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Antagonism in the human mineralocorticoid receptor

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Key residues of the human mineralocorticoid receptor (hMR) involved in the recognition of agonist and antagonist ligands were identified by alanine-scanning mutagenesis based on a homology model of the hMR ligand-binding domain. They were tested for their transactivation capacity and ability to bind agonists (aldosterone, cortisol) and antagonists (progesterone, RU26752). The three-dimensional model reveals two polar sites located at the extremities of the elongated hydrophobic ligand-binding pocket. Mutations of Gln776 and Arg817 in site I reduce the affinity of hMR for both agonists and antagonists and affect the capacity of hMR to activate transcription, suggesting that the C3-ketone group, common to all ligands, is anchored by these two residues conserved within the nuclear steroid receptor family. In contrast, mutations of Asn770 and Thr945 in the opposite site only affect the binding of agonists bearing the C21-hydroxyl group. The binding of hMR antagonists that exhibit a smaller size and faster off-rate kinetics compared with agonists is not affected. In the light of the hMR homology model, a new mechanism of antagonism is proposed in which the AF2-AD core region is destabilized by the loss of contacts between the antagonist and the helix H12 region.

Keywords: alanine-scanning mutagenesis/antagonist/human mineralocorticoid receptor/molecular modeling/nuclear receptor

Introduction

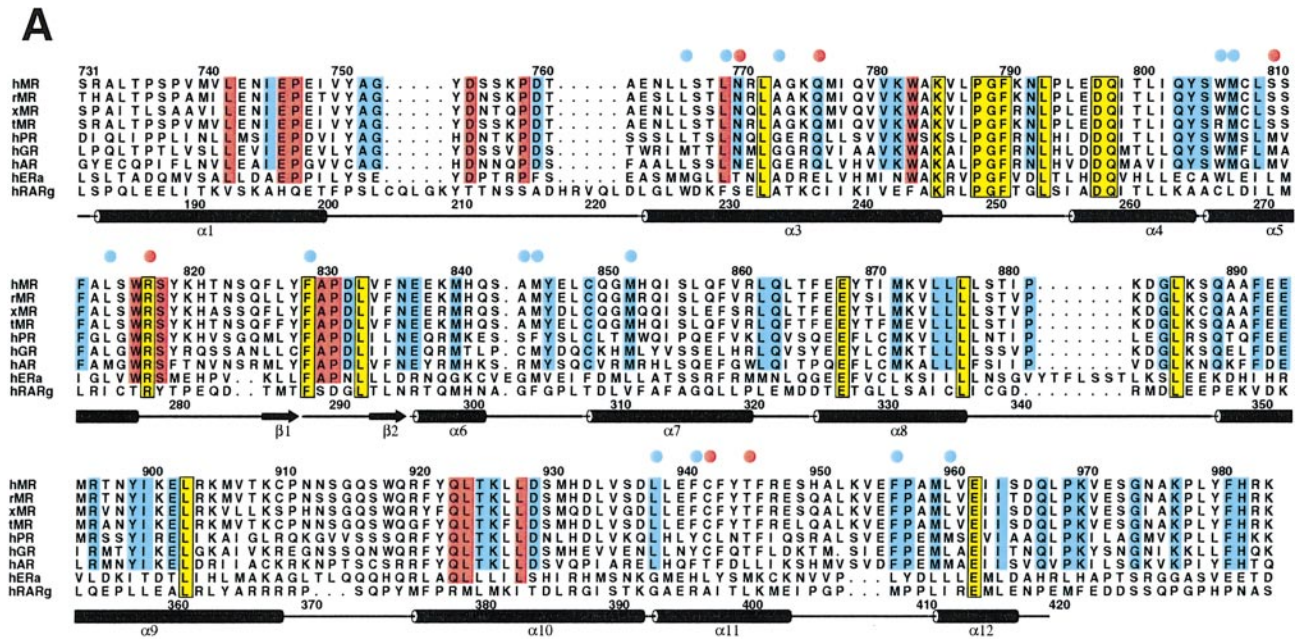
Aldosterone is a mineralocorticoid hormone that plays a major role in regulating sodium and potassium homeostasis (for reviews see Horisberger and Rossier, 1992; Bonvalet, 1998). It participates in the control of blood pressure and is implicated in some pathological disorders. Aldosterone exerts its effects by acting through a ligand-activated transcription factor, the mineralocorticoid receptor (MR). MR is a member of the nuclear receptor (NR) family that includes receptors for steroid and thyroid hormones, vitamin D3 and retinoic acids as well as numerous orphan receptors for which no ligands are known (Evans, 1988; Tsai and O'Malley, 1994; Mangelsdorf *et al*., 1995; Ribiero *et al*., 1995). NRs display a modular structure comprising five to six regions (A–F), with the N-terminal A/B region harboring an autonomous activation function, while the C and E regions correspond to the DNA-binding domain and the ligand-binding domain (LBD). Recently, the crystal structure of unliganded and liganded NR LBDs have been solved (Bourguet *et al*., 1995; Renaud *et al*., 1995; Wagner *et al*., 1995; Brzozowski *et al*., 1997). These crystal structures reveal a triple-layer antiparallel α-helical sandwich fold, with the major difference between the apo and holo states being the folding back of helix H12 towards the LBD core that leads to a more compact structure of the liganded LBD (Wurtz *et al*., 1996).

Aldosterone-dependent activation of gene transcription is thought to be a multistep process. Initially, aldosterone binds to the MR and causes a receptor *trans*-conformation within the LBD that is supposed to lead to the dissociation of the associated proteins from the receptor (Trapp and Holsboer, 1995; Couette *et al*., 1996). Then, the ligand-activated receptor binds as a dimer to the response elements present in the promoter region of target genes and initiates the hormone-mediated transcription through specific interactions with the transcription machinery (Lombès *et al*., 1993; Liu *et al*., 1995; Trapp and Holsboer, 1996). The antimineralocorticoid spirolactones, synthetic steroids with a C17 γ-lactonic ring, have been used for the past 30 years in the treatment of sodium-retaining states and as antihypertensive agents (Corvol *et al*., 1981; Sutanto and de Kloet, 1991). Mineralocorticoid antagonists bind to the receptor with an affinity identical to that of aldosterone and induce a receptor conformation that is transcriptionally silent, despite their smaller size compared with agonists (Couette *et al*., 1996). To determine how agonists and antagonists interact with the human MR (hMR) and to understand the consequence of their interaction on the receptor transactivation function, we constructed a three-dimensional (3D) model of the hMR-LBD, taking into account the homology of MRs with the other members of the NR superfamily and the crystal structure of the human retinoic acid receptor-gamma ligand-binding domain (hRARγ-LBD). We then explored the role of polar amino acid residues located in the LBP by alanine-scanning mutagenesis. These mutants were tested for their transactivation and ligand-binding capacities.

Results

Sequence alignment

The MR family shows a high degree of sequence conservation (82%). Comparison of MRs with human progesterone, glucocorticoid and androgen receptors (hPR, hGR and hAR) reveals a lower sequence identity (48%) but a strong



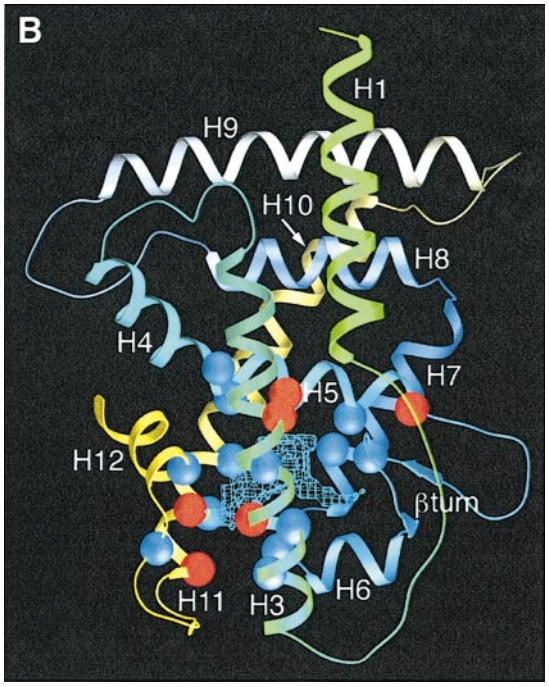


Fig. 1. Sequence alignment and homology model of the hMR-LBD. (**A**) The alignment includes MRs from numerous organisms and also hGR, hPR and hAR. The organism abbreviations are: h, *Homo sapiens*; r, *Rattus norvegicus*; x, *Xenopus laevis*; t, *Tupaia belangeri*. The sequences of the hERα and hRARγ for which crystal structures have been determined are also included. The alignment was derived as described in Materials and methods. The sequence numbering above and below the alignment are for the hMR and the hRARγ, respectively. Identical residues in the whole alignment are highlighted in yellow. Highly conserved residues between only hMR, hGR, hPR and hAR are boxed in blue, and in red for the steroid family. The secondary structure information shown below the alignment corresponds to the hRARγ crystal structure. hMR residues closer than 4.5 Å to the ligand are indicated by blue or red colored dots for hydrophobic or polar residues, respectively. The figure was prepared using ALSCRIPT (Barton, 1993). (**B**) Scheme showing the overall fold of the hMR-LBD with α-helices drawn as ribbons and β-strands as arrows. The putative ligand-binding cavity is depicted as a blue chicken wire surface. Residues in the close vicinity (4.5 Å) of the ligand are indicated by blue and red spheres for hydrophobic and polar residues, respectively. The figure was produced with SETOR (Evans, 1993).

sequence similarity (Figure 1A). The hRARγ and the human estrogen receptor α-isoform (hERα) sequences (Renaud *et al.*, 1995; Brzozowski *et al.*, 1997), whose LBD crystal structures have been solved recently, are included in the alignment for comparison. They share a much lower sequence identity (<20%) with MRs. Nevertheless, all 11 helices observed in hRARγ are well matched and represent the anchoring points for the alignment process. Highly conserved amino acids are located in the regions that belong to the characteristic NR signature (Wurtz *et al.*, 1996). Furthermore, the most variable regions correspond to loops connecting the secondary structure elements.

Model building

Previous reports have shown that the NR LBDs share a common architecture. Taking into account the sequence alignment in Figure 1A, we constructed a 3D model of the hMR-LBD using the crystal structure of the holo hRARγ-LBD as our template (Figure 1B). The loops that differ in length between hMR and hRARγ were kept as generated by the Modeller program (two additional residues between helix H5 and the β-turn, one residue between helices H8 and H9, three residues between helices H9 and H10 and between helices H11 and H12). The only exception is the loop between helices H1 and H3 (loop L1–3), where a 10 residue deletion is observed in hMR compared with hRARγ. This loop has been constrained to follow as closely as possible the Cα-trace of the crystal structure in the Modeller calculations. It adopts an extended conformation sufficient to link helices H1 and H3 (Figure 1B). Statistics, calculated with PROCHECK (Laskowski *et al.*, 1993), show that >97% of the residues in the Ramachandran plot are in the most favored or

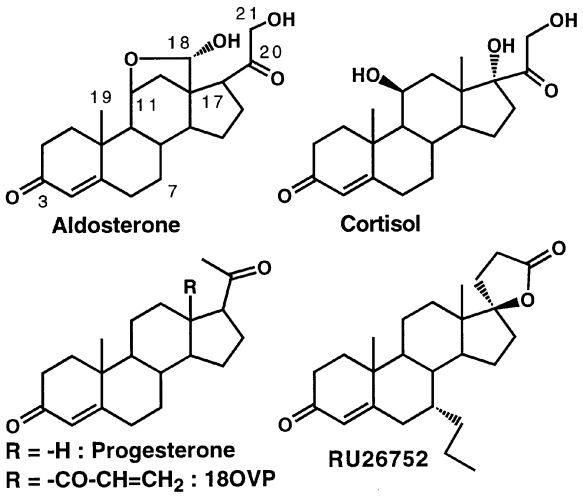


Fig. 2. hMR steroid agonist [aldosterone, cortisol and 18-oxo-18-vinylprogesterone, (18OVP)] and antagonist (progesterone and RU26752) ligands.

allowed regions and that side chain stereo-parameters are inside the range or better than the statistics derived from a set of crystal structures of at least 2.0 Å resolution. In addition, the program PROSAII (version 3.0; Hendlich *et al.*, 1990) gives a combined Z-score (Cβ and surface potentials) of –7.3, a value close to the range observed for RARγ and RXRα (–9.7 and –8.1, respectively). These results suggest that our model is of good quality despite the low sequence identity, and is suitable for further analysis.

As in the hRARγ-LBD crystal structure, the hMR-LBP is delineated by the helices H5, H7, H11 and H12, the β-turn and the loops L6–7 and L11–12 (Figure 1B). The probe-occupied volume of 469 Å^3, as calculated with VOIDOO (Kleywegt and Jones, 1994), is larger than that of the hRARγ (418 Å^3) but consistent with the size of aldosterone (303 Å^3, as calculated with GRASP; Nicholls *et al.*, 1991) compared with the all-*trans* retinoic acid (278 Å^3). The cavity is lined with 20 residues, of which 14 contribute to the hydrophobic nature of the cavity: Leu766, Leu769 and Ala773 (helix H3); Trp806, Met807 and Leu814 (helix H5); Phe829 (β-turn); Ala844 and Met845 (loop L6–7); Met852 (helix H7); Leu938 and Phe941 (helix H11); Phe956 (loop L11–12); and Leu960 (helix H12), which are highlighted by blue filled circles in Figure 1A and B. Five polar residues (Figure 1A and B, red filled circles) are located at the two extremities of the cavity: one site (site I) is composed of Gln776 (helix H3) and Arg817 (helix H5), and the other site (site II) comprises Asn770 (helix H3), Cys942 and Thr945 (helix H11). An additional polar residue, Ser810 in helix H5, is located in the middle of the cavity. The organization of the binding pocket is consistent with the two polar extremities of aldosterone (the C3-ketone group on the A-ring on one hand and the C20-ketone and the C21-hydroxyl groups on the other hand; Figure 2).

Agonist and antagonist binding to mutant hMRs

In order to determine the ligand orientation in the LBP and to probe the role of the polar residues in the agonist– and antagonist–hMR interaction, we tested the ability of various steroids to bind to mutant hMRs in which the polar residues were substituted by alanine (N770A, Q776A, S810A, R817A and T945A). The tested steroids were chosen for their structural characteristics (Figure 2). All the steroids harbor a ketone group at the A-ring C3-position. Aldosterone and cortisol, two mineralocorticoid agonists, have in common the C20-ketone and C21-hydroxyl groups, whereas aldosterone is characterized by an 11–18 hemiketal bridge and cortisol by C11β- and C17α-hydroxyl groups. Progesterone, a mineralocorticoid antagonist (Wambach and Higgins, 1978; Rafestin-Oblin *et al.*, 1991; Souque *et al.*, 1995), harbors only the C20-ketone group, and RU26752, a synthetic mineralocorticoid antagonist (Ulmann *et al.*, 1985), a C17 γ-lactonic ring.

Table I. Steroid dissociation constant at equilibrium (K_d) for the wild-type and mutant hMRs

	Aldosterone	Cortisol	Progesterone
WT	0.52 ± 0.03	0.87 ± 0.13	1.04 ± 0.06
N770A	–	–	1.19 ± 0.05
Q776A	6.71 ± 0.94	37 ± 10	5.82 ± 0.69
S810A	0.67 ± 0.06	0.76 ± 0.03	1.38 ± 0.08
R817A	9.69 ± 0.80	–	19.2 ± 3.80
T945A	3.87 ± 0.29	4.4 ± 0.3	1.21 ± 0.05

Wild-type (WT) and mutant hMRs were synthesized in the rabbit reticulocyte lysate. The lysate was 2-fold diluted with TEGWD buffer and incubated with increasing concentrations of [^{3}H]aldosterone, [^{3}H]cortisol or [^{3}H]progesterone for 4 h at 4°C. Bound and unbound steroids were separated by charcoal–dextran, and the K_d values (nM) were determined by computer analysis; –, undetected.

Mutant hMRs were transcribed and translated *in vitro* in the rabbit reticulocyte lysate. An electrophoretic analysis of all the ^{35}S-labeled mutant hMRs revealed a similar expression level for the protein band corresponding to a molecular mass of ~110 kDa (data not shown).

The affinities of tritiated aldosterone, cortisol and progesterone for the mutant hMRs were determined by Scatchard analysis. The K_d values are reported in Table I and the representative Scatchard plots are depicted in Figure 3A. Aldosterone binds to the wild-type hMR with an affinity of 0.52 nM, which is in the range of those previously reported (Arriza *et al.*, 1987; Binart *et al.*, 1991). Q776A, R817A and T945A display a lower affinity for aldosterone compared with the wild-type hMR, the K_d values ranging from 3 to 10 nM (Table I). The ability of hMR to bind cortisol also decreases after substitution of Thr945, Gln776 and Arg817 by alanine, but the effect was more pronounced than that observed for aldosterone binding. Indeed, T945A and Q776A have affinities for cortisol that are 5- and 40-fold lower, respectively, than the wild-type hMR, and the R817A mutant was unable to bind cortisol with a detectable affinity. Mutation of Asn770 to alanine completely abolishes the ability of hMR to bind aldosterone and cortisol.

The measured affinity of progesterone for the wild-type hMR was 1 nM, a value in good agreement with that already published for the recombinant hMR expressed using the baculovirus system (Souque *et al.*, 1995). The affinities of Q776A and R817A for progesterone were ~6-and 20-fold lower, respectively, than that of the wild-type hMR. In contrast, the affinity for progesterone of T945A and N770A was identical to that of the wild-type. Similarly, the affinity for RU26752 of T945A and N770A was at the same level as that of the wild-type hMR (data not shown). Mutation of

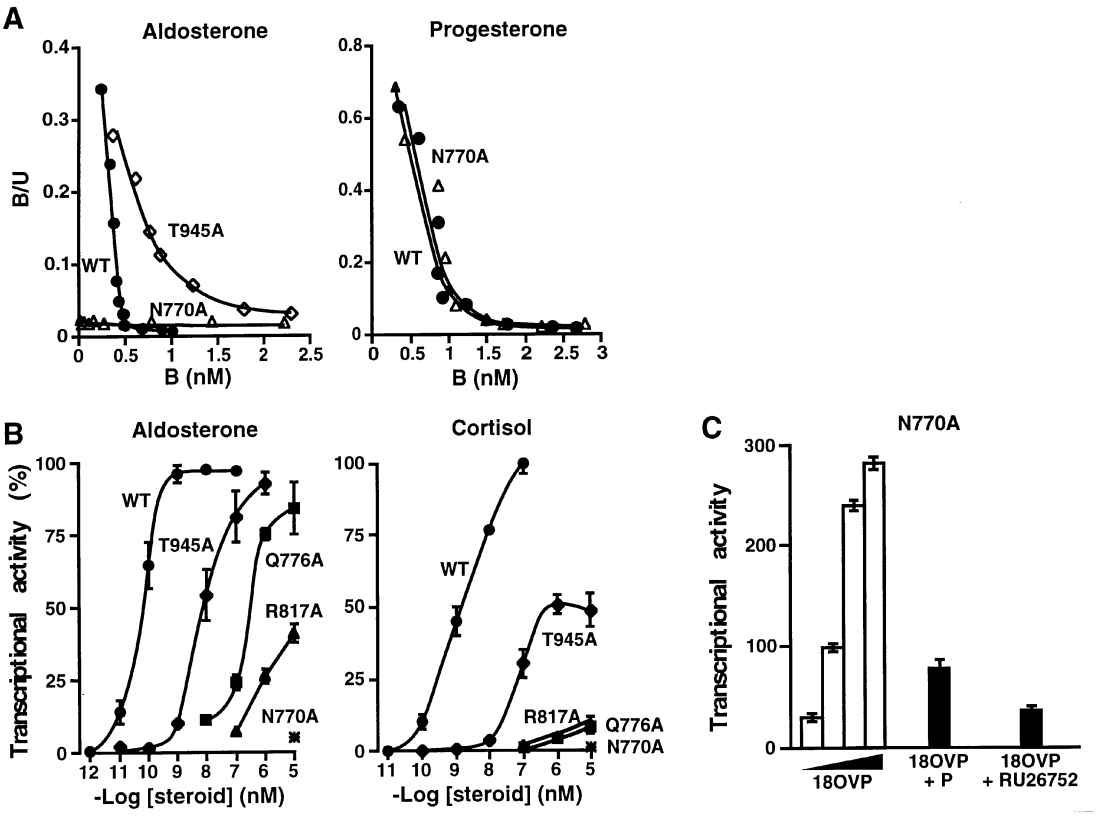


Fig. 3. Binding affinity and transactivation capacity of the wild-type and mutant hMRs. (**A**) Scatchard plot of [^{3}H]aldosterone and [^{3}H]progesterone binding to the wild-type and mutant hMRs. The wild-type or mutant hMRs were synthesized *in vitro* in a rabbit reticulocyte lysate. The lysate was diluted 2-fold with TEGWD buffer and incubated with increasing concentrations (0.1–100 nM) of [^{3}H]aldosterone (left) or [^{3}H]progesterone (right) for 4 h at 4°C. Bound (B) and unbound (U) steroids were separated by the dextran–charcoal method. (**B**) Transcriptional activation of luciferase activity by wild-type and mutant hMRs. COS-7 cells were transfected with wild-type or mutant hMR expression vectors, pFC31luc as reporter plasmid and a β-galactosidase internal reporter to correct for transfection efficiency. Before harvesting, cells were treated for 24 h with aldosterone from 10^{-12} to 10^{-5} M (left) or with cortisol from 10^{-11} to 10^{-5} M (right). Transactivation was determined by luciferase activity, normalized to the internal β-galactosidase control and is expressed as a percentage of wild-type activity at 10^{-7} M. Each point is the mean ± SEM of three separate experiments. (**C**) Transcriptional activation of luciferase activity by the N770A mutant. COS-7 cells were transfected with the mutant N770A expression vector and treated with 18OVP from 10^{-9} to 10^{-6} M or with 10^{-7} M 18OVP plus 10^{-5} M progesterone (P) or 10^{-5} M RU26752. Transactivation was determined by luciferase activity, normalized to the internal β-galactosidase control and is expressed in arbitrary units. Each point is the mean ± SEM of three separate experiments.

Ser810 to alanine does not alter the binding of aldosterone, cortisol or progesterone (Table I). Altogether, these results are in favor of an interaction of Gln776 and Arg817 with the C3-ketone group present in all the tested steroids and of an interaction of Asn770 and Thr945 with the 17β-substituent of corticosteroids.

Transactivation properties of mutant hMRs

To analyze the effect of the mutations on the hMR activity, mutant and wild-type cDNAs were transiently transfected into COS-7 cells together with a reporter plasmid containing the mouse mammary tumor virus (MMTV) promoter upstream of the luciferase gene. Dose–response curves were generated by adding increasing concentrations of aldosterone or cortisol to transfected cells (Figure 3B). Aldosterone increases the luciferase activity of the wild-type hMR in a dose-dependent function, with an ED_{50} value of ~0.1 nM. Substitution of Ser810 by alanine does not modify the aldosterone-induced transactivation function of hMR (data not shown). Substitution of Thr945 and Gln776 by alanine induces a shift in the dose–response curve of the aldosterone-induced luciferase activity towards higher concentrations, with ED_{50} values of ~10 nM for T945A and ~100 nM for Q776A. The aldosterone-mediated transactivation function was very low for R817A and almost undetectable for N770A (Figure 3B, left). A good correlation was observed between the decrease in the binding affinity of the mutant hMRs for aldosterone and the decrease in their transactivation capacity.

Cortisol acts as a potent inducer of the hMR activity, stimulating the hMR transactivation to the same level as aldosterone. Nevertheless, the sensitivity of hMR to cortisol (ED_{50} ~1 nM) was lower compared with aldosterone (Figure 3B, left and right), a result in good agreement with previous observations (Arriza *et al*., 1988; Rupprecht *et al*., 1993; Lombès *et al*., 1994). The cortisol-mediated transactivation function was lowered after substitution of Thr945 by alanine. Addition of high concentrations of cortisol (up to 10^{-5} M) to transfected cells did not stimulate the transactivation function of T945A maximally. Substitution of Gln776, Arg817 and Asn770 by alanine dramatically altered the cortisol-mediated transactivation function of hMR. Thus, the decrease in cortisol affinity that is observed after alanine substitution of Thr945, Gln776, Arg817 and Asn770 is correlated with the decrease in the cortisol-mediated transactivation function.

Since T945A and N770A were able to bind progesterone with an affinity similar to that of the wild-type hMR, we hypothesized that 18-oxo-18-vinylprogesterone (18OVP), a progesterone derivative previously shown to stimulate the transactivation function of hMR (Souque *et al.*, 1995), would keep its binding characteristics and agonist properties when acting through N770A. The 18OVP-N770A complex induces the luciferase activity with an ED_{50} of $\sim 5 \times 10^{-8}$ M, a value close to that observed with the wild-type receptor (Figure 3C; Souque *et al.*, 1995). Furthermore, progesterone and RU26752 at a concentration of 10^{-5} M were both able to antagonize the 18OVP-mediated activity of N770A. These results indicate that substitution of Asn770 does not alter the agonist activity of 18OVP nor does it modify the binding and antagonist features of progesterone and RU26752.

Ligand docking

The alanine-scanning mutagenesis revealed different ligand-binding and transactivation capacity patterns for the selected ligands. Especially, the substitution of Ser810 by alanine does not have any effect either on the ligand-binding affinity or the transactivation properties of the receptor, suggesting that this residue is not critical for ligand binding. In contrast, the substitution of Arg817 or Gln776 by alanine induces a dramatic decrease in the affinity of the ligands irrespective of their agonist or antagonist behavior. This supports the idea that Arg817 and Gln776 interact with the C3-ketone group, present in all the agonist and antagonist compounds. In the case of T945A and N770A, only the binding of agonists harboring a C21-hydroxyl group is affected, suggesting an interaction between Asn770 and Thr945 and the 17β-substituent of these ligands.

Taking these results into account we manually docked aldosterone into the LBP using the probe-accessible and van der Waals calculated volumes as guides (Materials and methods). This aldosterone–hMR-LBD complex was then refined further (Materials and methods). The A-ring of aldosterone forms close contacts with Ala773 (helix H3, 3.4 Å), Leu814 (helix H5, 3.5 Å) and Phe829 (β-turn, 3.4 Å) (Figure 4A). In particular, Phe829 makes π interactions with the α,β-unsaturated ketone. Furthermore, Gln776 (helix H3) and Arg817 (helix H5) form strong hydrogen bonds with the C3-ketone group (2.9 and 3.1 Å, respectively; Figure 4A and B). The estradiol– and raloxifene–hERα-LBD crystal structures have shown the presence of water molecules in the vicinity of the C3 position. Similarly, we placed one water molecule, which acts as a hydrogen bond donor with the C3-ketone group (2.7 Å) and the Gln776 carbonyl group (2.7 Å) and as a hydrogen bond acceptor with the Arg817 guanidinium group (3.0 Å; Figure 4B).

At the other extremity of the cavity, Phe956 (loop L11–12) is close to the C21-hydroxyl group, and the Thr945 γ-methyl group (helix H11) is in a van der Waals contact with the C20-ketone and the C21-hydroxyl groups. The Thr945 hydroxyl group seems to be involved in the stabilization of loop L11–12 by forming a hydrogen bond with Ser949 (3.0 Å between both oxygen atoms) (Figure 4A and C). In addition, the C21-hydroxyl group is implicated in two hydrogen bonds: one with the steroid C20-carbonyl (2.7 Å) and the other with the Asn770 δ-amide groups (2.9 Å). The C20-carbonyl heavy atom is at a distance of 3.1 Å from the Cys942 sulfhydryl heavy atom forming a hydrogen bond; the angle formed between the C20-carbonyl group and the sulfhydryl hydrogen has a value of 117°. Similar short cysteine–main chain ketone group distances and angles are observed in well-refined protein crystal structures (resolution ⩽2Å, 204 interactions with distances ranging from 2.9 to 3.4 Å and angles close to 115°; Iditis V3.0, Oxford Molecular Ltd; Figure 4A and C). The hemiketal hydroxyl group is also close to the Cys942 sulfhydryl (3.4 Å), but no direct hydrogen bond is involved in this interaction. Note that near the hemiketal moiety, the LBP can accommodate a water molecule which acts as hydrogen bond acceptor for the 18-hydroxyl group (1.7 Å) and as a hydrogen bond donor to the sulfhydryl atom of Cys942 (2.3 Å) and Met807 (2.3 Å).

Finally, the core of the steroid forms numerous short van der Waals contacts. The α-face is close to Leu769 (helix H3, 3.7 Å) and Ala844 (L6–7, 3.8 Å) and the β-face is tightly packed against helix H5. In particular the C18-hemiketal and C19-methyl groups fit well in the probe-accessible volume and are close to Met807 and Trp806, respectively (4.2 and 3.5 Å, respectively; Figure 4A). The D-ring is blocked by Leu938 (helix H11, 3.4 Å), Phe941 (helix H11, 3.8 Å), Ala844 (loop L6–7, 3.8 Å) and Met845 (loop L6–7, 3.8 Å; Figure 4A).

All the other ligands (cortisol, progesterone, RU26752 and 18OVP) were docked according to the aldosterone position, and the different complexes were refined. Due to the absence of the 11–18 hemiketal bridge, cortisol rotated around its C3–C17 axis with an angle of ~40°. The 21- and 17β-hydroxyl groups form hydrogen bonds with the Asn770 δ-carboxyl and amide groups, respectively (3.0 and 2.8 Å, respectively). Progesterone and RU26752, lacking the C17- and C21-hydroxyl groups, are unable to interact with Asn770. The C20-carbonyl group of progesterone and the 17β-oxygen atom of the RU26752 γ-lactonic ring could be involved in a hydrogen bond with Cys942 (3.2 Å; Figure 4D). In addition, the RU26752 7α-propyl substituent can be accommodated in a crevice observed in the probe-accessible volume, which is bounded by Leu769 (helix H3, 3.7 Å), Leu814 (helix H5, 3.8 Å), Phe829 (β-turn, 4 Å), Met840 (helix H6, 3.9 Å) and Met845 (loop L6–7, 3.7 Å). The C18-ketone of 18OVP could form a hydrogen bond with Cys942 (3.0 Å), and the C18-vinyl substituent forms close contacts with Trp806 (helix H5, 3.7 Å), Met807 (helix H5, 3.6 Å) and Leu960 (helix H12, 3.6 Å).

Discussion

The hMR homology model, based on the hRARγ crystal structure, reveals two polar sites located at each extremity of the LBP: site I contains Gln776 and Arg817, two polar residues highly conserved among the steroid family; and site II has three putative hydrogen bonding partners, Asn770, Cys942 and Thr945, conserved only in MR, GR and PR. Stereochemically, ligand docking can be achieved in two different ways, the A-ring being oriented to site I or site II. The ambiguity cannot be resolved with the model alone. To discriminate between the two orientations,

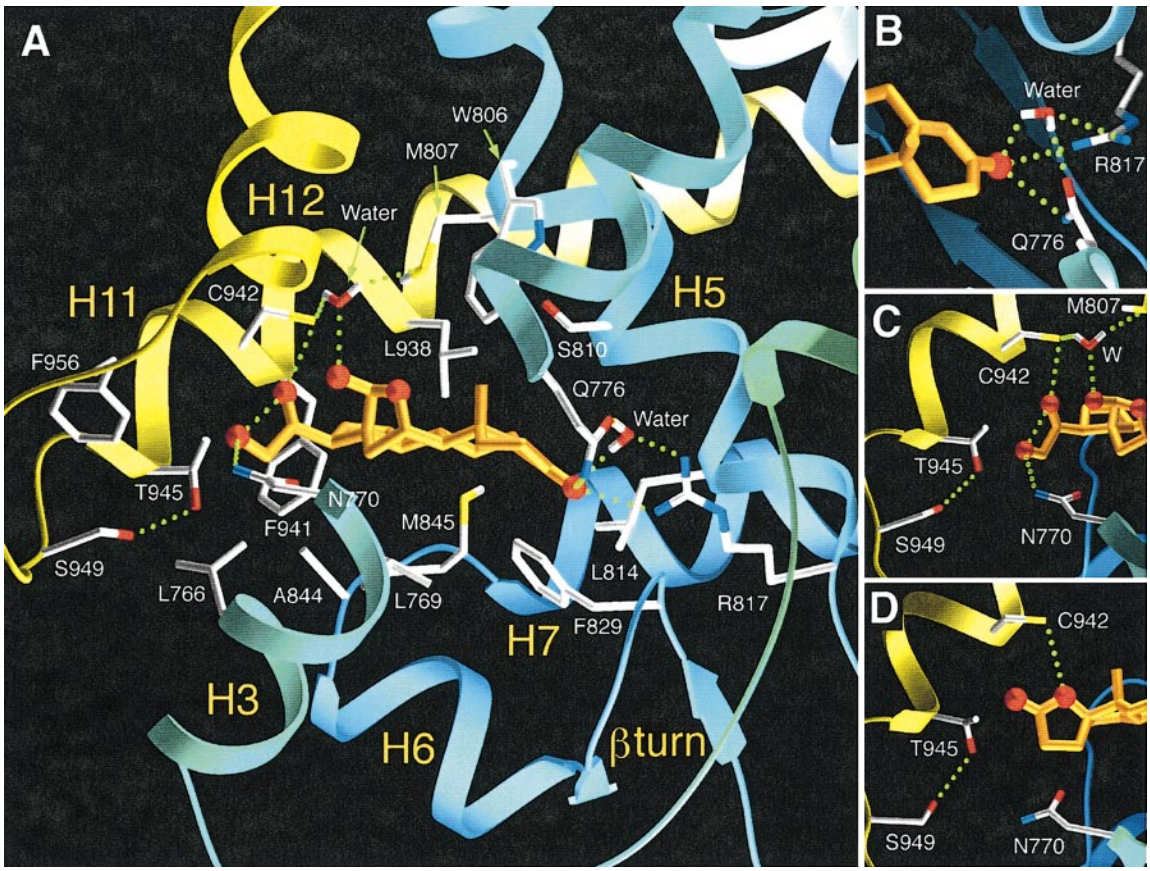


Fig. 4. Ligand docking in the hMR -ligand-binding pocket. The hMR-LBD backbone is drawn as ribbons, and selected residue side chains in close contact with the ligand are depicted in white (4.5 Å cut-off). The ligand is colored in orange, with its oxygen atoms in red. The hydrogen bonding networks anchoring the A-ring (site I) and the D-ring (site II), as discussed in the text, are depicted as green dashed lines. (**A**) Close up view of aldosterone in the ligand-binding niche. Close up view of (**B**) the aldosterone A-ring anchoring in site I with Gln776, Arg817 and the water molecule, (**C**) the aldosterone D-ring interaction in site II with Asn770, Cys942 and Thr945 and (**D**) the contact of the RU26752 γ-lactonic ring with the site II residues. The figures were produced with SETOR (Evans, 1993).

we substituted the polar residues by alanine within each site. The analysis of the transactivation capacity of the mutant hMRs together with their ability to bind mineralocorticoid agonists (aldosterone, cortisol) and antagonists (progesterone, RU26752) led to an unambiguous positioning of the ligands.

Ligand-anchoring sites

We have shown that substitution of Gln776 and Arg817 by alanine induces a decrease in the affinity for the ligands with an α,β-unsaturated ketone in the A-ring. The conservation of these amino acids in hPR, hGR and hAR suggests that the cognate ligands, also characterized by the A-ring α,β-unsaturated ketone, are anchored similarly in their LBP. This allows us to propose that, in all these receptors, the A-ring is directed towards site I. This orientation agrees with that of estradiol and the antagonist raloxifene in the hERα crystal structures where the phenolic A-ring hydroxyl group forms hydrogen bonds with the Glu353 carboxylate group (Gln776 in hMR) and the Arg394 guanidinium charged group (Arg817 in hMR) (Brzozowski *et al*., 1997). Furthermore, in such an orientation the 3D model shows that Ser810 (helix H5) in hMR is close to the A-ring β-face. In the rat GR and human PR, the corresponding residues, Met622 and Met759, have been photoaffinity labeled with triamcinolone acetonide and promegestone (R5020), both photoreactive at the A-ring level (Carlstedt-Duke *et al.*, 1990; Strömstedt *et al.*, 1990).

The ligand orientation was confirmed by mutagenesis of polar residues at the other extremity of the LBP (site II). Asn770 mutation abolishes the binding of 21-hydroxylated mineralocorticoids (aldosterone and cortisol), whereas binding of ligands lacking this substituent (progesterone, RU26752 and 18OVP) was not affected. Note that the progesterone derivative 18OVP lacking the 21-hydroxyl group is still acting as a full agonist through hMR. The 3D model suggests that the C18-enone oxygen could form a hydrogen bond with Cys942 (helix H11), the vinyl group being in close contact with helix H12 and stabilizing its positioning. Mutation of Cys942 has been shown to alter the binding of aldosterone and progesterone (B.Lupo, personal communication). Altogether, these results support the proposal that site II anchors the D-ring, Cys942 makes a hydrogen bond with the C20-carbonyl group and Asn770 with the C21-hydroxyl moiety. Thr945 interacts, but more weakly, with the same mineralocorticoid ligands. Indeed, its substitution by alanine induces only a 6-fold decrease in steroid binding, which results in a shift towards higher concentrations in the aldosterone- and cortisol-induced transactivation functions. These data rule out a direct contact with the ligand

through a hydrogen bond similar to that of Asn770 (Figure 4A and C), but favor a van der Waals contact involving the γ-methylene group. The Thr945 hydroxyl group could then contribute to the stabilization of loop L11–12 through a hydrogen bond with Ser949, as observed in the homology model.

Since cortisol and aldosterone, the natural glucocorticoid and mineralocorticoid compounds, bear the same 17β-substitutent, it is reasonable to postulate that in site II, the same contacts occur in the interaction between cortisol and hGR. We have shown that substitution of Asn770 and Thr945 by alanine does not alter the progesterone binding, suggesting that Cys942 is the only site II polar group interacting with this ligand. It would be interesting to identify the hPR residues involved in progesterone binding to elucidate why progesterone behaves as an agonist when acting through its cognate receptor whereas it has antimineralocorticoid and antiglucocorticoid properties (Rousseau *et al.*, 1972). The presence of a 17β-hydroxyl group in testosterone and estradiol suggests a different anchoring of these ligands in their cognate receptor. Indeed, in the hERα crystal structure, the estradiol 17β-hydroxyl contacts His524 in helix H11 (Brzozowski *et al*., 1997).

Mechanism of antagonism

Both agonist and antagonist hMR ligands have in common the A-ring bearing a C3-ketone group which, as suggested by this study, is anchored by the site I residues, Gln776 and Arg817. The major difference between the mineralocorticoid agonists and antagonists resides in the D-ring, where only the agonist ligands exhibit the 21-hydroxyl group. This part of the ligand is in the vicinity of the second site composed of Asn770, Cys942 and Thr945. As revealed by the 3D model, these residues form critical contacts with the ligand, especially a hydrogen bond between the C21-hydroxyl group and Asn770. In addition, this C21 polar group is also in van der Waals contacts with Phe956 in loop L11–12.

It has been shown recently that, upon agonist binding, NRs undergo a *trans*-conformation described as a mouse trap or related mechanism which results in the precise positioning of helix H12 which allows TIFs/co-activators to bind to NRs. In such an active complex, all-*trans* retinoic acid fits nicely the size and shape of the LBP. It is worth stressing that in the hRARγ complexes the side chains lining the cavity adopt the same geometries with all-*trans* and 9-*cis* retinoic acids and also with a synthetic agonist ligand (Klaholz *et al*., 1998), suggesting a perfect fit between agonist ligands and the LBP. For antagonists, it has been suggested that their action is achieved by steric hindrance which would displace helix H12. Such a mechanism has been confirmed by the recently solved raloxifene–hERα complex crystal structure, which showed that the protrusion of the antagonist repositions helix H12. Raloxifene is larger than estradiol, in support of the bulky strategy for the antagonist design. In contrast, the hMR antagonists considered in this study are smaller than the agonist ligands, and to our knowledge most hMR antagonists fall into this category, suggesting a different helix H12 destabilization compared with the previous mechanism. Our data, especially those of the Asn770 and Thr945 mutations, suggest that helix H12 is destabilized by disrupting or loosening ligand–protein contacts, especially in the loop L11–12 and helix H12 region. Intrusion of solvent molecules can accelerate the process. These lost contacts probably destabilize helix H12 from its active position. Experimental evidence corroborates such a hypothesis: the antagonists progesterone and RU26752, both dissociate more rapidly from the receptor than does aldosterone (nine and 20 times for progesterone and RU26752, respectively; Souque *et al.*, 1996).

It is worth underlining that cortisol, an hMR agonist, dissociates more rapidly from the hMR complex as compared with aldosterone (approximately twice as quickly; Souque *et al.*, 1996) and that aldosterone, in a complex with the T945A mutant, exhibits faster dissociation kinetics compared with the wild-type receptor (data not shown). Note that both aldosterone and cortisol exhibit similar affinities for hMR, but are characterized by a 10-fold difference in their transactivation capacities in favor of aldosterone (ED_{50} of 0.1 and 1 nM for aldosterone and cortisol, respectively). Similar data were obtained for other agonist ligands and for various MR species (Couette *et al.*, 1992; Rafestin-Oblin *et al.*, 1992; Lombès *et al.*, 1994; Souque *et al.*, 1996). These observations could again incriminate different stabilization of helix H12 in a way similar to the hMR antagonists but to a lesser extent. This modulation of the transactivation capacity of aldosterone and cortisol, and probably other agonists, is probably due to the different functional groups, the 11–18 hemiketal group for aldosterone and the 11β- and 17α-hydroxyl groups for cortisol, which could induce slightly different ligand orientations in the pocket. In our model, cortisol rotates around the C3–C17 axis and forms an additional hydrogen bond with the 17α-hydroxyl group and Asn770 (helix H3).

In conclusion, this work identified novel mutations affecting the binding of hMR agonists more severely than that of antagonists. The mutations, N770A and T945A, are clustered around the D-ring and close to the helix H12 region. In the light of the homology model described herein, it appears that the stabilization of the holo-conformation is achieved through interactions between the agonist C21-hydroxyl group and these residues. The observation that antagonists are characterized by a smaller size and faster off-rate kinetics compared with agonists suggests a novel antagonism mechanism by which the loss of critical ligand–protein contacts destabilizes the AF2-AD core region. This mechanism may be extended to other receptors, where the ligand contributes to the stabilization of the helix H12 region. Such a mechanism could represent an alternative to the bulky ligand strategy to design novel antagonist ligands.

Materials and methods

Sequence alignment

The ClustalW 1.5 package (Thompson *et al.*, 1994) was used with default parameters to align the MR sequences to the other members of the steroid family and the hRARγ sequences. First, the hGR, hPR, hAR and MR sequences were aligned and then the hERα and the hRARγ were added to the previous sequences in a profile alignment.

Model building and ligand docking

A model of the hMR-LBD was first generated by homology with hRARγ using the Modeller package (version 2.0; Sali and Blundell, 1993). The

homology model is based on the sequence alignment shown in Figure 1A and using the hRARγ crystal structure as a template. Ligands were positioned manually in the pocket using the probe-accessible and van der Waals volumes as guides (see section below). The side chains in the vicinity of the ligand were positioned in a favorable orientation using a rotamer library of the O package (Jones *et al.*, 1991). The Charmm package (QUANTA/CHARMM package, Molecular Simulation Inc., Burlington, MA) was used for all the calculations. The complexes were energy minimized in 2000 steps with a dielectric constant of 2, using the Powell procedure. Hydrogen bonds were defined by upper bound harmonic distance restraints (60 kcal $Å^{-2}$ force constant) during the minimization process for the following groups: Gln776 (NE2–H22) and O3 (2.0 Å); Arg817 (NH1–H12) and O3 (2.0 Å); Gln776 (OE1) and Arg817 (NH2–H22) (2.0 Å); H1 on H_2O and O3 (1.8 Å); H2 on H_2O and Gln776 (OE1) (1.8 Å); O on H_2O and Arg817 (NH2–H22) (2.0 Å); Cys942 (HG) and O20 (2.3 Å); and Cys942 (SG) and H on hemiketal hydroxyl (Å), Asn770 (ND2–H22) and H on 21-OH (1.8 Å). To maintain the overall structure of the LBD, the position of Cα atoms of residues 732–749 (helix H1), 763–785 (helix H3), 795–816 (helix H4), 826–828 (strand β1), 834–836 (strand β2), 837–842 (helix H6), 849–861 (helix H7), 866–877 (helix H8), 921–948 (helices H9 and H10) and 958–965 (helices H11 and H12) were harmonically restrained (30 kcal $Å^{-2}$ force constant).

Determination of the cavity volumes

The cavity volume of the binding niche was calculated with VOIDOO (Kleywegt and Jones, 1994), a program for computing molecular volumes and for studying cavities in macromolecules such as proteins. Three types of volume can be generated: the van der Waals, the probe-occupied and probe-accessible cavities. The van der Waals cavity is the molecule's van der Waals surface complement which gives valuable information about the size of the binding niche and the crevices. The two other algorithms use a probe-sphere with a 1.4 Å radius (the radius of a water molecule). The contacts between the probe- sphere and the van der Waals protein surface delimit the probe-occupied cavity which is similar to the Connolly-type surface (Connolly, 1993). The probe-accessible cavity is calculated in the same way as the probe-occupied cavity, but the cavity is described by the volume accessible to the center of the probe-sphere. This last cavity representation is very helpful for manually docking a ligand in its binding pocket as most of the apolar heavy atoms should remain inside this volume.

Steroids

[1,2-^{3}H]aldosterone (40–60 Ci/mmol), [1,2-^{3}H]cortisol (50–60 Ci/mmol) and [1,2,6,7-^{3}H]progesterone (80–110 Ci/mmol) were purchased from Amersham (Les Ulis, France). Non-radioactive aldosterone, cortisol and progesterone were obtained from Sigma (St Louis, MO). Unlabeled and ^{3}H-labeled RU26752 (50–60 Ci/mmol) were provided by Roussel-Uclaf Laboratories (Romainville, France). To avoid steroid adsorption, steroid solutions prepared in ethanol were dried and the steroids resuspended in 50% polyethylene glycol 300 prepared in TEG buffer (20 mM Tris–HCl pH 7.4, 1 mM EDTA, 10% glycerol) to give a final concentration of 5% in the lysate.

Expression and reporter constructs

A 3.6 kb *Hin*dIII fragment of the hMR cDNA containing the entire coding sequence of the hMR was excised from the plasmid 3750 (Arriza *et al*., 1987) provided by R.Evans (Salk Institute, San Diego, CA) and subcloned into the expression vector pcDNA3 (Invitrogen, NV leek, The Netherlands), thus creating the plasmid pchMR. pFC31Luc, which contains the MMTV promoter driving the luciferase gene, was obtained from H.Richard-Foy (LMBE, Toulouse, France).

Site-directed mutagenesis

The 3.6 kb *Hin*dIII fragment containing the entire coding sequence of the hMR was subcloned in the pAlter-1 vector. Each mutation was created by site-directed oligonucleotide mutagenesis using the Altered Sites *in vitro* Mutagenesis System (Promega, Charbonnières, France). Purified oligonucleotides were purchased from Genset (Paris, France).The primers used were: 5′-CTCTCCACGCTC GCC CGC TTA GCAGGC-3′ for N770A; 5′-TTAGCAGGCAAAGCGATGATCCAAGTC-3′ for Q776A; 5′-GCCTTGAGCTGGGCATCGTACAAACAT-3′ for R817A; 5′-TTCTGCTTCTACGCCTTCCGAGAGTCC-3′ for T945A; and 5′-TGGATGTGTCTAGACTCATTTGCCTTG-3′ for S810A.

The desired mutations were identified by direct sequencing. Insert-encoding mutant sequences were subcloned in the expression vector pcDNA3 for *in vitro* expression of the mutant receptors in the rabbit reticulocyte lysate or subsequent transfections in COS-7 cells.

Coupled cell-free transcription and translation

Plasmids (1 μg) containing cDNA coding for the wild-type or mutant hMRs were *in vitro* expressed using the T7-coupled rabbit reticulocyte lysate system purchased from Promega (Charbonnières, France) according to the manufacturer's instructions. The reactions were conducted with unlabeled or ^{35}S-labeled methionine in the translation mixture depending on the experiment.

Steroid-binding characteristics at equilibrium

After translation of the wild-type or mutant hMRs, the lysate was diluted 2-fold with ice-cold TEGWD buffer (20 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20 mM sodium tungstate and 10% glycerol) and incubated for 4 h at 4°C with increasing concentrations of [^{3}H]aldosterone, [^{3}H]cortisol or [^{3}H]progesterone (0.1–100 nM). Bound (B) and unbound (U) steroids were separated by the dextran–charcoal method. Bound steroid was measured by counting the radioactivity of the supernatant. The evolution of B as a function of U was analyzed as previously described (Claire *et al*., 1978), and the dissociation constant at equilibrium, K_d, was calculated.

Cell culture and transfection

COS-7 cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco-BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum. Ten μg of the wild-type or mutant pchMR plasmid DNA, 10 μg of pFC31Luc and 5 μg of pSVβ were transfected into COS-7 cells (4×10^6–5×10^6 cells in 500 μl of cold phosphate-buffered saline) by electroporation in a cell porator (330 V, 300 μF; Life Technologies). After electroporation, cells were put on ice for 5 min and then transferred into 6-well dishes with DMEM supplemented with 10% charcoal-stripped fetal calf serum. The tested steroids were added to the cells 12 h after transfection. After a 24 h incubation, cell extracts were assayed for luciferase (de Wet *et al.*, 1987) and β-galactosidase activity (Herbomel *et al.*, 1984). To standardize for transfection efficiency, the relative light units, obtained in the luciferase assay, were divided by the optical density obtained in the β-galactosidase assay.

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References

Arriza,J.L., Weinberger,C., Cerelli,G., Glaser,T.M., Handelin,B.L., Housman,D.E. and Evans,R.M. (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science*, **237**, 268–275.

Arriza,J.L., Simerly,R.B., Swanson,L.W. and Evans,R.M. (1988) The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron*, **1**, 887–900.

Barton,G.J. (1993) ALSCRIPT: a tool to format multiple sequence alignments. *Protein Eng.*, **6**, 37–40.

Binart,N., Lombès,M., Rafestin-Oblin,M.E. and Baulieu,E.E. (1991) Characterization of human mineralocorticosteroid receptor expressed in the baculovirus system. *Proc. Natl Acad. Sci. USA*, **88**, 10681–10685.

Bonvalet,J.P. (1998) Regulation of transport by steroid hormones. *Kidney Int.*, **53**, 49–55.

Bourguet,W., Ruff,M., Chambon,P., Gronemeyer,H. and Moras,D. (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature*, **375**, 377–382.

Brzozowski,A.M., Pike,A.C.W., Dauter,F., Hubbard,R.E., Bonn,T., Engström,O., Öhman,L., Greene,G.L., Gustafsson,J.A. and Carlquist,M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, **389**, 753–758.

Carlstedt-Duke,J., Strömstedt,P.E., Persson,B., Cederlund,E., Gustafsson,J.A. and Jörnvall,H. (1988) Identification of hormone-interacting amino acid residues within the steroid binding domain of the glucocorticoid receptor in relation to other steroid hormone receptors. *J. Biol. Chem.*, **263**, 6842–6846.

Claire,M., Rafestin-Oblin,M.E., Michaud,A., Corvol,P., Venot,A., Roth-Meyer,C., Boisvieux,J.F. and Mallet,A. (1978) Statistical test of models and computerised parameter estimation for aldosterone binding in rat kidney. *FEBS Lett.*, **88**, 295–299.

Connolly,M.L. (1993) The molecular surface package. *J. Mol. Graphics*, **11**, 139–141.

Corvol,P., Claire,M., Oblin,M.E., Geering,K. and Rossier,B.C. (1981) The mechanism of the antimineralocorticoid effects of spirolactones. *Kidney. Int.*, **20**, 1–6.

Couette,B., Lombès,M., Baulieu,E.E. and Rafestin-Oblin,M.E. (1992) Aldosterone antagonists destabilize the mineralocorticosteroid receptor. *Biochem. J.*, **282**, 697–702.

Couette,B., Fagart,J., Jalaguier,S., Lombès,M., Souque,A. and Rafestin-Oblin,M.E. (1996) The ligand induced conformational change of the mineralocorticoid receptor occurs within its heterooligomeric structure. *Biochem. J.*, **315**, 421–427.

de Wet,J.R., Wood,K.V., Deluca,M., Helsinki,D.R. and Subramani,S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.*, **7**, 725–737.

Evans,R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science*, **240**, 889–895.

Evans,S.V. (1993) SETOR: hardware lighted three-dimensional solid model representations of macromolecules. *J. Mol. Graphics*, **11**, 134–138.

Hendlich, M., Lackner, P., Weitckus, S., Floeckner, H., Froschauer, R., Gottsbacher, K., Casari, G. and, Sippl, M.J. (1990) Identification of native protein folds amongst a large number of incorrect models. The calculation of low energy conformations from potentials of mean force. *J. Mol. Biol.*, **216**, 167–180.

Herbomel,P., Bourachot,B. and Yanif,M. (1984) Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell*, **39**, 653–662.

Horisberger,J.D. and Rossier,B.C. (1992) Aldosterone regulation of gene transcription leading to control of ion transport. *Hypertension*, **19**, 221–227.

Jones,T.A., Zou,J.Y., Cowan,S.W. and Kjeldgaard (1991) Improved methods for binding protein models in electron density maps and the location of errors in these models. *Acta Crystallogr.*, **A7**, 110–119.

Klaholz,B.P., Renaud,J.P., Mitschler,A., Zusi,C., Chambon,P., Gronemeyer,H. and Moras,D. (1998) Conformational adaptation of agonists to the human nuclear receptor RAR-γ. *Nature Struct. Biol.*, **5**, 199–202.

Kleywegt,G.J. and Jones,T.A. (1994) Detection, delineation, measurement and display of cavities in macromolecular structures. *Acta Crystallogr.*, **D50**, 178–185.

Laskowski,R.A., MacArthur,M.W., Moss,D.S. and Thorntorn,J.M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.*, **26**, 283–291.

Liu,W.H., Wang,J., Sauter,N.K. and Pearce,D. (1995) Steroid receptor heterodimerization demonstrated *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 12480–12484.

Lombès,M., Binart,N., Rafestin-Oblin,M.E., Joulin,V. and Baulieu,E.E. (1993) Characterization of the interaction of the human mineralocorticoid receptor with hormone response elements. *Biochem. J.*, **302**, 557–583.

Lombès,M., Kenouch,S., Souque,A., Farman,N. and Rafestin-Oblin,M.E. (1994) The mineralocorticoid receptor discriminates aldosterone from glucocorticoids independently of the 11 beta-hydroxysteroid dehydrogenase. *Endocrinology*, **135**, 834–840.

Mangelsdorf,D.J. *et al*. (1995) Overview: the nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.

Nicholls,A., Sharp,K.A. and Honig,B. (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins*, **11**, 281–296.

Rafestin-Oblin,M.E., Couette,B., Barlet-Bas,C., Cheval,L., Viger,A. and Doucet,A. (1991) Renal action of progesterone and 18-substituted derivatives. *Am. J. Physiol.*, **260**, 828–832.

Rafestin-Oblin,M.E., Lombès,M., Couette,B. and Baulieu,E.E. (1992) Differences between aldosterone and its antagonists in binding kinetics and ligand-induced hsp90 release from mineralocorticosteroid receptor. *J. Steroid Biochem. Mol. Biol.*, **41**, 815–821.

Renaud,J.P., Rochel,N., Ruff,M., Vivat,V., Chambon,P., Gronemeyer,H. and Moras,D. (1995) Crystal structure of the RAR-gamma ligand-binding domain bound to all-*trans* retinoic acid. *Nature*, **378**, 681–689.

Ribeiro,R.C.J., Kushner,P.J. and Baxter,J.D. (1995) The nuclear hormone receptor gene superfamily. *Annu. Rev. Med.*, **46**, 443–453.

Rousseau,G.G., Baxter,J.D. and Tomkins,G.M. (1972) Glucocorticoid receptor: relations between steroid binding and biological effects. *J. Mol. Biol.*, **67**, 99–115.

Rupprecht,R., Reul,J.M.H.M., van Steensel,B., Spengler,D., Soder,M., Berning,B., Holsboer,F. and Damm,K. (1993) Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. *Eur. J. Pharmacol.*, **247**, 145–154.

Sali,A. and Blundell,T.L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.*, **234**, 779–815.

Souque,A., Fagart,J., Couette,B., Davioud,E., Sobrio,F., Marquet,A. and Rafestin-Oblin,M.E. (1995) The mineralocorticoid activity of progesterone derivatives depends on the nature of the Cc-18 substituent. *Endocrinology*, **136**, 5651–5658.

Souque,A., Fagart,J., Couette,B. and Rafestin-Oblin,M.E. (1996) Sulfhydryl groups are involved in the binding of agonists and antagonists to the human mineralocorticoid receptor. *J. Steroid Biochem. Mol. Biol.*, **57**, 315–321.

Sutanto,W. and De Kloet,E.R. (1991) Mineralocorticoid receptor ligands—biochemical, pharmacological, and clinical aspects. *Med. Res. Rev.*, **11**, 617–639.

Strömstedt,P.E., Berkenstam,A., Jörnvall,H., Gustafsson,J.A. and Carlstedt-Duke,J. (1990) Radiosequence analysis of the human progestin receptor charger with [^{3}H]promegestone. *J. Biol. Chem.*, **265**, 12973–12977.

Thompson,J.D., Higgins,D.G. and Gibson,T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.

Trapp,T. and Holsboer,F. (1995) Ligand-induced conformational changes in the mineralocorticoid receptor analyzed by protease mapping. *Biochem. Biophys. Res. Commun.*, **215**, 286–291.

Trapp,T. and Holsboer,F. (1996) Heterodimerization between mineralocorticoid and glucocorticoid receptors increases the functional diversity of corticosteroid action. *Trends Pharmacol. Sci.*, **17**, 145–149.

Tsai,M.J. and O'Malley,B.W. (1994) Molecular mechanism of action of steroid/thyroid receptor superfamily. *Annu. Rev. Biochem.*, **63**, 451–486.

Ulmann,A., Bertagna,C., Le Go,A., Husson,J.M., Tache,A., Sassano,P., Menard,J. and Corvol,P. (1985) Assessment of the anti-mineralocorticosteroid effects of RU28318 in healthy men with induced exogenous and endogenous hypermineralocorticism. *Eur. J. Clin. Pharmacol.*, **28**, 531–535.

Wagner,R.L., Apriletti,J.W., McGrath,M.E., West,B.L., Baxter,J.D. and Fletterick,R.J. (1995) A structural role for hormone in the thyroid hormone receptor. *Nature*, **378**, 690–697.

Wambach,G. and Higgins,J.R. (1978) Antimineralocorticoid action of progesterone in the rat: correlation of the effect on electrolyte excretion and interaction with renal mineralocorticoid receptors. *Endocrinology.*, **102**, 1686–1693.

Wurtz,J.M., Bourguet,W., Renaud,J.P., Vivat,V., Chambon,P., Moras,D. and Gronemeyer,H. (1996) A canonical structure for the ligand-binding domain of nuclear receptors. *Nature Struct. Biol.*, **3**, 87–94.

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