

# Human mineralocorticoid receptor interacts with actin under mineralocorticoid ligand modulation

Stéphan Jalaguier\*, Dominique Mornet, Danielle Mesnier, Jean J. Léger, Gilles Auzou

*Institut National de la Santé et de la Recherche Médicale, Unité 300, Faculté de Pharmacie, 15 Avenue Charles Flahault, 34060 Montpellier, France*

Received 8 March 1996

**Abstract** The human mineralocorticoid receptor of the steroid receptor family contains a modular structure with domain E which is considered to be a hormone binding domain. Recombinant protein approaches enabled us to clearly determine that this domain is also able to interact with F-actin ( $K_d$  about 2  $\mu$ M) and G-actin. Moreover, it was revealed that this mineralocorticoid receptor domain/actin interaction was modulated by specific mineralocorticoid ligands. Agonist (aldosterone) steroid binding almost totally (91%) abolished the interaction with F-actin, while antagonist (progesterone) binding allowed more than 30% of this binding. Steroid modulation of the interaction between domain E and actin indicated that this actin binding is specific and could be essential for cellular mineralocorticoid receptor activity.

**Key words:** Mineralocorticoid receptor; Hormone binding domain; Actin; *Hsp*; Aldosterone; Progesterone

## 1. Introduction

The mineralocorticoid receptor (MR) belongs to the steroid receptor family. It contains ligand-activated transcription factors that mediate specific gene expression [1]. The untransformed cytoplasmic MR is associated with a set of heat shock proteins (hsp) including hsp90 [2–5] and probably hsp70 [6], hsp56 [7] and p23 [8] as for other steroid receptors. After ligand binding, the receptor is translocated to the nucleus but the mechanism underlying this translocation is not yet known.

One of the major constituents of the cytoplasmic proteins is actin, a highly conserved and ubiquitous protein. Actin monomers (globular actin or G-actin) are able to assemble in cells to form actin polymers (filamentous actin or F-actin). This assembly forms a highly dynamic actin cytoskeleton in most cells.

It was previously shown that hsp90 was able to bind actin [9]. In addition, the ATPase domains of both hsp70 and actin have a common three-dimensional structure [10]. These two observations led us to investigate whether an interaction between MR and actin could occur and further its regulation. As MR is poorly expressed naturally, we undertook our investigation with the recombinant hormone binding domain (HBD or domain E) of the human mineralocorticoid receptor (hMR), shown to be the interaction domain between hMR and hsp90 [11]. Using co-sedimentation assays and ELISA

tests, we have shown that hMR directly interacts with actin *in vitro* through its HBD. Moreover, this interaction was modulated by the mineralocorticoid agonist and antagonist. Here we demonstrate that aldosterone (agonist) binding to the receptor markedly destabilized the HBD/F-actin interaction, while progesterone (antagonist) binding allowed stronger interaction of the recombinant HBD with F-actin.

## 2. Materials and methods

### 2.1. Protein preparations

Actin was purified from rabbit skeletal muscle as previously described [12] with slight modifications [13]. F-actin was stored at 4°C in buffer A (2 mM Tris-HCl pH 8.0, 10 mM KCl, 0.1 mM  $\text{CaCl}_2$  and 1 mM  $\text{NaN}_3$ ). Preparation of G-actin was previously described [14]. F and G-actin were titrated by spectrophotometric measurement in a Kontron Uvikon 930 using actin  $\epsilon$  1% at 280 nm = 1.1. Construction of plasmids encoding the HBD recombinant protein fused to the C-terminal part of the maltose binding protein (MBP) and its purification are described elsewhere [11]. Concentration of the fusion protein MBP-HBD was measured by the Bradford assay using BSA for calibration. HBD was separated from MBP by incubation of the purified MBP-HBD with Factor Xa for 120 min at room temperature.

### 2.2. Co-sedimentation assays

Co-sedimentations of F-actin with MBP-HBD were performed in an Airfuge (Beckman). 8  $\mu$ M of F-actin (i.e. 4 molar excess as the best conditions to demonstrate complete co-sedimentation of MBP-HBD by F-actin, Fig. 1A) was added to 2  $\mu$ M of MBP-HBD. The mixture was incubated for 20 min at room temperature before centrifugation at 28 psi for 30 min in a buffer containing 50 mM Tris HCl, pH 7.5, 50 mM KCl, 0.1 mM  $\text{CaCl}_2$  and 1 mM  $\text{NaN}_3$ . Control experiments consisted of sedimentation of MBP-HBD and F-actin alone. After centrifugation, the samples were analyzed as follows: the total mixture before co-sedimentation, the supernatant and pellet resuspended in the buffer described above were boiled in an equal amount of SDS loading buffer. The samples were then subjected to a 12% SDS-PAGE and visualized by Coomassie blue staining. Recombinant MBP-HBD was added to F-actin at different concentrations, as described in Fig. 2A. Densitometric analysis of the gels was performed.

### 2.3. Gel electrophoresis and immunoblotting

Proteins were separated by a 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). MBP was detected by 4E8 and HBD by 18C7, two monoclonal antibodies developed in our laboratory. Monoclonal antibody fixation was revealed by a rabbit anti-mouse antibody coupled with alkaline phosphatase (from Jackson Immuno Research).

### 2.4. Immunological techniques

Binding of HBD recombinant proteins to actin was carried out using a solid-phase immunoassay (ELISA).

100  $\mu$ l/well of G-actin to a final concentration of 1  $\mu$ M was adsorbed on microtiter plates overnight at 4°C. After rinses with PBS-Tween 0.1% and PBS alone, saturation with 3% BSA in PBS was performed for 1 h at 37°C. Serially diluted recombinant MBP-HBD was then incubated in the wells for 1 h at 37°C. After rinses in PBS-Tween 0.1% and PBS alone, the wells were incubated with 50  $\mu$ l of 4E8 antibody (dilution 1/5). Following three rinses in PBS-Tween 0.1% and one in PBS alone, the wells were incubated for 1 h at

\*Corresponding author. Fax: (33) (67) 04-21-40.

**Abbreviations:** MR, mineralocorticoid receptor; hMR, human mineralocorticoid receptor; HBD, hormone binding domain; MBP, maltose binding protein; hsp, heat shock protein; GR, glucocorticoid receptor.

37°C with 50 µl of alkaline phosphatase conjugated anti-mouse secondary antibody diluted to 1/5000 in PBS-5% dried milk. After three washes in PBS-Tween 0.1% and three other washes in NaCl 0.15 M, the wells were incubated for 1 h at 37°C in the presence of 100 µl of *para*-nitrophenylphosphate (Sigma 104) diluted to 1 mg/ml in diethylethanolamine.

### 2.5. Steroid binding experiments

The purified MBP-HBD (about 400 nM concentration) was incubated for 90 min at 30°C with untreated reticulocyte lysate (Promega) fourfold diluted in TEGW buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 20 mM sodium tungstate and 10% glycerol), supplemented with ATP and MgCl<sub>2</sub> to 1 mM with an ATP regenerating system consisting of 3 mM phosphocreatine (Sigma) and 5 units/ml of creatine phosphokinase (Sigma). 75 µl samples were then incubated for 1 h with either [<sup>3</sup>H]aldosterone or [<sup>3</sup>H]progesterone at 100 nM concentration, in the presence or absence of 50 µM of the radioinert steroid. Bound and free hormones were separated by centrifugation after prior incubation in a dextran/charcoal suspension (4% Norit/0.4% dextran). F-actin was then added at a final concentration of 4 µM and incubated for 20 min at room temperature. After centrifugation at 28 psi for 30 min, radioactivity from the pellet and the supernatant was counted in a Beckman spectrometer following the addition of 3 ml of scintillant fluid (Ultima Gold from Packard). The results are expressed as a percentage of total radioactivity.

## 3. Results

### 3.1. Binding of recombinant hMR with actin

The recombinant hMR, consisting of hMR HBD (domain E corresponding to amino acids 729–984) fused to the maltose binding protein (MBP), was constructed and purified as described elsewhere [11]. The purified MBP-HBD protein (4 µM concentration) was analyzed after Airfuge centrifugation in the absence or presence of 16 µM F-actin. The Coomassie blue stained gel shown in Fig. 1A, lane 3, revealed that MBP-HBD interacted with F-actin. As a control, F-actin alone (Fig. 1A, lane 1) was completely pelleted whereas MBP-HBD, when centrifuged alone, totally remained in the supernatant (Fig. 1A, lane 2). This result revealed a direct interaction between MBP-HBD and F-actin.

We used two monoclonal antibodies to characterize the specificity of the interaction, 18C7 which is HBD-specific, and 4E8 which is MBP-specific. HBD could be separated from MBP by digestion of the recombinant MBP-HBD with Factor Xa. MBP-HBD digested by Factor Xa (4 µM) was centrifuged with F-actin (20 µM). The sedimentation results were analyzed with the monoclonal antibodies 18C7 and 4E8 (see Fig. 1B). As indicated in Fig. 1B on the right panel, MBP was not able to sediment with F-actin. In contrast, all of the HBD was recovered pelleted (Fig. 1B, left panel). These results excluded any MBP-actin interaction and clearly revealed that the interaction was HBD-specific.

We further characterized the interaction through a saturation analysis of the two proteins. Increasing concentrations of F-actin were added to a fixed concentration of MBP-HBD (4 µM). The total mixture, supernatant and pellet were analyzed by SDS-PAGE. Densitometric analyses of the Coomassie blue stained gels allowed quantitation of the pelleted recombinant protein, i.e. interacting with F-actin. From these data, a saturation curve was deduced, which is presented in Fig. 2A. The saturation curve highlighted the specificity of the interaction. Saturation was obtained for a MBP-HBD/F-actin molar ratio of about 1/7. The apparent  $K_d$  deduced from Fig. 2 was about 2 µM. As a control, sedimentation of 4 µM MBP-HBD alone is shown in Fig. 2A (inset). The Coomassie blue stained

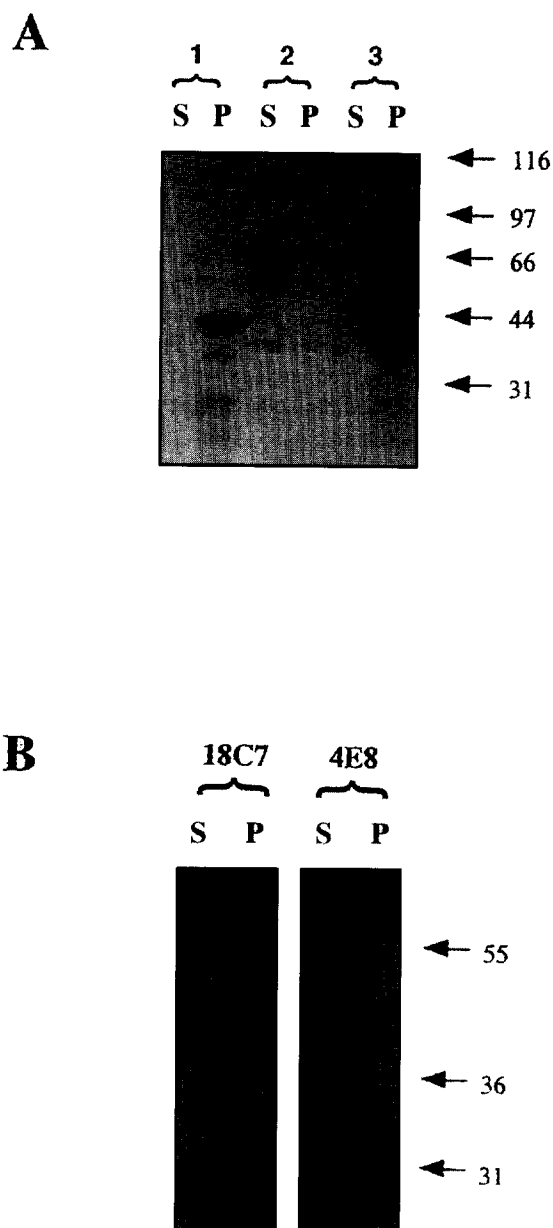


Fig. 1. Characterization of the MBP-HBD/F-actin interaction. A: Co-sedimentation assays between F-actin and the recombinant MBP-HBD protein. 8 µM of F-actin was sedimented alone (1), 2 µM MBP-HBD was also sedimented alone (2) and 8 µM of F-actin was co-sedimented with 2 µM MBP-HBD (3) after incubation as described in section 2. The samples corresponding to supernatants (S) and pellets (P) were boiled in an equal volume of SDS loading buffer and then electrophoresed on 12% SDS-PAGE before visualization by Coomassie blue staining. B: Characterization of the interaction specificity. 4 µM of MBP-HBD was digested with Factor Xa. 16 µM of F-actin was added to the digestion volume. Co-sedimentation assays with F-actin were performed as described in section 2. The nitrocellulose membrane, corresponding to the supernatant (S) and pellet (P), was incubated with fivefold diluted 18C7 (left panel), an anti-hMR HBD monoclonal antibody, or with fivefold diluted 4E8, an anti-MBP monoclonal antibody (right panel). Monoclonal antibody fixation was revealed by a rabbit anti-mouse antibody coupled with alkaline phosphatase.

gel revealed that MBP-HBD was not able to sediment alone at this concentration.

As the glucocorticoids were shown to stabilize actin filaments [15] we investigated the possibility that MBP-HBD

would also interact with G-actin. G-actin was thus adsorbed on a microtiter plate at a constant concentration of 1  $\mu\text{M}$ . The interaction between increasing concentrations of MBP-HBD and coated G-actin was analyzed by ELISA technique, as described in section 2.4. Fig. 2B presents the saturation curve of G-actin with MBP-HBD. The curve profile presents a plateau, which reveals a specific interaction between MBP-HBD and G-actin.

### 3.2. Ligand modulation of MBP-HBD/F-actin interaction

To adopt a steroid binding conformation, MBP-HBD has to be associated with a set of hsp [12]. The purified recombinant protein was thus incubated in reticulocyte lysate, which is particularly rich in these different hsps. As mentioned in

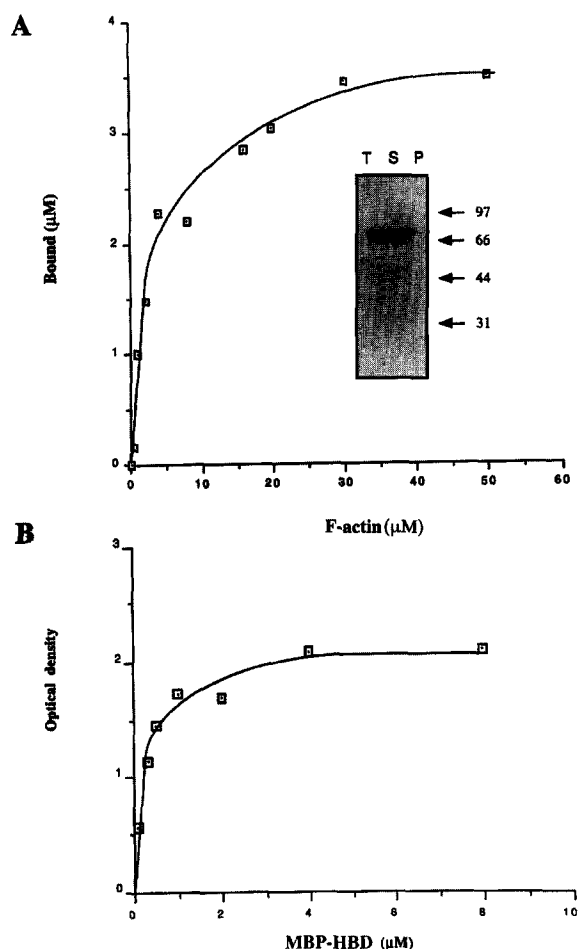


Fig. 2. Saturation analyses of the actin/MBP-HBD interaction. A: Saturation analysis with F-actin. 4  $\mu\text{M}$  of MBP-HBD was incubated with increasing concentrations of F-actin (from 0.3 to 50  $\mu\text{M}$ ). Incubation and preparation of the samples were as described in Fig. 1. Densitometric analysis of the gels was performed to quantify the Coomassie blue stained band. Inset: Sedimentation assay with only 4  $\mu\text{M}$  HBD. The recombinant MBP-HBD was prepared in the usual buffer and then centrifuged as previously described. The samples indicated correspond to the total mixture before centrifugation (T), supernatant (S) and pellet (P). B: Saturation analysis with G-actin. G-actin was adsorbed on a microtiter plate at a constant concentration of 1  $\mu\text{M}$ . Increasing concentrations of MBP-HBD were then added to interact with G-actin. The complex formed between the two components was measured by ELISA as described in section 2, using fivefold diluted 4E8, a monoclonal anti-MBP antibody. Monoclonal antibody fixation was revealed by an alkaline phosphatase, conjugated anti-mouse secondary antibody.

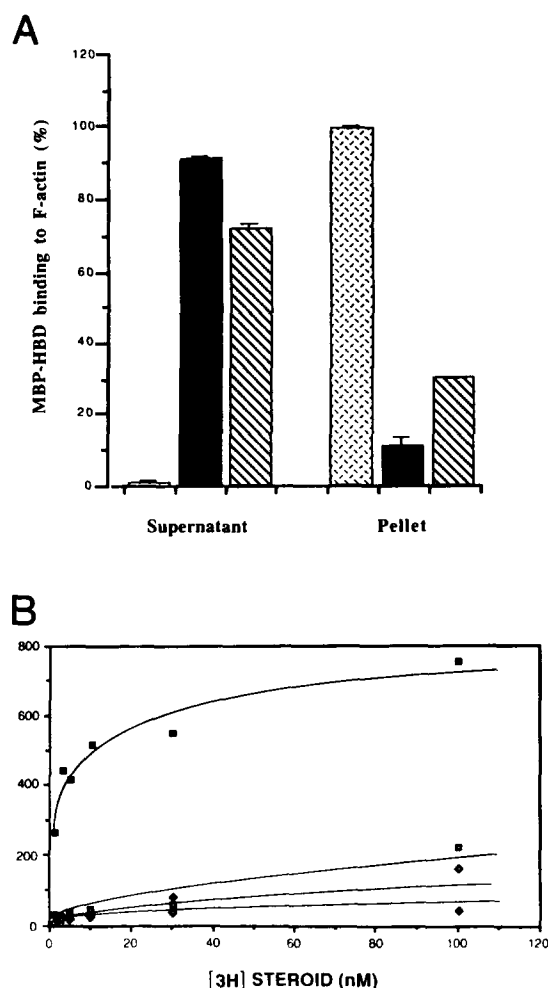


Fig. 3. Ligand modulation of the MBP-HBD/F-actin interaction. A: Steroid binding experiments were performed as indicated in section 2. Results were expressed as a percentage of the total radioactivity before centrifugation except for the direct interaction between MBP-HBD and F-actin measured in the absence of reticulocyte lysate where results are the expression of densitometric analysis of a Coomassie blue stained gel. Results indicated were for the non-ligand bound MBP-HBD (cross-hatched bar), [ $^3\text{H}$ ]aldosterone-MBP-HBD complex (black bar) and [ $^3\text{H}$ ]progesterone-MBP-HBD complex (hatched bars). The data shown are the means  $\pm$  SE of three separate experiments. B: Dose-response investigation of the MBP-HBD/F-actin interaction. Steroid binding experiments were performed as indicated in section 2 with increasing aldosterone and progesterone concentrations (from 1 nM up to 100 nM). The curves represent the radioactivity counted either in the supernatant (solid squares, with aldosterone; hollow squares, with progesterone) or the pellet (solid diamonds, with aldosterone; hollow diamonds, with progesterone).

section 2.5, [ $^3\text{H}$ ]aldosterone (MR agonist) or [ $^3\text{H}$ ]progesterone (MR antagonist) was then added to the mixture. As previously described [11], the amount of receptor incubated in reticulocyte lysate is much greater than that actually able to bind [ $^3\text{H}$ ]steroids by measuring the radioactivity associated with such receptors. The ligand free receptor, in its oligomeric form, could not be investigated in these conditions. The [ $^3\text{H}$ ]steroid-MBP-HBD complex was co-sedimented with F-actin. As indicated in Fig. 3A, only about 9% of the total radioactivity was pelleted, indicating that the [ $^3\text{H}$ ]aldosterone-MBP-HBD complex co-sedimented poorly with F-actin. In contrast, the interaction between the

[<sup>3</sup>H]progesterone-MBP-HBD complex and F-actin was stronger because about 33% of the total radioactivity was pelleted. As a control, MBP-HBD without incubation in reticulocyte lysate almost totally co-sedimented with F-actin. This result clearly indicated that the interaction was specifically modulated by the mineralocorticoid ligands. In addition, the interaction was probably not due to a distinct  $K_d$  of aldosterone or progesterone, since we have previously shown [11] that they had the same affinity for MBP-HBD. We also performed a dose-response experiment (Fig. 3B) where, after incubation in reticulocyte lysate, increasing aldosterone and progesterone concentrations (from 1 to 100 nM) were added to a fixed F-actin concentration (4  $\mu$ M). The results indicate saturable curves for both aldosterone and progesterone and for supernatants and pellets, strengthening the specificity of the interaction. Moreover, the results obtained with the dose-response curves confirmed those obtained in Fig. 3A. Indeed, the curves demonstrated that the [<sup>3</sup>H]progesterone-MBP-HBD/F-actin interaction is stronger than that of [<sup>3</sup>H]aldosterone-MBP-HBD/F-actin.

#### 4. Discussion

In the present study, using a recombinant protein technique and taking advantage of the fact that the soluble construct of the mineralocorticoid receptor domain E can be specifically cleaved from the MBP part, we demonstrated that actin binding is restricted to this domain of the hMR. Direct co-sedimentation assays between MBP-HBD and F-actin revealed a  $K_d$  of 2  $\mu$ M for interactions. In addition, we determined that G-actin was also able to bind to hMR, as shown by ELISA techniques. Although a previous study showed that the glucocorticoid receptor (GR), another member of the steroid receptor superfamily, bound actin filaments [16] via hsp90, we describe here a direct interaction between a steroid receptor and actin.

Our first results showed a non steroid-binding conformation of the HBD. Indeed, in order to bind its ligands, MR has to be associated with a variety of proteins, particularly hsp90 and probably hsp70, hsp56 and p23, as is the case for other members of the steroid family such as GR. We thus found that a 'nude' receptor could directly interact with F-actin.

We also tried to determine whether ligand binding could regulate this interaction. MBP-HBD recovered a steroid binding conformation through incubation in reticulocyte lysate [11]. In these conditions, the interaction was stronger with the [<sup>3</sup>H]progesterone-MBP-HBD complex than with [<sup>3</sup>H]aldosterone-MBP-HBD complex. The aldosterone-bound MR is widely described as being associated with hsp90 [2–5], as for the GR [17–19]. Furthermore, hsp90 was shown to be an actin binding protein [9,20,21]. Hsp90 also binds MR through its HBD [11], as for GR [22–24]. We suggest that the hsp90-HBD association in the reticulocyte lysate masks the interaction site with F-actin. Progesterone, a mineralocorticoid antagonist, is thought to induce a conformational change of the receptor that differs from that induced by aldosterone [25,26]. We thus propose that the better interaction observed between the [<sup>3</sup>H]progesterone-MBP-HBD complex and F-actin is due to the availability of the F-actin binding site. It was previously shown that progesterone binding induced a looser conformation of the ligand binding domain, contrary to aldosterone binding which induced a very tight

conformation of the binding structure able to withstand high protease concentrations [26]. The better interaction described with progesterone bound MBP-HBD could thus have been facilitated by the loose structure of the ligand binding domain, whereas the aldosterone-MBP-HBD structure was too tight to allow such an interaction with F-actin. Moreover, ligand modulation of the interaction revealed that hsp90 adopted a distinct conformation depending on whether it was associated with GR or MR. The direct interaction between hMR and F-actin cannot be extended to other steroid receptors. Indeed, GR interacts with F-actin via hsp90, while both progesterone [27,28] and estrogen receptors [29,30] are nuclear, and F-actin is mainly cytoplasmic.

However, the role of the F-actin/steroid receptor interaction remains unclear. It was previously hypothesized [16,31] for GR that the hsp complex acted as a transport particle or transportosome for bidirectional shuttling of GR into and out of the nucleus. This shuttling could be modulated by interactions with F-actin. In the same way, shuttling of the receptor from the cytosol to the nucleus might be partly directed by actin and modulated by mineralocorticoid ligands, as seen above. Moreover, the affinity of F-actin for MBP-HBD was about 2  $\mu$ M, which is the same affinity as hsp90 for F-actin [20]. Both hsp90 and F-actin are widely represented in the cytosol. The three proteins could therefore compete to interact. We hypothesize that F-actin is also an anchoring protein of the non-oligomeric form of the MR. Anchoring would then be regulated by hsp association and ligand binding with the receptor.

Another protein, calmodulin, was observed to interact with the related GR [32]. This protein is thus a potential MR interacting protein and could modulate such interactions. In addition, many associations between actin and its interacting proteins are modulated by nucleotides. This remains to be investigated for the MR.

**Acknowledgements:** This research was supported by grants from the Institut National de la Santé et de la Recherche Médicale.

#### References

- [1] Evans, R.M. (1988) *Science* 240, 889–895.
- [2] Rafestin-Oblin, M.E., Couette, B., Radanyi, C., Lombes, M. and Baulieu, E.E. (1989) *J. Biol. Chem.* 264, 9304–9309.
- [3] Binart, N., Lombes, M., Rafestin-Oblin, M.E. and Beaulieu, E.E. (1991) *Proc. Natl. Acad. Sci. USA* 88, 10681–10685.
- [4] Alnemri, E.S., Maksymowych, A.B., Robertson, N.M. and Litwack, G. (1991) *J. Biol. Chem.* 266, 18072–18081.
- [5] Caamaño, C.A., Morano, M.I., Patel, P.D., Watson, S.J. and Akil, U. (1993) *Biochemistry* 32, 8589–8595.
- [6] Sanchez, E.R., Hirst, M., Scherrer, L.C., Tang, H.-Y., Welsh, M. J., Harmon, J.M., Simons S.S. Jr., Ringold, G. M. and Pratt, W.B. (1990) *J. Biol. Chem.* 265, 20123–20130.
- [7] Sanchez, E.R., Faber, L.E., Henzel, W.J. and Pratt W. B. (1990) *Biochemistry* 29, 5145–5152.
- [8] Hutchison, K.A., Stancato, L.F., Owens-Grillo, J.K., Johnson, J.L., Krishna, P., Toft, D.O. and Pratt, W.B. (1995) *J. Biol. Chem.* 270, 18841–18847.
- [9] Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H. and Yahara, I. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8054–8058.
- [10] Bork, P., Sander, C. and Valencia, A. (1992) *Proc. Natl. Acad. Sci. USA* 89, 7290–7294.
- [11] Jalaguier, S., Mesnier, D., Léger, J.J. and Auzou, G. (1996) *J. Steroid Biochem. Mol. Biol.* (in press).
- [12] Spudich, J.A. and Watt, S. (1971) *J. Biol. Chem.* 246, 4866–4871.

- [13] Eisenberg, E. and Kielly, W.W. (1974) *J. Biol. Chem.* 242, 1140–1145.
- [14] Bonet-Kerrache, A., Fabbriozio, E. and Mornet, D. (1994) *FEBS Lett.* 355, 49–53.
- [15] Castellino, F., Heuser, J., Marchetti, S., Brono, B. and Luini, A. (1992) *Proc. Natl. Acad. Sci. USA* 89, 3775–3779.
- [16] Miyata, Y. and Yahara, I. (1991) *J. Biol. Chem.* 266, 8779–8783.
- [17] Bresnick, E.H., Dalman, F.C., Sanchez, E.R. and Pratt, W. B. (1989) *J. Biol. Chem.* 264, 4992–4997.
- [18] Cadepond, F., Schweizer-Groyer, G., Segard-Maurel, I., Jibard, N., Hollenberg, S.M., Giguère, V., Evans, R.M. and Baulieu, E.E. (1991) *J. Biol. Chem.* 266, 5834–5841.
- [19] Ohara-Nemoto, Y., Strömstedt, P-E., Dahlman-Wright, K., Nemoto, T., Gustafsson, J-A. and Carlstedt-Duke, J. (1990) *J. Steroid Biochem. Mol. Biol.* 37, 481–490.
- [20] Nishida, E., Koyasu, S., Sakai, H. and Yahara, I. (1986) *J. Biol. Chem.* 261, 16033–16036.
- [21] Kellermayer, M S.Z. and Csermely, P. (1995) *Biochem. Biophys. Res. Commun.* 211, 166–174.
- [22] Pratt, W.B., Jolly, D.J., Pratt, D.V., Hollenberg, S. M., Giguere, V., Cadepond, F.M., Schweizer-Groyer, G., Catelli, M.-G., Evans, R.M. and Baulieu, E.-E. (1988) *J. Biol. Chem.* 263, 267–273.
- [23] Howard, K.J., Holley, S.J., Yamamoto, K.R. and Distelhorst, C. W. (1990) *J. Biol. Chem.* 265, 11928–11935.
- [24] Dalman, F.C., Scherrer, L.C., Taylor, L.P., Akil, H. and Pratt, W.B. (1991) *J. Biol. Chem.* 266, 3482–3490.
- [25] Couette, B., Lombes, M., Baulieu, E.E. and Rafestin-Oblin, M. E. (1992) *Biochem. J.* 282, 697–702.
- [26] Couette, B., Fagart, J., Jalaguier, S., Lombès, M., Souque, A. and Rafestin-Oblin, M.E. (1996) *Biochem. J.* (in press).
- [27] Guiochon-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat, M. and Milgrom, E. (1991) *EMBO J.* 10, 3851–3859.
- [28] Tuohimaa, P., Pekki, A., Bläuer, M., Joensuu, T., Vilja, P. and Ylikomi, T. (1993) *Proc. Natl. Acad. Sci. USA* 90, 5848–5852.
- [29] King, W.J. and Green, G.L. (1984) *Nature* 307, 745–747.
- [30] Perrot-Applanat, M., Logeat, F., Groyer-Picard, M.T. and Milgrom, E. (1985) *Endocrinology* 116, 1473–1484.
- [31] Pratt, W.B. (1993) *J. Biol. Chem.* 268, 21455–21458.
- [32] Ning, Y.-M. and Sanchez, E.R. (1995) *Biochem. Biophys. Res. Commun.* 208, 48–54.