Differential intracellular localization of human mineralocorticosteroid receptor on binding of agonists and antagonists

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The effect of aldosterone and antimineralocorticoids on the intracellular localization of human mineralocorticosteroid receptor (hMR) was studied using a new monoclonal anti-peptide antibody FD4. This antibody was directed against the peptide hMR-(412–422). As demonstrated by ultracentrifugation analysis, immunoprecipitation assays and Western blot, FD4 recognized both the native and denatured form of the receptor overexpressed in the baculovirus expression system. In whole-cell assays, the amount of hMR recovered in high-salt extracts was significantly lower after exposure to the antimineralocorticoid ZK91587 than to aldosterone, suggesting a lack of nuclear MR translocation. FD4 was also used for immunohistochemical studies on hMR-expressing High Five cells. In the absence of hormone, immunoreactive hMR was detected almost exclusively

in the cytoplasmic compartment of cells. After aldosterone exposure, intense nuclear immunostaining appeared in a time-dependent manner, consistent with stable nuclear localization of the receptor. Immunohistochemistry showed that antimineralocorticosteroids (ZK91587, SC9420, 18-vinylprogesterone) predominantly maintained a cytoplasmic distribution of hMR and inhibited its aldosterone-dependent nuclear localization. Thus, in our model, the nuclear/cytoplasmic partition of hMR is drastically different in the presence of antagonists from that in the presence of aldosterone. This phenomenon may contribute to their mechanism of action by preventing productive interaction of antagonist–receptor complex with specific DNA sequences in aldosterone target cells.

INTRODUCTION

The mineralocorticosteroid receptor (MR) is a regulatory transcriptional factor that mediates aldosterone action within target tissues (see ref. [1] for a review). However, a number of questions remain unanswered about the structure and function of this receptor, most notably the nature and physiological significance of its interaction with receptor-associated proteins (hsp90, hsp70, p59, etc.) and the identity of specific target sequences and transcription factors involved in the modulation of receptor activity. Another important aspect of MR physiology is its subcellular localization and the influence of ligands on its nuclear/cytoplasmic distribution. Studies on MR have been hampered for many years by its very low abundance. This is the reason why the native MR protein has not yet been purified, thus preventing generation of specific antibodies by classic immunological procedures. One alternative approach is the anti-idiotypic strategy, which has facilitated the production of H10E antibody [2]. This monoclonal antibody recognizes the ligand-binding site of MR and has been used to detect it in the rabbit kidney [3,4], heart and blood vessels [5]. The deduced primary sequence of the cDNA encoding the human MR (hMR) [6] prompted us to use the anti-peptide approach to raise specific antibodies that could be directed against regions of the receptor other than the ligand-binding domain. We were particularly interested in producing monoclonal antibodies able to recognize multiple forms of the hMR, i.e. native and denatured forms, a valuable property often missing from polyclonal anti-peptide antibodies [7–9].

We report here on the production and characterization of the monoclonal antibody FD4 directed against a preselected sequence of hMR, located at its N-terminal domain, the least-conserved region among members of the steroid-hormone receptor super-

family. This anti-peptide antibody interacts with both native and denatured forms of the receptor as demonstrated by density-gradient analyses, immunoprecipitation assays, Western-blot and immunohistochemical experiments.

Although antimineralocorticosteroid compounds bind with high affinity to MR, their antagonistic effects seem to intervene at several molecular levels of the signal-transduction pathway. To investigate the effects of various agonist and antagonist ligands on the nuclear/cytoplasmic localization of hMR, immunohistochemical studies have been performed with the new monoclonal anti-peptide antibody. In the present paper, we provide evidence that the nature of the ligand markedly modifies the intracellular distribution of hMR, giving additional support for a specific function for MR in distinguishing between aldosterone and antimineralocorticosteroids.

MATERIALS AND METHODS

Peptides

The primary sequence of hMR was analysed by computerized methods in a search for suitable peptides. Peptide sequences deduced from hMR cDNA [6] were selected for their high index of immunogenicity and potential for producing specific antibodies. These sequences are not found in the primary structure of other steroid receptors, in particular the glucocorticosteroid receptor (GR). A high score for hydrophilicity, a high probability of flexibility, protrusion and secondary structure of peptide sequences predicted a large immunogenic response [10]. One peptide designated RU54869 was synthesized by Roussel-Uclaf Laboratories and corresponds to the sequence hMR-(412–422). It was conjugated to keyhole limpet haemocyanin or to BSA using glutaraldehyde [11].

Abbreviations used: MR, mineralocorticosteroid receptor; hMR, human MR; GR, glucocorticosteroid receptor; AcNPV, *Autographa californica* nuclear polyhedrosis virus.

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Steroids

[1,2-³H]Aldosterone (40–60 Ci/mmol) and unlabelled aldosterone were from Amersham and Sigma (St. Louis, MO, U.S.A.) respectively. [³H]ZK91587 and unlabelled ZK91587 were purchased from NEN (Les Ulis, France). SC9420 was a gift from Searle Laboratories, Chicago, IL, U.S.A. 18-Vinylprogesterone was a gift from Dr. A. Marquet (Paris VII University, France).

Monoclonal antibodies

Balb/c mice were immunized intraperitoneally with about 200 µg of peptide hMR-(412–442) conjugated to keyhole limpet haemocyanin in complete Freund's adjuvant, and boosted intraperitoneally with the same amount of conjugate in incomplete Freund's adjuvant every 2 weeks. Finally, about 50 µg of conjugate in PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) was administered both intravenously and intraperitoneally. Three days after the last boost, spleen cells were fused with the non-secreting myeloma cell line P3x63-Ag8.653 by a previously described method [2]. The hybridoma supernatants were screened for the presence of anti-peptide antibodies by e.l.i.s.a. Positive clones were expanded and monoclonal antibody-producing hybridomas were obtained by subcloning the cells three times by the limiting dilution technique. Ascites fluid was obtained by injecting intraperitoneally about 10⁶–10⁷ cells into mice pretreated with 0.2 ml of pristane (Sigma). The monoclonal antibody FD4 was purified by affinity chromatography. Then 10 mg of peptide hMR-(412–422) conjugated to BSA was coupled to CNBr-activated Sepharose. Ascites fluid containing FD4 was recycled through the affinity column overnight at 4 °C. The column was extensively washed with 100 ml of PBS and eluted with 0.2 M glycine, pH 2.8. Fractions of volume 1 ml were collected and immediately neutralized with 60 µl of saturated K₂HPO₄. Fractions containing FD4 were pooled and dialysed against PBS at 4 °C. Purified FD4 had a final concentration of ~1 mg/ml.

E.l.i.s.a.

Polystyrene microtitre plates (96-well; Corning) were coated with 250 ng of peptide hMR-(412–422) conjugated to BSA (100 µl/well) in 0.1 M NaHCO₃, pH 9.3, overnight at 4 °C. After three washes with PBS containing 0.1% Tween 20, diluted antiserum or hybridoma supernatants were added and incubated for 2 h at 37 °C. The presence of antibodies was detected by peroxidase-labelled goat anti-mouse or anti-rabbit IgG antibodies diluted in PBS. The substrate solution consisted of 15 mg of *o*-phenylenediamine dichloride (Sigma) in 20 ml of 0.1 M citrate/phosphate buffer, pH 4.8, in the presence of 10 µl of 30% H₂O₂. After 10 min, the colour development was stopped by the addition of 50 µl of 4 M H₂SO₄. Absorbance at 490 nm was determined in a Microplate EL308 reader (BioTek Instruments). For competition studies, antibodies were preincubated for 1 h at room temperature with increasing concentrations of peptide.

Expression of hMR in the baculovirus system

Wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV), recombinant baculovirus AcNPV-hMR and insect Sf21 cells were used as previously described [12]. *Trichoplusia ni* (SB1-4) cells, designated High Five cells, were purchased from British Bio-technology Products Ltd., Oxford, U.K. Sf21 cells and High Five cells were grown in TNM-FH medium (Sigma) supplemented with 10% stripped fetal calf serum (Sérovial,

Gelrolbrun, France) as monolayers and in suspension in spinner flasks respectively. Insect cells were routinely infected with recombinant baculovirus at a multiplicity of infection greater than 10. At 24–48 h after infection, the cells were rinsed with PBS and homogenized in TEGW buffer [20 mM Tris/HCl, 1 mM EDTA, 10% (v/v) glycerol and 20 mM sodium tungstate, pH 7.4]. Cytosol was obtained after centrifugation at 4 °C for 15 min at 15000 *g*. hMR was measured by aldosterone-binding assays as described elsewhere [12].

Metabolic labelling of hMR

Sf21 cells (2.7 × 10⁷) were infected with the recombinant baculovirus AcNPV-hMR. At 40 h after infection the cells were rinsed with methionine-free medium and incubated with 500 µCi of [³⁵S]methionine (1000 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) for 6 h at 27 °C. The cells were then rinsed twice with cold PBS and lysed by four cycles of freeze-thawing in 1.5 ml of P buffer (50 mM potassium phosphate buffer, pH 7.6, 20 mM sodium tungstate, 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride, 5 µg/ml leupeptin, 5 µg/ml antipain). The homogenates were centrifuged at 15000 *g* at 4 °C for 15 min and the supernatant was considered to be the cytosol fraction.

Immunoprecipitation

Cytosol samples containing the [³⁵S]methionine-labelled hMR were incubated overnight with antibodies at 4 °C. Protein A-Sepharose (Pharmacia LKB, Uppsala, Sweden) was added and the incubation continued for 4 h at 4 °C. After extensive washing with P buffer, the pellets were incubated with sample buffer containing SDS and boiled for 4 min before loading on to a 7.5% polyacrylamide gel. After electrophoresis, the gel was fixed with propan-2-ol/water/acetic acid (25:65:10, by vol.) for 30 min, and then treated for 15 min with Amplify (Amersham). The gel was dried and subjected to autoradiography.

Glycerol density-gradient ultracentrifugation

Sedimentation analysis was performed on a linear 15–40% glycerol density gradient in 20 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 20 mM sodium tungstate. Glucose oxidase (Boehringer, Mannheim, Germany) (*s*_{20,w} = 7.9S) and horseradish peroxidase (Sigma type VI) (*s*_{20,w} = 3.6S) were used as internal standards. Samples were centrifuged at 50000 rev./min at 4 °C in an SW60 Beckman rotor for 18 h.

Western blotting

Samples were subjected to electrophoresis on a 7.5% polyacrylamide gel. Standards consisting of Rainbow markers (Amersham) were myosin (200 kDa), phosphorylase *b* (97.4 kDa), BSA (69 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa). Gels were transferred to nitrocellulose membranes which were subsequently blocked with 10% non-fat dried milk in PBS for 1 h at room temperature. The membranes were incubated overnight at 4 °C with the appropriate dilution of purified FD4. hMR was visualized using the Vectastain ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA, U.S.A.).

Whole-cell assays

At 48 h after infection, Sf21 cells infected with AcNPV-hMR were rinsed twice with serum-free TNM-FH medium and incu-

bated with 50 nM [^3H]aldosterone or [^3H]ZK91587 for 1 h at 27 °C. The cells were then rinsed three times with cold PBS and homogenized in TEGW buffer containing 1 mM phenylmethanesulphonyl fluoride, 1 mM dithiothreitol, 5 $\mu\text{g}/\text{ml}$ antipain and 5 $\mu\text{g}/\text{ml}$ leupeptin as protease inhibitors. Cytosol fractions were obtained after 15 min centrifugation at 15000 g . The pellets were rinsed once with an equal volume of TEGW buffer and homogenized in this buffer containing 0.4 M KCl and kept for 30 min at 4 °C. After 15 min centrifugation at 15000 g , the supernatants were denoted high-salt extracts. Both cytosolic and nuclear fractions were assayed for receptor content by glycerol gradient ultracentrifugation as described above. The results are expressed as fmol of receptor/ 10^6 cells.

Immunocytochemistry

High Five insect cells infected with either the wild-type virus or AcNPV/hMR recombinant baculovirus, or mock-infected, were rinsed with PBS and fixed directly in Petri dishes using Bouin's fluid. After 7 min of *in situ* fixation, the cells were washed several times with 70% ethanol and dehydrated with increasing concentrations of ethanol (80, 90, 95, 100%). The cells were then rinsed with butan-1-ol and embedded in Paraplast directly in the Petri dish. After cooling to 4 °C, the paraffin-embedded cells were removed from the Petri dish. Tangential sections were prepared and processed for immunohistochemistry with purified FD4 antibody and the avidin-biotin-peroxidase system using the biotinylated secondary antibody at a dilution of 1:200 according to the manufacturer's (Vector Laboratories) recommendations. This procedure presented several methodological advantages over fluorescence techniques, which include permeabilizing treatment with detergent agents known to lead to artefacts such as redistribution of intracellular antigens [13]. After immunostaining, histological examination was performed by two independent observers in blind experiments, each counting about 1000 cells. The positively staining cells were counted and classified into three different categories: C for exclusive cytoplasmic staining; C + N when cytoplasmic and nuclear staining were of comparable intensity; N for unique nuclear staining. The results were tested for statistical significance using the χ^2 test.

RESULTS

Production and characterization of FD4 antibody

A peptide corresponding to Gly-412-Ser-422 of hMR has been successfully used to raise a polyclonal antibody A4 [12] which displayed interesting immunological properties. We decided to use the same immunizing peptide to generate monoclonal antibodies. From two fusions, 15 positive hybridomas producing anti-peptide antibodies were detected by e.l.i.s.a. Of the clones that stabilized in culture, one, designated FD4, was subcloned and further characterized. This monoclonal antibody is an IgG₁ with a κ light chain. Preliminary experiments showed that cellular extracts containing recombinant hMR increased the sedimentation coefficient of [^{35}S]methionine-labelled FD4 antibody. This precipitation was inhibited in the presence of an excess of peptide hMR-(412-422) (results not shown). These results provided the first evidence that FD4 interacts specifically with hMR under native conditions.

The ability of purified FD4 to interact with hMR was further determined by density-gradient analysis. Figure 1 shows that tungstate-stabilized hMR labelled with [^3H]aldosterone sedimented as a $9.6 \pm 0.4 \text{ S}$ (mean $\pm$ S.D.; $n = 5$) entity in the presence of an unrelated antibody. In contrast, incubation with FD4 resulted in a shift of [^3H]aldosterone-hMR complexes to

$11.3 \pm 0.3 \text{ S}$ (mean $\pm$ S.D.; $n = 5$), consistent with a specific recognition of the untransformed state of the receptor. As FD4 interacts with the native form of hMR, we performed immunoprecipitation experiments. Sf21 cells infected with the recombinant virus AcNPV-hMR were metabolically labelled with [^{35}S]methionine, and cellular extracts incubated with FD4 or the polyclonal anti-hMR antibody A4 were used as positive control. Figure 2 shows the cytosolic (lane 1) and total (lane 7) ^{35}S -labelled proteins from infected insect cells. A4 was able to

Figure 1 Interaction of the monoclonal antibody FD4 with tungstate-stabilized hMR

Cytosol fractions were prepared from Sf21 insect cells infected with recombinant baculovirus in TEGW buffer as described in the Materials and methods section. Cytosol samples were incubated with [^3H]aldosterone for 4 h at 4 °C. [^3H]aldosterone-hMR complexes (1.5 pmol) were then incubated for an additional 4 h with either 1 μg of control antibody (○) or FD4 (●). The samples were charcoal-treated and centrifuged on a 15–40% glycerol gradient in TEW buffer and run at 18 h at 50000 rev./min. Arrows indicate the sedimentation of standards [GO, glucose oxidase (7.9S); PO, peroxidase (3.6S)].

Figure 2 Immunoprecipitation of hMR by the monoclonal antibody FD4

Infected Sf21 cells were metabolically labelled with [^{35}S]methionine as described in the Materials and methods section. ^{35}S -labelled proteins from whole-cell lysate (lane 7) or cytosolic fraction (lane 1) were analysed on a 7.5% acrylamide gel. Portions of cytosol were incubated overnight at 4 °C with preimmune serum (lane 2), A4 antibody (lane 3), MOPC31C (a control murine IgG₁) (lane 4), 7C10 [a monoclonal anti-(rabbit hsp90)] (lane 5) or FD4 antibody (lane 6) and then incubated with Protein A-Sepharose on an end-over-end rotator for 3 h at 4 °C. After centrifugation, the pellets were washed and boiled in SDS buffer before electrophoresis. The gel was then subjected to autoradiography.

Figure 3 Western-blot analysis of recombinant hMR with FD4

Protein (10 μ g) from cytosolic fractions of uninfected insect cells (lane 1) or infected cells (lanes 2 and 3) were subjected to a SDS/PAGE (7.5% gels) and transferred to a nitrocellulose membrane. Blots were probed with purified FD4 (1:400 dilution) (lanes 1 and 2) or with the same dilution of purified FD4 preincubated with free peptide hMR-(412–422) (100 μ g/ml) (lane 3). The positions of molecular-mass markers (kDa) are indicated on the left.

Figure 4 Immunodetection of recombinant hMR in High Five insect cells with the monoclonal antibody FD4

The expression of hMR was examined by an indirect immunohistochemical technique. High Five cells were either infected with recombinant AcNPV–hMR (**a**, **b**, **c**) or mock-infected (**d**). Immunostaining was obtained after incubation with purified FD4 (dilution 1:500) (**a** and **d**) or FD4 preincubated with free peptide hMR-(412–422) (100 μ g/ml) (**b**) or with MOPC31C, an unrelated control IgG₁ (**c**). Note that in the absence of hormone (**a**), most immunoreactive hMR was localized in the cytoplasmic compartment of infected cells. (Magnification $\times$ 437.)

immunoprecipitate a unique $\sim$ 120 kDa protein corresponding to hMR (lane 3). Preimmune serum did not have any effect (lane 2). Although FD4 was an IgG₁ known to bind weakly to Protein

Figure 5 Distribution of hMR after exposure of insect cells to aldosterone or ZK91587

At 48 h after infection by AcNPV–hMR, Sf21 cells were incubated with 50 nM [³H]aldosterone (▨) or [³H]ZK91587 (■) for 1 h at 27 °C. The amount of labelled receptor recovered in both the cytosol and the nuclear fraction was measured as described in the Materials and methods section. Results, expressed as fmol/10⁶ cells, are means $\pm$ S.E.M. of five independent experiments.

A, it immunoprecipitated recombinant hMR under the experimental conditions used in our assay (lane 6). Murine antibodies MOPC31C (Sigma) and 7C10 {an anti-(rabbit hsp90) antibody [14]}, both IgG₁ molecules, were used as negative controls (lanes 4 and 5 respectively).

Using Western-blot analysis (Figure 3), purified FD4 recognized a single polypeptide band of $\sim$ 120 kDa in the cytosol fraction of infected cells (lane 2). This immunodetection was completely abolished after preincubation of FD4 with an excess of free peptide hMR-(412–422) (lane 3). Cytosol fractions of non-infected cells did not contain any immunoreactive material (lane 1), confirming the specificity of the antibody.

FD4 was also used to immunodetect hMR by means of immunocytochemistry. Indirect immunocytochemical experiments were performed in High Five cells which can be grown as monolayers that attach firmly to flasks, a useful property for *in situ* fixative procedures and inclusion. Under steroid-free conditions, purified FD4 produced intense immunostaining in the cytoplasmic compartment of the infected cells (Figure 4a). This immunostaining was extinguished after preincubation of the antibody with an excess of free peptide hMR-(412–422) (Figure 4b). No specific staining was detected in infected cells with an unrelated antibody (Figure 4c) nor in uninfected High Five cells with FD4 antibody (Figure 4d), indicating that FD4 specifically detects the recombinant hMR. It is noteworthy that the percentage of positive cells varied between 50 and 90, and the relative intensity of immunolabelling depended on the infection state of the insect cells, which is a function of the viral stock used, the time and the multiplicity of infection.

Influence of ligands on the intracellular distribution of hMR

We compared the subcellular distribution of hMR after exposure of cells to [³H]aldosterone or [³H]ZK91587, an aldosterone antagonist [15], by measuring the amount of labelled receptor recovered in both the low-salt extract referred to as the cytosol fraction, and the high-salt extract, considered to be the nuclear fraction. Figure 5 shows that the [³H]aldosterone–hMR complexes were mostly detected in the nuclear fraction, whereas after incubation with ZK91587, most of the hMR was recovered in the

Figure 6 Aldosterone-dependent nuclear localization of hMR in insect cells

High Five cells were infected with recombinant baculovirus AcNPV-hMR. At 24 h after infection, the cells were incubated without (a) or with 50 nM aldosterone for 1 h (b) or 24 h (c) and processed for immunohistochemistry as described in the Materials and methods section. In the absence of hormone, the immunoreactive hMR was mostly localized in the cytoplasm (a). After 1 h of incubation with aldosterone, immunoreactive hMR was clearly redistributed over the nuclei of infected cells (b). After 24 h of incubation, strong and almost exclusively nuclear immunostaining was observed (c). (Magnification $\times 400$.)

cytosol fraction, suggesting an impaired nuclear translocation of the receptor after antagonist binding.

The effects of aldosterone on the intracellular localization of hMR were also examined by immunohistochemistry. When compared with the hormone-free condition (Figure 6a), incubation with 50 nM aldosterone for 1 h induced clearly visible

Figure 7 Quantitative analysis of the intracellular distribution of hMR after aldosterone and SC9420 treatment

At 36 h after infection, High Five cells were incubated for 4 h without (hatched bars) or with 50 nM aldosterone (solid black bars), 1 μ M SC9420 (dotted bars) or aldosterone plus SC9420 (solid grey bars) and processed for immunohistochemistry. For each hormonal treatment, the positive cells were classified into three different categories (C, cytoplasmic; C+N, cytoplasmic and nuclear; N, nuclear) by two independent observers in blind experiments. One example of each type of immunolabelling is presented at the bottom of the Figure. (Magnification $\times 700$.) Results are expressed as a percentage of the positive cells.

nuclear staining (Figure 6b). After 24 h of incubation with the hormone (Figure 6c), nuclear staining was accompanied by a concomitant decrease in cytoplasmic labelling, indicating an aldosterone-induced nuclear localization of the recombinant hMR. Time-course studies indicated that the percentage of cells showing exclusive nuclear staining increased during the first 4 h and remained stable for the following 24 h.

The subcellular immunolocalization of hMR was examined after exposure to spironolactone (SC9420), another aldosterone antagonist [16], alone or in combination with aldosterone. The subcellular distribution of the immunolabelling was quantitatively analysed by classifying the positively staining cells into three categories: C, exclusively cytoplasmic labelling; C+N, nuclear and cytoplasmic immunostaining; N, predominantly nuclear labelling. Figure 7 details the results of this analysis and gives an example of each category of subcellular immunolabelling (C, C+N and N). In the absence of hormone, 85 % of the positive cells showed cytoplasmic labelling (C category). After 4 h treatment with 50 nM aldosterone, 50 % of the cells displayed exclusive nuclear labelling (N). In contrast, immunoreactive hMR was recovered mostly in the cytoplasmic compartment after treatment with 1 μ M SC9420. Simultaneous treatment with aldosterone and SC9420 led also to predominantly cytoplasmic labelling, with only 10 % of the cells showing nuclear staining (N).

Table 1 summarizes the results of the subcellular localization of hMR in cells treated for 4 h with 1 μ M aldosterone or three

Table 1 Influence of aldosterone and mineralocorticoid antagonists on the intracellular distribution of hMR in infected insect cells

Insect High Five cells were infected with recombinant baculovirus AcNPV-hMR. At 36 h after infection, the cells were incubated for 4 h at 27 °C in the absence or presence of 1 μ M steroid [aldosterone, antimineralocorticosteroids (SC9420, ZK91587 and 18-vinylprogesterone)] and processed for immunohistochemistry. Results are expressed as the percentage of positively staining cells in each immunolabelling category (C, cytoplasmic; C+N, cytoplasmic and nuclear; N, nuclear). * $P < 0.001$ when compared with the hormone-free condition. † $P < 0.001$ when compared with the aldosterone treatment.

	Percentage of positive cells		
	C	C+N	N
Hormone-free	85.6	13.1	1.3
Aldosterone	24.3	15.7	60.0*
SC9420	52.4	37.5	10.1*†
ZK91587	52.2	28.5	19.3*†
18-Vinylprogesterone	74.2	16.5	9.3*†

antimineralocorticosteroid compounds, SC9420, ZK91587 and 18-vinylprogesterone [17], as expressed by the percentage of cells in the C, C+N and N categories. As compared with aldosterone, the aldosterone antagonists were unable to induce similar nuclear localization of hMR (10–20% compared with 60%). These values are statistically different from those observed after exposure to aldosterone ($P < 0.001$). Kinetic studies of the subcellular distribution of hMR indicate that aldosterone was the only steroid that induced a stable nuclear localization of hMR for up to 24 h.

DISCUSSION

In the present paper, we have described the production and characterization of a new monoclonal antibody directed against a synthetic peptide derived from hMR. This peptide, GGNSKI-NSDSS, is poorly conserved among species, as the corresponding sequence of rat MR differs at 4 of the 11 residues [18]. The monoclonal antibody raised against this peptide (FD4), an IgG₁, recognized the denatured form of hMR as demonstrated by Western-blot analysis. It was also useful for the detection of recombinant hMR in immunohistochemistry. Moreover, FD4 interacted with the native form of the receptor, a property that allows it to immunoprecipitate hMR whether free or bound to the ligand. By gel mobility-shift assay, we have previously shown that hMR binds specifically to oligonucleotides containing glucocorticoid response elements. Preincubation of hMR with FD4 promoted a supershift of hMR–DNA complexes, indicating that this monoclonal antibody also recognized the DNA-bound state of the receptor [19]. Together, our results demonstrate that FD4 recognizes both the denatured and native forms of the receptor without affecting its ligand- and DNA-binding properties, suggesting that the epitope located in the N-terminal region of hMR is not in the close vicinity of heat-shock-protein-binding sites and zinc fingers. The recognition by FD4 of the native form of hMR constitutes an additional advantage over existing polyclonal antibodies, namely MinRec2 directed against peptide A672–E687 of hMR [7], hMRsN [9] directed against D87–E96, and MinRec4 [8] raised against a fusion protein encompassing G352–S519 of hMR. Besides FD4, H10E, a monoclonal anti-idiotypic antibody, interacts with the ligand-binding domain of MR [2] and has been used to immunolocalize this receptor in the kidney and cardiovascular system [3–5]. However, the usefulness of this antibody

is somewhat limited, as the integrity of the steroid-binding domain of the MR is absolutely required for adequate immunoreactivity. Thus FD4 was the most suitable tool to use for examination of the subcellular distribution of hMR.

MR belongs to the superfamily of so-called 'nuclear receptors', although its intracellular localization and regulation by aldosterone agonists and antagonists remain poorly characterized. In the absence of hormone, indirect immunodetection with FD4 showed that the recombinant hMR is localized, almost exclusively, in the cytoplasmic compartment of infected insect cells, whereas some immunoreactive hMR could be detected in the nucleus. Our results confirmed the data obtained by both immunofluorescence techniques [9,12] and confocal laser scanning microscopy [20] in the baculovirus overexpression system. Moreover, the aldosterone-induced nuclear translocation of the endogenous receptor has also been demonstrated in mouse macrophage cells by immunocytochemistry [20].

In contrast with oestrogen and progesterone receptors located in the nucleus [21,22], the cytoplasmic distribution of unliganded MR resembles that reported for GR by most authors [13,23–26]. The presence of unliganded GR in the nucleus has recently been described [27–29]. However, it has been argued that the intracellular localization of GR is largely dependent on the experimental conditions (fixative procedures, presence of detergent, tumoral or overexpressing cellular models) [27]. Indeed, these apparently discrepant observations may reflect the nuclear–cytoplasmic shuttling of GR [30]. The recycling mechanism by which the receptor passively diffuses into the cytoplasm and is actively translocated back to the nucleus may in fact be a general phenomenon in all experimental models for steroid receptors [30,31].

We have examined whether aldosterone or its antagonists modify the nucleocytoplasmic distribution of hMR. We showed that aldosterone-bound receptor was predominantly localized in the nucleus, suggesting that the hormone might de-repress the activity of putative nuclear localization signals. As far as GR is concerned, two hormone-dependent nuclear localization signals have been described, one (NL1) located in the hinge region of receptors next to the C-terminal site of the DNA-binding domain and the other (NL2) included in the ligand-binding domain [24,26]. NL1, defined as a 28-amino acid fragment, contains the nuclear localization signal of the Simian Virus 40 large T-antigen [32]. Such signals remain to be identified for MR but, by sequence alignment with other steroid receptors, the segment 660–688 of hMR may correspond to the NL1 motif.

In contrast with aldosterone, antimineralocorticosteroids SC9420, 18-vinylprogesterone and ZK91587 did not promote nuclear accumulation of hMR. These results are in agreement with previous biochemical studies which reported that, unlike aldosterone–receptor complexes, receptors labelled with spiro-lactones (SC26304 or prorenone) were not recovered in the nucleus after subcellular fractionation [33,34]. These latter observations have been interpreted to reflect an absence of nuclear translocation of MR and/or by a low affinity of antagonist–receptor complexes for nuclear acceptors [35]. In the light of our data, we propose that antagonist–receptor complexes are translocated into the nucleus but aldosterone antagonists are unable to keep the receptor in the nuclear compartment. The alteration of receptor recycling kinetics is likely, as we were able to observe a weak but significant nuclear staining after treatment with antagonists, a result consistent with the detection of some specific nuclear binding of spironolactone by autoradiography [36].

Our results demonstrate that the intracellular distribution of hMR is differentially regulated by aldosterone and antimineralocorticosteroids. Distinct changes in the receptor con-

formation and/or in its phosphorylation state, for instance, induced by these ligands could be responsible for the modification of the nuclear/cytoplasmic distribution of the receptor. Anti-mineralocorticosteroids have been shown to destabilize the hetero-oligomeric structure of MR, dissociating very rapidly from the receptor and facilitating the release of the associated protein, hsp90 [37,38]. We propose that the mechanism of action of antimineralocorticosteroids involves rapid dissociation kinetics, making stable ligand-receptor complexes unsustainable, and the absence of nuclear retention, both properties co-operating to prevent productive interaction of the receptor with specific DNA sequences in mammalian aldosterone target cells.

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REFERENCES

- Johnson, J. P. (1992) *Pharmacol. Ther.* **53**, 1–29
- Lombès, M., Edelman, I. S. and Erlanger, B. F. (1989) *J. Biol. Chem.* **264**, 2528–2536
- Lombès, M., Farman, N., Oblin, M. E., Baulieu, E. E., Bonvalet, J. P., Erlanger, B. F. and Gasc, J. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1086–1088
- Farman, N., Oblin, M. E., Lombès, M., Delahaye, F., Westphal, H. M., Bonvalet, J. P. and Gasc, J. M. (1991) *Am. J. Physiol.* **260**, C226–C233
- Lombès, M., Oblin, M. E., Gasc, J. M., Baulieu, E. E., Farman, N. and Bonvalet, J. P. (1992) *Circ. Res.* **71**, 503–510
- Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Houseman, D. E. and Evans, R. M. (1987) *Science* **237**, 268–275
- Krozowski, Z. S., Rundle, S. E., Wallace, C., Castell, M. J., Shen, J. H., Dowling, J., Funder, J. W. and Smith, A. I. (1989) *Endocrinology* **125**, 192–198
- Fukushima, K., Sasano, H., Nagura, H., Sasaki, I., Matsuno, S. and Krozowski, Z. S. (1991) *Tohoku J. Exp. Med.* **165**, 155–163
- Alnemri, E. S., Maksymowych, A. B., Robertson, N. M. and Litwack, G. (1991) *J. Biol. Chem.* **266**, 18072–18081
- Walter, G. (1986) *J. Immunol. Methods* **88**, 149–161
- Avrameas, S. (1969) *Immunochemistry* **6**, 43–52
- Binart, N., Lombès, M., Rafestín-Oblin, M. E. and Baulieu, E. E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10681–10685
- Wikström, A., Bakke, O., Okret, S., Brönnegård, M. and Gustafsson, J. A. (1987) *Endocrinology* **120**, 1232–1242
- Radanyi, C., Lombès, M., Renoir, J. M., Delahaye, F. and Baulieu, E. E. (1992) *J. Steroid Biochem. Mol. Biol.* **42**, 863–874
- Losert, W., Bittler, D., Buse, M., Casals-Stenzel, J., Haberey, M., Laurent, H., Nickish, K., Schillinger, E. and Wiechert, R. (1986) *Drug Res.* **36**, 1583–1600
- Kagawa, C. M. (1960) *Endocrinology* **67**, 125–132
- Rafestín-Oblin, M. E., Couette, B., Barlet-Bas, C., Cheval, L., Viger, A. and Doucet, A. (1991) *Am. J. Physiol.* **260**, F828–F832
- Patel, P. D., Sharma, T. G., Goldman, D. J. and Watson, S. J. (1989) *Mol. Endocrinol.* **3**, 1877–1885
- Lombès, M., Binart, N., Oblin, M. E., Joulin, V. and Baulieu, E. E. (1993) *Biochem. J.* **292**, 577–583
- Robertson, N. M., Schulman, G., Karnik, S., Alnemri, E. and Litwack, G. (1993) *Mol. Endocrinol.* **7**, 1226–1239
- King, W. J. and Greene, G. L. (1984) *Nature (London)* **307**, 745–747
- Gasc, J. M., Renoir, J. M., Radanyi, C., Joab, I., Tuohimaa, P. and Baulieu, E. E. (1984) *J. Cell Biol.* **99**, 1193–1201
- Gustafsson, J. A. (1987) *Endocrinol. Rev.* **8**, 185–234
- Picard, D. and Yamamoto, K. R. (1987) *EMBO J.* **6**, 3333–3340
- Cidlowski, J. A., Bellingham, D. L., Powell-Oliver, F. E., Lubahn, D. B. and Sar, M. (1990) *Mol. Endocrinol.* **4**, 1427–1437
- Cadepond, F., Gasc, J. M., Delahaye, F., Jibard, N., Schweizer-Groyer, G., Segard-Maurel, I., Evans, R. and Baulieu, E. E. (1992) *Exp. Cell Res.* **201**, 99–108
- Gasc, J. M., Delahaye, F. and Baulieu, E. E. (1989) *Exp. Cell Res.* **181**, 492–504
- Martins, V. R., Pratt, W. B., Terracio, L., Hirst, M. A., Ringold, G. M. and Housley, P. R. (1991) *Mol. Endocrinol.* **5**, 217–225
- Brink, M., Humbel, B. M., DeKloet, E. R. and Van Driel, R. (1992) *Endocrinology* **130**, 3575–3581
- DeFranco, D. B., Qi, M., Borror, K. C., Garabedian, M. J. and Brautigan, D. L. (1991) *Mol. Endocrinol.* **5**, 1215–1228
- Guiochon-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat, M. and Milgrom, E. (1991) *EMBO J.* **10**, 3851–3859
- Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M. and Milgrom, E. (1989) *Cell* **57**, 1147–1154
- Marver, D., Stewart, J., Funder, J. W., Feldman, D. and Edelman, I. S. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1431–1435
- Claire, M., Oblin, M. E., Michaud, A. and Corvol, P. (1979) *Endocrinology* **104**, 1194–1200
- Corvol, P., Claire, M., Oblin, M. E., Geering, K. and Rossier, B. (1981) *Kidney Int.* **20**, 1–6
- Bonvalet, J. P., Blot-Chabaud, M. and Farman, N. (1991) *Endocrinology* **128**, 280–284
- Rafestín-Oblin, M. E., Lombès, M., Couette, B. and Baulieu, E. E. (1992) *J. Steroid Biochem. Mol. Biol.* **41**, 815–821
- Couette, B., Lombès, M., Baulieu, E. E. and Rafestín-Oblin, M. E. (1992) *Biochem. J.* **282**, 697–702