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Mechanistic aspects of mineralocorticoid receptor activation

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Mechanistic aspects of mineralocorticoid receptor activation. Aldosterone exerts its biological effects through binding to mineralocorticoid receptor (MR). Ligand binding induces a receptor *trans*conformation within the ligand-binding domain and dissociation of associated proteins from the receptor. The ligand-activated receptor binds as a dimer to the response elements present in the promoter region of target genes and initiates the transcription through specific interactions with the transcription machinery. The glucocorticoid hormone cortisol binds to the human MR (hMR) with the same affinity as aldosterone, but is less efficient than aldosterone in stimulating the hMR *trans*activation. The antimineralocorticoid spirolactones also bind to the hMR but induce a receptor conformation that is transcriptionally silent. In this report, we describe the key residues involved in the recognition of agonist and antagonist ligands and propose a two-step model with a dynamic dimension for the MR activation. In its unliganded state, MR is in an opened conformation in which folding into the ligand-binding competent state requires both the heat shock protein 90 and the C-terminal part of the receptor. An intermediate complex is generated by ligand binding, leading to a more compact receptor conformation. This transient complex is then converted to a transcriptionally active conformation in which stability depends on the steroid-receptor contacts.

Aldosterone acts via a ligand-activated transcription factor, the mineralocorticoid receptor (MR). The MR is a member of the nuclear receptor (NR) family that includes receptors for steroid and thyroid hormones, vitamin D_3, and retinoic acids, as well as numerous orphan receptors for which no ligands are known [1–4]. MR has a modular structure comprising five regions (A through E; Fig. 1). The N-terminal A/B region harbors an autonomous activation function. The central C-domain (DBD) is highly conserved and is composed of two zinc fingers involved in DNA binding and receptor dimerization. A hydrophilic region forms a hinge between the DBD and C-terminal ligand-binding domain (LBD). The LBD mediates numerous functions, including ligand binding, interaction with heat-shock proteins, dimerization, nuclear targeting, and hormone-dependent activation.

Several crystal structures of NR LBDs have been described: the unliganded form (apo receptor) of the retinoid X receptor α (RXRα) and (thiazolidinedione)-peroxisome proliferator-activated receptor γ (PPARγ) and the agonist-bound form (holo receptor) of all-*trans*-retinoic acid receptor γ (RARγ), thyroid receptor α (TRα), estrogen receptor α (ERα), progesterone receptor (PR), and PPARγ [5–11]. All of these NR LBDs have a common fold, with 11 to 12 α helices (numbered H1 through H12) and one β turn arranged as an antiparallel α helical "sandwich" in a three-layer structure [12]. The LBD adopts a more compact structure when a ligand is bound. The helix H11 is repositioned in continuity with H10, and helix H12, which encompasses the autonomous activating domain (AF-2AD), is folded back toward the LBD core. The repositioning of H12, together with additional structural changes, such as bending of H3, brings it into a distinct receptor environment, creating an interface suitable for NR coactivator binding [13]. Many coactivators that interact with the NRs in a ligand-dependent manner have been identified. These include steroid receptor-coactivator 1 (SRC-1), transcriptional intermediary factor II (TIF-II)/GRIP-1, receptor-interacting proteins of molecular weights 140 (RIP-140), TIF-1, and CBP/p300 [14, 15].

The aldosterone-dependent activation of gene transcription is thought to be a multistep process. Aldosterone initially binds to MR and causes a receptor *trans*conformation within the LBD that is believed to lead to the dissociation of the associated proteins from the receptor [16, 17]. The ligand-activated receptor then binds, as a dimer, to the response elements in the promoter regions of target genes and initiates hormone-mediated transcription through specific interactions with the transcription machinery [18–20]. The way in which aldosterone acts through its own receptor has been a puzzle for some time for two main reasons: aldosterone and cortisol both bind to the human MR (hMR) with the same affinity, and the plasma glucocorticoid concentration is 100- to 1000-fold higher than that of aldosterone. Consequently, most of the hMR should be occupied by glucocorticoid, resulting in sodium retention.

Key words: aldosterone, antimineralocorticoid, steroid receptor, ligand binding.

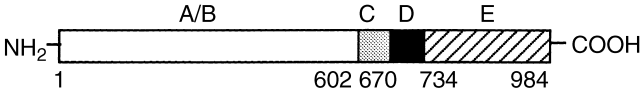

Fig. 1. Schematic representation of the human mineralocorticoid receptor (hMR) structural organization.

The role of the 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) in mineralocorticoid/glucocorticoid selectivity has been established over the past decade [21, 22]. This enzyme metabolizes 11β-hydroxylated-glucocorticoids (cortisol and corticosterone), but not aldosterone, into 11-keto derivatives (cortisone and 11-dehydrocorticosterone) that have a very low affinity for the MR [23]. This enzyme is present in aldosterone-sensitive tissues [24, 25], and mutations that inactivate this enzyme are responsible for the syndrome of apparent mineralocorticoid excess, resulting in sodium retention and severe arterial hypertension [26]. Ex vivo experiments have revealed that the MR is more sensitive to aldosterone than to cortisol [27–31]. However, the mechanisms by which MR is differentially stimulated by the two corticosteroid hormones remain unknown.

The antimineralocorticoid spirolactones, synthetic steroids with a C17 γ-lactone ring, have been used for the past 30 years in the treatment of sodium-retaining states and as antihypertensive agents [32, 33]. Mineralocorticoid antagonists bind to the receptor with an affinity identical to that of aldosterone and induce a receptor conformation that is transcriptionally silent [17].

In this report, we studied ligand–hMR interactions by using a three-dimensional model of the hMR-LBD [34], and analyzed the hMR activation process by examining the ligand-induced hMR conformational changes and the receptor–coactivator interaction.

FOLDING REQUIREMENTS OF hMR FOR LIGAND BINDING

On the basis of sequence homology among NRs, the hMR-LBD has been defined as the sequence from position 734 to 984 [27]. However, this sequence has not been shown to be the minimum region that can bind agonists and antagonists. A series of mutant hMRs truncated at either the amino- or carboxy-terminal end of the receptor was analyzed for its ability to bind agonists and antagonists and to interact with the heat shock protein hsp90, a chaperone protein that is a prerequisite for ligand binding to hMR [35–37]. The MR-LBD (MR734-984) does not interact with hsp90 and has a lower aldosterone-binding capacity than the entire receptor (Table 1). Adding a short amino acid sequence, the five residues upstream of the LBD, led to a truncated receptor (MR729-984) that interacts with hsp90 and has the same aldosterone-binding capacity as the entire hMR. MR729-984 and MR734-984 both have a lower affinity for aldosterone than the entire receptor (Table 1). MR611-984, which is devoid of the A/B region, has an affinity similar to that of the entire hMR (Table 1). Thus, the sequence Thr-Ile-Ser-Arg-Ala (729 through 733) upstream of the LBD is required for hsp90 and steroid binding, and a longer sequence is needed for folding the receptor into a high-affinity hormone-binding state.

Table 1. Aldosterone dissociation constant at equilibrium (K_d) and maximum number of aldosterone binding sites (*N*) for entire and truncated human mineralocorticoid receptors (hMRs)

	K_d	*N*
	nmol/L	
MR	0.52 ± 0.03	0.43 ± 0.13
MR 611–984	1.34 ± 0.13	0.48 ± 0.01
MR 729–984	2.93 ± 0.29	0.38 ± 0.02
MR 734–984	2.90 ± 0.62	0.12 ± 0.01

The entire or truncated hMRs were synthesized *in vitro* in rabbit reticulocyte lysate. The lysate was diluted twofold with TEGWD buffer (20 mmol/L Tris-HCl, 1 mmol/L EDTA, 10% glycerol, 20 mmol/L sodium tungstate and 1 mmol/L dithiothreitol, pH 7.4) and incubated with increasing concentrations (0.1 to 100 nmol/L) of [^{3}H]-aldosterone for four hours at 4°C. Bound and unbound aldosterone were separated by dextran-charcoal and the K_d and *N* values were determined by computer analysis.

Short segments of the C-terminal part of the receptor were deleted because large truncations completely abolished its steroid-binding capacity. These mutant hMRs [MRΔ(C1), MRΔ(C2), MRΔ(C3), and MRΔ(C4)] lack the last one, two, three, or four amino acids [38]. Deletion of the last one or two amino acids decreases aldosterone and cortisol binding by 30%, as well as the binding of antagonists such as RU26752. Deletion of the last three amino acids [MRΔ(C3)] resulted in a 70% decrease in steroid binding, and deletion of the last four amino acids abolishes the steroid binding, although the interaction with hsp90 is not affected. The lack of ligand binding to the carboxy-terminal truncated hMRs was due to misfolding of the receptor, as shown by the different sensitivity to trypsin of MRΔ(C4) when compared with that of the full-length receptor. These results support the idea that a short sequence upstream the LBD is necessary for the interaction of hMR with hsp90, and that hsp90 and the C terminus of hMR are both essential for folding of the receptor into a ligand-binding competent state.

IDENTIFICATION OF hMR AMINO ACIDS INTERACTING WITH MINERALOCORTICOID AGONISTS AND ANTAGONISTS

The crystal structure of the LBD of several NRs has shown a common fold with 11 to 12 α helices and one β turn arranged as an antiparallel α helical "sandwich" in a three-layer structure [12]. As MR purification and crystallization appeared to be very difficult, a three-dimensional model of the hMR-LBD has been constructed by

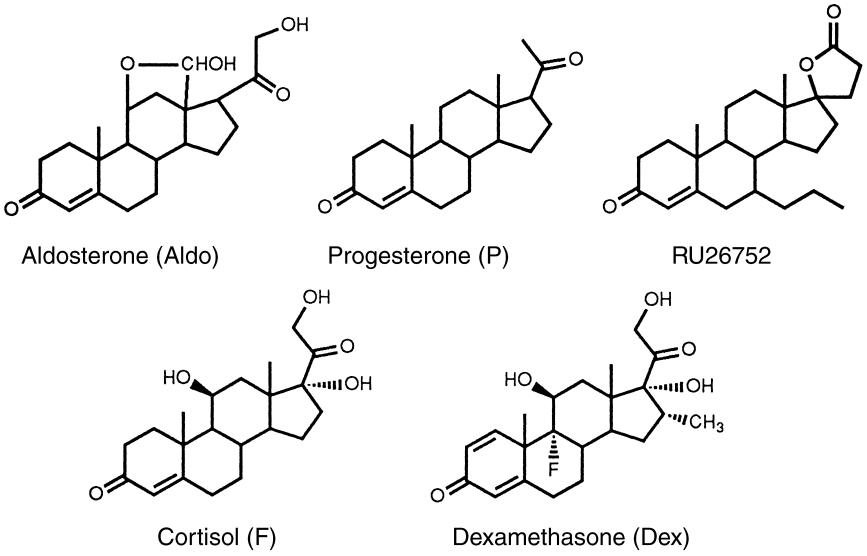

Fig. 2. Human mineralocorticoid receptor (hMR) steroid agonist (aldosterone, cortisol, and dexamethasone) and antagonist (progesterone and RU26752) ligands.

taking as a template the crystal structure of the human retinoic acid receptor γ LBD (hRARγ-LBD) [34] and refined by using crystal structure of the human PR (Fagart et al, manuscript in preparation). This model has allowed the pointing out of the amino acids delimiting the ligand-binding cavity. Two polar sites are located at the extremities of the elongated hydrophobic ligand-binding pocket. The site I is composed of Gln776 (helix H3) and Arg817 (helix H5), and the site II comprises Asn770 (helix H3), Cys942, and Thr945 (helix H11). 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 on the other hand). We replaced polar residues of the ligand-binding pocket by alanine and tested the ability of the mutant hMRs (N770A, Q776A, R817A, and T945A) to interact with steroids selected for their agonist (aldosterone, cortisol) or antagonist (progesterone) properties and for their structures (Fig. 2). Aldosterone, cortisol, and progesterone have the C3 ketone group in common. Aldosterone and cortisol are characterized by a C21 hydroxyl group, whereas progesterone is lacking this hydroxyl group. Q776A and R817A display a lower affinity for aldosterone, cortisol, and progesterone compared with the wild-type hMR (Table 2). Progesterone binds to T945A and N770A with an affinity similar to that of the wild-type hMR. In contrast, substitution of Thr945 by alanine decreased the affinity of the receptor for aldosterone and cortisol, and replacement of Asn770 by alanine completely abolished the ability of hMR to bind aldosterone and cortisol. These results altogether support the proposal that site I and site II anchor the A-ring and D-ring, respectively. Within the site I, Gln776 and Arg817 interact with the steroid C3-ketone group, common to agonists and antagonists. Within the site II, Asn770 and Thr945 interact with the 17 β-substituent of corticosteroids. Asn770 makes a hydrogen bond with the C21-hydroxyl group and the 18-hemiketal group, and the Thr945 γ methyl group is in a Van der Waals contact with the C21-hydroxyl group. Thus, the agonist–hMR interaction is characterized by numerous contacts within the anchoring site II compared with the antagonist–hMR interaction.

Table 2. Steroid dissociation constant at equilibrium (K_d) for the wild-type and mutant hMRs

	Aldosterone	Cortisol	Progesterone	RU26752
WT	0.52 ± 0.03	0.87 ± 0.13	1.04 ± 0.06	1.31 ± 0.31
N770A	UN	UN	1.19 ± 0.05	ND
Q776A	6.71 ± 0.94	37 ± 10	5.82 ± 0.69	ND
R817A	9.69 ± 0.80	UN	19.2 ± 3.80	ND
T945A	3.87 ± 0.29	4.4 ± 0.3	1.21 ± 0.05	ND

Wild type (WT) and mutant hMRs were synthesized in the rabbit reticulocyte lysate. The lysate was twofold diluted with TEGWD buffer and incubated with increasing concentrations (0.1 to 100 nmol/L) of [^{3}H]-aldosterone, [^{3}H]-cortisol, [^{3}H]-progesterone or [^{3}H]-RU26752 for four hours at 4°C. Bound and unbound steroids were separated by charcoal-dextran and the K_d values were determined by computer analysis. Abbreviations are: UN, undetected; ND, not determined.

N770A, Q776A, and R817A were tested, by cotransfection assays, for their ability to respond to aldosterone and cortisol. At a concentration of 10^{-6} mol/L, cortisol was unable to stimulate the *trans*activation function of N770A, Q776A, and R817A, whereas at this concentration, it maximally stimulated the wild-type hMR function (Fig. 3). Aldosterone was unable to stimulate the *trans*-activation of N770A. In contrast, the aldosterone induced-*trans*activation activities of Q776A and R817A were 25 and 75% that of the wild-type receptor, respectively. These results are compatible with steroid-docking studies that revealed that cortisol underwent an approximate 40° rotation around its C3-C17 axis within the li-

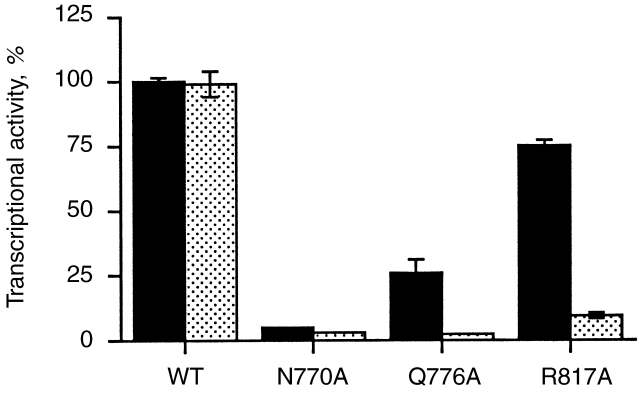

Fig. 3. Transcriptional activation of luciferase activity by wild-type and mutant hMRs. COS-7 cells were transfected with wild-type or mutant-hMR expression vectors, pFC31luc as the reporter plasmid, and a β-galactosidase internal reporter to correct for transfection efficiency. Before harvesting, cells were treated for 24 hours with 10^{-6} mol/L aldosterone (■) or cortisol (▨). *Trans*activation was determined by luciferase activity, normalized to the internal β-galactosidase control, and expressed as a percentage of wild-type activity at 10^{-6} mol/L. Each point is the mean ± SEM of three separate experiments.

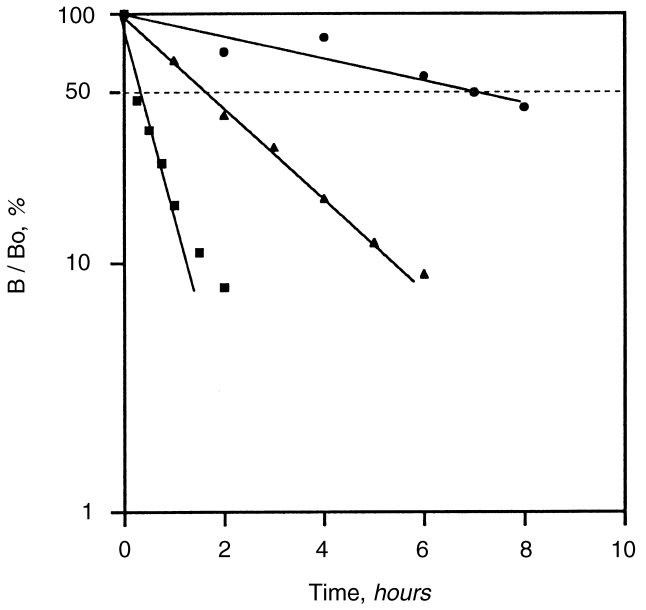

Fig. 4. Dissociation kinetics of [^{3}H]-aldosterone (●), [^{3}H]-cortisol (▲), and [^{3}H]-RU26752 (■) from the wild-type hMR. Wild-type hMR was produced by translation in vitro in the rabbit reticulocyte lysate. The lysate was diluted twofold with TEGWD buffer and incubated with 10 nmol/L [^{3}H]-aldosterone [^{3}H]-cortisol, or [^{3}H]-RU26752 for 30 minutes at 20°C. The end of this incubation period was time zero for kinetic analysis. The lysate was then divided into two. One sample was kept at 20°C to measure the stability of steroid-binding sites, and the other was incubated with 1 μmol/L unlabeled aldosterone, cortisol, or RU26752. Bound and free steroids were separated by dextran charcoal. Nonspecific binding was measured in parallel incubations for each incubation time. Results are corrected for receptor stability and are expressed as a percentage of the binding measured at zero time.

gand-binding pocket of the hMR compared with aldosterone [34].

hMR ACTIVATION PROCESS

The structure of the LBD of several NRs has been described [5–11]. The major difference between the unliganded and agonist-associated structures is the repositioning of the helix H12 that is folded back toward the LBD core in the agonist-associated conformation, creating an interface suitable for NR coactivator binding [13]. As some distinct contacts are involved in the interaction of MR with agonists and antagonists, we wondered how these distinct contacts modulate the hMR activity.

Aldosterone antagonists, such as progesterone and RU26752, bind to hMR with an affinity of the same order of magnitude as that of aldosterone. Nevertheless, they dissociate more rapidly from the receptor than does aldosterone, indicating that antagonist-hMR complexes are less stable than aldosterone-hMR complexes (Table 2 and Fig. 4). Proteolysis assays revealed that both agonist and antagonist binding to hMR induced changes in the LBD compaction [17]. Glutathione S transferase (GST) pull-down experiments showed that agonist-associated hMRs, but not unliganded or antagonist-associated hMR, are able to interact with RIP140, SRC1, and TIF-1α, three coactivators known to bind to NRs.

We propose that interactions between mineralocorticoid agonists and hMR, especially between the C21-hydroxyl group and the residues Asn770 and Thr945, are crucial for the stabilization of the helix H12 in its active state. The homology model revealed that the amide group of the Asn770 residue makes a hydrogen bond with the backbone oxygen atom of Glu955, a residue of the H11-H12 loop. The disrupting or loosening antagonist-receptor contacts probably destabilize helix H12 from its active position.

It is worth emphasizing that glucocorticoids, such as cortisol and dexamethasone, exhibit an affinity for hMR of the same order of magnitude as aldosterone (Table 2, data not shown for dexamethasone). Nevertheless, they dissociate more rapidly from the hMR than aldosterone, a result illustrated in Figure 4 for cortisol. On the other hand, *cis-trans*-cotransfection assays revealed that hMR was more sensitive to aldosterone than to cortisol and dexamethasone [27–31]. The differences in the ability of aldosterone and cortisol to stimulate hMR *trans*-activation function are probably due to the distinct contacts involved in the interaction of hMR with aldosterone and cortisol. In fact, in addition to the 21 hydroxyl group present in both steroids, aldosterone is characterized by an 11-18 hemiketal group and cortisol by the 11β- and 17α-hydroxyl groups.

In conclusion, we propose that the hMR activation is a multistep process with a dynamic dimension. An equilibrium model for hMR activation is proposed in

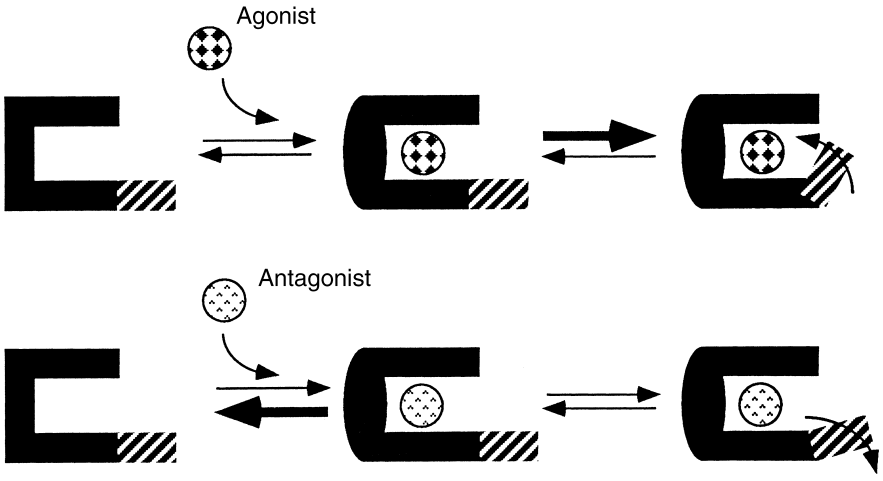

Fig. 5. An equilibrium model for hMR activation. In the absence of hormone, the hMR exists in a transcriptionally inactive conformation. After agonist binding, the receptor is converted to a transient inactive conformation and then to a transcriptionally active conformation. The contacts between the hMR and aldosterone in the helix H12 region stabilize hMR in its active conformation. After antagonist binding, the receptor adopts the transient inactive conformation. The loss of contacts between hMR and antagonists prevents the stabilization of H12 in its active position.

Figure 5. In the absence of ligand, the receptor exists in an open pocket conformation. Upon aldosterone binding, an intermediate complex is first generated, leading to a more compact receptor conformation. This transient complex is then converted to a transcriptionally active conformation by the folding back of the helix 12 toward the core of the hMR-LBD. The stabilization of the helix H12 in its active position is achieved through numerous contacts between aldosterone and hMR, namely between the agonist C21 hydroxyl group and the residue Asn770. The aldosterone 11-18 hemiketal group strengthens the hMR stabilization in its active conformation. In contrast, the 11β- or 17α-hydroxyl groups modify the steroid positioning within the ligand pocket and destabilize hMR active conformation.

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