Ligand-induced conformational change in the human mineralocorticoid receptor occurs within its hetero-oligomeric structure

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To determine the first steps involved in the mechanism of action of aldosterone and its antagonists, we analysed the ligand-induced structural changes of the human mineralocorticoid receptor (hMR) translated *in vitro*. Limited chymotrypsin digestion of the receptor generated a 30 kDa fragment. Following binding of a ligand to hMR, the 30 kDa fragment became resistant to chymotrypsin proteolysis, indicating a change in the receptor conformation. Differences in sensitivity to chymotrypsin of the 30 kDa fragment were observed after binding of agonists and antagonists to hMR, suggesting that these two classes of ligands induced different hMR conformations. Several lines of evidence allowed us to identify the 30 kDa fragment as the subregion encompassing the C-terminal part of the hinge region and the ligand-binding domain (LBD) of hMR (hMR 711–984). (1) The 30 kDa fragment is not recognized by FD4, an antibody directed against the N-terminal region of hMR. (2) Aldosterone remains associated with the 30 kDa fragment after chymotrypsin proteolysis of the aldosterone–hMR complex. (3) A truncated hMR, lacking the last 40 C-terminal amino acids (hMR 1–944), yields a 26 kDa proteolytic fragment. In addition, we showed that the unbound and the aldosterone-bound 30 kDa fragment were both associated with heat-shock protein (hsp) 90, indicating that the ligand-induced conformational change takes place within the hetero-oligomeric structure and that the 711–984 region is sufficient for hsp90–MR interaction. We conclude that the ligand-induced conformational change of the receptor is a crucial step in mineralocorticoid action. It occurs within the LBD, precedes the release of hsp90 from the receptor and is dependent upon the agonist/antagonist nature of the ligand.

INTRODUCTION

Aldosterone modulates cation transport, mainly in kidney and colon, by first binding to an intracellular receptor, the mineralocorticoid receptor (MR), that belongs to the superfamily of ligand-activated transcription factors [1]. Steroid receptors share a common modular structure which contains a variable N-terminal domain (A/B region), a highly conserved domain for DNA binding (DBD; C region) and a ligand-binding domain (LBD; E region). The D region is believed to provide a hinge between the DBD and the LBD [2]. It is supposed that the binding of hormone to receptor initiates a series of events that includes conformational changes, phosphorylation, dissociation of receptor-associated proteins, dimerization and DNA binding at hormone response elements. After DNA binding, the activated receptor can interact with the transcriptional machinery and regulate gene transcription.

Little is known about the MR as compared with other members of the nuclear receptor superfamily. Unliganded MR is detected almost exclusively in the cytoplasmic compartment of cells. Aldosterone modifies the intracellular distribution by inducing nuclear receptor translocation [3–5]. In its untransformed state, MR exists as a soluble hetero-oligomeric complex that includes the 90 kDa heat-shock protein, hsp90 [6]. Hsp56 and hsp70 are most likely also associated with human MR (hMR), as has been observed for the other steroid receptors [7–9]. Analysis of MR activation *in vitro* has been limited by the relative resistance of the cytosolic receptor to activation, by the lability of the activated MR and by its propensity to aggregate [10–12]. It has been reported that MR activation causes dissociation of hsp90 from the intact receptor [6] and that, after activation, both unliganded and liganded hMR bind to DNA–cellulose [12]. Taken together, this suggests that release of hsp90 from the receptor is necessary for the binding of MR to DNA, but that other steps are required to confer on the receptor its transcriptional activity.

A complementary approach to studying gene regulation by steroid receptors is the use of hormone antagonists. Spirolactones, the most widely used anti-mineralocorticoid antagonist compounds, act mainly by antagonizing the effect of aldosterone at the target-cell level [13]. At the molecular level, anti-mineralocorticoids bind MR with high affinity, dissociate rapidly and, in addition, they are able to destabilize hsp90–MR interaction [14,15]. Therefore, anti-mineralocorticoid activity cannot be explained by maintenance of the hsp90–MR interaction, as has been proposed for the anti-glucocorticoid activity of RU486 [16]. Upon antagonist binding, the receptor interacts with hormone-response elements [17] but does not activate transcription efficiently. Thus, it is likely that hormones and anti-hormones modify the receptor conformation in different ways. Agonists may induce conformational changes that expose the receptor transcription–activation domains, whereas antagonists do not impair MR binding to DNA but induce a conformation that is transcriptionally silent. It is now well established for members of the nuclear receptor superfamily that agonist and antagonist steroids modify receptor conformation differently [18–25]. Nevertheless, to date, no study has been reported for MR.

To elucidate the key steps involved in the mechanism of the action of aldosterone, we analysed the conformation of both free hMR and hMR associated with mineralocorticoid agonists or antagonists. Different approaches have been used to study protein conformation, such as affinity partitioning [26], circular dichroism [27], protease digestion [18,21–24,28–31] and mapping with antibody probes [20]. In the present paper, we use limited

Abbreviations used: hMR, human mineralocorticoid receptor; LBD, ligand-binding domain; DBD, DNA-binding domain; hsp, heat-shock protein.

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proteolysis assays with chymotrypsin to analyse the conformation of the hMR translated *in vitro*. This provides evidence that subsequent to ligand binding, hMR undergoes a conformational change which is dependent upon the nature of the ligand. The ligand-dependent conformational change is a very early event in the mechanism of the action of aldosterone that precedes the release of hsp90.

MATERIALS AND METHODS

Chemicals

[1,2-^{3}H]Aldosterone (40–60 Ci/mmol) and L-[^{35}S]methionine (1000 Ci/mmol) were purchased from Amersham (Les Ulis, France). ^{14}C-Labelled protein low-range molecular-mass markers were obtained from Bethesda Research Laboratories. Non-radioactive aldosterone, corticosterone, cortisol, progesterone and chymotrypsin were obtained from Sigma (St. Louis, MO, U.S.A.). Unlabelled and [^{3}H]RU26752 were provided by Roussel-Uclaf Laboratories (Romainville, France). Entensify was obtained from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Unlabelled spironolactone (SC9420) was provided by Searle Laboratories. TNT® T7-coupled rabbit reticulocyte lysate system was purchased from Promega (Charbonnières, France). To avoid steroid adsorption, steroid solutions prepared in ethanol were dried and re-suspended in 50 % (v/v) polyethylene glycol 300 prepared in TEG (see Buffers section for details), to give a 5 % (v/v) final concentration of polyethylene glycol 300 in the lysate.

Buffers

TEG buffer contained 20 mM Tris/HCl, 1 mM EDTA and 10 % (v/v) glycerol. TEGW buffer was TEG with 20 mM sodium tungstate. TEGWD was TEGW with 1 mM dithiothreitol. All buffers were adjusted to pH 7.4 at 25 °C.

DNA constructs

The plasmid encoding the recombinant hMR (1–944) was constructed as follows. The cDNA coding for hMR (1–944) was amplified by PCR with primers whose sequences were deduced from hMR cDNA [1]. Forward primer: 5′-CCC-AAGCTTATGGAGACCAAAGGCTACCAC-3′; reverse primer: 5′-CCCAAGCTTTCAGAAGCAGAATTCCAGC-3′. A *Hin*dIII sequence was added to the coding sequence (underlined). The PCR product, digested by *Hin*dIII, was inserted in the *Hin*dIII-digested parental phMR3750 vector under T7 polymerase control.

Coupled cell-free transcription and translation

Plasmids phMR3750 or hMR(1–944) (1 μg), containing cDNA coding for the full-length [1] or mutant hMR, were transcribed with T7 RNA polymerase and translated simultaneously in rabbit reticulocyte lysate for 1 h at 30 °C according to the manufacturer's instructions. Depending on the experiment, the reactions were conducted either with unlabelled or ^{35}S-labelled methionine in the translation mixture.

Steroid-binding characteristics at equilibrium

After translation of the hMR, the lysate was 4-fold diluted with ice-cold TEGWD buffer. Increasing concentrations of [^{3}H]aldosterone (0.1–100 nM) were added to 25 μl of lysate. After 4 h at 4 °C, total radioactivity was counted. Bound (B) and unbound (U) steroids were separated by the following dextran–charcoal treatment: 25 μl of lysate was stirred for 5 min with 50 μl of 4 % (w/v) Norit A/0.4 % (w/v) dextran–T70 in TEG buffer and centrifuged at 1500 ***g*** for 5 min. Bound steroid was determined by counting the radioactivity of the supernatant. The evolution of B as a function of U was analysed by a previously described computer method [32] and the dissociation constant K_d was calculated.

Sucrose gradient centrifugation

Samples were layered on top of a 5–20 % (w/v) sucrose gradient prepared in TEGW buffer. Gradients were centrifuged in a VTi 65.2 rotor at 4 °C for 2 h at 385000 ***g***. Three-drop fractions were collected by piercing the bottom of each tube and the radioactivity was counted. Aldolase (A, 7.9 S), BSA (4.6 S) and myoglobin (M, 2 S) were used as external sedimentation markers.

Limited proteolytic digestion of translated hMR

To 5 μl of ^{35}S-labelled translation mix, incubated both in the absence and presence of unlabelled steroid for 10 min at 20 °C, 0.5 μl of chymotrypsin (diluted in water) was added, followed, in both cases, by an additional incubation of either 10 or 60 min duration. A 1 μl aliquot was removed and mixed with 20 μl of denaturing loading dye, boiled for 5 min and immediately loaded onto an SDS/12.5 %-polyacrylamide gel. Following electrophoresis, the gels were fixed for 30 min in methanol/acetic acid/distilled water (30:10:60, by vol.) and then treated with Entensify and dried. Autoradiography was performed at −80 °C overnight.

Analysis of autoradiograms by scanning densitometry

Autoradiograms were scanned by image analysis (Optilab, Graftek, France). Results are given as signal intensity expressed in arbitrary units.

Miscellaneous

The protein concentration in the lysate was determined by the Bradford method, using BSA as standard [33]. The protein concentration of the rabbit reticulocyte lysate was about 50 mg/ml. Radioactivity was measured in an LKB liquid-scintillation spectrometer after the addition of 5 ml of OptiPhase 'HiSafe' (counting efficiency ~50 %).

RESULTS

Characterization of the hMR translated *in vitro*

We first examined the steroid-binding properties and the structure of hMR synthesized in the rabbit reticulocyte lysate. Preliminary experiments were performed to ensure that equilibrium was reached after 4 h incubation at 4 °C. Analysis of the binding characteristics at equilibrium revealed that [^{3}H]aldosterone and [^{3}H]RU26752, an aldosterone antagonist [34], bind to one class of specific sites with dissociation constants of 0.66 ± 0.30 nM ($n = 3$) and 0.75 ± 0.14 nM ($n = 3$) respectively (results not shown). These K_d values are in the range of those already reported for native [35] and recombinant hMR [36,37].

The structure of hMR translated *in vitro* was analysed on sucrose density gradient equilibrated in sodium tungstate-containing buffer. hMR has a sedimentation coefficient of 9.9 ± 0.5 S ($n = 4$). As shown in Figure 1A the [^{3}H]aldosterone–hMR complexes were shifted to 11.8 S after incubation with FD4 [5], a monoclonal antibody directed against an epitope in the N-terminal part of hMR (amino acid residues 412–422). Incubation

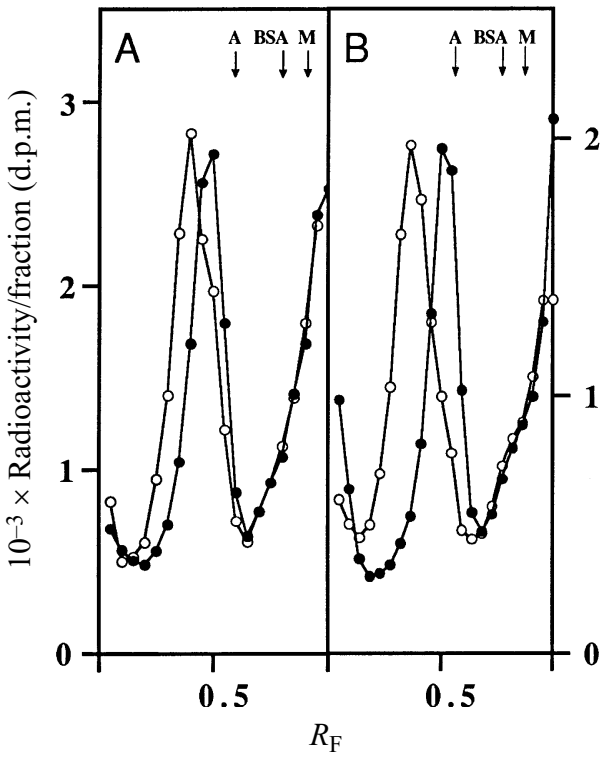

Figure 1 hMR translated *in vitro* is recognized by FD4, an anti-MR antibody, and by 7C10, an anti-hsp90 antibody

[^{3}H]Aldosterone–MR complexes (0.2 ml) recovered in TEGWD buffer were incubated for 1 h at 20 °C with 10 μl of undiluted control ascites (●) (**A** and **B**) or with 10 μl of FD4 (○), a monoclonal antipeptide antibody raised against the N-terminal part of hMR (**A**), or with 10 μl of 7C10 (○), a monoclonal antibody raised against rabbit hsp90 (**B**). After dextran–charcoal treatment, a 180 μl aliquot was layered on top of a 5–20% (w/v) sucrose gradient prepared in TEGW buffer. Gradients were centrifuged for 2 h at 385 000 ***g*** in a VTi 65.2 rotor at 4 °C. Sedimentation markers are indicated for aldolase (A, 7.9 S), BSA (4.6 S) and myoglobin (M, 2 S).

with 7C10 [38], a monoclonal anti-rabbit hsp90 antibody, shifted the [^{3}H]aldosterone–hMR complexes to 12.3 S (Figure 1B), indicating that the hMR expressed *in vitro* is associated with rabbit hsp90. The electrophoretic analysis of the ^{35}S-labelled hMR revealed a major band corresponding to a molecular mass of ~ 110 kDa (Figure 2, lane 1), a value close to that predicted from the cDNA sequence of hMR [1]. Taken together, these results indicate that the hMR synthesized *in vitro* has structural and ligand-binding properties indistinguishable from those of the native hMR.

Analysis of the hMR conformation by limited proteolysis

In order to identify the early steps in the mechanism of action of aldosterone, we analysed the conformation of free hMR as well as of hMR bound to various ligands. We used a limited proteolytic digestion assay, one of the methods normally used to study protein conformation in solution [2]. Figure 2 shows that treatment of the [^{35}S]methionine-labelled hMR for 10 min at room temperature with increasing concentrations of chymotrypsin resulted in the gradual digestion of the free hMR. A predominant band of ~ 30 kDa was observed with 3 μg/ml chymotrypsin (Figure 2, lane 3). This 30 kDa fragment was completely digested with a chymotrypsin concentration of 60 μg/ml (Figure 2, lane 6). In contrast, digestion of aldosterone-

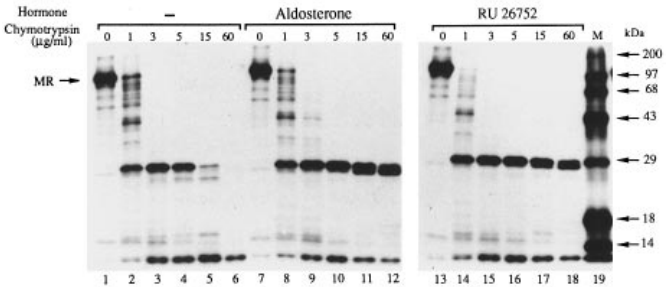

Figure 2 Ligands induce conformational changes in the hMR translated *in vitro*

[^{35}S]hMR, synthesized *in vitro*, incubated without (lanes 1–6) or with (lanes 7–12) 100 nM aldosterone, or with 100 nM RU26752 (lanes 13–18) was digested for 10 min at 20 °C by the indicated concentrations of chymotrypsin. An equal volume of water was added for the undigested controls (lanes 1, 7 and 13). The resulting digestion products were analysed on a 12.5% (w/v) acrylamide gel and subjected to autoradiography as described in the Materials and methods section. The sizes of molecular-mass markers (lane 19) are indicated on the right-hand side of the Figure.

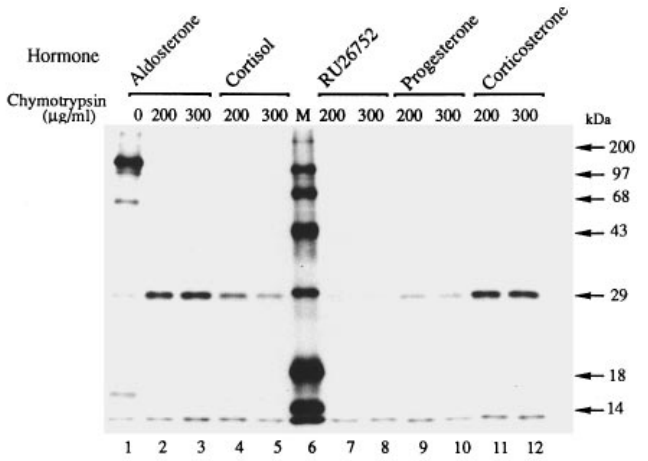

Figure 3 Agonists induce a more compact structure of the hMR translated *in vitro* than do antagonists

[^{35}S]hMR, synthesized *in vitro*, incubated with 100 nM agonists: aldosterone (lanes 1–3), cortisol (lanes 4 and 5), corticosterone (lanes 11 and 12), or antagonists: RU26752 (lanes 7 and 8) and progesterone (lanes 9 and 10) were digested for 1 h at 20 °C with the indicated concentrations of chymotrypsin. An equal volume of water was added for the undigested control (lane 1). Digestion products were analysed on a 12.5% (w/v) acrylamide gel and subjected to autoradiography as described in the Materials and methods section. The sizes of molecular-mass markers (lane 6) are indicated on the right-hand side of the Figure.

or RU26752-treated hMR with 60 μg/ml of chymotrypsin yielded a 30 kDa digestion-resistant fragment (compare lanes 12 and 18 with lane 6, Figure 2). Similar results were obtained with other mineralocorticoid agonists (cortisol and corticosterone) and aldosterone antagonists (spironolactone and progesterone) (results not shown). These results indicate that following agonist or antagonist binding to hMR, the 30 kDa fragment becomes resistant to proteolysis, suggesting that hMR is recovered as a more compact structure after ligand binding.

Treatment of ligand–hMR complexes with higher chymotrypsin concentrations (200 and 300 μg/ml) for 1 h revealed important differences among ligands (see Figure 3). After aldosterone (lanes 2 and 3) and corticosterone (lanes 11 and 12)

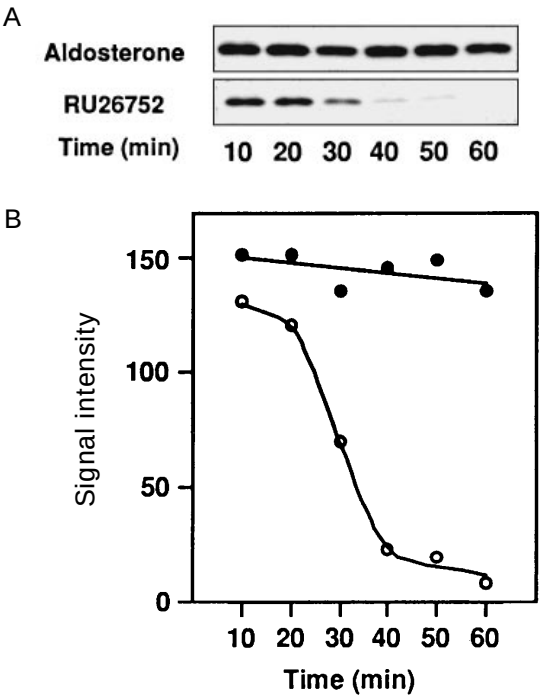

Figure 4 Aldosterone and RU26752 binding to hMR lead to different proteolysis kinetics of the 30 kDa fragment

[^{35}S]hMR, synthesized *in vitro*, incubated with 100 nM aldosterone or RU26752 was digested at 20 °C with 300 μg/ml of chymotrypsin for various periods of time. Digestion products were analysed on a 12.5% (w/v) acrylamide gel and subjected to autoradiography as described in the Materials and methods section. Autoradiograms are presented in (**A**). The band corresponding to the 30 kDa fragment was densitometrically scanned and quantified. The signal intensity, expressed in arbitrary units, of the aldosterone- (●) and RU26752- (○) associated 30 kDa fragment is plotted as a function of time (**B**).

binding to hMR, the 30 kDa fragment was completely resistant to chymotrypsin digestion. A somewhat lower proteolysis resistance was observed after cortisol binding, compared with what was observed with aldosterone and corticosterone (compare lanes 4 and 5 with lanes 2 and 3 and 11 and 12). In contrast, a higher sensitivity of the 30 kDa fragment to proteolysis was observed after incubation of hMR with antagonists; the digestion was complete after RU26752 binding (lane 8) and almost complete after progesterone binding to hMR (lane 10). Proteolysis kinetics of the RU26752– and aldosterone–hMR complexes were further performed using a 300 μg/ml concentration of chymotrypsin. The autoradiograms presented in Figure 4A were scanned as described in the Materials and methods section and the signal intensity corresponding to the 30 kDa fragment was plotted as a function of time (Figure 4B). After RU26752 binding to hMR the 30 kDa fragment disappeared in a time-dependent manner and was completely proteolysed after 60 min (see Figure 4). In contrast the 30 kDa fragment obtained from aldosterone–hMR complex was highly resistant to chymotrypsin. Taken together these results suggest that the binding of agonists and antagonists to hMR promote different receptor conformations.

Characterization of the 30 kDa fragment

The electrophoretic pattern of the chymotrypsin-treated hMR, presented in Figure 2, reveals a high degree of labelling of the 30 kDa fragment. By scanning the autoradiograms, the labelling intensity of the 30 kDa fragment was found to be $\sim 50\%$ of that of the entire receptor, suggesting that this fragment contains approximately half of the methionine residues present in the hMR. Examination of the hMR primary structure revealed that half of the 25 methionine residues are located in the N-terminal part of the receptor and the other half in the LBD. To identify the 30 kDa fragment, hMR was incubated with [^{3}H]aldosterone, then submitted to proteolysis with 15 μg/ml of chymotrypsin for 10 min to generate the 30 kDa fragment, and lastly analysed on a sucrose gradient. Under these proteolysis conditions, [^{3}H]aldosterone remains associated with the 30 kDa fragment (see Figure 5A). The [^{3}H]aldosterone-bound 30 kDa fragment displayed a sedimentation coefficient of 9.3 S that is unchanged after incubation of the complex with FD4, a monoclonal antibody that interacts with the N-terminal part of hMR. These results strongly suggest that the 30 kDa fragment contains the LBD. Chymotrypsin is known to cleave the C-terminal peptide-bond of aromatic residues (tryptophan, tyrosine and phenylalanine). There is only one chymotrypsin site in the hinge region of hMR (Tyr-710) and it is compatible with the generation of a 30 kDa fragment. This enzymic cleavage site is preceded by a non-classical sequence of eight proline residues which could allow the receptor to bend, thus facilitating the chymotrypsin attack. To confirm that the 30 kDa fragment represents the 711–984 region of hMR, we submitted to chymotrypsin proteolysis a mutant of hMR with the last 40 C-terminal amino acids deleted. As shown in Figure 5B, digestion of the hMR mutant (1–944) with chymotrypsin resulted in the formation of a 26 kDa fragment. This result allowed us to conclude that the 30 kDa fragment starts from Ile-711 in the hinge region and includes the entire LBD of hMR.

To elucidate whether the ligand-induced conformational change occurs before or after hsp90-dissociation from hMR, we examined the hetero-oligomeric structure of the 30 kDa fragment by using 7C10, an anti-hsp90 antibody. Figure 5A shows that the 9.3 S aldosterone-bound 30 kDa fragment was shifted by 7C10 to the 12.1 S region, indicating that hsp90 is associated with the 30 kDa fragment. Additional experiments were performed to ensure that the unliganded 30 kDa fragment was also associated with hsp90. The unliganded ^{35}S-labelled hMR was submitted to a 5 μg/ml chymotrypsin action for 10 min and loaded onto a sucrose gradient. The gradient fractions were analysed by SDS/PAGE, followed by autoradiography. Autoradiograms, presented in Figures 6A and 6B, were scanned as described above and the deduced profiles are presented in Figure 6C. The unliganded 30 kDa fragment sedimented as a major 9.4 S peak, which was shifted to the 11.4 S region after incubation with 7C10. It should be noted that the unliganded 30 kDa fragment was still able to bind [^{3}H]aldosterone with an affinity that is of the same order of magnitude as that observed with the intact receptor (results not shown). Taken together, our results indicate that both the free and aldosterone-bound 30 kDa fragments are associated with hsp90.

DISCUSSION

In the present paper we show that binding of a ligand to hMR induces a change in the receptor conformation that is dependent upon the agonist/antagonist nature of the ligand, allowing the proposition that ligand-induced conformational change plays a key role in the activation of MR.

Using *in vitro* translation of hMR mRNA in the reticulocyte lysate, we demonstrated that limited chymotrypsin-treatment of hMR leads to a 30 kDa fragment. Ligand binding to hMR

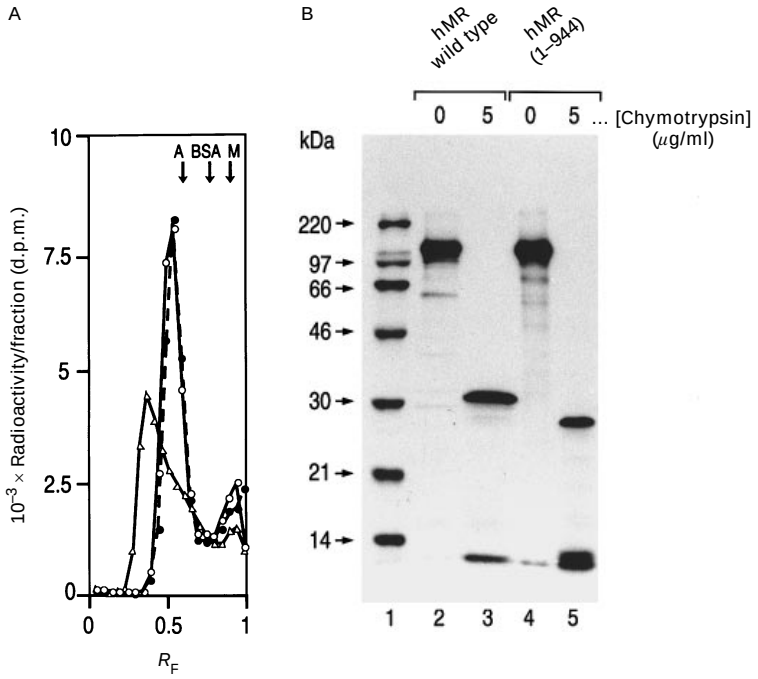

Figure 5 The 30 kDa fragment encompasses the LBD of hMR

(**A**) hMR, synthesized *in vitro*, was incubated for 10 min at 20 °C with 100 nM [^{3}H]aldosterone before digestion for 10 min at 20 °C with 15 μg/ml chymotrypsin. [^{3}H]Aldosterone-labelled 30 kDa fragment (0.2 ml) was incubated for 1 h at 20 °C with 10 μl of undiluted control ascites (●) or FD4 (○), the antipeptide antibody raised against the N-terminal part of hMR, or 7C10 (△), the anti-hsp90 antibody. After dextran–charcoal treatment, aliquots (180 μl) were layered on top of 5–20% (w/v) sucrose gradients prepared in TEGW buffer. Gradients were centrifuged for 2 h at 385000 ***g*** in a VTi 65.2 rotor at 4 °C. Sedimentation markers are indicated for aldolase (A, 7.9S), BSA (4.6S) and myoglobin (M, 2S). (**B**) ^{35}S-Labelled wild-type hMR or hMR (1–944), synthesized *in vitro*, were digested for 10 min at 20 °C with 5 μg/ml of chymotrypsin (lanes 3 and 5). An equal volume of water was added for the undigested controls (lanes 2 and 4). The resulting products were analysed on a 12.5% (w/v) acrylamide gel and were subjected to autoradiography as described in the Materials and methods section. The sizes of molecular-mass markers (lane 1) are indicated on the left-hand side of the Figure.

changed the proteolytic degradation pattern in such a way that the 30 kDa fragment became more resistant to chymotrypsin digestion, indicating that the binding of a ligand to hMR induces a tighter, more compact receptor structure that is less accessible to protease. The ligand-induced conformational change of hMR occurred within a region encompassing the C-terminal part of the hinge region and the LBD (711–984 hMR). This conclusion arose from two major observations. First, deletion of the 40 C-terminal amino acids of the receptor results in a 4 kDa reduction in the size of the resistant fragment. Secondly, the 30 kDa fragment recovered from the aldosterone–hMR complexes remains associated with aldosterone and furthermore, the 30 kDa fragment was able to bind aldosterone with binding parameters that were almost identical with those of the intact receptor. Ligand-induced conformational changes involving a region contained in or surrounding the LBD have already been observed for steroid [18–22,24–26,28,30], thyroid hormone [27,29] and retinoic acid [23,31] receptors. Protease-resistant conformations were also observed for yeast transcriptional activator [39] and herpes simplex virus DNA polymerase [40] after DNA binding.

The size of the proteolytic fragment observed after antagonist binding to hMR was not different from that observed after agonist binding, suggesting that the chymotrypsin sites exposed after agonist and antagonist binding are the same. Nevertheless, we observed that the sensitivity of the 30 kDa fragment to chymotrypsin was time- and dose-dependent. For instance, aldosterone- or corticosterone-bound 30 kDa fragment resists up to at least 300 μg/ml chymotrypsin, whereas after antagonist binding (RU26752 and progesterone), the 30 kDa fragment was almost completely digested by 200 μg/ml chymotrypsin. Thus, the difference in proteolytic sensitivity of the 30 kDa fragment after agonist and antagonist binding reveals differences in hMR conformation which lie in the three-dimensional arrangement of the same amino acid side-chains on the surface of the LBD. These results corroborate the previous observations made by Fritsch et al. for anti-oestrogens [22]. In contrast, different exposure of proteolytic sites has been reported for other steroid receptors after agonist and antagonist binding. In the case of progesterone, oestrogen and retinoic acid receptors, antagonists induced protection of a smaller fragment than that induced by agonists [18,21,23], whereas, for androgen receptor, protection of a large fragment, including the hinge and the C-terminal region of the receptor, was observed after anti-androgen binding [24]. Thus, even if some subtle differences are observed among the receptors, the ligand-induced conformational change, which occurs within the LBD and is dependent upon the nature of the ligand, seems to be a general feature for nuclear receptors.

The use of an anti-hsp90 antibody (7C10) allowed us to demonstrate that free and aldosterone-bound 30 kDa fragments were both associated with hsp90, leading to the conclusion that the ligand-induced conformational change was not a consequence of hsp90 release from hMR, but rather occured within the hetero-oligomeric structure of the receptor. Allan et al. [18] previously observed that agonist binding to progesterone receptor enhanced the resistance to proteolysis of a 30 kDa fragment generated from both unactivated and activated receptors. Thus

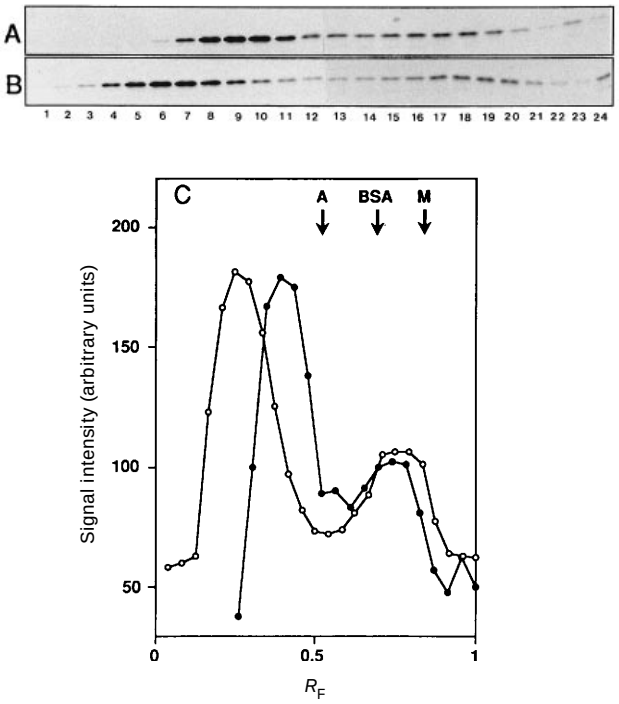

Figure 6 Unliganded 30 kDa fragment is associated with hsp90

[^{35}S]hMR, synthesized *in vitro*, was incubated for 10 min at 20 °C with 5 μg/ml of chymotrypsin. The resulting ^{35}S-labelled 30 kDa fragment (0.1 ml) was incubated for 1 h at 20 °C with 5 μl of undiluted control ascites or 7C10, the anti-hsp90 antibody. Aliquots (100 μl) were layered on top of a 5–20% (w/v) sucrose gradient prepared in TEGW buffer. Gradients were centrifuged for 2 h at 365000 ***g*** in a VTi 65.2 rotor at 4 °C. The fractions collected were analysed on a 12.5% (w/v) acrylamide gel. Autoradiograms corresponding to incubations with control ascites and 7C10 antibody are presented in (**A**) and (**B**) respectively. The fraction numbers, 1–24, are indicated below. The band corresponding to the 30 kDa fragment was densitometrically scanned and quantified. The profiles obtained in the presence of control ascites (●) and in the presence of 7C10 (○) are presented in (**C**). The sedimentation markers are indicated for aldolase (A, 7.9S), BSA (4.6S) and myoglobin (M, 2S).

our results are consistent with those of Allan et al. [18] in demonstrating that ligand-induced conformational change is a very early step in the mechanism by which the hormone activates the receptor and that such conformational change precedes the release of hsp90 from the receptor.

Our results also indicate that the 711–984 region of hMR is sufficient for the interaction between hsp90 and the receptor and that the 30 kDa fragment retains the secondary and tertiary structure necessary for steroid binding in solution. This contrasts with the negligible steroid-binding capacity of the bacterially produced N-terminal truncated MR [41,42]. The absence in HtpG, the *Escherichia coli* hsp90 homologous protein, of a region that is necessary for hsp90–receptor interaction might be responsible for these differences [41,43,44]. Indeed, the ligand-binding capacity of the mutated MR was restored by adding rabbit reticulocyte lysate [41,42]. Thus it seems clear that for MR, as for glucocorticoid receptor, hsp90 is a prerequisite for hormone binding. Indeed, Bresnick et al. have already reported that hsp90 is necessary for generation of the LBD conformation of glucocorticoid receptor that is appropriate for steroid binding [45].

In summary, our results bring new information on the sequence of the initial events involved in the mechanism of action of aldosterone. Binding of a ligand to hMR induces a conformational change that takes place in the C-terminal part of the receptor molecule. This change of conformation occurs while hMR is still associated with hsp90. Agonists and antagonists induce different hMR conformations, which in turn may differentially modulate protein–protein interactions. Finally, it is likely that the change in the conformation of hMR plays a key role in subsequent steps, most notably hsp90 release, receptor dimerization and receptor interaction with other factors necessary for building the final functional transcriptional complex.

We thank J. L. Arriza and R. Evans for providing us with the full-length hMR construct (phMR3750). Roussel Uclaf and Searle Laboratories are also acknowledged for giving us RU26752 and SC9420 respectively. We thank M.G. Catelli and J.P. Bonvalet for very useful discussions, E. Eugene for scanning analyses, A. Vandewalle for the design of the Figures and V. Lévêque for assistance in preparing the manuscript.

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Received 19 October 1995/30 November 1995; accepted 30 November 1995