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The Met852 Residue Is a Key Organizer of the Ligand-Binding Cavity of the Human Mineralocorticoid Receptor

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ABSTRACT

Spirolactones harboring various C7 substituents are aldosterone antagonists, and some of them are used in the treatment of essential hypertension. They bind to the human mineralocorticoid receptor and render it transcriptionally inactive. Structural analysis using a three-dimensional homology model of the ligand-binding domain of the receptor has revealed that the Met852 residue of the ligand-binding cavity faces the C7 substituent of spirolactones. We therefore tested the binding capacities of C7-substituted spirolactones in an in vitro system expressing either the mutant receptor, in which Met852 was replaced by alanine, or the wild-type receptor. The M852A mutation had almost no effect on the binding of C7-substituted spirolactones to mineralocorticoid receptor but dramatically reduced the capacity of the receptor to bind steroids with no C7 substituent (aldosterone, cortisol, deoxycorticosterone, and canrenone). *cis-trans* Cotransfection assays revealed that two spirolactones characterized by having a propyl group [7α-propyl-17α-hydroxy-3-oxo-preg-4-ene-21-carboxylic acid γ-lactone (RU26752)] or a thioacetyl group (spironolactone) at the C7 position acquired agonist properties when bound to the mutant receptor. In contrast, mexrenone and eplerenone, both of which harbor an acetyl group at the C7 position, retained antagonist properties when bound to the mutant receptor. Overall, these findings indicate that Met852 acts as an organizer residue that plays two major roles: 1) it allows steroids with no substituent at the C7 position to be accommodated within the ligand-binding cavity; and 2) it is involved in the steric hindrance that prevents C7-substituted spirolactones from folding the receptor in its active state.

The biological effects of aldosterone are mediated via the mineralocorticoid receptor (MR), a member of the nuclear receptor superfamily (Evans, 1988; Mangelsdorf et al., 1995). MR displays a modular structure comprising several separate domains with specific functions (Arriza et al., 1987). The N-terminal domain of the MR contains an autonomous activation function (AF-1) that is considered to be constitutively active (Rupprecht et al., 1993; Fuse et al., 2000) and plays a key role in the interaction with transcriptional coregulators (Tallec et al., 2003) and with the ligand-binding domain (LBD) (Rogerson and Fuller, 2003). The central DNA-binding domain is composed of two zinc-finger structures that are involved in DNA binding and receptor homodimerization (Liu et al., 1996). The LBD is involved in ligand binding (Fagart et al., 1998) and in the interaction with the heat shock protein 90 and transcriptional coactivators (Couette et al., 1998; Hellal-Levy et al., 2000). This region harbors a ligand-dependent activation function, AF-2 (Nemoto et al., 1993). The crystal structure of the MR-LBD has not yet been solved. However, three-dimensional homology models of the hMR-LBD have been generated from the crystal structures of nuclear receptors, making it possible to predict the three-dimensional organization of the MR-LBD and to see how agonist and antagonist ligands are docked within the ligand-binding cavity (Fagart et al., 1998; Auzou et al., 2000).

In the absence of the ligand, MR is predominantly located in the cytoplasm (Fejes-Toth et al., 1998). It is associated with a multiprotein complex composed of heat shock proteins and immunophilins (Rafestin-Oblin et al., 1989; Pratt and Toft, 1997). Heat shock protein 90 maintains the MR in an inactive state and in a ligand-binding–competent state (Couette et al., 1998). The binding of aldosterone to the MR induces changes in the receptor conformation (Trapp and Holsboer, 1995; Couette et al., 1996) that trigger the translocation of the receptor into the nucleus (Fejes-Toth et al., 1998), the recruitment of transcriptional coactivators (Hellal-Levy et al., 2000), and interaction of the MR in dimer form

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ABBREVIATIONS: MR, mineralocorticoid receptor; LBD, ligand-binding domain; AR, androgen receptor; AF, activation function; TEGWM buffer, Tris-HCl/EDTA/sodium tungstate/β-mercaptoethanol/glycerol; h, human; RU26752, 7α-propyl-17α-hydroxy-3-oxo-preg-4-ene-21-carboxylic acid γ-lactone.

with DNA sequences located in regulatory regions of aldosterone-regulated genes.

Spironolactone and eplerenone, a newly synthesized C7-substituted spirolactone, are MR antagonists as effective as other antihypertensive agents for treating so-called essential hypertension. They improve survival in heart failure and have beneficial effects in preventing the development of cardiac fibrosis and renal damage in patients with essential hypertension (Pitt et al., 1999, 2003; Weinberger et al., 2002; McMahon, 2003). These compounds are also used to treat primary aldosteronism (Gordon et al., 1993; Gittler and Fajans, 1995). Although spironolactone is an effective MR blocker, it has some undesirable side effects. Indeed, spironolactone has both antagonist effects mediated via the androgen receptor and agonist effects mediated via the progesterone receptor (McMahon, 2003). Eplerenone is more selective in its effects but has relatively little affinity for the MR (McMahon, 2003). Spirolactones compete with aldosterone to bind to the MR and render the receptor transcriptionally inactive (Corvol et al., 1978). Several steps in MR function are impaired as a result of spirolactone binding. Spironolactone binds to the MR with a high affinity, but it dissociates very rapidly from the receptor, preventing the stabilization of the antagonist-receptor complex and thereby preventing its interaction with the transcriptional coactivators (Hellal-Levy et al., 2000). The rate of nuclear translocation of MR upon spirolactone binding is slower, and the final nuclear-to-cytoplasmic ratio in steady state is lower than with aldosterone (Fejes-Toth et al., 1998).

The antagonist activity of the spirolactones has been linked to the presence of a γ-lactone substituent at the steroid C17 position, which characterizes all spirolactone molecules (Corvol et al., 1978). Another characteristic of spirolactones is the presence of a C7 side chain, which seems to modulate their antagonist efficiency. In this study, we explored how the nature of this steroid C7 substituent affects the antagonist potency of spirolactones. On the basis of a three-dimensional homology model of the ligand-binding domain of the human MR, the Met852 residue that faces the C7 steroid position was replaced by an alanine, and the ability of the mutant receptor MR_{A852} to bind spirolactones with various C7 substituents was tested. *cis-trans* Cotransfection assays were also performed to determine the ability of C7-substituted spirolactones to activate or inactivate MR_{A852}. We show that all spirolactones bind to MR_{A852}, unlike steroids with no substituent at the C7 position, and that they act as antagonist or agonist ligands depending on the nature of this C7 substituent.

Materials and Methods

Chemicals. [1,2-^{3}H]Aldosterone (40–60 Ci/mmol) was purchased from Amersham Biosciences Inc. (Saclay, France), and [1,2-^{3}H]RU26752 (50–60 Ci/mmol) was kindly provided by Aventis (Paris, France). Aldosterone, cortisol, and deoxycorticosterone were purchased from Sigma-Aldrich (St. Louis, MO). Eplerenone, canrenone, and spironolactone were provided by Pfizer Inc. (New York. NY). Mexrenone was a gift from G. Auzou (Institut National de la Santé et de la Recherche Médicale U540, Montpellier, France). RU26752 was from Aventis. Dulbecco's minimum essential medium and all other compounds used for cell culture were from Invitrogen (Cergy Pontoise, France). All other products for the biochemical studies were from Sigma-Aldrich.

Expression and Reporter Constructs. The expression plasmid pchMR contains the entire coding sequence of hMR (Fagart et al., 1998). The mutation M852A was created on the recombinant pchMR using the QuikChange procedure from Stratagene (Amsterdam, The Netherlands). The sets of primers (MWG-BIOTECH AG, Ebersberg, Germany) were as follows: forward primer, 5′-GAACTATGCCAGGGGATGCACCAAATCAGCCTTC-3′; reverse primer, 5′-GAAGGCTGATTTGGTGCATCCCCTGGCATAGTTC-3′. To ensure that there was no additional mutation, the Bpu1102I-AflII fragment of the resulting MR construct was subcloned into pchMR after being sequenced. The plasmid pcβgal was constructed by cutting out the HindIII-BamHI fragment coding for the β-galactosidase from plasmid pSVβ (Promega, Charbonnieres, France), and inserting it into pcDNA3 (Invitrogen). pFC31Luc contains the mouse mammary tumor virus, a promoter that drives the luciferase gene (Gouilleux et al., 1991).

Cultured Cells and Transfection Procedures. COS-7 cells were cultured in T175 flasks with Dulbecco's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were maintained in medium supplemented with 10% charcoal-stripped fetal calf serum for 4 h before and then throughout the transfection procedure. Subconfluent cells were transfected by the phosphate calcium precipitation method. The phosphate solution, prepared for one T175 flask, contains 15 μg of one of the receptor expression vectors (pchMR or $pchMR_{A852}$), 30 μg of pFC31Luc, and 6 μg of pcβgal in HEPES-buffered saline 1× supplemented with 160 mM $CaCl_2$. Twelve hours after transfection, cells were rinsed with phosphate-buffered saline, trypsinized, and replated in six-well plates. The steroids to be tested were added to the medium of transfected cells 4 h after seeding. After incubating for 24 h, cell extracts were assayed for luciferase (de Wet et al., 1987) and β-galactosidase activities (Herbomel et al., 1984). To standardize the transfection efficiency, the relative light units obtained in the luciferase assay, were divided by the optical density obtained in the β-galactosidase assay.

Coupled Cell-Free Transcription and Translation. Plasmids (1 μg) containing cDNA encoding the full-length human MR or the mutant MR_{A852} were transcribed for 90 min at 30°C using T7 RNA polymerase and translated into the rabbit reticulocyte lysate system purchased from Promega according to the manufacturer's instructions.

Steroid-Binding Studies. MR and MR_{A852} were expressed in vitro using the T7-coupled rabbit reticulocyte lysate system. The lysates containing the human MR or MR_{A852} were diluted 4-fold with TEGWM buffer (20 mM Tris-HCl, 1 mM EDTA, 20 mM sodium tungstate, 1 mM β-mercaptoethanol, and 10% glycerol, pH 7.4) and incubated for 4 h at 0°C with 5×10^{-9} M [^{3}H]aldosterone or [^{3}H]RU26752 either alone or with unlabeled competitors (5×10^{-7} M). Bound and free steroids were separated by the dextran-charcoal method: 25 μl of lysate was stirred for 5 min with 50 μl of 4% Norit A and 0.4% Dextran-T70 in 20 mM Tris-HCl, 1 mM EDTA, and 10% glycerol, pH 7.4, and centrifuged at 4500*g* for 5 min at 4°C. The radioactivity was determined in an LKB liquid scintillation spectrometer after adding 5 ml of OptiPhase HiSafe (PerkinElmer Life and Analytical Sciences, Boston, MA).

Steroid-Binding Characteristics at Equilibrium. The reticulocyte lysates containing the human MR or the mutant MR_{A852} were diluted 4-fold with TEGWM buffer and incubated with 3×10^{-10} to 3×10^{-7} M [^{3}H]RU26752 for 4 h at 0°C. Bound and free steroids were separated by the dextran-charcoal method described above. Bound steroid was measured by counting the radioactivity of the supernatant. The change in bound as a function of unbound was analyzed as described previously (Claire et al., 1979), and the dissociation constant at equilibrium, K_d, was calculated.

Kinetic Experiments. The human MR and the mutant MR_{A852} were expressed in vitro as described above. The lysates were diluted 4-fold with TEGWM buffer and incubated with 10^{-8} M [^{3}H]RU26752 for 2 h at 0°C. One half of the labeled lysate was kept at 0°C and was

used to determine the stability of the [^{3}H]RU26752-MR complexes, and the other half was incubated with 10^{-6} M RU26752 for various periods. Bound and free steroids were separated using dextran-charcoal. Parallel incubations of [^{3}H]RU26752 with in vitro-expressed β-galactosidase were performed to calculate the nonspecific binding. The findings were corrected for receptor stability and were expressed as a percentage of the binding measured at time 0.

Results

Effect of Steroid Substituents on the Mineralocorticoid Antagonist Activity of Spirolactones. The various spirolactones depicted in Fig. 1 were tested for their ability to activate the transiently expressed MR or to inhibit the aldosterone-induced MR activity using *cis-trans* cotransfection assays performed in COS-7 cells with pchMR and a reporter plasmid containing mouse mammary tumor virus promoter upstream of the luciferase gene. As reported previously (Arriza et al., 1987), aldosterone stimulates the MR transactivation activity in a dose-dependent manner, with a maximum induced activity for 10^{-9} M aldosterone (data not shown). All of the spirolactones tested, including canrenone, eplerenone, mexrenone, RU26752, and spironolactone, displayed at 10^{-6} M very low agonist activities corresponding to less than 10% of the maximum aldosterone-induced MR activity (data not shown). The antagonist potency of the spirolactones was then tested by incubating MR-transfected COS-7 cells with 10^{-9} M aldosterone in the presence of increasing concentrations of spirolactones (10^{-9} to 10^{-5} M). They all inhibited the aldosterone-induced MR activity in a dose-dependent manner (Fig. 2 and Table 1). RU26752 and spironolactone, with a 7α-propyl group and a 7α-thioacetyl group, respectively, were the two most potent antagonists (IC_{50} values, $\sim 3 \times 10^{-8}$ and $\sim 5 \times 10^{-8}$ M, respectively). Mexrenone, which has a 7α-acetyl group, and canrenone, which has no C7 substituent, were both less potent inhibitors of the aldosterone-induced activity (IC_{50} values, $\sim 2 \times 10^{-7}$ M and 3×10^{-7} M, respectively). Eplerenone, characterized by having a 7α-acetyl group and a 9α-11α-epoxy group, was the least potent antagonist ($IC_{50} \sim 2 \times 10^{-6}$ M). Thus, the efficiency of the antagonist activity of spirolactones depended closely on the nature of the C7 substituent. The 9α-11α-epoxy group present on eplerenone further reduced the antagonist activity of this compound compared with its parent compound mexrenone.

Binding of Agonist and Antagonist Ligands to the Mutant MR_{A852}. The three-dimensional MR model generated by using the crystallographic data from nuclear receptors as a template (Fagart et al., 1998; Auzou et al., 2000) predicted that the steroid C7 substituent faces Met852, a residue of the H7 helix. This finding led us to generate a mutant receptor by substituting alanine for methionine (MR_{A852}), which we used to test the role of this residue in the accommodation of spirolactones in the ligand-binding pocket of the receptor. In vitro-expressed MR and MR_{A852} were tested for their ability to bind [^{3}H]aldosterone and [^{3}H]RU26752. The binding of the two tritiated steroids to β-galactosidase was also determined to provide an estimation of the nonspecific binding. [^{3}H]Aldosterone binding to

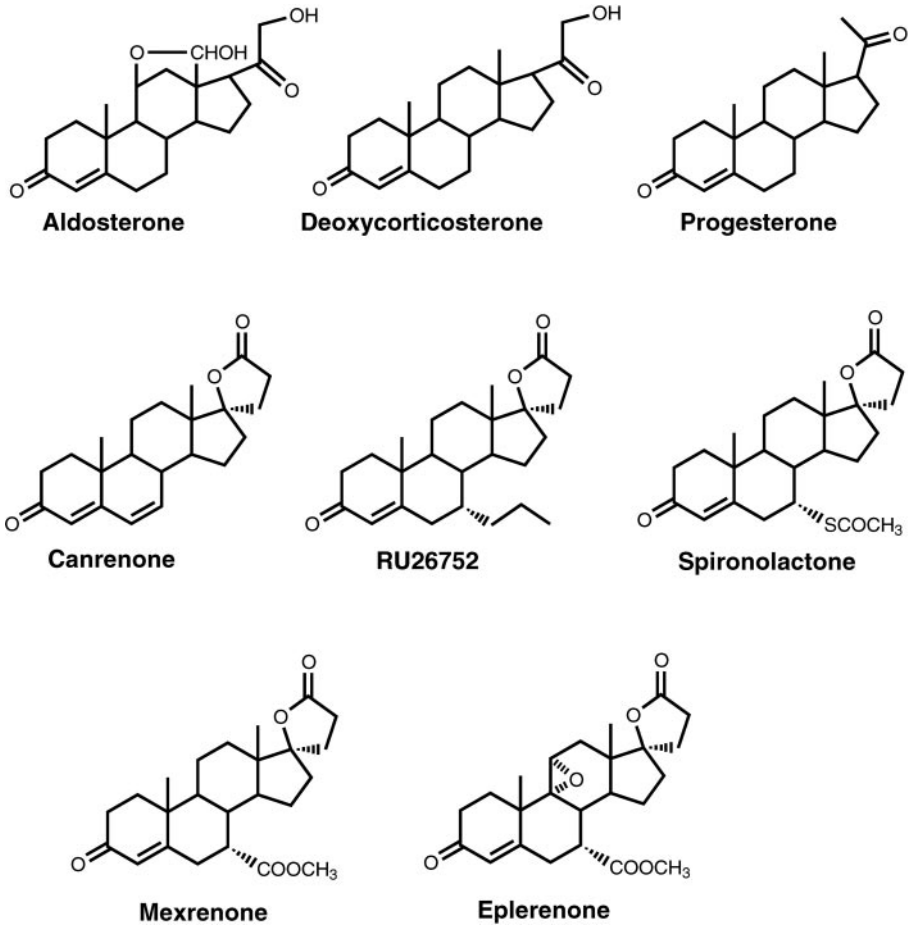

Fig. 1. Structural formulae of ligands.

MR_{A852} was very low, nearly the same as the binding of [^{3}H]aldosterone to β-galactosidase, which was less than 10% of the [^{3}H]aldosterone binding to MR (Fig. 3A). In contrast, the binding of [^{3}H]RU26752 to MR_{A852} was equivalent to 60% of the binding to MR. As a control, the nonspecific binding of [^{3}H]RU26752 to β-galactosidase was equivalent to 10% of the binding of the synthetic steroid to MR (Fig. 3A). Scatchard plot analyses revealed that [^{3}H]RU26752 bound to MR_{A852} and MR with very similar affinity values (0.71 versus 0.94 nM) (Fig. 3B). The half-lives of the RU26752-MR_{A852} and RU26752-MR complexes were also calculated from their dissociation kinetics (Fig. 3C). RU26752 dissociated much more slowly from MR_{A852} ($t_{1/2}$ = 4.5 h) than from MR ($t_{1/2}$ = 1.5 h). This finding suggested that the M852A mutation reinforced the stability of the RU26752-MR complexes without altering the affinity of the synthetic steroid for the receptor.

The fact that RU26752 is able to bind to MR_{A852}, whereas aldosterone is not, raises the question of whether other MR ligands without a C7 substituent, such as aldosterone, or with a C7 substituent, such as RU26752, are able to bind to the mutant MR_{A852}. Because most of the steroids to be tested were available as unlabeled steroids, their abilities to inhibit the binding of [^{3}H]RU26752 to the mutant MR_{A852} and the wild-type MR were measured. Aldosterone, cortisol, deoxycorticosterone, and canrenone, all of which lack a C7 substituent, inhibited the binding of [^{3}H]RU26752 to MR by more than 80%. In contrast, aldosterone, cortisol, and canrenone were unable to inhibit the binding of [^{3}H]RU26752 to MR_{A852}. (Fig. 4, left). Deoxycorticosterone displayed a low affinity for MR_{A852}, because it inhibited the binding of [^{3}H]RU26752 to MR_{A852} by ~50% (Fig. 4, left). The ability of C7-substituted spirolactones to inhibit the binding of [^{3}H]RU26752 to the mutant MR_{A852} and the wild-type MR was also tested. Eplerenone displayed low affinity, because it was able to displace only ~50% of the [^{3}H]RU26752 bound to MR and was virtually unable to inhibit [^{3}H]RU26752 binding to the mutant MR_{A852}. (Fig. 4, right). Mexrenone, spironolactone, and RU26752 all displayed high affinity toward both MR and the mutant MR_{A852}, because they displaced more than 70% of the [^{3}H]RU26752 bound to both receptors (Fig. 4, right). Thus, the M852A mutation either dramatically reduced the ability of the receptor to bind ligands without C7 substituents (such as deoxycorticosterone and canrenone) or completely abolished it (aldosterone and cortisol). In contrast, spirolactones with a C7 substituent were still bound to the receptor despite the M852A mutation.

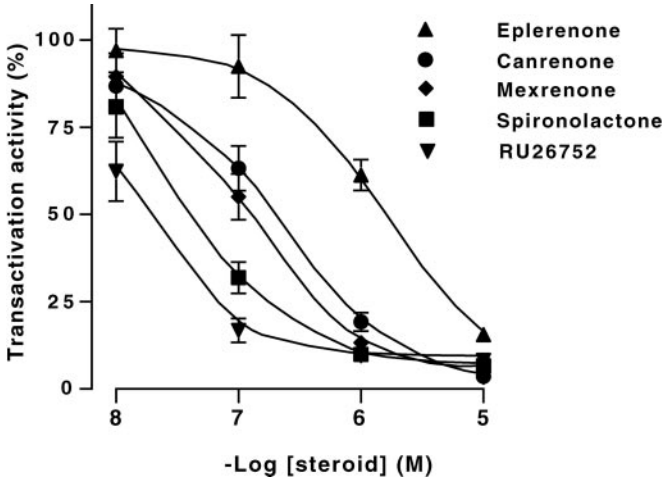

Fig. 2. Effect of spirolactones on aldosterone-induced MR transactivation activity. COS-7 cells were transfected with pchMR, an hMR expression vector, with pFC31luc as the reporter plasmid, and a β-galactosidase internal reporter to correct for transfection efficiency. Before being harvested, cells were treated for 24 h with 10^{-9} M aldosterone in the absence or presence of various concentrations (10^{-8} to 10^{-5} M) of canrenone, eplerenone, mexrenone, spironolactone, and RU26752. The MR transactivation activity was determined by luciferase activity, relative to the internal β-galactosidase control, and is expressed as a percentage of MR activity in response to aldosterone alone. Values are the mean ± S.E.M. of two to six separate experiments.

TABLE 1

Spirolactone concentrations required to inhibit aldosterone-induced MR and spirolactone-induced MR_{A852} activities by 50%

Steroid	Steroid Substituent	IC_{50}	
		MR	MR_{A852}
		M	
RU26752	7α-CH_2-CH_2-CH_3	3×10^{-8}	(agonist)
Spironolactone	7α-$SCOCH_3$	5×10^{-8}	(agonist)
Mexrenone	7α-$COOCH_3$	2×10^{-7}	5×10^{-8}
Canrenone	C6–C7 insaturation	3×10^{-7}	10^{-5}
Eplerenone	7α-$COOCH_3$, 9α-11α-epoxy	2×10^{-6}	10^{-6}

Effects of the M852A Mutation on the MR Transactivation Properties. We have previously shown that antagonist ligands dissociate more rapidly from MR than aldosterone does (Fagart et al., 1998). Because RU26752 dissociates more slowly from MR_{A852} than from the wild-type receptor, the question arises of whether RU26752 and the other spirolactones act as agonist or antagonist ligands when bound to MR_{A852}. *cis-trans* Cotransfection assays showed that both spironolactone and RU26752 activate MR_{A852} in a concentration-dependent manner (Fig. 5A). They displayed nearly the same efficiency, with their maximum activity induced by 10^{-8} M and with an ED_{50} value of ~5×10^{-10} M. Mexrenone had low agonist activity, inducing 15% of the maximum MR_{A852} activity at a concentration of 10^{-7} M (Fig. 5A). The other spirolactones, canrenone and eplerenone, were unable to activate the mutant MR_{A852} within the range of concentrations tested (10^{-11} to 10^{-7} M) (Fig. 5A). The ability of the spirolactones to inhibit the spironolactone-induced activity of MR_{A852} was also examined. As shown in Fig. 5B, mexrenone, eplerenone, and to a lesser extent canrenone were able to antagonize the spironolactone-induced activity of MR_{A852} with the following potencies: mexrenone (IC_{50} ~ 5×10^{-8} M) > eplerenone (IC_{50} ~ 10^{-6} M) > canrenone (IC_{50} ~ 10^{-5} M) (Table 1). The inability of mexrenone to completely inhibit spironolactone-induced MR_{A852} activity was probably caused by the agonist activity of this compound (Fig. 5A). Thus, three spirolactones (canrenone, mexrenone, and eplerenone) retained their antagonist properties even when bound to the mutant MR_{A852} (Table 1). In contrast, when either RU26752 or spironolactone was bound to MR_{A852}, it became a potent MR_{A852} agonist.

Competition experiments also revealed that deoxycorticoterone, but not aldosterone or cortisol, was able to inhibit the binding of [^{3}H]RU26752 to the mutant MR_{A852} by 50%. Deoxycorticoterone differs from aldosterone and cortisol by having no substituent at the C11 position. Its ability to act as an agonist and/or antagonist when bound to MR_{A852} was further examined. Deoxycorticoterone did not activate the mutant receptor MR_{A852} (data not shown). In contrast, it did display

antagonist activity when bound to MR_{A852}, because it produced dose-dependent inhibition (IC_{50}, 5×10^{-8} M) of spironolactone-induced MR_{A852} activity (Fig. 5B). Thus, deoxycorticosterone, which has no C7 or C11 substituents, is still able to bind to MR_{A852} but behaves as an MR_{A852} antagonist.

Ligands Docking within the hMR Ligand-Binding Domain. Three-dimensional models of the MR-LBD have been generated using the crystallographic data of nuclear receptors as the template (Fagart et al., 1998; Auzou et al., 2000). In these models, the ligand-binding pocket is delineated by the H3, H5, H7, H11, and H12 helices, the first β-turn, and the H6–H7 and H11–H12 loops. The cavity is lined by 20 residues, 14 of which contribute to the hydrohobic nature of the cavity. Five polar residues are located at the two extremities of the cavity: Gln776 (H3) and Arg817 (H5) at one extremity, and Asn770 (H3), Cys942, and Thr945 (H11) at the other. Another polar residue is located in the middle of the cavity, Ser810 (H5). Aldosterone can be easily accommodated within the ligand-binding cavity (Fig. 6A). The C7 carbon is located at a distance of 3.9 Å from the Met852 side chain, a distance compatible with the formation of van der Waals bonds. When spironolactone is docked in the ligand-binding cavity of the MR-LBD, its C7 substituent points toward a region defined by Ser811 (H5), Phe829 (β-turn), and Met852 (H7). Because of the short distance be-

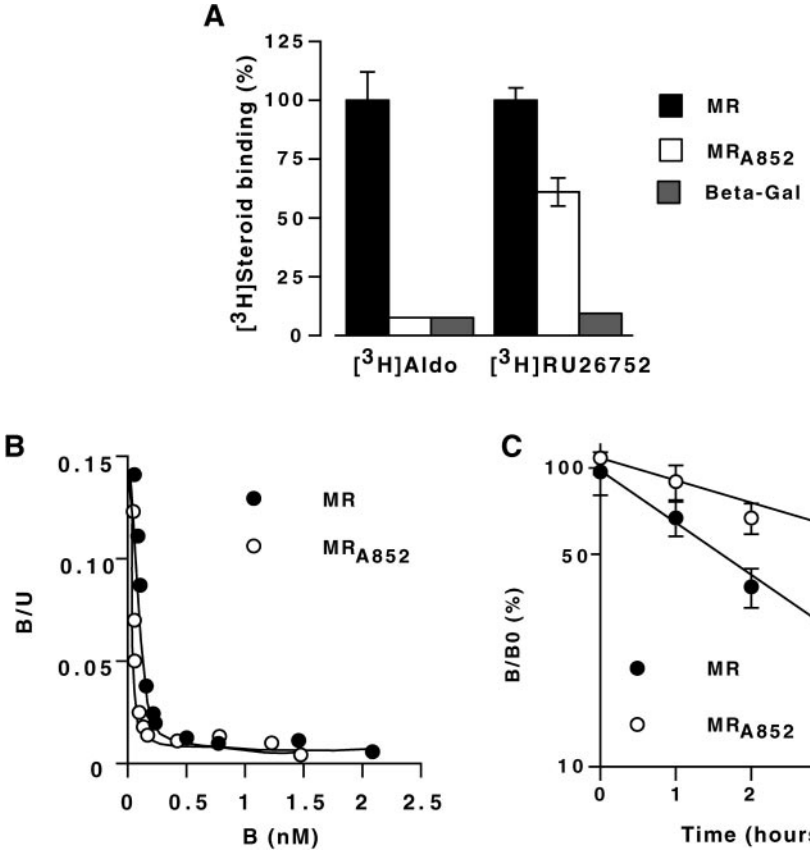

Fig. 3. Binding of aldosterone and RU26752 to MR and MR_{A852}. MR, MR_{A852}, or β-galactosidase was synthesized in vitro in rabbit reticulocyte lysate, and the lysate was diluted 4-fold with TEGWM buffer. A, binding of aldosterone and RU26752 to MR and MR_{A852}. MR, MR_{A852}, or β-galactosidase produced by translation in vitro was incubated with 5×10^{-9} M [^{3}H]aldosterone or [^{3}H]RU26752 for 4 h at 4°C. Bound (B) and unbound (U) steroids were separated by the dextran-charcoal method. Results are expressed as the percentage of tritiated steroid bound to MR. Values are the mean ± S.E.M. of three separate experiments. B, Scatchard plot of the binding of [^{3}H]RU26752 to MR and MR_{A852}. MR and MR_{A852} produced by translation in vitro were incubated with increasing concentrations of [^{3}H]RU26752 (3×10^{-10} to 3×10^{-7} M) for 4 h at 4°C. B and U steroids were separated by the dextran-charcoal method. C, dissociation kinetics of [^{3}H]RU26752 from MR and MR_{A852}. MR, MR_{A852}, or β-galactosidase produced by translation in vitro was incubated with 10^{-8} M [^{3}H]RU26752 for 2 h at 4°C. The end of this incubation period was taken as time 0 for the kinetic analysis. One aliquot was kept at 4°C to measure the stability of the steroid-receptor complexes, and another aliquot was incubated with the unlabeled RU26752 (10^{-6} M). The bound and free steroids were separated by the dextran-charcoal method. Nonspecific binding was measured simultaneously for each test time by incubating [^{3}H]RU26752 with another β-galactosidase. Results were corrected for receptor stability and were expressed as a percentage of the binding measured at time 0. Values are the mean ± S.E.M. of three separate experiments.

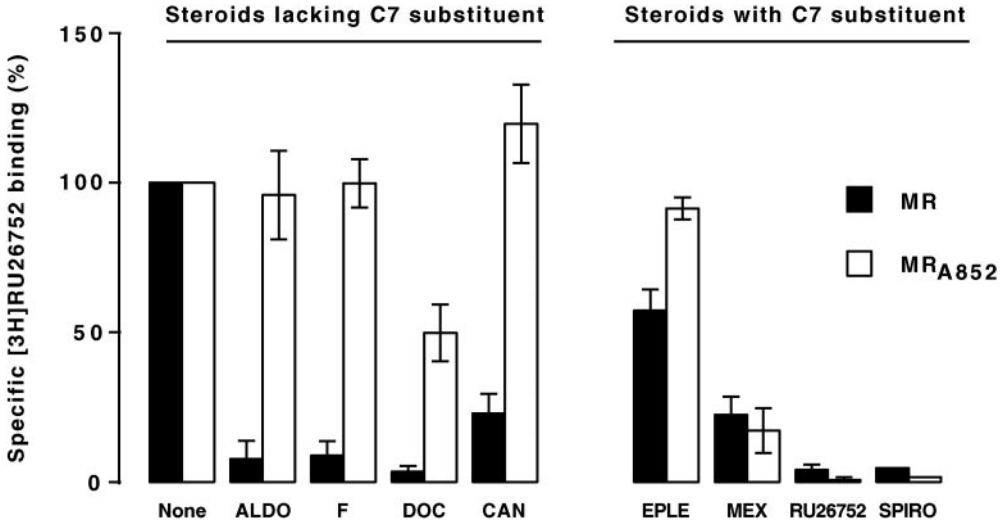

Fig. 4. Steroid competition with the binding of [^{3}H]RU26752 to MR and MR_{A852}. MR, MR_{A852}, or β-galactosidase was synthesized in vitro in rabbit reticulocyte lysate, and the lysate was diluted 4-fold with TEGWM buffer. The diluted lysates were incubated with 5×10^{-9} M [^{3}H]RU26752 in the absence or presence of a 5×10^{-7} M concentration of the steroids tested for 4 h at 0°C. The bound (B) and unbound (U) steroids were separated by the dextran-charcoal method. Results are expressed as the percentage of the specific binding of [^{3}H]RU26752 to MR. The bars correspond to the mean ± S.E.M. of three separate experiments.

tween the spironolactone C7 substituent and Met852, it seemed unlikely that spirolactone can adopt the same position as aldosterone within the ligand-binding cavity (data not shown). Replacing Met852 by an alanine within the ligand-binding pocket created a groove opposite the C7 substituent, which allowed the accommodation of the spirolactone C7 side chain and facilitated the accommodation of the molecule. In this position, the ketone function of the spironolactone lactonic ring is in a favorable position to make hydrogen bonds with Asn770 (2.9 Å) and Thr945 (3.1 Å) (Fig. 6B).

Discussion

The antagonist properties of spirolactones are believed to be linked to the γ-lactone substituent at the C17 position that characterizes all spirolactones. The findings of the present study provide some evidence suggesting that Met852, a residue of the ligand-binding cavity of the human mineralocorticoid receptor, may play a crucial role in accommodating ligands with no C7 substituent and in conferring the antagonist activity of the C7-substituted spirolactones.

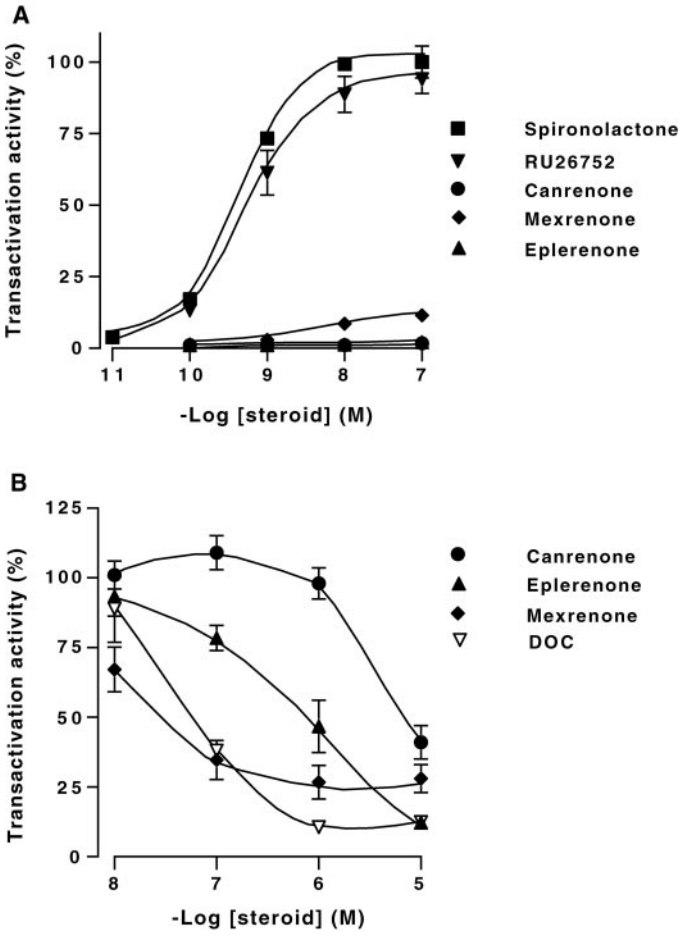

Fig. 5. Transactivation properties ofMR_{A852}. COS-7 cells were transfected with pchMR_{A852}, an MR_{A852} expression vector, with pFC31luc as reporter plasmid and a β-galactosidase internal reporter to correct for transfection efficiency. A, before being harvested, the cells were treated for 24 h with various concentrations (10^{-11} M to 10^{-7} M) of canrenone, eplerenone, mexrenone, RU26752, or spironolactone The MR_{A852} transactivation activity was determined from the luciferase activity, normalized in terms of the internal β-galactosidase control, and expressed as a percentage of the MR_{A852} activity in response to 10^{-7} M spironolactone. Each point is the mean of two to six separate experiments. B, before being harvested, the cells were treated for 24 h with 10^{-8} M spironolactone in the absence (100%) or presence of various (10^{-8} to 10^{-5} M) concentrations of canrenone, deoxycorticosterone, eplerenone, mexrenone, or progesterone. The MR_{A852} transactivation activity was determined from the luciferase activity, normalized in terms of the internal β-galactosidase control, and expressed as a percentage of the MR_{A852} activity in response to 10^{-9} M spironolactone. The values reported are the mean ± S.E.M. of two to six separate experiments.

Mutagenesis analysis guided by a three-dimensional model of the MR-LBD has made it possible to identify the residues of the ligand-binding cavity involved in anchoring the polar functions of aldosterone (Fagart et al., 1998). Gln776 (H3) and Arg817 (H5) are involved in anchoring the ketone at the C3 position, and Thr945 is involved in anchoring the 20-ketone function of aldosterone. Asn770 (H3) forms hydrogen bonds with the 18- and 21-hydroxyl functions. However, the involvement of the hydrophobic residues in steroid binding remains to be demonstrated. Docking aldosterone within the ligand-binding cavity of the MR-LBD homology model has shown that the C7 carbon is well-placed to establish van der Waals contacts with the Met852 side chain. This contact seems to be crucial for accommodating ligands with no C7 substituent within the MR ligand-binding cavity, as revealed by the ligand-binding experiments performed using the mutant MR_{A852}. In fact, we found that the M852A mutation completely abolished the binding of aldosterone, cortisol, and canrenone and reduced the binding of deoxycorticosterone by more than 50%. Met852 is conserved in the androgen receptor (AR), the glucocorticoid receptor, and the progesterone receptor (Wurtz et al., 1996). The crystal structures of the steroid receptor LBDs complexed with an agonist ligand have identified van der Waals contacts between the ligand C7-carbon (dihydrotestosterone, dexamethasone, and progesterone) and the methionine residue of the corresponding receptor (AR-Met787, glucocorticoid receptor Met646, and progesterone receptor 801) (Williams and Sigler, 1998; Matias et al., 2000; Bledsoe et al., 2002). It is interesting that the M787V mutation in the human AR that is associated with a complete androgen insensitivity syndrome dramatically reduced the capacity of the AR to bind androgen ligands (Nakao et al., 1992). This makes it tempting to postulate that the methionine facing the C7 carbon may play a crucial role in accommodating ligands within the ligand-binding cavity of the steroid receptors, with the exception of the estrogen receptor, which has a leucine residue instead of methionine at the corresponding position (Wurtz et al., 1996; Brzozowski et al., 1997).

Here, we show that spironolactone and RU26752 lose their antagonist activities when bound to the mutant MR_{A852}, whereas two other spirolactones, mexrenone and eplerenone, still display antagonist properties when associated with the mutant MR_{A852}. We also provide evidence that deoxycorticosterone, an MR agonist, displays an antagonist activity when bound to the mutant MR_{A852}. These observations raise the question of whether the M852A mutation can modify the steroid-receptor contacts that are required to stabilize the MR in its active state. Establishing the crystal structures of several nuclear receptors in their inactive and active states has greatly added to our understanding of the process by which steroid receptors are activated. The major difference between the antagonist-associated and agonist-associated states of the steroid receptors lies in the location of the H12 helix that harbors the ligand-activated transactivation function, known as AF-2 (Moras and Gronemeyer, 1998; Bour-

guet et al., 2000). In the active state, the position of the H12 helix unmasks an interface suitable for nuclear-receptor coactivator binding, whereas in the inactive state, the H12 helix covers this region. The hMR-LBD homology model of the MR-LBD and mutagenesis studies have identified several contacts in the region of the H12 helix and in the loop between the H11 and H12 helices involved in stabilizing the hMR in the active state (Fagart et al., 1998; Hellal-Levy et al., 2000). One of these contacts is the strong hydrogen bond between the oxygen atom of the Glu955 and Asn770 in the H3 helix, a residue that also forms a hydrogen bond with the 21-hydroxyl function, which characterizes all MR agonist ligands, such as aldosterone, cortisol, and deoxycorticosterone (Hellal-Levy et al., 2000). We have shown that the Asn770 residue plays a crucial role in stabilizing the MR in its active state and, conversely, that the antagonist activity of spirolactones is related to their inability to make contact with Asn770 (Fagart et al., 1998). As far as RU26752 and spironolactone are concerned, Met852 is probably the only residue that impairs the ability of these steroids to establish contact with Asn770. Indeed, both RU26752 and spironolactone, which normally act as antagonists when bound to the wild-type MR, actually activate the receptor carrying the M852A mutation. Additional constraints are probably involved in the cases of mexrenone and eplerenone, because these two molecules retain their antagonist properties when bound to the mutant $MR_{A852.}$ The orientations of the C7 side chains of the spirolactones tested in this study are quite distinct (Fig. 7 shows a schematic representation). The ketone of the thioacetyl group of spironolactone and the propyl group of RU26752 are probably close to Met852. In contrast, the ketone function of the mexrenone carboxymethyl group is directed toward Phe829, and its methyl group is close to Met852. These orientations suggest that the positions of RU26752 and spironolactone would be impaired only by Met852, whereas that of mexrenone would be impaired by both Met852 and Phe829. We observed that the switch from antagonist to agonist activity as a result of the M852A mutation is accompanied by an increased in the stability of the RU26752-receptor complexes. This makes it is tempting to

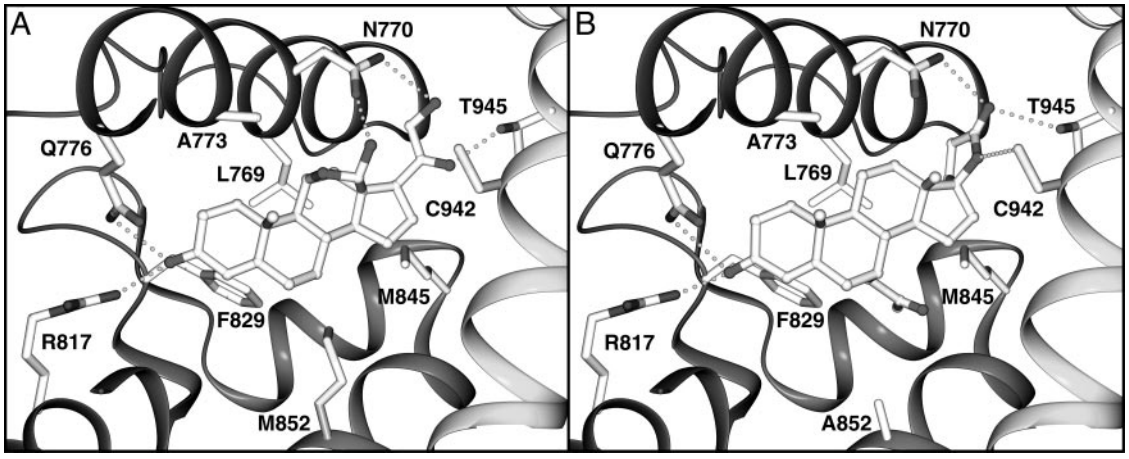

Fig. 6. Steroid docking within the hMR ligand-binding domain. The positioning of aldosterone in the ligand-binding pocket of MR (A) and of spironolactone in the ligand-binding pocket of MR_{A852} (B) is shown. The helices are depicted as ribbons, and only selected side chains in the vicinity of the ligands are shown. The hydrogen bonds between the steroid polar groups and the hMR residues are depicted as green dots. The figure was generated using Dino (Dino, Vizualizing Structural Biology, 2002; http: //www.dino3d.org).

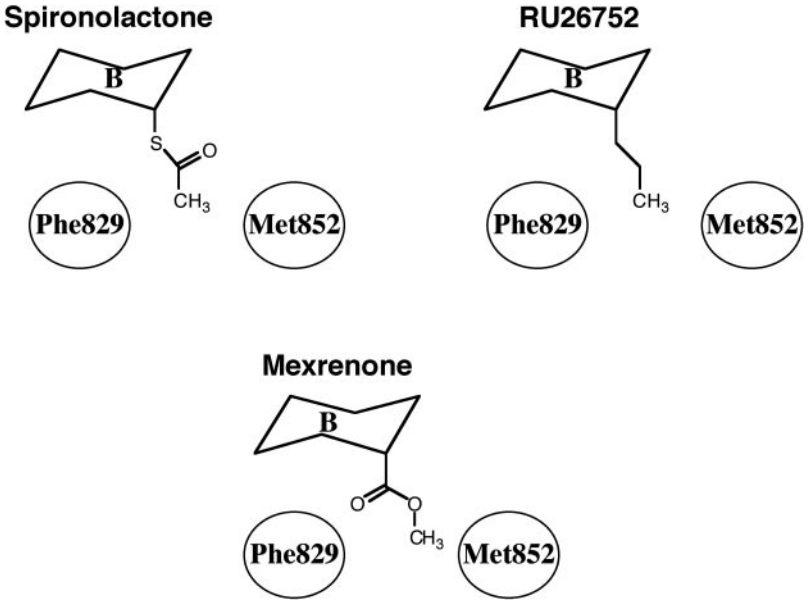

Fig. 7. Schematic representation of the C7 substituents of spirolactones within the ligand-binding domain of the MR. The orientation of the C7 substituent of the steroid B ring within the groove formed by the residues Phe829 and Met852 is shown for spironolactone, RU26752, and mexrenone.

propose that the contact between the Asn770 of the MR_{A852} and the ketone function of the RU26752 lactone ring is directly responsible for the stabilization of the active complex. The three-dimensional homology model also indicates that the Leu769 facing the steroid C11-carbon could induce further steric hindrance, which could be responsible for the fact that eplerenone displays less antagonist activity than mexrenone (Fig. 2).

Despite the fact that deoxycorticosterone acts as a potent MR agonist, this steroid seems to be unable to activate the mutant MR_{A852}. It is likely that replacing the Met852 residue by an alanine may modify the structural organization of the ligand-binding cavity and, as a consequence, the accommodation of ligands. Aldosterone and cortisol, both of which have C11 substituents, are unable to bind to the mutant MR_{A852}, whereas deoxycorticosterone, which has no C11 substituent, still binds to the mutant receptor MR_{A852}. The antagonist property of deoxycorticosterone when bound to MR_{A852} might be caused by the inability of its C21 hydroxyl group to contact Asn770. It has been proposed that maximal MR transactivation activity requires close contact between Asn770 and the C21 hydroxyl group of aldosterone, and that steroid substitution of C21 hydroxylated steroids can modify their accommodation within the ligand-binding cavity and, as a consequence, their ability to establish contact with Asn770. This is true for corticosterone, which has a 11-hydroxyl group, and is less effective than deoxycorticosterone in activating MR, and also for 11-dehydrocorticosterone, which displays antagonist features when bound to MR (Farman and Rafestin-Oblin, 2001; Bocchi et al., 2003).

In conclusion, the present findings have provided evidence suggesting that the Met852 residue acts as an organizer of the ligand-binding cavity. It allows C7-lacking steroids, such as aldosterone and cortisol, to be accommodated, and it is involved in the steric hindrance that prevents C7-substituted spirolactones from folding the receptor in its active state. This work has identified new determining factors for the MR activation process and provides new insights relevant to the design of new MR antagonists.

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