A Bacterially Expressed Mineralocorticoid Receptor Is Associated *in Vitro* with the 90-Kilodalton Heat Shock Protein and Shows Typical Hormone- and DNA-Binding Characteristics†

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ABSTRACT: A recombinant system was developed for generation of steroid–receptor complexes *in vitro*. The DNA- and steroid-binding domains of the rat mineralocorticoid receptor were expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase. The identity of the expressed recombinant protein was confirmed by Western blot analysis. Protein preparations purified by affinity chromatography, avoiding the use of detergents or high ionic strength buffers, exhibited negligible steroid binding. However, after incubation of these preparations with rabbit reticulocyte lysate, known to promote the association of isolated steroid receptors with heat shock proteins, the [^{3}H]aldosterone-binding activity gradually increased. This temperature-dependent effect reached a maximum after 1 h at 30 °C and was favored by ATP supplementation (B_{max} = 22 ± 3 pmol/mg of protein). The apparent K_d value for aldosterone (0.6 ± 0.2 nM) and the steroid-binding specificity of the recombinant protein were in accordance with those reported for the native mineralocorticoid receptor. The sedimentation and DNA–cellulose-binding characteristics of the radioactive complexes were also in agreement with those reported for the native heteromeric receptor. Complexes sedimented at 8.9 ± 0.2 or 4.2 ± 0.2 S in sucrose gradients containing 20 mM sodium molybdate or 0.4 M KCl, respectively. Monoclonal antibody 8D3 against the 90-kDa heat shock protein (hsp90) was able to bind to the 8.9S complexes, increasing its sedimentation coefficient. Treatment of the complexes with 100 mM sodium thiocyanate, known to activate the native receptor to a DNA-binding state, caused a 79% increase in DNA–cellulose binding over the control values. This last effect was prevented when 20 mM molybdate was also present. This is the first report of the use of a recombinant steroid receptor for the generation *in vitro* of complexes showing typical characteristics of native receptors associated to hsp90. This novel strategy is apt to facilitate future structural studies of steroid receptors.

Adrenal steroids exert their biological effects on the genome via intracellular receptor molecules. Mammalian tissues contain two different types of these receptors (Reul & deKloet, 1986). Those with high affinity for both the physiologically occurring mineralocorticoid aldosterone and the glucocorticoid corticosterone represent the mineralocorticoid receptors (MR).[1] Those with lower affinity for these natural steroids are the classical, dexamethasone-binding glucocorticoid receptors (GR) (Moguilewski & Raynaud, 1980; Krozowski & Funder, 1983; McEwen et al., 1986). Depending on its localization, MR has been shown to play at least two distinct physiological roles. For example, in kidney, MR acts as a receptor for aldosterone and is involved in the control of the water/salt balance. In contrast, in brain limbic neurons, MR acts as a glucocorticoid receptor that is thought to be involved in the regulation of the resting levels of cortisol/corticosterone (McEwen et al., 1986; deKloet & Reul, 1987; Arriza et al., 1988). Yet, despite the physiological importance of MR, its specific characteristics in comparison with the GR and the other steroid receptors remain largely unstudied.

The MR is a member of the superfamily of ligand-regulated transcription factors, which includes the other steroid hormone receptors as well as the thyroid hormone, vitamin D, and retinoic acid receptors (Evans, 1988). Our laboratory has isolated a MR cDNA from a rat hippocampus library (Patel et al., 1989) that is 90% homologous at the amino acid level to that for the human kidney MR (Evans, 1988). Sequence homologies of MR with other steroid receptors support a molecular organization based on functional domains (Krozowski & Funder, 1983; Arriza et al., 1987; Evans, 1988). Indeed, there is significant amino acid identity between MR and GR in the DNA-binding (94%) and the steroid-binding domains (57%) that is reflected in the partial overlapping of the ligand-binding specificities of both receptors (Arriza et al., 1987).

As with most of the steroid receptors (Pratt, 1987), MR is found in cytosolic preparations associated with the 90-kDa heat shock protein, hsp90, forming heteromeric complexes that typically sediment at 8–9 S upon centrifugation in density gradients (Rafestin-Oblin et al., 1989). The complexes detected by this analysis can be considered as core units derived from larger complexes of yet undefined composition and stoichiometry (Bresnick et al., 1990). The relative instability of these native receptor complexes has been a limitation for their isolation and structural characterization. This situation is aggravated for MR because of its low expression levels in different tissues and cell lines. On the other hand, there is now strong evidence that the interaction with hsp90 is necessary for appropriate steroid binding in the case of GR (Bresnick et al., 1989; Dalman et al., 1989; Meschini et al., 1990; Nemoto et al., 1990), and possibly MR as well.

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[1] Abbreviations: MR, mineralocorticoid receptor(s); GR, glucocorticoid receptor(s); hsp90, 90-kDa heat shock protein; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEDGM, 20 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, 10% glycerol, and 20 mM sodium molybdate, pH 7.6 at 0 °C; TEDG, same as TEDGM but without molybdate.

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The overexpression of recombinant MR and GR in insect cell lines (Srinivasan & Thompson, 1990; Alnemri et al., 1991a,b; Binart et al., 1991), and of GR in yeast (Schena & Yamamoto, 1988; Picard et al., 1990; Wright et al., 1990), has yielded receptor preparations that are functionally similar to the native counterparts. In these expression systems, the host cells seem to provide all the factors needed for proper receptor synthesis and final assembly. In contrast, recombinant glucocorticoid (Nemoto et al., 1990; Ohara-Nemoto et al., 1990), progesterone (Eul et al., 1989; Power et al., 1990), and androgen (Young et al., 1990; Roehrborn et al., 1992; Nemoto et al., 1992) receptors generated in bacteria consistently exhibit hydrodynamic characteristics that are inconsistent with the formation of definite heteromeric complexes (Ohara-Nemoto et al., 1990; Eul et al., 1989; Power et al., 1990; Young et al., 1989; Roehrborn, 1992; Nemoto et al., 1992). For GR, in possible agreement with its particular structural requirements, the steroid-binding affinity is reduced about 100 times as well (Ohara-Nemoto et al., 1990). Yet, bacterial expression systems potentially offer higher levels of expression and greater ease of purification as compared to eukaryotic systems.

The recent discovery that reticulocyte lysate can promote the association of isolated steroid receptors with heat shock proteins (Smith et al., 1990, 1992; Scherrer et al., 1990) prompted us to employ these cell-free conditions on recombinant steroid receptors overexpressed in bacteria. Using a purified recombinant fusion protein containing the DNA- and steroid-binding domains of the rat MR, we have been able to form *in vitro*, and for the first time using a recombination preparation, steroid–receptor complexes associated to hsp90 evidencing hydrodynamic and binding characteristics indistinguishable from those of the native heteromeric receptors. Unlike the yeast, eukaryotic cell transfection, and baculovirus expression systems, that yield final products already assembled into functional complexes, the bacterial system can be used as a source of protein that can be readily purified and subsequently treated with exogenous factors *in vitro*, under more controlled conditions. In this way, the applications of this recombinant cell-free system for the functional and structural characterization of steroid receptors could be further extended to the interactions with proteins and factors required for its assembly and processing.

EXPERIMENTAL PROCEDURES

Construction of the Protein Expression Vector. In order to express a MR derivative as a C-terminal fusion protein of glutathione *S*-transferase (GST), a 3098 bp *Stu*I/*Stu*I fragment of the rat MR cDNA (Patel et al., 1989) was isolated, ligated to *Sal*I linkers (Promega), and then inserted into a *Sal*I-digested pGEX-KG vector (Smith & Johnson, 1988; Dixon, 1991). Plasmids with proper orientation of the insert were selected, digested with *Bam*HI, and finally religated. The resulting construct, pMRBam/Sal, has an open reading frame of 1830 nucleotides coding for a fusion protein encompassing, from N- to C-terminal, the complete GST sequence, a fragment of 6 amino acids containing a potential thrombin cleavage site, and the rMR sequence from Gly596 to Lys981. The plasmid was then used to transform *Escherichia coli* HB101 strain. For transformation, the cells were rendered competent to incorporate plasmid DNA by the calcium chloride/rubidium chloride procedure (Kushner, 1978). Transformants were identified on the basis of their acquired ampicillin resistance and were further characterized by DNA restriction analysis and sequencing.

Expression and Purification of the Fusion Protein. Overnight cultures of *E. coli* (HB101 strain) transformed with either parental or recombinant pGEX-KG plasmids were processed as previously described (Smith & Johnson, 1988) with some modifications, particularly in the screening of protein expression, and in the protein purification in the absence of detergent. Briefly, cells were diluted 1:10 in fresh medium and grown for 1 h at 37 °C before isopropyl β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM concentration. After 3 h of further growth, the cells were pelleted at 5000*g* for 10 min and washed 3 times with 50 volumes of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4, pH 7.3). The pellets were resuspended in MTPBS (7–20 mL/L of culture), and cells were lysed by sonication in an ice bath (3 cycles of 10 s at 60 W). The lysate was centrifuged at 50000*g* for 10 min.

For affinity purification, the lysate supernatant was mixed with a 50% (v/v) suspension of glutathione–agarose beads (sulfur-linked, Sigma) in MTPBS, at a ratio of 3 mL of suspension per liter of culture, and incubated at 4 °C for 30 min. The beads were washed 3 times with 10 volumes of ice-cooled MTPBS and finally eluted by incubation for 20 min with 5 mM glutathione in 50 mM Tris-HCl (pH 7.8).

Fast screening of the transformants was performed in crude and affinity-adsorbed samples by SDS–PAGE (Laemmli, 1970), followed by Coomassie blue (Sigma) staining. For screening of crude samples, the cell pellets (1 mL of culture) were directly resuspended in 0.2 mL of water and boiled for 5 min in the presence of 1 volume of electrophoresis sample buffer containing 5% (v/v) β-mercaptoethanol. For screening of affinity-adsorbed samples, the cell pellets (5 mL of culture) were treated as described above for purification except that the elution step was replaced by boiling the bead pellets for 5 min in 0.1 mL of electrophoresis sample buffer with β-mercaptoethanol.

Antibodies. The immune serum anti-GST/VH1 (Guan et al., 1991) was kindly provided by Dr. Jack Dixon (Department of Biological Chemistry, University of Michigan Medical School). The rabbit anti-MR antibody was generated in our laboratory using a synthetic peptide corresponding to positions 830–841 in the rMR sequence. The 8D3 IgM monoclonal antibody, an immune ascites containing hsp90-reactive IgM (Perdew, 1988), was kindly provided by Dr. Gary Perdew (Department of Foods and Nutrition, Purdue University).

Electroblotting. Proteins analyzed by SDS–PAGE (Laemmli, 1970) were transferred onto Immobilon-P (Millipore) membranes using a Bio-Rad Transblot apparatus, equipped with a refrigerating coil, in a buffer consisting of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.5. The efficiency of the transfer process was evaluated by including at least one lane of prestained molecular weight markers (Amersham). Transfer was made at 4 °C and 200 V (20 V/cm) for 2–5 h. Membrane strips were eventually saved for staining (10 min) in 0.1% (w/v) Coomassie blue, 10% (v/v) methanol, and 5% (v/v) acetic acid, followed by destaining in a similar solution without the dye.

Immunostaining. After electroblotting, membrane blots were incubated for 2 h at 20 °C in 3% (w/v) bovine serum albumin (BSA) in TBS buffer (0.9% NaCl/20 mM Tris-HCl, pH 7.4) and washed 3 times (10 min each) with wash solution (0.1% BSA in TBS). The blots were then incubated for 2 h at 20 °C with immune antisera against GST-VH1 (0.2%) or MR (0.3%) in TBS buffer containing 1% (w/v) BSA and 0.05% (v/v) Tween-20. Following three washes (5 min each) in wash solution, the blots were incubated for 2 h at 20 °C with 0.1% peroxidase-coupled sheep anti-rabbit IgG (Boehringer) in TBS buffer containing 1% BSA and 0.05% (v/v) Tween-20. After three washes (5 min each) with wash

solution, the blots were finally incubated for 1–5 min in a solution containing 0.8 mM 4-chloro-1-naphthol in 0.02% (v/v) hydrogen peroxide.

Reticulocyte Lysate Treatment. Untreated rabbit lysate (Promega) was added to the purified preparations (1:1) and incubated at 0 or 30 °C for 0–90 min. When indicated, supplementation of the mixture prior to the incubation was effected with ATP and $MgCl_2$ to 1 mM, with or without an ATP-regenerating system. This system consisted of 3 mM phosphocreatine (disodium salt, Sigma) and 5 units/mL creatine phosphokinase (rabbit muscle, Sigma).

Steroids. [1,2,6,7-^{3}H]Aldosterone (75 Ci/mmol) and [1,2,3,4-^{3}H]dexamethasone (70–110 Ci/mmol) were obtained from Amersham. The following unlabeled steroids were obtained from Sigma: aldosterone, cortisol, corticosterone, deoxycorticosterone, progesterone, testosterone, 17β-estradiol, dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadene-3,20-dione), and spironolactone [7-(acetylthio)-17-hydroxy-3-oxopregn-4-ene-21-carboxylic acid, γ-lactone]. The GR antagonist RU38486 [11β-[4-(dimethylamino)phenyl]-17β-hydroxy-17α-(1-propynyl)-11β,17β-estra-4,9-dien-3-one] and the GR agonist RU26988 [11β,17β-dihydroxy-17α-(1-propynyl)-11β,17β-androsta-1,4,6-trien-3-one] were a gift from Roussel-UCLAF (Romainville, France).

Steroid-Binding Assays. Binding was performed in TEDGM and TEDG buffers (20 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, and 10% glycerol with or without 20 mM sodium molybdate, respectively, pH 7.6 at 0 °C). For saturation binding assays, aliquots (50 μL) of the reticulocyte-treated purified preparation were incubated at 0–2 °C with [^{3}H]-aldosterone or [^{3}H]dexamethasone (0.1–60 nM) in the presence or absence of a 1000-fold molar excess of the corresponding radioinert steroid (each condition in triplicate). After 5–16 h, bound and free hormones were separated by addition of an ice-cold dextran/charcoal suspension in TEDG (1% Norit A/0.1% dextran). The tubes were incubated for 10 min at 0–2 °C and centrifuged. The supernatants were removed and counted in a Packard scintillation counter. For competition assays, aliquots of the reticulocyte-treated purified preparation were incubated as described above with 1 nM [^{3}H]aldosterone in the presence or absence of 0.1–1000 nM unlabeled steroid. In all cases, the specific binding (B) was calculated as the difference between the radioactivity bound in the absence and in the presence of the radioinert steroid.

Sucrose Gradient Analysis. Linear sucrose gradients (10–30%), prepared in 20 mM Tris-HCl (pH 7.6 at 0 °C) containing either 20 mM sodium molybdate or 400 mM potassium chloride, were preequilibrated at 4 °C. [^{3}H]-Aldosterone–receptor complexes obtained as described before in TEDM or TED, respectively, were applied on top of the 5-mL gradients. Alternatively, prior to gradient loading, complexes obtained in TEDM were treated for 1 h at 4 °C with 50 μL of either 8D3 monoclonal antibodies against hsp90 or control ascites. Following centrifugation at 50000g for 14 h at 2 °C, seven-drop fractions were collected from bottom to top and counted for radioactivity.

DNA–Cellulose-Binding Assays. The technique was adapted from Luttge and Emadian (1988). Briefly, [^{3}H]aldosterone–receptor complexes generated in buffers TEDM and TED (same as TEDGM and TEDG without glycerol) were adjusted to 300 mM KCl or 100 mM NaSCN. Control, KCl-treated, and NaSCN-treated samples were incubated for 30 min at 0 °C. All samples were then adjusted to 5 mM molybdate concentration, and the newly dissociated aldosterone was removed with the dextran/charcoal suspension as described above. Double-stranded calf thymus DNA–cellulose (Sigma) was prepared overnight in TDE buffer. Aliquots of the slurry (30 mg of DNA–cellulose) were added to the samples and incubated for 60 min at 0 °C. Assay tubes were gently resuspended every 10 min. The [^{3}H]aldosterone–receptor/DNA–cellulose complexes were then pelleted, washed in TDE and transferred to scintillation vials for determination of bound radioactivity.

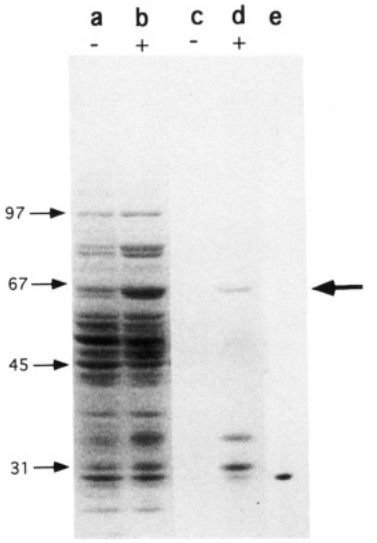

FIGURE 1: Bacterial expression of the GST–MR fusion protein. Aliquots of samples from IPTG-induced (+) or noninduced (–) bacteria were analyzed by SDS–PAGE on a 10% (w/v) polyacrylamide gel and stained with Coomassie blue. The positions of molecular mass markers (kDa) and of the induced fusion protein are indicated by arrowheads at the left and right sides of the panel, respectively. Lanes a and b contain supernatants of bacterial extracts. Lanes c and d contain affinity-purified preparations. Lane e contains affinity-purified GST obtained from bacteria infected with the parental pGEX-KG plasmid.

Data Analysis and Statistics. For saturation-binding studies, the binding parameters, dissociation constant (K_d), and binding maximum (B_{max}) were derived from Scatchard analysis. Data regarding DNA–cellulose binding were analyzed by one-way analysis of variance (ANOVA) and subsequently by the Scheffé F and Fisher's protected least significant difference tests, as described in the Stat View 512$^+$ program manual.

Miscellaneous. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS

Bacterial Expression of the Recombinant MR. The pMRBam/Sal construct, bearing an insert coding for 386 amino acid residues of the rMR sequence encompassing the putative DNA- and steroid-binding domains, was obtained as described under Experimental Procedures. As shown in Figure 1, extracts from bacteria transformed with this plasmid and induced with isopropyl β-D-thiogalactopyranoside (IPTG) revealed the synthesis of a novel protein of 65 kDa (lane b). This molecular mass is consistent with the one predicted for a fusion protein composed of glutathione S-transferase (GST, 25 kDa) linked to the N-terminal truncated MR (40 kDa). Densitometric scanning of the Coomassie blue-stained gels revealed that the 65-kDa protein represented 3–5% of the total proteins present in the bacterial extract. The fusion protein was soluble after bacterial lysis and could be readily purified by a single step of affinity chromatography on glutathionine S–agarose, using mild elution conditions and

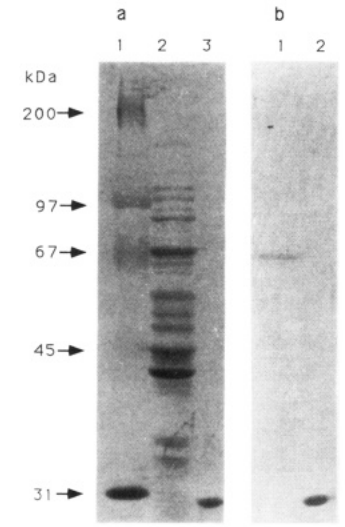

FIGURE 2: Western immunoblotting analysis. Duplicate aliquots of extracts from IPTG-induced bacteria were analyzed by SDS–PAGE on a 10% (w/v) polyacrylamide gel. After electroblotting, membranes were divided to obtain the individual samples, and were either stained with Coomassie blue (panel a) or immunostained using the GST-reactive immune serum anti-GST/VH1 (panel b). Lane a2 and b1 contain the extracts. Lane a3 and b2 contain affinity-purified GST. Prestained molecular mass markers are included in lane a1.

avoiding the use of detergents (see Experimental Procedures). In the purified preparation, together with the species of interest, two other major bands were visible in the low molecular mass region (Figure 1, lane d). These bands were only observed after IPTG induction and were not sensitive to the addition of proteolysis inhibitors before or after bacterial rupture. In addition, both bands completely disappeared after thrombin treatment, giving rise to immunopositive GST and unresolved low molecular mass peptides that migrated with the electrophoretic front. Therefore, the low molecular mass peptides that migrated with the electrophoretic front. Therefore, the low molecular mass species are not bacterial contaminants or fusion protein degradation products. They are likely to be early termination products of translation of plasmid-encoded sequences corresponding, from N- to C-terminal, to GST, a thrombin recognition site, and short additional sequences including the N-terminal part of the MR insert. The proportion of low molecular mass bands in relationship with the 65-kDa band varied in different preparations and in different strains of transformed bacteria. The case showed in Figure 1 corresponds to one of the highest ratios obtained.

In order to confirm that the IPTG-induced proteins were indeed fusions with GST, duplicate aliquots of extracts from induced bacteria, analyzed by SDS–PAGE and electrotransferred to membranes, were either stained with Coomassie blue (Figure 2a, lane 2) or immunostained using GST-reactive antibodies (Figure 2b, lane 1). Among the multiple proteins evidenced by Coomassie blue staining, only three of them were immunodetected, with molecular masses coincident to those of the major bands observed in purified preparations. In the photograph shown, only the predominant 65-kDa band is visible (Figure 2b, lane 1), while the two lower molecular mass bands are not perceptible. The 65-kDa band was the only one evident when an antibody raised against a synthetic peptide corresponding to the C-terminal part of the MR sequence was used for the immunodetection (data not shown).

Generation of [^{3}H]Aldosterone-Binding Activity by Reticulocyte Lysate. To determine whether the bacterially produced MR construct can bind its cognate or related ligands, incubations of the purified preparations were performed with [^{3}H]aldosterone and [^{3}H]dexamethasone in concentrations

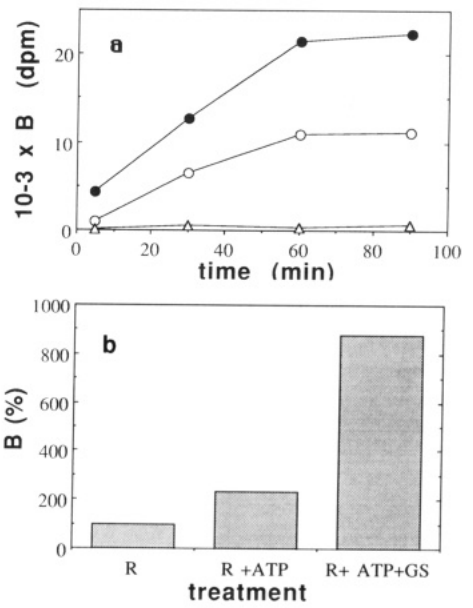

FIGURE 3: [^{3}H]Aldosterone-binding activity of the GST–MR purified preparation. Steroid-binding assays using 20 nM [^{3}H]aldosterone were performed on 50-μL aliquots of the preparation pretreated with rabbit reticulocyte lysate (R) as described under Experimental Procedures. Panel a: time course of R treatment. The purified preparation was preincubated with R for different periods a 0 (△) or 30 (○, ●) °C, with (●) or without (△, ○) the addition of 1 mM ATP/$MgCl_2$. Panel b: effect of supplementation of R with ATP and the ATP-regenerating system. The purified preparation was preincubated for 60 min at 30 °C with R alone, R plus 1 mM ATP/$MgCl_2$, or R plus 1 mM ATP/$MgCl_2$ and the ATP-regenerating system (GS). The steroid-binding activity of the resulting preparations is expressed as the percentage of the specific binding (B, %) obtained by treatment with R alone (100%).

up to 60 nM. Negligible specific binding was detected in these assays. However, after pretreatment with rabbit reticulocyte lysate at 30 °C, these preparations showed [^{3}H]-aldosterone-binding activity (Figure 3a,b). This activating effect was gradual, reaching maximum [^{3}H]aldosterone binding after 60 min of treatment. The supplementation of the incubation media with ATP to 1 mM concentration, in the absence or in the presence of an ATP-regenerating system, increased the maximum binding activity by 1.5- and 8-fold, respectively (Figure 3b). When similar preparations were incubated either alone at 37 °C or with reticulocyte lysate at 0 °C, the steroid-binding activity was negligible. No detectable binding was obtained when a nonrelated GST fusion protein (GST–dopamine receptor fragment) was incubated with reticulocyte lysate at 0 or 30 °C.

Characteristics of Steroid Binding. The purified preparations, treated with reticulocyte lysate in the presence of an ATP-regenerating system, were incubated with increasing concentrations of [^{3}H]aldosterone. As shown in Figure 4, the specific steroid binding was saturable. Scatchard analysis of the data (Figure 4, inset) indicated a linear plot consistent with a single class of sites. The value calculated for the apparent equilibrium dissociation constant (K_d, 4 °C) was 0.6 ± 0.2 nM ($n = 5$). The correspondent binding capacity values varied from 17 to 32 pmol/mg of purified protein.

To further explore the hormone-binding specificity of the system, we performed [^{3}H]aldosterone competition assays using different steroids. As seen in Figure 5 steroids known to interact with high affinity with the MR were all potent competitors of [^{3}H]aldosterone. The order of potency of these steroids (with the apparent K_i values given in parentheses, estimated from IC_{50} interpolations) was corticosterone (0.2 nM) ≥ deoxycorticosterone (0.3 nM) ≥ aldosterone (0.5 nM)

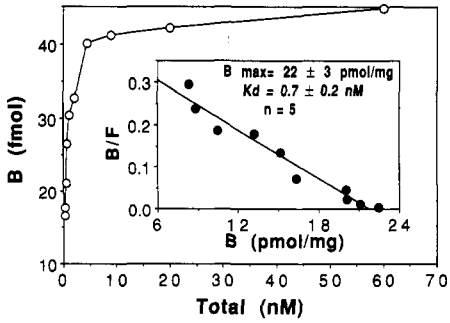

FIGURE 4: Saturation analysis of steroid binding. Aliquots of the GST–MR purified preparations (2 μg of protein), pretreated for 60 min at 30 °C with R (supplemented with ATP and the ATP-regenerating system), were incubated with [^{3}H]aldosterone (0.10–60 nM). The specific binding (B) was measured as described under Experimental Procedures. *Inset*: Scatchard plot of the saturation data. The binding capacity (B_{max}) was calculated as the amount of steroid bound (femtomoles) per milligram of protein of the purified preparation present in the aliquot. Values of B_{max} and K_d are expressed as the mean (plus and minus the standard error) of five independent experiments using different purified preparations.

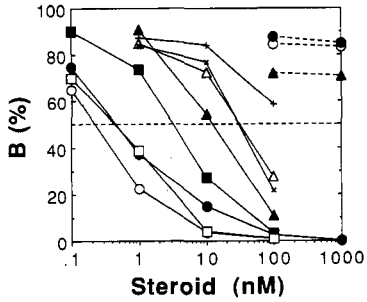

FIGURE 5: Steroid-binding specificity. Aliquots of the GST–MR purified preparations (2 μg of protein), pretreated for 60 min at 30 °C with R (supplemented with ATP and the ATP-regenerating system), were incubated with 1 nM [^{3}H]aldosterone in the presence of the amounts of nonradioactive steroids indicated on the abscissa: corticosterone (O—O); deoxycorticosterone (□—□); aldosterone (●—●); cortisol (■—■); spironolactone (▲—▲); progesterone (×—×); dexamethasone (△—△); RU26988 (O- - -O); RU38486 (●- - -●); testosterone (+—+); 17β-estradiol (▲- - -▲). The ordinate represents the radioactivity bound (B) as a percentage of that bound in the absence of nonradioactive steroids. Values were corrected for nonspecific binding (radioactivity bound in the presence of a 1000-fold excess of nonradioactive aldosterone).

> cortisol (1.4 nM) > spironolactone (6.5 nM) > progesterone (16 nM) > dexamethasone (18 nM). Neither synthetic steroids with selective high affinity for GR, like RU26988 (>1 mM) and RU38486 (>1 mM), nor sex steroid hormones like testosterone (>100 nM) or 17β-estradiol (>1 mM) competed efficiently.

Sedimentation Characteristics of the Steroid-Bound Receptor Complexes. In cellular extracts and under certain conditions, i.e., low ionic strength and/or in the presence of transition-metal anions like molybdate, most steroid receptors form large stable complexes of sedimentation coefficient 7–10 S that do not preferentially bind to DNA. In the case of native MR, the complexes are dissociated on gradients containing 0.4 M KCl, showing a shift in the sedimentation coefficient from 8.5 to 4.3 S (Rafestin-Oblin et al., 1989). Therefore, we decided to determine if the hydrodynamic characteristics of the [^{3}H]aldosterone–GST–MR protein complexes generated upon reticulocyte lysate incubation were in accordance with these findings. The sedimentation coefficient of receptor complexes prepared in the presence of molybdate was determined on a sucrose gradient in the absence or presence of KCl. As shown in Figure 6 the radioactive complexes sedimented at 8.9 ± 0.2 S (n = 4) in a gradient

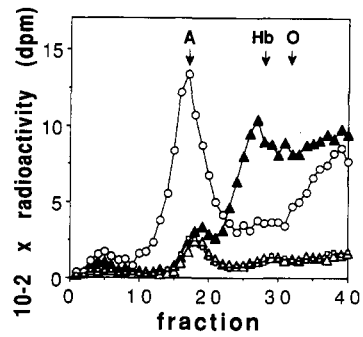

FIGURE 6: Sucrose gradient sedimentation of GST–MR complexes: effect of dissociating conditions. [^{3}H]Aldosterone–GST–MR complexes (0.2 mL), obtained in TEDM as described under Experimental Procedures in the absence (O, ▲) or in the presence (□, △) of a 100-fold excess of nonradioactive aldosterone, were applied on top of 5-mL sucrose gradients (10–30%). The gradients contained 20 mM Tris-HCl (pH 7.6) and either 20 mM sodium molybdate (O, △) or 400 mM KCl (▲, □). Following centrifugation at 50000g for 14 h at 2 °C, seven-drop fractions were collected from bottom to top and counted for radioactivity. The arrows on the top of the figure indicate the positions of external sedimentation standards: A, β-amylase (8.9 S); Hb, horse hemoglobin (4.2 S); O, ovalbumin (3.6 S).

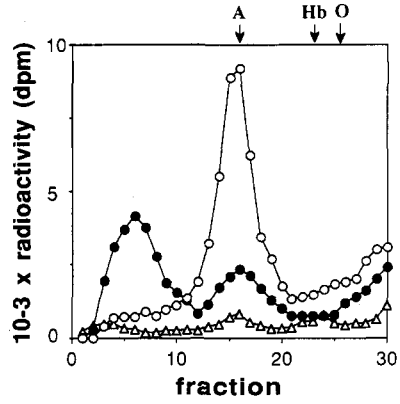

FIGURE 7: Anti-hsp90 monoclonal antibody 8D3 binds to the 9S complexes of GST–MR. [^{3}H]Aldosterone–GST–MR complexes (0.2 mL) obtained in TEDM were incubated for 1 h at 4 °C with 50 μL of undiluted control ascites (O) or 8D3 monoclonal antibody against hsp90 (●) and applied on top of 5-mL sucrose gradients (10–30%), prepared in 20 mM Tris-HCl (pH 7.6 at 0 °C) containing 20 mM sodium molybdate. Complexes generated in the presence of a 100-fold excess of nonradioactive aldosterone were also incubated with 8D3 antibody and run as a control (△). Following centrifugation at 50000g for 14 h at 2 °C, seven-drop fractions were collected from bottom to top and counted for radioactivity. The arrows on the top of the figure indicate the positions of external sedimentation standards: A, β-amylase (8.9 S); Hb, horse hemoglobin (4.2 S); O, ovalbumin (3.6 S).

containing 20 mM molybdate. Less than 3% of the radioactivity associated to the 8.9S peak sedimented at the bottom of the gradient, probably representing high molecular mass aggregates containing specific complexes. When the gradient contained 0.4 M KCl instead of molybdate, the complexes sedimented at 4.2 ± 0.3 S (n = 4). In either case, when the complexes were formed in the presence of a 100-fold excess of aldosterone, the radioactivity associated with the peaks virtually disappeared, evidencing saturable binding characteristics.

Binding of the 8D3 Monoclonal Antibody against hsp90 to the Steroid-Bound Receptor Complexes. In order to provide additional evidence of the association of hsp90 with the receptor complexes, sedimentation-shift analysis on sucrose gradients was performed. As seen in Figure 7, addition of 8D3 ascites to receptor complexes generated in the presence of molybdate increased their sedimentation coefficient, in relation to control ascites, from 8.9 to 12 S, respectively.

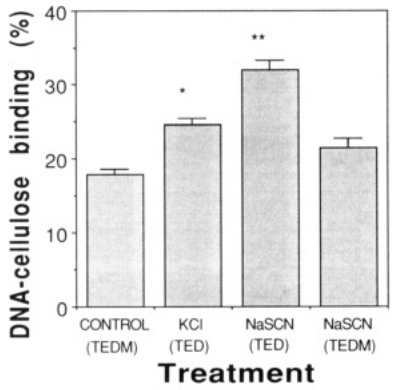

FIGURE 8: DNA–cellulose binding of GST–MR complexes: effect of different salt treatments. [^{3}H]Aldosterone–receptor complexes generated in TED or TEDM buffer (same as TEDGM and TEDG without glycerol) were adjusted to 300 mM KCl or 100 mM NaSCN. Control (TEDM, no adjustment), KCl (TED), NaSCN (TED), and NaSCN (TEDM) samples were preincubated for 30 min at 0 °C, further processed, and finally incubated with DNA–cellulose as described under Experimental Procedures. The ordinate represents the percentage of the [^{3}H]aldosterone–receptor complexes that bound to DNA–cellulose (mean ± SEM of six replicates per group). (One asterisk) $P < 0.001$ vs control; (two asterisks) $P < 0.001$ vs control and NaSCN (TEDM).

Effect of Different Salt Treatments on the DNA-Binding Characteristics of the Steroid-Bound Complexes. Recent studies have demonstrated that incubation of [^{3}H]aldosterone–MR complexes generated in mouse brain cytosol in the presence of the chaotropic anion thiocyanate increases the fraction of receptors retained by DNA–cellulose (Luttge & Emadian, 1988). Using similar conditions (Figure 8), we preformed radioactive complexes in the absence of molybdate. We then treated them with 100 mM sodium thiocyanate for 30 min at 30 °C. This led to a significant increase in DNA binding (79%) relative to untreated complexes. Similar treatment using 0.3 M KCl instead of thiocyanate produced a significant but less marked effect (37%). Both salts caused a significant dissociation of the [^{3}H]aldosterone complexes in relation to controls. The effect of thiocyanate was completely prevented when complexes were preformed in the presence of 20 mM molybdate.

DISCUSSION

In this paper, we explored the possibility of using a bacterially produced steroid receptor as a model for conducting structural and mechanistic studies at a reductionist level. As a first step, these studies require the development of a system suitable for expression of high amounts of steroid-binding protein. Here we show that *E. coli* can be express a recombinant GST–MR fusion construct to levels of 3–5% of the total soluble proteins (3–5 mg/L of cell culture). These levels of expression are approximately 3–4 orders of magnitude higher than those corresponding to any MR-expressing tissue. In addition, the use of GST as the carrier protein allows us to combine good levels of expression of soluble fusion protein with a simple and mild purification step. Thus, the presence of 5 mM glutathione in the eluant is sufficient for specific protein desorption from the glutathione–agarose matrix.

Beside the amounts of expressed protein and the suitability for simple purification procedures, additional factors like protein structure and biological properties are to be considered before selecting any expression system. Previous attempts at using bacterial systems for studying steroid receptors generated products with hydrodynamic and/or steroid-binding characteristics different from the native counterparts (Eul et al., 1989; Ohara-Nemoto et al., 1990; Power et al., 1990; Young et al., 1990; Roehrborn et al., 1992; Nemoto et al., 1992), casting doubt on the validity of the approach.

Our results show that the recombinant MR can be isolated in a non-steroid-binding state, and subsequently converted *in vitro*, by treatment with reticulocyte lysate, to a form exhibiting typical MR characteristics. The [^{3}H]aldosterone-binding affinity and specificity of the resulting preparations are in good agreement with values previously reported either for the native MR in rat brain (Moguilewsky & Raynauld, 1980; Veldhuis et al., 1982) or for recombinant MR expressed in mammalian (Arriza et al., 1987) and insect (Alnemri et al., 1991b; Binart et al., 1991) cell lines. The binding capacity for the same ligand (17–32 pmol/mg of purified protein) is the highest ever reported for a purified MR preparation. However, an estimate of the maximum capacity expected for the amount of fusion protein treated with lysate reveals that the experimental capacity is still 1% of the calculated value. This may result either from a low efficiency of the lysate treatment process, *per se*, or from a limited availability of the fusion protein in a structure suitable for lysate modification. A combination of both causes, and/or the presence of limiting amounts of unknown lysate factors that are required for the process is also possible.

The MR characteristics of the lysate-induced [^{3}H]aldosterone complexes of the fusion protein are further corroborated by its hydrodynamic and DNA-binding behavior. A sedimentation coefficient of 8.9 S is obtained in sucrose gradients containing 20 mM sodium molybdate. This coefficient value and the shift to 4.2S species observed in gradients containing 0.4 M KCl are similar to the characteristics reported for cytosolic complexes of the chick intestine MR in association with hsp90 (Rafestin-Oblin et al., 1989). The increase in DNA–cellulose binding of the radioactive complexes in response to treatments with KCl and the chaotropic anion thiocyanate is also in agreement with previous results using preparations of mouse brain MR (Luttge & Emadian, 1988). Also in accordance with these results is the effect of molybdate in completely suppressing the positive effect of thiocyanate.

Addition of rabbit reticulocyte lysate has been previously shown to promote the temperature- and energy-dependent reassociation of immunopurified progesterone and glucocorticoid receptors with heat shock proteins (Smith et al., 1990, 1992; Scherrer et al., 1990). Interestingly, significant reconstitution only occurred when purified preparations of receptors were used, the results being consistent with the presence of unknown inhibitory factors in the crude cytosolic preparations. We also used purified preparations in our protocol, a fact which may have contributed to our successful results. The positive effect on steroid-binding generation observed upon supplementation of the reticulocyte lysate with Mg^{2+}/ATP, alone or in combination with an ATP-regenerating system, suggests that the process in energy-dependent. In agreement with our finding, a strict Mg^{2+}/ATP requirement for assembly of progesterone receptor with heat shock proteins has been recently reported by Toft and co-workers (Smith et al., 1992). We are presently conducting experiments to more precisely define the nucleotide specificity and lysate factors involved in the process.

Our results using the 8D3 antibody against hsp90 evidence the association of this non-steroid-binding protein to the receptor complexes. Therefore, since the lysate-untreated purified preparation does not bind steroid, these data represent the first indication that the MR, like the GR (Bresnick et al., 1989; Dalman et al., 1989; Meschini et al., 1990; Nemoto et al., 1990), requires the association of hsp90 for proper steroid recognition. The participation of rabbit heat shock protein-

(s) during reticulocyte lysate incubation of the purified MR is strongly supported by the characteristics of the process and products. These characteristics include the temperature and ATP requirements (Scherrer et al., 1990; Smith et al., 1990, 1992) for the steroid-binding generation and the hydrodynamic behavior of the bacterially generated receptor complexes. However, in addition to the association with hsp90, at this time we cannot rule out the possibility of other receptor modifications induced by lysate treatment, e.g., phosphorylation. Our findings can also explain, at least in part, why a purified GR–fusion construct generated in bacteria (Bonifer et al., 1989), bearing the complete DNA- and steroid-binding domains, does not bind steroid. To more strictly correlate the functional requirements of the MR versus GR, we have recently used our bacterial system for the production of a GST–GR fusion receptor (unpublished results). Interestingly, as is the case with MR, this construct showed the same dependence on lysate treatment to evidence proper structure and steroid binding.

In conclusion, our results demonstrate that the combination of bacterial production of MR fusion protein with subsequent *in vitro* treatment with reticulocyte lysate can be used for generation of a receptor with properties similar to its authentic counterpart. Therefore, possible bacterial posttranslational modifications do not significantly interfere with the generation of the final functional products.

Interestingly, and at odds with MR or GR overexpression using baculovirus systems (Alnemri et al., 1991a,b; Binart et al., 1991) isomeric forms of the expressed steroid-binding proteins are not evident when bacterial systems are used (Eul et al., 1989; Nemoto et al., 1990, 1992; Ohara-Nemoto et al., 1990; Power et al., 1990; Young et al., 1990; Roehrborn et al., 1992). The structural homogeneity of the products, together with their potential and correct function, is factor of importance for future attempts at receptor crystallography.

Finally, the data is this report strongly suggest the first-time occurrence of sequential assembly *in vitro* of a recombinant steroid receptor. The findings also facilitate future studies on the mechanism and factors required for receptor assembly and function. In addition, the recombinant characteristics of the system extend its use to structure–function analysis of the receptor by site-directed mutagenesis.

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