Involvement of the N-terminal region of the human mineralocorticoid receptor hormone-binding domain in agonist and antagonist binding as revealed by a new monoclonal antibody

Stéphan JALAGUIER[‡], Brigitte LUPU^{*‡§}, Gérald HUGON^{*}, Marie-Edith RAFESTIN-OBLIN[†] and Gilles AUZOU^{*}

^{*}INSERM U300, Faculté de Pharmacie Bat. K, 15 Avenue Charles Flahault, 34060 Montpellier Cedex 2, France, and [†]INSERM U246, Faculté de Médecine Xavier Bichat, 16 Rue Henri Huchard, BP 416, 75870 Paris Cedex 18, France

To gain a better understanding of the mechanism of binding to the human mineralocorticoid receptor (hMR), we developed a new monoclonal antibody (mAb) raised against the hormone-binding domain (HBD). For this purpose, mice were immunized with a fusion protein including the sequence Thr⁷²⁹–Lys⁹⁸⁴ of hMR. After ELISA screening, mAb 18C7 was selected for its specificity towards the HBD. This antibody recognized both the denatured and native MR forms, as well as the hetero-oligomeric MR form and the transformed MR state. By using several HBD subfragments, the mAb 18C7 epitope was located in the N-terminal region of the HBD from Thr⁷²⁹ to Leu⁷⁶⁵. We then studied the effect of the antibody on aldosterone and progesterone binding to the hMR. When 18C7 was incubated with liganded MR, it was able to partly displace (20%) the hormone from its binding site. When 18C7 was incubated with MR before aldosterone or progesterone, the antibody inhibited 75–80% of the

binding. The effect of 18C7 on the binding was similar with both hormones. A sucrose gradient analysis indicated the simultaneous presence of two kinds of receptor complexes: the steroid–MR complex and the antibody–MR complex. After its associated proteins, especially the heat-shock protein hsp90, had been cross-linked with the hMR by dimethylpimelimidate, 18C7 was still able to react with the receptor. Our results indicated that the epitope recognized by 18C7 was directly implicated in hormone binding. The lack of steroid binding of HBD mutants with the Thr⁷²⁹–Leu⁷⁶⁵ sequence deleted [Jalaguier, Mesnier, Léger and Auzou (1996) *J. Steroid Biochem. Mol. Biol.* **57**, 43–50] supports this hypothesis. Because of the similar behaviours of aldosterone and progesterone, we conclude that the N-terminal Thr⁷²⁹–Leu⁷⁶⁵ region of the HBD is similarly involved in the binding of both hormones.

INTRODUCTION

The human mineralocorticoid receptor (hMR) is a member of the steroid hormone receptor family [1]. The hMR, as part of this family [2,3], has been characterized at the molecular level by the presence of functional domains related to ligand binding, DNA binding and transcriptional regulation. Owing to its long N-terminal region, the hMR is larger than other steroid receptors with its 984-residue sequence. It is most closely related to the glucocorticoid receptor (GR): their DNA-binding domains share approximately 94% similarity, whereas their C-terminal hormone-binding domains (HBDs) show 57% similarity. In addition to providing structural features for the hormone-binding site, the 250-residue C-terminus is assumed to be responsible for multiple functions of this receptor because it harbours a ligand-dependent transcriptional activation factor and a hormone-dependent nuclear localization signal. This domain is also involved in the receptor dimerization and the binding of heat-shock proteins (hsps), and recently it has been shown to bind actin [4].

As for the other members of the family, unliganded hMR is a soluble hetero-oligomeric complex that includes hsp90 [5,6] and probably other receptor-associated proteins (hsp70 and hsp56)

(reviewed in [7,8]). The presence of these associated proteins is necessary for binding the physiological hMR ligand, the steroid hormone aldosterone [6,9], as well as the natural antagonist progesterone and synthetic anti-mineralocorticoid spirolactones. After binding the ligand, a conformational change occurs in the C-terminal part of the hMR, and it has been demonstrated that agonists and antagonists induce different hMR conformations [10,11]. Then dissociation of hsp90 occurs, a process known as activation or transformation, which provides the DNA-binding form of hMR.

MR antibodies were mainly produced for the purpose of localizing the receptor and were raised against preselected peptide sequences located on the N-terminal region [12–14] or derived from the hinge region [15]. Only one monoclonal antibody (mAb H10E) recognized the ligand-binding site of hMR [16], but because H10E was elicited as an anti-idiotypic antibody against aldosterone, its usefulness has been somewhat limited.

New probes were required for functional studies of the structurally complex HBD. We thus developed a new mAb (18C7) to that domain by immunizing mice with the glutathione S-transferase (GST)–HBD fusion protein, which links GST with the 255-residue C-terminus of hMR from Thr⁷²⁹ to Lys⁹⁸⁴ [9]. The 18C7 epitope, located in the N-terminal part of the HBD,

Abbreviations used: DMP, dimethylpimelimidate; GST, glutathione S-transferase; GR, glucocorticoid receptor; HBD, hormone-binding domain; hMR, human mineralocorticoid receptor; hsp, heat-shock protein; mAb, monoclonal antibody; MBP, maltose-binding protein; MR, mineralocorticoid receptor.

[‡] Considered jointly as first authors.

[§] To whom correspondence should be addressed.

was demonstrated to be a common region involved in the binding of aldosterone (mineralocorticoid agonist) and progesterone (mineralocorticoid antagonist).

MATERIALS AND METHODS

Chemicals

D-[1,2-³H(N)]Aldosterone (52 Ci/mmol) was purchased from DuPont–New England Nuclear (Les Ulis, France). [1,2,6,7-³H]Progesterone (92 Ci/mmol), L-[³⁵S]methionine (more than 1000 Ci/mmol) and [¹⁴C]methylated protein molecular mass markers (10–100 µCi/mg) were obtained from Amersham (Les Ulis, France). Unlabelled aldosterone and progesterone were from Sigma (Saint Quentin Fallavier, France). DEAE-cellulose DE52 was from Whatman (Clifton, NJ, U.S.A.). The TNT T7 Coupled Reticulocyte Lysate System was from Promega (Charbonnières, France). Dimethylpimelimidate (DMP) was from Sigma. Amplify was purchased from Amersham. All other chemicals were of reagent grade and obtained from commercial sources.

Plasmids and vectors

The pHMR3750 plasmid, containing the full-length hMR-coding sequence under control of the T7 promoter, and the pRShMR plasmid, containing the hMR coding sequence under control of the Rous sarcoma virus promoter, were gifts from Dr. R. M. Evans and Dr. J. L. Arriza (The Salk Institute, La Jolla, CA, U.S.A.) [1].

pIH902, a vector of the pMAL-2 series, including the *malE* gene, which encodes the maltose-binding protein (MBP), was purchased from New England Biolabs (Ozyme, Montigny-le Bretonneux, France).

Buffers

TEG buffer contained 20 mM Tris/HCl, 1 mM EDTA and 10% (v/v) glycerol. TEGW and TEGMo buffers were TEG containing 20 mM sodium tungstate or 20 mM sodium molybdate respectively. TEGK buffer was TEG supplemented with 0.4 M KCl. All buffers were adjusted to pH 7.4 at 25 °C.

Antibody generation

The mouse mAb 18C7 was generated by injecting mice with a total bacterial inclusion body preparation of the fusion protein GST–HBD[T729–K984] [9]. Primary screening was performed by ELISA with bacterial recombinant GST–HBD, MBP–HBD [9], GST and MBP used as antigens. One hybridoma was immunoreactive against both GST–HBD and MBP–HBD, but not against the unfused GST and MBP. It was clonally purified by limiting dilution. The resulting mAb (18C7) was subtyped by ELISA by using a mouse-hybridoma subtyping kit (Boehringer Mannheim, Meylan, France): 18C7 was an IgG1 with a κ light chain. After ascite production, 18C7 was purified at 2 mg/ml concentration by chromatography on a DEAE-cellulose (DE52) column.

Construction and expression of receptor fragments

Several HBD fragments were produced as fusion proteins with MBP by cloning inserts into the pIH902 vector in accordance with a previously described protocol [9].

Fragments HBD[L766–K984] and HBD[T729–E940] were as previously described [9].

Fragments HBD[T729–G915] and HBD[L766–G915] were obtained by PCR amplification of the parental HBD cDNA with the following primers, thus introducing the *Bam*HI (5' site) and *Hind*III (3' site) cloning sites: 5'-CCCGGATCCACAATCTCAGAGCGCTCACA-3' (forward primer for [T729–G915] with a *Bam*HI site underlined), 5'-CCCGGATCCCTCTCCACGCTCAACCGCTTA-3' (forward primer for [L766–G915] with a *Bam*HI site underlined) and 5'-CCCAAGCTTTACCCAGAATTGTTGGGACACTTAG-3' (reverse primer for both fragments with a *Hind*III site underlined).

After digestion with *Bam*HI and *Hind*III, the PCR product was inserted into the previously digested pIH902.

Fragments HBD[T729–L862] and HBD[L862–K984] were obtained by cleavage of the parental HBD cDNA (digestion with *Bam*HI–*Pst*I and *Hind*III–*Pst*I respectively). Both fragments were inserted into pIH902, previously digested with *Bam*HI or *Hind*III respectively, followed by a blunting step with Klenow fragment.

After electrotransformation of *Escherichia coli* TB1 strains with the corresponding plasmid, the recombinant proteins were expressed as previously described [9].

The binding of aldosterone and progesterone was assayed with the HBD fragments by the protocol previously described [9].

hMR transcription and translation *in vitro*

Full hMR was expressed *in vitro* directly in rabbit reticulocyte lysate: pHMR3750, containing the full-length hMR-coding sequence under control of the T7 promoter, was transcribed with T7 RNA polymerase and simultaneously translated in rabbit reticulocyte lysate for 1 h at 30 °C in accordance with the protocol recommended by Promega. When specified, MR was labelled by incorporation of L-[³⁵S]methionine.

Covalent cross-linking

The cross-linking conditions were as described by Rafestin-Oblin et al. [5]. The hMR expressed in the rabbit reticulocyte lysate system was diluted (1:1, v/v) in cold TEGMo. To 100 µl aliquots of MR was added one-tenth vol. of 2.2 M triethanolamine (pH 8.8) to adjust the pH and then one-fifth vol. of 0.1 M DMP freshly prepared in 0.2 M triethanolamine (pH 8.8). The reaction mixture was incubated for 30 min at 10 °C. The reaction was stopped by the addition of 0.2 M triethanolamine (pH 8.8) to block any remaining unreacted imidate groups.

Ligand binding assays

Ligand binding assays were performed as previously described [11]. Briefly, the crude translation medium was diluted with 1 vol. of ice-cold TEGW buffer. Aliquots (25 µl) of this medium were incubated for 4 h at 4 °C with the tritiated ligand at a saturating concentration (10 nM [³H]aldosterone or 10 nM [³H]progesterone). mAb 18C7 was added as described and incubated at 4 °C for 4 h. Non-specific binding was determined by the addition of 1 µM unlabelled steroid. Bound steroid was separated from free steroid by using the dextran-charcoal treatment described previously [5]. After the addition of 3 ml of Ultima Gold solution (Packard, Rungis, France), radioactivity was measured in a Beckman liquid-scintillation counter.

Sucrose density gradient experiments

Sedimentation analysis was performed on a linear 5–25% (w/v) sucrose gradient prepared in TEG or TEGK buffer, with or

without 20 mM tungstate or molybdate ions depending on the experiment. Gradients were centrifuged at 180000 *g* in a SW41 rotor (Beckman, France) for 15 h at 4 °C. After the bottom of each tube had been pierced, three-drop fractions were collected and radioactivity was counted. Catalase (C, 11.3 S), aldolase (A, 7.9 S) and BSA (B, 4.6 S) were used as external sedimentation markers.

Transfection of COS-7 cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (w/v) charcoal-treated calf serum, 5 mM Hepes (pH 7.4), 2 mM glutamine, 100 i.u./ml penicillin and 100 µg/ml streptomycin. The cells were plated and transfected on 10 µg/ml glass coverslips pretreated with poly(L-lysine) (100000 cells per coverslip). COS-7 cells were transfected with 5 µg of the expression plasmid pRShMR and 20 µg of n-[1(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate (Boehringer Mannheim, Meylan, France) in accordance with the manufacturer's instructions. After 24 h of incubation with DNA, the cells were treated for 4 h with the indicated hormones at 100 nM final concentration. Cells were washed three times with cold PBS, fixed for 15 min in 4 % (w/v) paraformaldehyde in PBS, rehydrated by three washes with PBS, and permeabilized with 1 % (v/v) Triton X-100 in PBS for 1 min. After additional washes with PBS, free aldehydes were reduced with 0.05 % NaBH₄ in PBS for 30 min at 4 °C [17], subsequently washed with PBS, and incubated with 2 % (v/v) calf serum for 30 min at 37 °C.

Immunohistochemistry

Sections (10 µm) of frozen rabbit kidney were cut in a cryostat at -20 °C. Indirect immunofluorescence was performed as described below.

Immunofluorescence

Treated COS cells or kidney sections were incubated for 1 h at 37 °C with mAb 18C7 as primary antibody [mAb 18C7 at 1:100 dilution in PBS supplemented with 0.05 % Tween-20 and 1 % (w/v) BSA]. Bound antibody was detected by staining with a fluorescein-conjugated affinity-purified goat anti-(mouse IgG) (Sigma) diluted 1:200 in PBS supplemented with 0.05 % Tween-20 and 1 % (w/v) BSA, and incubated for 30 min at 37 °C. A Nikon Optiphot-2 microscope was used for epifluorescence microscopy and microphotography.

Immunoblot analysis

Discontinuous SDS/PAGE [10 % (w/v) gel] was performed as described by Laemmli [18]. After electrophoresis, proteins were transferred to nitrocellulose filters (Schleicher and Schuell, Ecquevilly, France) in a Bio-Rad Trans Blot apparatus (Bio-Rad Laboratories, Ivry-sur-Seine, France) at 50 V for 2 h in 20 % (v/v) methanol/192 mM glycine/0.1 % SDS/25 mM Tris (pH 8.3). After transfer, the filters were blocked with 3 % (w/v) non-fat dried milk in Tris-buffered saline (TBS) supplemented with 0.05 % Tween-20. Immunoblots were probed with mAb 18C7 (1:1000 in TBS supplemented with 0.05 % Tween-20 and 3 % non-fat dried milk). Immune complexes were revealed with an alkaline phosphatase-conjugated affinity-pure rabbit anti-(mouse IgG) (Jackson, Interchim, Montluçon, France) diluted 1:5000 in TBS supplemented with 0.05 % Tween-20.

Autoradiography

After electrophoresis of the L-[³⁵S]methionine-labelled samples by discontinuous SDS/PAGE (10 % gel), the gels were fixed in 7 % (v/v) acetic acid for 30 min. Signals were enhanced by fluorography: the gels were soaked in Amplify for 30 min then rinsed briefly in water and dried down. They were exposed to autoradiography on Hyperfilm-MP (Amersham) at -70 °C.

RESULTS AND DISCUSSION

Anti-HBD antibody production and characterization

To direct the immunogenic response towards the hMR HBD, we immunized mice with the recombinant fusion protein GST-HBD, in which GST is linked to the 255 residues of HBD (Thr⁷²⁹-Lys⁹⁸⁴), which was previously prepared in our laboratory [9]. Among the several hybridomas producing antibodies detected by ELISA, one hybridoma (mAb 18C7) reacted with the two fusion proteins GST-HBD and MBP-HBD, but not with GST and MBP, thus indicating that 18C7 is specific to the HBD.

The interaction of mAb 18C7 with denatured HBD was examined by immunoblot analysis. mAb 18C7 recognized both the GST-HBD (55 kDa; Figure 1, lane 2) and MBP-HBD (70 kDa; Figure 1, lane 4), but no bands were observed with GST or with MBP (Figure 1, lanes 3 and 5 respectively). Some additional bands corresponding to unidentified degradation products were observed. Because the high similarity in the HBD sequences of both hMR and human GR (hGR) was responsible for strong cross-reactivities between the two receptors, we tested the antibody reactivity towards hGR. Immunoblot analysis provided evidence of 18C7 cross-reactivity with hGR (results not shown).

Several HBD fragments were produced (see the Materials and methods section) to fully localize the epitope recognized by 18C7. These fragments were HBD mutants deleted at the N-terminal end, at the C-terminal end or at both ends. In agreement with the previously published results by Jalaguier et al. [9], none of these fragments was able to bind aldosterone or progesterone (results not shown). By immunoblot analysis, mAb 18C7 recognized only the fragments including the N-terminal region of

Figure 1 Immunoblot analysis of recombinant MR fragments

MR fragments were expressed as fusion proteins with GST or MBP, then subjected to SDS/PAGE (10 % gel) and transferred to nitrocellulose. Immunodetection was performed with 18C7 as described in the Materials and methods section. Lane 2, GST-HBD; lane 4, MBP-HBD. Lanes 3 and 5 correspond respectively to GST and MBP deprived of any fused protein. Lanes 6–11, recombinant HBD fragments fused to MBP: lane 6, Leu⁷⁶⁶-Lys⁹⁸⁴; lane 7, Thr⁷²⁹-Glu⁹⁴⁰; lane 8, Thr⁷²⁹-Gly⁹¹⁵; lane 9, Leu⁷⁶⁶-Gly⁹¹⁵; lane 10, Thr⁷²⁹-Leu⁸⁶²; lane 11, Leu⁸⁶²-Lys⁹⁸⁴. The molecular masses (in kDa) of the protein markers in lane 1 are indicated at the left.

Figure 2 Immunodetection of MR in rabbit kidney cortex by 18C7

Sections were immunostained with mAb 18C7 as described in the Materials and methods section. In kidney cortex immunostaining was observed along the collecting tubules (CT) (A), with no staining in the glomerulus (GL) (B). Magnification $\times 40$.

the HBD, e.g. fragments Thr⁷²⁹–Glu⁹⁴⁰ (Figure 1, lane 7), Thr⁷²⁹–Gly⁹¹⁵ (Figure 1, lane 8) and Thr⁷²⁹–Leu⁸⁶² (Figure 1, lane 10). The antibody did not react with fragments including the C-

terminal region of the HBD, e.g. Leu⁷⁶⁶–Lys⁹⁸⁴ (Figure 1, lane 6) and Leu⁸⁶²–Lys⁹⁸⁴ (Figure 1, lane 11). It also did not recognize Leu⁷⁶⁶–Gly⁹¹⁵ (Figure 1, lane 9), which includes the central region of the HBD. These results highlighted the epitope recognized by mAb 18C7 along the hMR: it encompasses the N-terminal region of the HBD from Thr⁷²⁹ to Leu⁷⁶⁵.

With an immunohistochemical technique, mAb 18C7 revealed immunoreactive structures in sections of rabbit kidney cortex. As shown in Figure 2(A), hMR was detected in the collecting ducts but no immunostaining was observed in the proximal tubules or in the glomerulus (Figure 2B). The immunostaining distribution observed with 18C7 was in close agreement with that revealed by other anti-MR antibodies [13,19]. Similar immunostaining was also obtained with rat kidney sections (results not shown). The ability of 18C7, an anti-human MR, to interact with both rabbit and rat MR demonstrated its interspecies cross-reactivity.

We further examined the labelling of hMR by 18C7 in transiently transfected COS-7 cells. In the absence of ligand the receptor was strongly detected in the cytoplasm (Figure 3B). Under the conditions of our experiment, after 4 h of incubation of the cells with aldosterone, hMR was revealed by 18C7 in the nucleus only, where the receptor is supposed to be in its transformed DNA-bound state (Figure 3C). A similar effect was observed after 4 h of incubation with the antagonist progesterone (Figure 3D). Under these experimental conditions, 18C7 confirmed that hMR is functionally involved in nuclear translocation of the mineralocorticoid agonist (aldosterone) and antagonist (progesterone). Our results are in total agreement with those of Robertson et al. [13], even though Lombès et al. [14] established that the intracellular distribution of hMR expressed in High Five cells was differentially regulated by aldosterone and anti-mineralocorticoids.

Influence of mAb 18C7 on steroid binding

It was previously demonstrated that the hMR expressed *in vitro* in reticulocyte lysate had steroid-binding properties identical with those of the native receptor [11,20]. The effect of mAb 18C7 on steroid binding was examined by using the recombinant full

Figure 3 Immunocytochemical localization of hMR transfected into COS-7 cells

pRShMR-transfected COS-7 cells were incubated for 4 h in the absence (B) or presence of 100 nM aldosterone (C) or 100 nM progesterone (D). Cells were processed for immunofluorescence by using 18C7 antibody as described in the Materials and methods section. Untreated cells (A) are shown as controls. Magnification $\times 200$.

Figure 4 Effect of mAb 18C7 on ligand binding to MR

[³H]Aldosterone–MR complexes were incubated for 4 h at 4 °C with 10 μ l of serial dilutions of 18C7 in a final volume of 100 μ l (●). hMR was incubated for 4 h at 4 °C with 10 μ l of serial dilutions of 18C7, followed by a second incubation with 10 nM [³H]aldosterone (□) or [³H]progesterone (▲) for 4 h at 4 °C. Non-specific binding was measured in the presence of 1 μ M unlabelled steroid. Bound and free steroid were separated by the dextran-charcoal technique. Results are means of four separate experiments and expressed as a percentage of specific [³H]hormone binding measured in the absence of antibody.

hMR expressed in a reticulocyte lysate coupled transcription–translation system. In our experiments two incubation conditions were examined: the steroid (agonist or antagonist) was incubated with hMR either before or after incubation with 18C7.

hMR was first incubated with 10 nM [³H]aldosterone for 4 h at 4 °C and the aldosterone–hMR complex was subsequently incubated for 4 h at 4 °C with increasing amounts of mAb 18C7. The antibody was able to displace [³H]aldosterone binding in a dose-dependent manner (Figure 4). At 1 μ M 18C7, 20% of the bound radioactivity was displaced from the complex. A similar result was obtained when both hormone and antibody were incubated simultaneously with the receptor (results not shown).

When hMR was incubated with increasing concentrations of 18C7 before incubation with [³H]aldosterone, the antibody inhibited aldosterone binding. The percentage inhibition of bound radioactivity was dependent on the antibody concentration. In these conditions, 1 μ M 18C7 was able to prevent [³H]aldosterone binding with 82% efficiency of inhibition (Figure 4).

A similar effect of 18C7 was observed on progesterone binding. When hMR was first incubated with 18C7 and then with 10 nM [³H]progesterone, steroid binding was inhibited by the antibody in a dose-dependent manner. Figure 4 shows that a maximum inhibitory effect of 75% was induced by 1 μ M 18C7.

These results indicate that when incubated with hMR, mAb 18C7 was able to block the access of both aldosterone and progesterone to their binding site in a similar fashion.

Sucrose density gradient analysis

The ability of mAb 18C7 to interact with native hMR was further determined by sucrose density gradient analysis in the presence of sodium tungstate, which is known to stabilize the hetero-oligomeric MR form [5]. Figure 5 shows that the tungstate-stabilized hMR labelled with 10 nM [³H]aldosterone sedimented as a 10.5 S entity. When the [³H]aldosterone–MR complex was incubated with various amounts of mAb 18C7, no shift was observed and, under these conditions, no marked

Figure 5 mAb 18C7 does not co-sediment with the [³H]aldosterone–MR complex

[³H]Aldosterone–MR complexes (100 μ l) recovered in TEGW buffer were incubated for 4 h at 4 °C with 24 μ l of undiluted control ascites (○) or with 6 μ l (▲) or 24 μ l (●) of 18C7. After dextran-charcoal treatment, a 100 μ l aliquot was layered on top of a 5–25% (w/v) sucrose gradient prepared in TEGW buffer. Gradients were centrifuged for 15 h at 180 000 *g* in a SW41 rotor at 4 °C. Sedimentation markers are indicated: aldolase (a, 7.9 S) and BSA (b, 4.6 S).

decrease in the bound radioactivity could be detected whatever the amount of 18C7. However, when the incubation conditions were changed (18C7 was first incubated with hMR and then with [³H]aldosterone), a slight decrease (30%) in bound radioactivity was observed (results not shown). These results indicate that the antibody did not co-sediment with the [³H]aldosterone–MR complex. They also suggest that 18C7 was partly able to block the access of aldosterone to its binding site.

In the above experiments, the liganded hMR was detected by using [³H]aldosterone as tracer. To favour the concomitant detection of both the unliganded and liganded forms of the receptor, hMR was metabolically labelled with [³⁵S]methionine during its expression in the lysate system. The gradient profiles shown in Figure 6(A) indicate that the unliganded [³⁵S]hMR sedimented at 10.5 S. After incubation with 18C7, the peak was shifted at 11.5 S, demonstrating that 18C7 co-sedimented with native unliganded hMR. Figure 6(A) also shows that the sedimentation profile of the aldosterone-liganded [³⁵S]hMR matched that of the unliganded hMR with a sedimentation coefficient of 10.5 S.

Sedimentation of [³⁵S]hMR in the presence of both aldosterone and 18C7 was then evaluated by incubating the antibody first and then the steroid. In this case, two overlapping peaks were

Figure 6 mAb 18C7 co-sediments with unliganded MR

[³⁵S]hMR, synthesized *in vitro*, was incubated in the absence of ligand (A, ○), in the presence of 10 nM unlabelled aldosterone (A, ●; B, ●) or in the presence of 20 μl of 18C7 (A, ▲; B, ▲). [³⁵S]hMR (B, □) was incubated first with 20 μl of 18C7 and then with 10 nM aldosterone. All incubations were conducted at 4 °C for 4 h. A 100 μl aliquot was layered on top of a 5–25% (w/v) sucrose gradient prepared in TEGW buffer. Gradients were centrifuged for 15 h at 180 000 *g* in a SW41 rotor at 4 °C. Sedimentation markers are indicated: catalase (c, 11.3 S) and aldolase (a, 7.9 S).

observed at approx. 11.5 S and 10.5 S (Figure 6B), indicating the presence of two simultaneous complexes.

In the presence of progesterone, 18C7 had a similar effect on hMR: a large shoulder encompassing both forms of the complexed receptor was observed at approx. 11 S (results not shown).

These overall results indicate that 18C7 was able to compete for the aldosterone-binding site, as well as for that of progesterone.

Covalent cross-linking

As the association of hsp90 with hMR is required for a competent steroid-binding site [6,9], it was necessary to determine whether 18C7 acted directly on the steroid-binding site or through hsp binding. hMR and its associated proteins were thus covalently linked and the effect of 18C7 on steroid binding was examined by gradient analysis.

We covalently cross-linked the receptor-associated proteins on [³⁵S]hMR with DMP, an irreversible cross-linking reagent that was demonstrated to promote covalent binding between the chick MR and hsp90 without altering aldosterone binding [5]. To verify the efficiency of coupling in our assays, a parallel experiment was conducted with the hsp-deprived transformed hMR state. The gradient analyses were performed in the presence of sodium molybdate, which allows hMR transformation at high salt concentrations (0.4 M KCl) [5].

Under low salt conditions (Figure 7A), the molybdate-stabilized [³⁵S]hMR sedimented at 10 S. After treatment with DMP, the cross-linked hMR sedimented at the same position (results not shown). When the cross-linked hMR was subsequently incubated with 18C7, the peak shifted from 10 S to 11 S. This shift was identical with that observed after the incubation of untreated hMR with 18C7.

Under high salt conditions (0.4 M KCl; Figure 7B), the non-cross-linked hMR incubated with 18C7 sedimented at 8.8 S, whereas the cross-linked hMR, after incubation with 18C7, had

Figure 7 mAb 18C7 reacts with the cross-linked MR

(A) [³⁵S]hMR, synthesized *in vitro*, was incubated in TEG containing 20 mM sodium molybdate in the absence (○) or presence of 20 μl of 18C7 (▲). After treatment with DMP, [³⁵S]hMR was incubated with 20 μl of 18C7 (□). A 100 μl aliquot was layered on top of a 5–25% (w/v) sucrose gradient prepared in TEGMo buffer. (B) Sample aliquots were incubated as in (A) but in the presence of 0.4 M KCl. The samples were analysed on sucrose gradients containing molybdate and 0.4 M KCl. Gradients were centrifuged for 15 h at 180 000 *g* in a SW41 rotor at 4 °C. Sedimentation markers are indicated: catalase (c, 11.3 S), aldolase (a, 7.9 S) and BSA (b, 4.6 S).

shifted to form a large peak at approx. 10.5 S. As a control for the transformation process, the non-cross-linked hMR was analysed under high salt conditions. No peak could be detected in the 4 S area because of the large amount of free [³⁵S]methionine. Fractions corresponding to the 10.5 S peak, the 8.8 S peak and the masked area at approx. 4–5 S were then analysed by autoradiography after SDS/PAGE (Figure 8). Fractions 5–15 (peak at 10.5 S) migrated as a 250 kDa entity, which corresponded well to the hMR cross-linked with hsp90. Fractions 10–16 (peak at 8.8 S) mainly migrated at 110 kDa, indicating that it was the transformed form of the receptor that was recognized by 18C7. Fractions 12–20 migrated as a major 110 kDa entity (peak at approx. 5 S according to the autoradiograph pattern), indicating the presence of the transformed hMR state.

These results indicate that 18C7 recognition of hMR was not hindered by cross-linked proteins such as hsp90. They also demonstrate that 18C7 was able to react with the 4 S transformed hMR state.

The overall results of the present study demonstrate that the new mAb described in this paper (18C7), raised against the HBD of hMR, recognized both the denatured and native hMR forms. It also reacted with both the hetero-oligomeric 10 S structure of the receptor as well as the transformed 4 S state. It then provided an important tool for direct examination of the HBD structure and function. When the effect of the antibody was tested on hormone binding, 18C7 seriously impaired both aldosterone and progesterone binding. When incubated before the steroid, mAb 18C7 was able to prevent 75–80% of hormone binding to hMR. Furthermore the sucrose density gradient analysis revealed the concomitant presence of two complexes with hMR: the steroid-hMR complex and the antibody-hMR complex. The presence of a ternary complex steroid-hMR-antibody was ruled out by the fact that 18C7 did not co-sediment with the liganded [³H]aldosterone-hMR. As 18C7 was able to react in a similar way with the hMR, cross-linked or not with the hsp90, our results

Figure 8 Mab 18C7 interacts with the transformed hMR state

The collected ^{35}S -labelled fractions of the high-salt (0.4 M KCl) gradients corresponding to the 10.5 S peak (A), the 8.8 S peak (B) and the area at approx. 4 S to 5 S (C) detected in Figure 7(B) were subjected to SDS/PAGE (10% gel). After fixation and enhancement of signals by fluorography, the gels were dried and then exposed to an autoradiography film. The positions of molecular mass markers (in kDa) are shown at the left.

clearly demonstrate that the 18C7 epitope, located in the N-terminal part of the HBD, is directly involved in steroid binding.

It is now agreed that the hormone-binding pocket of steroid receptors is made up of residues from throughout the HBD. Furthermore it had been previously suggested that aldosterone and spironolactone bound to a common site [21]. These hypotheses are strengthened by our present study. Indeed, the three HBD mutants with the N-terminal Thr⁷²⁹–Leu⁷⁶⁵ sequence deleted were unable to bind aldosterone or progesterone. These results demonstrate clearly that the Thr⁷²⁹–Leu⁷⁶⁵ sequence is part of a module involved in the joint binding of agonists and antagonists. Recently, Wurtz et al. [22] proposed a canonical structure for the ligand-binding domain of the whole nuclear receptor family: the anti-parallel α -helical sandwich. From their report, six helices (H3 to H5 and H8 to H10) form the core of the ligand-binding domain of retinoic acid receptors. As a comparison with this model, the hMR sequence Thr⁷²⁹–Leu⁷⁶⁵ includes helix H1, the loop between H1 and H3, and the first three amino acids of helix H3. Although not directly implicated in the six helices constituting the core binding site, this region encompasses H1, which is supposed to be involved in key contacts with the ligand-binding core through five amino acid residues (Leu⁷⁴², Glu⁷⁴³, Ile⁷⁴⁵, Glu⁷⁴⁶ and Ile⁷⁴⁹). In addition to H1, the hMR sequence Thr⁷²⁹–Leu⁷⁶⁵ also comprises the loop between H1 and H3, which is probably involved in bending H3 during ligand binding. It would now be interesting to determine whether the site recognized by 18C7 is involved in the receptor conformation or whether there are direct contacts with the steroid.

We thank: Dr. Jean Léger for his constructive and helpful comments on this manuscript; Dr. Françoise Pons for her assistance with the immunohistochemistry; Anne-Marie Cathiard for her help with COS cell transfection; Danielle Mesnier for excellent skilful assistance in the expression of recombinant proteins in *E. coli*; and Patrick Atger for skilful photographic work. This research was supported by grants from the French Institut National de la Santé et de la Recherche Médicale.

REFERENCES

- 1 Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E. and Evans, R. M. (1987) *Science* **237**, 268–275
- 2 Evans, R. M. (1988) *Science* **240**, 889–895
- 3 Tsai, M.-J. and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63**, 451–486
- 4 Jalaguier, S., Mornet, D., Mesnier, D., Léger, J. J. and Auzou, G. (1996) *FEBS Lett.* **384**, 112–116
- 5 Rafestin-Oblin, M.-E., Couette, B., Radanyi, C., Lombès, M. and Baulieu, E. E. (1989) *J. Biol. Chem.* **264**, 9304–9309
- 6 Caamano, C. A., Morano, M. I., Patel, P. D., Watson, S. J. and Akil, H. (1993) *Biochemistry* **32**, 8589–8595
- 7 Pratt, W. B. (1993) *J. Biol. Chem.* **268**, 21455–21458
- 8 Smith, D. F. and Toft, D. O. (1993) *Mol. Endocrinol.* **7**, 4–11
- 9 Jalaguier, S., Mesnier, D., Léger, J. J. and Auzou, G. (1996) *J. Steroid Biochem. Mol. Biol.* **57**, 43–50
- 10 Trapp, T. and Holsboer, F. (1995) *Biochem. Biophys. Res. Commun.* **215**, 286–291
- 11 Couette, B., Fagart, J., Jalaguier, S., Lombès, M., Souque, A. and Rafestin-Oblin, M.-E. (1996) *Biochem. J.* **315**, 421–427
- 12 Fukushima, K., Sasano, H., Nagura, H., Sasaki, I., Matsuno, S. and Krozowski, Z. S. (1991) *Tohoku Exp. Med.* **165**, 155–163
- 13 Robertson, N. M., Schulman, G., Karnik, S., Alnemri, E. and Litwack, G. (1993) *Mol. Endocrinol.* **7**, 1226–1239
- 14 Lombès, M., Binart, N., Delahaye, F., Beaulieu, E. E. and Rafestin-Oblin, M.-E. (1994) *Biochem. J.* **302**, 191–197
- 15 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 (Baltimore)* **125**, 192–198
- 16 Lombès, M., Edelman, I. S. and Erlanger, B. F. (1989) *J. Biol. Chem.* **264**, 2528–2536
- 17 Moncman, C. L., Rindt, H., Robbins, J. and Winkelman, D. A. (1993) *Mol. Biol. Cell* **4**, 1051–1067
- 18 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 19 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
- 20 Schulman, G., Daniel, V., Cooper, M., Alnemri, E. S., Maksymowych, A. B. and Litwack, G. (1992) *Receptor* **2**, 181–193
- 21 Couette, B., Lombès, M., Beaulieu, E. E. and Rafestin-Oblin, M.-E. (1992) *Biochem. J.* **282**, 697–702
- 22 Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Chambon, P., Moras, D. and Gronemeyer, H. (1996) *Nature Struct. Biol.* **3**, 87–94