labeling data or treatment with specific reagents that intact thiols influence the ligand binding properties of steroid receptors (*8*). Contrary to other members of the steroid receptor family, no affinity labeling experiments have been conducted on MR which could have assigned amino acid residues located at the steroid binding surface of the receptor. More recently, a previous study on hMR treated with methyl methane thiosulfonate (MMTS), a reversible cysteine specific modifying reagent, revealed that the integrity of the thiol groups is necessary for the binding of both agonists and antagonists to hMR (*9*). These data suggest that some cysteine residue(s) may be located in the ligand binding

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[1] Abbreviations: hAR, human androgen receptor; hER, human estradiol receptor; hGR, human glucocorticoid receptor; hMR, human mineralocorticoid receptor; hPR, human progesterone receptor; HBD, hormone binding domain; GRE, glucocorticoid response elements; wt, wild-type.

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	Helix 5	Helix 7	Helix 8	Helix 9	Helix 11
hMR	C808	C849	I871	C910	C942
hGR	F602	C643	C665	R704	C736
hPR	S757	C789	C820	R859	C891
hAR	G743	C784	C806	K845	T877
hER	E385	F425	C447	A493	L525

FIGURE 1: Location of the four cysteines of the hMR HBD and their analogous residues in the other human steroid receptors. Helices are shown according to the model described by Wurtz et al. (*7*) for nuclear receptors.

pocket of hMR or at least participate in the ligand binding process.

The aim of the present work was to identify which of the four cysteine residue(s) present in the hMR HBD (C808, C849, C910, or C942) are important for steroid binding and to further investigate the role that these cysteine(s) play in the binding of agonists and antagonists. Our approach was to produce in vitro point mutations with subsequent studies of steroid binding and transactivation properties of the mutants. Cysteines at positions 808, 849, 910, and 942 were individually mutated into serine residues. Serine was selected because it represents a specific isoatomic substitution of sulfur to oxygen, thus allowing assessment of the importance of cysteine thiols for ligand recognition, while minimizing the possibility of drastic changes in the secondary structure or conformation of the protein.

MATERIALS AND METHODS

Chemicals. [1,2-^{3}H]Aldosterone (47 Ci/mmol), [1α,2α-(n)-^{3}H]progesterone (49 Ci/mmol), L-[^{35}S]methionine (>1000 Ci/mmol), and [^{14}C]methylated protein molecular-mass markers (10−100 mCi/mg) were obtained from Amersham (Les Ulis, France). [1,2-^{3}H]Cortisol (51.9 Ci/mmol) was from NEN. Unlabeled aldosterone, progesterone, and hydrocortisone were from Sigma (Saint Quentin Fallavier, France). Tritiated and unlabeled RU26752 were a kind gift from Roussel Uclaf (Romainville, France). Synthesis of the 11β-Allenyl-3-oxo-19-nor-17α-pregna-4,9-diene-21,17-carbolactone (FH151) was previously described (*10*). Oligonucleotide primers were synthesized by either Eurogentec (Seraing, Belgium) or OLIGO-Express (Paris, France). The TNT T7 coupled reticulocyte lysate system, the Profection mammalian transfection system, and the Dual-Luciferase reporter assay system were from Promega (Charbonnieres, France). Amplify was purchased from Amersham. UltimaGold was from Packard France (Rungis, France). All other chemicals were reagent grade and obtained from commercial sources.

Buffers. TEG buffer contains 20 mM Tris/HCl, 1 mM EDTA, and 10% (v:v) glycerol. TEGWD buffer is TEG containing 20 mM sodium tungstate supplemented with 1 mM dithiothreitol. All buffers were adjusted to pH 7.4 at 25 °C.

Plasmids and Vectors. The phMR3750 plasmid, containing the full-length hMR-coding sequence under control of the T7 promoter, was a kind gift from Dr. R. M. Evans and Dr. J. L. Arriza (The Salk Institute, La Jolla, CA).

The plasmid CMV-MR containing the full length coding region of hMR downstream of the human cytomegalovirus strong promoter (*11*) and the plasmid TAT-TK-luc containing the firefly luciferase gene downstream of the tyrosine aminotransferase (2 GRE) under the control of the minimal promoter thymidine kinase from herpes simplex virus (*12*) were kind gifts from Dr. M. Pons (INSERM U439, Montpellier, France).

The pRL-TK vector from Promega, intended for use as an internal control reporter of transfection, contains a cDNA encoding *Renilla* luciferase under control of the herpes simplex virus thymidine kinase (HSV-TK) promoter.

The pCI mammalian expression vector from Promega contains the human cytomegalovirus (CMV) major immediate-early gene enhancer/promoter region.

DNA Constructs. The hMR receptor fragment [G595-K984] was obtained by PCR amplification from phMR3750 as matrix using the following primers which introduced a *Xho*I site at the 5′-end and a *Sal*I site at the 3′-end:

5′-AGC TGC TCG AGA TGG GAT CTT CAA GAC CTT C-3′ (forward primer with a *Xho*I site underlined)

5′-CGG CTG TCG ACT CAC TTC CGG TGG AAG TAG AG-3′ (reverse primer with a *Sal*I site underlined).

After purification, the 1170 bp fragment was inserted in the pCI vector previously digested by *Xho*I and *Sal*I. The sequence was confirmed by automated fluorescent sequencing (Genome Express, Grenoble).

In Vitro Mutagenesis. Point mutations were performed by oligonucleotide site-directed mutagenesis of the hMR-[G595-K984] cDNA using the QuikChange site-directed mutagenesis kit from Stratagene. The mutant oligonucleotide primers, each complementary to opposite strands of the plasmid, were annealed and extended during temperature cycling by means of *Pfu* DNA polymerase. Selection of mutated DNA was performed by treatment of the product with *Dpn*I, which digests the methylated parental DNA template. Mutated DNA was then transformed into electrocompetent JM109 *E. coli* by electroporation. Transformants were screened by sequencing (Genome Express, Grenoble, France).

The oligonucleotide primer pairs used for cysteine mutagenesis into serine were as follows (forward primers are only presented, reverse primers are complementary and reverse):

C808S : 5'-CAG TAT TCT TGG ATG TCT CTA TCA TCA TTT GCC-3'

C849S : 5'-GTA TGA ACT ATC CCA GGG GAT GC-3'

C910S : 5'-GGT AAC TAA GTC TCC CAA CAA TTC TGG-3'

C942S : 5'-GCT GGA ATT CTC CTT CTA CAC CTT CC-3'

In Vitro Receptor Transcription and Translation. The wild-type and mutant receptors were expressed in vitro directly in rabbit reticulocyte lysate using the TNT T7 quick-coupled transcription/translation system from Promega. Plasmids containing the relative coding sequences under control of the T7 promoter were transcribed with T7 RNA polymerase and simultaneously translated in rabbit reticulocyte lysate for 1 h at 30 °C in accordance with the protocol recommended by the manufacturer. When specified, receptors were labeled by incorporation of L-[^{35}S]methionine. In this case, aliquots (5 μL) of the in vitro translation products were solubilized in 20 μL of SDS sample buffer and analyzed on a 10% SDS-polyacrylamide gel by electrophoresis. The

gels were fixed, dried, and exposed to a phosphoimaging screen (Fujix).

Limited Proteolytic Digestion of Translated Receptors. Chymotrypsin (100 μg/mL final concentration) was added to 10 μL of [^{35}S]-labeled translation mix and incubated for 30 min at 20 °C. Aliquots (5 μL) of each sample were then mixed with 20 μL of denaturating loading dye, boiled for 5 min, and immediately loaded onto a SDS/12.5% polyacrylamide gel. Following electrophoresis, the gels were fixed, dried, and exposed to a phosphoimaging screen (Fujix).

Hormone Binding Studies. Binding assays were performed on crude translation medium diluted accordingly (one-fourth for the full hMR, one-tenth for G595-K984 wt and C910S, one-twentieth for C808S, and one-half for C849S and C942S, C942S was also assayed undiluted) with ice-cold TEGWD buffer. Aliquots of this medium (25 μL) were incubated at 4 °C for 4 h with increasing concentrations of the tritiated ligand. Nonspecific binding was determined by the addition of 100-fold excess of radioinert corresponding ligand. Bound steroid was separated from free steroid by the addition of two ice-cold volumes of dextran-coated charcoal (1% activated charcoal, 0.1% dextran T-70 in TEGW). Samples were treated for 5 min at 4 °C and then centrifuged at 12 000 $\times$ *g* for 4 min at 4 °C. Radioactivity in the supernatant was measured by liquid scintillation counting. The affinity of the hormone for the receptor was determined by Scatchard analysis (*13*). Competition studies were performed by displacing a saturating concentration of [^{3}H]aldosterone with increasing concentrations of radioinert steroids.

Cell Culture. Monkey kidney cells (CV-1) were obtained from American Type Culture Collection (ATCC) and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Cergy Pontoise, France) with L-glutamine, supplemented with 3% fetal bovine serum and 100 U/mL penicillin and streptomycin. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. Twenty-four hours prior to transfection, cells were removed from their culture flasks by trypsinization, resuspended in supplemented medium, and plated in 6-well plates (3×10^5 cells/well).

Transfection and Luciferase Assays. CV-1 cells were transfected by the calcium phosphate method using the Profection mammalian transfection system from Promega. Three hours prior to transfection, the medium was replaced by culture medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS). Cells were transfected with 1 μg/well of the expression vectors indicated, 4 μg/well of the luciferase reporter plasmid, and 0.5 μg/well of the pRL-TK luciferase control vector. Twenty-four hours after transfection, the medium was replaced and the transfectants were treated with hormones as indicated from a 1000$\times$ stock in ethanol. Luciferase activity in cell lysates was determined with the Dual-Luciferase reporter assay system from Promega. Both firefly and *Renilla* luciferase activities in the lysate were determined in a Wallac LKB 1251 luminometer. *Renilla* luciferase activity was assayed and used to correct for differences in transfection efficiency between individual wells. All transfections were performed in duplicate in two or more independent experiments.

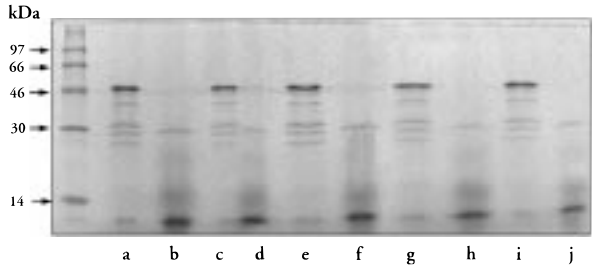

FIGURE 2: In vitro transcription and translation of G595-K984 (wt) and mutants. Proteins were expressed in reticulocyte lysate in the presence of [^{35}S]methionine as described in Materials and Methods. Native (lanes a, c, e, g, and i) and 100 μg/mL chymotrypsin-treated (lanes b, d, f, h, and j) receptor fragments are shown: G595-K984 (wt) (a, b), C808S (c, d), C849S (e, f), C910S (g, h), and C942S (i, j). The positions of molecular mass markers (in kDa) are shown at the left.

RESULTS

Expression of hMR, G595-K984 Fragment Receptor and Mutants. All mutations were performed within the hMR fragment G595-K984 encompassing the DBD, the hinge region, and the entire HBD. This fragment is hereafter referred to as G595-K984 (wt). Mutations of cysteines 808, 849, 910, and 942 individually substituted by serine within the G595-K984 fragment are referred to as C808S, C849S, C910S, and C942S, respectively.

Coupled in vitro transcription and translation of the full hMR and the G595-K984 (wt) fragment and its mutants was first performed to analyze the expression of each protein. The full hMR migrated as expected as a band with a molecular mass of 110 kDa (data not shown), whereas the G595-K984 (wt) and its mutants migrated as a major 41 kDa band (Figure 2). The wt and mutated G595-K984 receptors were expressed at similar levels: after scanning the gel using a phosphoimager, the data did not show any significant variation between the expression level of the different proteins. The lower molecular weight species also detected for each protein were presumably degradation products, incomplete or alternative translational products. Treatment of the [^{35}S]methionine-labeled translation products with 100 μg/mL chymotrypsin for 30 min at 20 °C resulted in protein digestion leading to a main fragment migrating as a 30 kDa band for each mutant. This 30 kDa fragment was previously described after digestion of native full hMR and characterized in the study of Couette et al. (*4*). This demonstrates that mutation of cysteine residues did not modify the conformation of the receptor regarding accessibility to enzyme attack.

Hormone Binding and Affinity. All mutants expressed in vitro in the reticulocyte lysate were tested for their ability to bind [^{3}H]aldosterone. The G595-K984 (wt) and mutants C808S, C849S, and C910S were able to bind aldosterone but not mutant C942S (data not shown). We then determined the maximum binding level for each mutant according to various protein concentrations by diluting the expression lysate medium (data not shown). The same binding level was obtained for the different mutants when diluting the lysate medium one-fourth for the full hMR, one-tenth for G595-K984 (wt) and C910S, one-twentieth for C808S and one-half for C849S.

The affinities of the different mutants for aldosterone were then determined by Scatchard analyses (Figure 3 and Table 1). The affinity of G595-K984 (wt) for aldosterone,

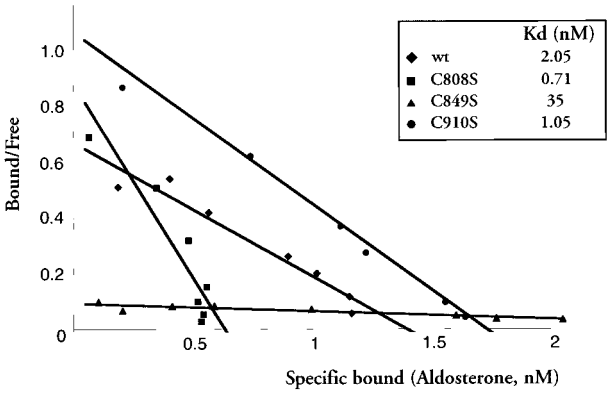

FIGURE 3: Determination of dissociation constant (Kd) of aldosterone binding to G595-K984 (wt) and mutants. Specific binding of [^{3}H]aldosterone to wt and mutants expressed in vitro in reticulocyte lysate was determined as described in Materials and Methods. The graph is one of the three or more Scatchard plots which were used to calculate the average Kd values presented in Table 1. Each point represents the average of triplicate determinations.

Table 1: Affinity of Aldosterone and Progesterone Binding to Wild Type and Mutants[a]

hMR mutant	aldosterone (Kd)	progesterone (Kd)
G595-K984	1.89 ± 0.58 (n = 6)	0.99 (n = 2)
C808S	0.53 ± 0.20 (n = 4)	0.37 (n = 2)
C849S	28.68 ± 9.15 (n = 3)	14.49 (n = 1)
C910S	1.69 ± 0.90 (n = 3)	1.02 (n = 1)
C942S	N.M.[b]	N.M.[b]

[a] Binding affinity (Kd) for G595-K984 (wt) and mutants expressed in vitro in reticulocyte lysate. Kd values were calculated by Scatchard analysis of equilibrium binding data (Figure 3). The average values are listed along with sem when more than two experiments were performed. The number of experiments (n) is indicated. Each experiment consists of triplicate at each hormone concentration. [b] N.M., not measurable.

characterized by a Kd of 1.89 ± 0.58 nM (n = 6), was actually slightly lower than the full hMR affinity (Kd about 1 nM, (*1*)). Mutant C910S bound with a similar affinity with a Kd of 1.69 ± 0.90 nM (n = 3), whereas the affinity of the C808S mutant was about 3-fold better than G595-K984 (wt) with a Kd of 0.53 ± 0.20 nM (n = 4). On the other hand, the C849S mutant exhibited a drastically decreased affinity with a Kd of 28.68 ± 9.15 nM (n = 3). No affinity could be determined for the binding-failure C942S mutant.

The binding of progesterone, a mineralocorticoid antagonist, was also examined (Table 1). The G595-K984 (wt) bound progesterone with a 2-fold higher affinity than aldosterone, in agreement with previous data for the entire hMR (*12*). C808S, C849S, and C910S were also characterized by a better affinity for progesterone. C942S failed to bind [^{3}H]progesterone. Moreover, this mutant failed to bind any tritiated steroid tested; it was also unable to bind [^{3}H]-cortisol and the synthetic mineralocorticoid antagonist, [^{3}H]-RU26752 (data not shown).

We further characterized the interaction of agonists and antagonists with C808S, C849S, and C910S. The ability of aldosterone, cortisol, progesterone, RU26752, and FH151 (a synthetic spirolactone antagonist (*10*) with an allenic substituent on the 11β position of the steroid) to compete for [^{3}H]aldosterone binding was examined.

When a saturating concentration of 5 nM [^{3}H]aldosterone was incubated with wt G595-K984, progesterone was the best competitor (Table 2; IC_{50} of 1.20), followed by the two synthetic spirolactones FH151 and RU26752 (IC_{50} of 3.74 and 4.08 nM, respectively), aldosterone (IC_{50} of 5.92), and cortisol (IC_{50} of 6.98). The same general pattern was also observed with mutants C808S and C910S (Table 2). However, when C849S was incubated with a saturating 50 nM [^{3}H]aldosterone concentration, a change in the specificity order was observed. The synthetic spirolactone FH151 proved to be the best competitor (IC_{50} of 11.65 nM) followed by progesterone (IC_{50} of 44.66 nM) and then aldosterone, RU26752, and cortisol (IC_{50} around 75−80 nM).

Transcriptional Activation Properties of Mutant Receptors. The transactivating abilities of the wild-type and mutant receptors were analyzed by measuring luciferase activity in CV-1 cells cotransfected with the corresponding expression plasmid and the glucocorticoid-responsive reporter gene TAT-TK-luc, a reporter plasmid containing two glucocorticoid response elements from the tyrosine aminotransferase (TAT) upstream of a thymidine kinase (TK)-luciferase reporter gene (*12*).

When CV-1 cells were transiently transfected by G595-K984 (wt) or the full hMR and then treated with a 10 nM aldosterone concentration, the transactivation potency of G595-K984 (wt) was 20% that observed for the full hMR (not shown), which was in agreement with the results of previous studies (*11*, *15*). Maximum transcriptional activation was observed with 1 nM aldosterone for G595-K984 and its mutants C808S and C910S, while 100 nM aldosterone was needed for C849S to achieve activation (Figure 4). No luciferase activation was observed with C942S (not shown). We assume that the higher transactivation level observed for C808S is mainly the result of a higher expression of this protein in CV-1 cells rather than a stronger activation.

The effect of agonists and antagonists on the luciferase gene transactivation was then studied (Figure 5). Transiently transfected CV-1 cells were then treated with an aldosterone concentration able to saturate each mutant (10 nM for G595-K984 (wt), C808S, and C910S and 100 nM for C849S), and the luciferase activity was determined. With the various mutants, the transactivation activities were nearly identical. Transactivation of mutants in response to 10 or 100 nM cortisol, a mineralocorticoid agonist, was also measured (Figure 5). While cortisol activated transcription of full hMR at a higher level than aldosterone (not shown), this higher cortisol effect was not observed with G595-K984 and the mutants. The maximum ability of each mutant was essentially equivalent to that of G595-K984 (wt) and similar to that observed with aldosterone. With the C849S mutant, 100 nM cortisol was not sufficient to reach the same level of transcription. No reporter gene activation was observed when mutant C942S was treated with 100 nM aldosterone or cortisol. Subsequently, transiently transfected CV-1 cells were first treated with a saturating aldosterone concentration and then incubated with 1 mM progesterone. This progesterone concentration completely abolished the transcription activation induced by aldosterone on C808S, C849S, and C910S (Figure 5). At this concentration, progesterone did not show any significant agonistic effect (Figure 5). Mutant C942S was also insensitive to progesterone.

Table 2: Relative Binding Affinity of Cysteine Mutants for Steroids[a]

hMR mutant	aldosterone (IC_{50})	progesterone (IC_{50})	cortisol (IC_{50})	RU26752 (IC_{50})	FH151 (IC_{50})
G595-K984	5.95 ± 0.24	1.29 ± 0.09	6.98 ± 0.29	4.08 ± 0.32	3.74 ± 0.21
C808S	4.87 ± 0.12	1.66 ± 0.12	4.54 ± 0.22	3.33 ± 0.07	3.26 ± 0.10
C849S	75.57 ± 13.74	44.66 ± 1.89	81.00 ± 8.60	78.00 ± 8.16	11.65 ± 1.63
C910S	4.80 ± 0.43	2.23 ± 0.10	6.21 ± 0.70	4.80 ± 0.43	3.76 ± 0.14

[a] In vitro expressed receptors were incubated with 5 nM (G595-K984, C808S, and C910S) or 50 nM (C849S) [^{3}H]aldosterone and then incubated with increasing concentrations of the corresponding unlabeled steroid as described under Materials and Methods. IC_{50} values were calculated from dose-response curves and are reported in nanomolar concentrations. The average values are listed along with sem for three experiments.

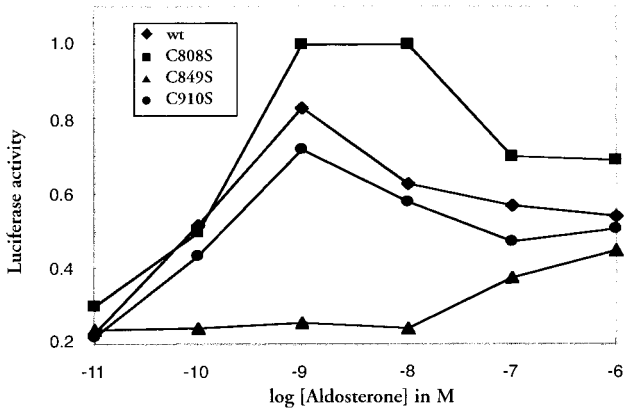

FIGURE 4: Transcriptional activity of G595-K984 (wt) and mutants in response to aldosterone. CV-1 cells were cotransfected with an expression vector encoding either wild-type or mutant G595-K984 receptor fragment and a reporter plasmid, TAT-TK-luc, according to the method described under Materials and Methods. The cells were then treated with increasing aldosterone concentrations. After cell lysis, the corrected firefly luciferase activity (arbitrary units, see Materials and Methods) was plotted as a function of the concentration of aldosterone added to each well. All values were determined from an average of three independent transfections. The error bars unshown for reasons of clarity were comprised between 0.04 and 0.15 (luciferase arbitrary units).

DISCUSSION

To assess the role of cysteine residues in the hMR HBD, we produced the various single cysteine to serine mutants (C808S, C849S, C910S, and C942S) in the receptor fragment G595-K984 encompassing the DNA binding domain, the hinge region, and the hormone binding domain of hMR, and their binding and transcriptional properties were determined.

Substitution of cysteine for serine at the C910 position generated a mutant receptor with properties that were similar to those of the G595-K984 (wt) receptor.

This mutation did not alter ligand binding and transcriptional activity, indicating that C910 is not crucial for these functions. Furthermore, this cysteine residue is not involved in the specificity of the receptor. There is no homologous cysteine residue in the other steroid receptors at this position located in helix H9 (Figure 1) which in the model described by Wurtz et al. (*7*) does not seem to be directly involved in the ligand binding pocket.

As observed for C910, there is also no cysteine residue equivalent to C808 in the other steroid receptors (Figure 1). While not substantially modifying transcriptional activity of the G595-K984 fragment, mutation into serine at this position led to a 3-fold increase in binding affinity. Most mutations in the HBD of steroid receptors reduce or abolish receptor activity. However, there are some reports of GR mutants with increased activity (for a review, see ref *16*). For example, mutations at position C656 of rGR (*17*, *18*) or C638 (*19*) of hGR created a "super receptor" with higher sensitivity to the ligand. It had been inferred that the presence of a cysteine at this position could attenuate the steroid binding affinity of the GR (*8*) or that this position would contact a cofactor which the mutant could more efficiently displace (*19*). However, inasmuch as the C808S mutation did not significantly modify the transcriptional activity of the receptor (Figures 4 and 5), it is not likely that this position contacts a cofactor involved in the transcriptional machinery. As serine is both a smaller and more polar residue than cysteine, C808 which is located in helix H5 (one of the helices most involved (*7*, 20) in contacting the steroid) might rather participate in the development of the binding pocket and possibly attenuate the steroid binding affinity through steric hindrance.

The introduction of a serine at C849 located in helix H7 of the hMR HBD drastically reduced the affinity for both agonists and antagonists. Furthermore, it significantly decreased the hormonal sensitivity of the receptor.

Except for ER, the homologous positions of hMR C849 were also cysteine residues in the other steroid receptors: C643 in hGR, C784 in hAR, C789 in hPR (Figure 1).

Introduction of a serine residue at the corresponding position in the GR [(C643 in hGR (*19*), C661 in rGR (*18*), and C649 in mGR (*21*)] induced no drastic loss of hormone binding activity (a 4-fold loss in binding affinity for rGR C661S (*18*), whereas hGR C643S (*19*) essentially maintained wild-type affinity); the mutation of this position in mGR to a glycine indicated a very slight but reproducible increase of about 2.5-fold in the IC_{50} of dexamethasone (*21*). Altogether, these results indicate that C643 is not crucial for the ligand binding process of GR, but it is nevertheless involved in maintaining specificity to glucocorticoids (*19*). Furthermore, this cysteine residue had been demonstrated to be part of the vicinal thiol pair in the hGR jointly with C638 (*8*, *18*) and the presence or absence of an intramolecular disulfide between a vicinally spaced pair of cysteine SH groups in the HBD was demonstrated to be one factor determining steroid binding. From the primary structure of this receptor, a unique bithiol is observed in the HBD (C656 and C661 of rGR corresponding to C638 and C643 of hGR). There is no thiol pair equivalent in hMR since the effect of arsenite, a specific reagent for vicinal dithiols, provides a way to distinguish between GR and MR (*22*).

The ER crystal structure (*20*) is currently the only available structure for a steroid receptor HBD which could be a model for the ligand pocket environment. In the crystal, the C849 (hMR) equivalent position is F425 (hER) which lies close to I424. This latter residue makes nonpolar contacts with the D-ring of the steroid, which was already predicted by Wurtz's canonical model for nuclear receptors (*7*). The introduction of serine, a polar residue, close to L848 of hMR

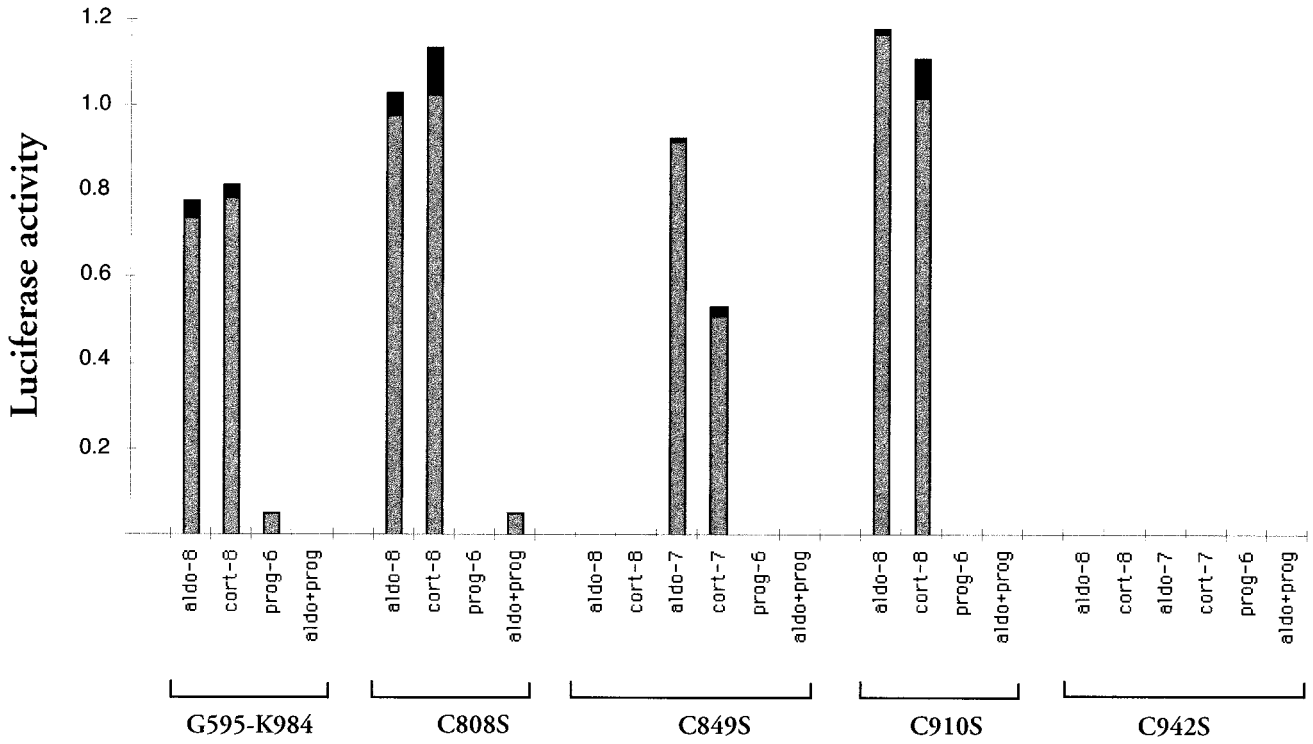

FIGURE 5: Transactivation properties of G595-K984 (wt) and mutants in response to agonists and antagonists. CV-1 cells were as described in Figure 4 legend. Cells were treated with the indicated steroid concentration (10 nM aldosterone, 10 nM cortisol, 1 mM progesterone, or 10 nM aldosterone + 1 mM progesterone) as described under Materials and Methods. The two mutants C849S and C942S were also treated with 100 nM aldosterone and 100 nM cortisol. All values (luciferase arbitrary units, see Materials and Methods) were determined from an average of three independent transfections. The black tip on each column represents the error bar.

might trigger some change in the conformation, causing the drastic loss of affinity and sensitivity of C849S.

Introduction of a serine at position C942 in helix 11 of hMR generated a mutant receptor unable to bind any assayed steroid and which was devoid of any transcriptional activity. The corresponding position in hGR (C736) and its equivalent in rGR (C754) and in mGR (C742) were previously demonstrated by photoaffinity labeling to lie in close proximity to the bound hormone molecule thus highlighting a direct role of Cys736 in hormone binding (*23*, *24*, for a review, see ref *16*). However, the homologous cysteine residue C891 in hPR is not photoaffinity labeled suggesting a putative role for this position in steroid specificity (*24*). A mutant C742G (mGR) had been previously described as an unstable and almost completely nonfunctional protein (*25*). After randomization of residue 736 by oligonucleotide-directed mutagenesis, only three substitutions were found to result in significant activity (C736A, S, T) although they altered both receptor function and steroid binding affinity to GR (*26*).

In other respects, ER crystal structure data (*20*) demonstrate that the estradiol D-ring makes nonpolar contacts with L525 (equivalent ER position to C942 of MR), and they indicate that the 17β hydroxyl of the estradiol D-ring makes a single hydrogen bond with the former residue (His 524) in H11.

Due to the slight steroid molecule differences in their relative ligands, the 3-ketosteroid receptors are actually more closely related to each other than to the estrogen receptor. The part of the steroid molecule which might interact with C942 of hMR could be either the 3-keto group in the A ring or the 20-keto group in the D-ring. Consequently, all steroids known to bind hMR, such as aldosterone, progesterone, cortisol, and synthetic spironolactones described in this study, bear both keto groups. From the ER structure, we assume that the group of the steroid contacted by C942 is preferentially the 20-keto group. On the basis of the ER structure, we also consider that C942 might be involved with the 20-keto group through a hydrogen bond in the same way as the 17β hydroxyl of the estradiol with His 524. The lack of steroid binding by mutant C942S indicates that substitution of SH into OH dramatically jeopardizes hormone binding probably due to the introduction of the small polar hydroxyl group of the side chain. A hydrogen bond with a sufhydryl group might be better tolerated in the hydrophobic environment of the cavity while the hydroxyl of serine, as it is more polar, could be hydrated with water molecules, thus preventing good contacts between steroid and amino acid. We therefore conclude that the polar hydroxyl group in the serine side chain interferes with hydrophobic interactions that generally take place during ligand binding, precluding any contact. Altogether, our data highlight the importance of the C942 position for the ligand binding process, indicating it as a putative anchorage point of the molecule in the cavity of hMR.

In conclusion, none of the cysteine−serine substitutions that are described in this study induced a change in the specificity of the receptor, contrasting with results obtained with similar mutations in other steroid receptors. Furthermore, another related mutation, I871C (helix H8, Figure 1), did not modify the binding specificity of hMR even though the homologous position in hGR (C665) is a candidate for glucocorticoid versus mineralocorticoid selectivity (*19*). The mutation I871C only induced a slight decrease in the binding

affinity for both agonists and antagonists (a Kd of 4 nM for aldosterone and 1.5 nM for progesterone were calculated from Scatchard analyses, data not shown). These overall results indicate that cysteine residues in the hMR are not responsible for ligand specificity but two of them, C849 and C942, actively participate in the ligand binding process for both agonists and antagonists. Results obtained for the mutant receptor C942S support nuclear receptor models, assigning a direct role to that position in ligand binding, and our results indicate that this direct interaction most likely occurs through a hydrogen bond between the amino acid and the 20-keto group of the receptor, which could account for one of the anchorages of the steroid in hMR.

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