Characterization of human mineralocorticosteroid receptor expressed in the baculovirus system

(aldosterone/transcription factor/90-kDa heat shock protein/anti-idiotypic antibody/anti-peptide antibody)

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ABSTRACT   To investigate the structure and function of the mineralocorticosteroid receptor (MR), one has to circumvent the major difficulty related to its very low abundance. For this purpose, the full-length human MR (hMR) has been produced using the efficient baculovirus system. The recombinant protein is overexpressed in Sf9 insect cells at a concentration of $\approx$2 pmol/mg of protein, which is 50–100 times more than the concentration in aldosterone target tissues. It binds aldosterone with high affinity ($K_d \approx 1$ nM) and clearly displays a mineralocorticosteroid specificity as evidenced by competition studies with steroid ligands and by the monoclonal anti-idiotypic antibody H10E interacting with the steroid-binding domain of MR. After [^{35}S]methionine labeling, a single polypeptide band at $\approx$120 kDa is detected and further identified as hMR by immunoblotting with A4, an anti-peptide antibody. Sedimentation analyses show that the native form of MR is recognized by A4 and B174, an antibody to the 90-kDa heat shock protein, since they both induce a shift of the receptor from 9S to 11S sedimentation coefficient. These results clearly demonstrate that MR is a heterooligomer containing the insect equivalent of the 90-kDa heat shock protein. This 9S receptor complex is converted to a 4S form during high-salt gradient ultracentrifugation, suggesting that MR can undergo a complete in vitro transformation. Immunofluorescence studies indicate that hMR, which is almost exclusively a cytoplasmic protein in Sf9 cells, is translocated to the nucleus after aldosterone exposure. Therefore, the recombinant hMR seems to behave as the native receptor. Given the possibility of large-scale protein production, the baculovirus system should prove useful in studies of the molecular basis of MR function as a transcription factor.

Structural and functional studies on mineralocorticosteroid receptor (MR) have always been hampered mostly because of its extremely low abundance within aldosterone target tissues. This protein belongs to the superfamily of thyroid/steroid hormone receptors known as hormone-dependent transcription factors (1). However, even if some of the biochemical properties of MR have been defined (2), many questions remain to be elucidated concerning the various steps between hormone binding, the activation process, and transcriptional gene regulation. By in vitro analyses, MR in its untransformed state has been detected as a heterooligomeric entity (9S form) that includes a 90-kDa heat shock protein (hsp90) (3). Protein components known to be associated with other steroid receptors (4–6) may also be part of the multimolecular MR complex but have not yet been clearly defined. Moreover, only a few studies have examined the activation, or transformation, process leading to a 4S form of the receptor able to bind DNA (7, 8). Structural MR modifications occurring during this transformation step involve

the release of hsp90 from the receptor (3, 9). Finally, studies on productive interaction of MR with hormone response elements and the nature of target genes remain rather limited (10–13).

To explore the structure–function relationships of MR, we have produced the full-length human MR (hMR) by using the baculovirus expression system (14, 15). This system, which allows generation of large quantities of recombinant protein (16), is extremely valuable as compared with transient expression, already achieved with hMR (9), which is time-consuming and tedious. In addition, unlike the Escherichia coli and yeast expression systems, which may generate solubilization problems and/or drastic decrease in hormonal affinities (17–19), the baculovirus expression system seems suitable for production of functional proteins as already described for insulin (20), epidermal growth factor (21), glucocorticosteroid (22–24), and thyroid hormone (25) receptors. The expressed receptor has been characterized and identified using specific antibodies directed against the binding subunit of MR and the associated protein hsp90. We demonstrate that the tested physicochemical characteristics of the recombinant receptor cannot be distinguished from those of the native protein.

MATERIALS AND METHODS

Materials. [1,2-^{3}H]Aldosterone (40–60 Ci/mmol; 1 Ci = 37 GBq) and L-[^{35}S]methionine (1000 Ci/mmol) were purchased from the Radiochemical Centre Amersham. RU486 was from Roussel Uclaf. ZK91587 was from NEN. Other steroids were purchased from Sigma. Steroid adsorption was prevented by the use of PEG 300 (3). The plasmid phMR3750 (10) was obtained from R. M. Evans and J. L. Arriza (Salk Institute). The monoclonal anti-idiotypic antibody H10E (26) was used as diluted ascites. The A4 rabbit polyclonal anti-peptide antibody was prepared in the laboratory and raised against a synthetic peptide in the N-terminal domain of hMR (27). The B174 rabbit polyclonal anti-peptide antibody (C. Radanyi, J. M. Renoir, and E.E.B., unpublished work) was raised against the C-terminal portion of hsp90.

Viruses and Cells. Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant viral stocks were produced and assayed in Spodoptera frugiperda (Sf9) cells. The cells were grown in TNM-FH medium (15) containing 10% stripped fetal bovine serum, either as monolayers or in suspension in spinner flasks.

Construction of Baculovirus Transfer Vector and Isolation of Recombinant Virus. The phMR3750 plasmid, which contains the entire hMR coding sequence, was cleaved by Xma III and Afl II, which cut 9 base pairs (bp) upstream and 35 bp downstream of the MR coding sequence, respectively. This

Abbreviations: MR, mineralocorticosteroid receptor; hMR, human MR; hsp90, 90-kDa heat shock protein; AcNPV, Autographa californica nuclear polyhedrosis virus.
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fragment was inserted into the BamHI site of the transfer vector pVL941 (28), after all ends were made blunt by treatment with T4 DNA polymerase, leading to the recombinant pVL941-hMR. The recombinant baculovirus AcNPV-hMR was produced by in vivo homologous recombination. The resultant viral pool was screened and occlusion-negative viruses were plaque-purified as described (15).

Expression of hMR. Sf9 cells were routinely infected with the recombinant virus at a multiplicity of infection > 10. After infection, the cells were rinsed with phosphate-buffered saline (PBS) and homogenized, with a glass–glass Potter-type homogenizer, in TEG buffer [20 mM Tris·HCl/1 mM EDTA/ 10% (vol/vol) glycerol, pH 7.4 at 20°C] or TEGMo or TEGW buffer (20 mM sodium molybdate or tungstate in TEG buffer). Cytosols were obtained after centrifugation at 4°C at 105,000 × g for 45 min.

Binding Assays. Cytosol was incubated for 3–4 hr at 4°C with 10 nM [³H]aldosterone in the absence or presence of a 100-fold excess of unlabeled aldosterone. Bound and unbound steroid were separated by the dextran/charcoal technique (3). Values of steroid binding parameters were measured at equilibrium. The specificity of binding was assessed by competition studies.

Metabolic Labeling and Immunoblotting of Infected Sf9 Cells. Sf9 cells (6 × 10⁵ per well) were infected with AcNPV-hMR virus. Mock-infected and wild-type-infected cells were used as controls. After 1 hr of viral absorption, the inoculum was replaced with fresh medium. After 24 or 48 hr of infection, the medium was removed and the cells were incubated for 5 hr with 10 μCi of [³⁵S]methionine in methionine-free TNM-FH medium. Cells were rinsed with PBS and then suspended in SDS sample buffer. The lysates were submitted to electrophoresis and the proteins were electroblotted on a nitrocellulose membrane that was blocked with 10% nonfat dry milk in PBS for 1 hr at 37°C and then incubated with A4 poly clonal antibody. The presence of hMR was visualized using the Vectastain ABC immunoperoxidase system (Vector Laboratories). The same membrane was also autoradiographed at −80°C for 18–72 hr.

Sedimentation Gradient Ultracentrifugation. Glycerol gradients were prepared in the appropriate buffer as mentioned in the figure legend and ultracentrifugation was performed as described (3).

Immunohistochemical Studies. Baculovirus-infected Sf9 cells were cultured on small glass coverslips, washed in PBS, fixed in 3% paraformaldehyde in PBS for 30 min at room temperature, and permeabilized with 1% (vol/vol) Triton X-100 in PBS for 5 min. The A4 antibody (1:500 dilution) was applied for 30 min and followed by the fluorescein-coupled goat anti-rabbit IgG antibody (1:20 dilution) for an additional 30 min. The coverslips were counterstained with Evans blue and mounted in Moviol (Hoechst). The specificity of immunostaining was assessed by a nonimmune serum.

RESULTS AND DISCUSSION

Steroid Binding Characteristics of the Expressed hMR. Infection of Sf9 cells with the recombinant virus AcNPV-hMR resulted in the expression of hMR as demonstrated by high aldosterone binding capacity observed in the cytosol of infected cells. No specific aldosterone binding was detected in the cytosol of mock-infected or wild-type-infected Sf9 cells. Time-course studies indicated that aldosterone binding was significant 24 hr after infection (1.3 pmol/mg of protein) and reached a maximum 48 hr after infection (1.7 pmol/mg of protein). Specific aldosterone binding was almost undetectable 3 or 4 days after infection. This time course of hMR expression, as measured by steroid binding, was in accordance with the results of the [³⁵S]methionine labeling and immunoblotting experiments (see below). Consequently, we routinely used a 40- to 48-hr infection period. Scatchard analysis revealed that aldosterone bound to the expressed hMR with high affinity (Fig. 1A). One class of specific sites was detected with a K_d value of 0.96 ± 0.08 nM, a value identical to that reported for the hMR expressed in the transfected COS cells system (9, 10) and in the same range as the native hMR found in the kidney (29), colon (30), or the human cell line HT29 (31). The binding capacity was 1.91 ± 0.10 pmol/mg of protein, a receptor concentration about 50–100 times higher than that generally recovered in the cytosol of aldosterone target tissues (2). In the presence of sodium tungstate (TEGW buffer) in the cytosol, the binding

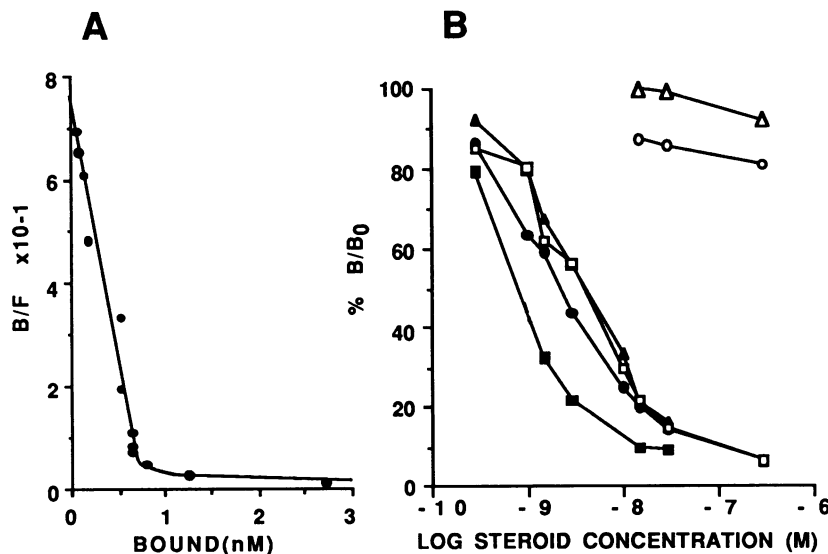**A**   **B**

FIG. 1. Steroid binding characteristics of the expressed hMR in Sf9 cells. (A) Scatchard plot of aldosterone binding to recombinant MR. Diluted cytosol aliquots were incubated with 0.1–300 nM [³H]aldosterone for 3 hr at 4°C. Bound (B) and free (F) hormone were separated by treatment with dextran-coated charcoal. Each point is an average of triplicate determinations. (B) Steroid specificity. Diluted cytosol was incubated for 3 hr at 4°C with 3 nM [³H]aldosterone either alone or in the presence of various concentrations of unlabeled competitors: aldosterone (●), cortisol (▲), deoxycorticosterone (■), ZK91587 (□), estradiol (○), and RU486 (△). Results are expressed as the percentage of binding measured with [³H]aldosterone alone (mean of three different experiments).

capacity was increased by 40% and 300% as compared with that in TEGMo and TEG buffer respectively, further confirming the high stabilizing effect of sodium tungstate (9). The high level of hMR expression that can be reached with the baculovirus system constitutes by itself, for practical reasons, a breakthrough for further studies of this rare steroid receptor.

The expressed hMR clearly displayed mineralocorticoid specificity as demonstrated by competition assays (Fig. 1*B*). Unlabeled aldosterone competed with [^{3}H]aldosterone for binding to the hMR, with an IC$_{50}$ of ≈1 nM consistent with the K_d value. Deoxycorticosterone, cortisol, and ZK91587, an antimineralocorticosteroid compound, all were potent competitors for the recombinant hMR. The high intrinsic affinity of hMR for cortisol *in vitro* has already been demonstrated with transfected COS cells (9, 10). This high affinity of MR for glucocorticosteroids may not be involved in *in vivo* steroid action, where the existence of the 11β-hydroxysteroid dehydrogenase in target cells confers an aldosterone selectivity on MR (32, 33). In contrast, estradiol and RU486, a synthetic steroid that does not bind to MR (3, 34), were unable to displace [^{3}H]aldosterone from the recombinant MR. The steroid binding specificity of the expressed protein, characteristic of MR, was further confirmed by the ability of the monoclonal anti-idiotypic antibody H10E to completely abolish [^{3}H]aldosterone binding to the receptor (see below). This immunological recognition of the functional steroid-binding domain gives additional evidence that the expressed receptor in insect cells has a conformation identical to that of the *bona fide* protein.

Identification of hMR by Metabolic Labeling and Western Blot Analysis. The nature of the hMR protein produced in insect cells was examined after metabolic labeling and compared with the Western blot analysis (Fig. 2). Sf9 cells infected with the recombinant virus AcNPV-hMR were labeled with [^{35}S]methionine 24 or 48 hr after infection. After lysis, various amounts of cellular proteins were submitted to electrophoresis and immunoblotted. The nitrocellulose membrane was then autoradiographed. Fig. 2*A* shows the pattern of ^{35}S-labeled proteins. Many bands corresponding to the newly synthesized proteins were detected 24 hr after infec-

tion (lanes 1–3). At 48 hr, when 1/10th as much material was loaded, a single protein band with a molecular mass of ≈120 kDa was seen (lanes 4–8). As expected, in cells infected with the wild-type virus (lane 9) the major identified cellular protein was polyhedrin (≈30 kDa), whose expression is directly controlled by its own promoter and can be synthesized to constitute up to 50% of the total protein. Although controlled by this strong promoter, hMR expression never reached such a level. Mock-infected cells (lane 10) did not display any significant protein bands; however, the bands became detectable after longer exposure of the membrane or when the dried gels were directly autoradiographed. The ≈120-kDa protein band fit exactly with the signal revealed after immunoblotting with the A4 polyclonal antibody (Fig. 2*B*). The immunoreactive hMR visible at both infection periods was strongest at 48 hr postinfection. The width of this band may correspond to the large amount of protein or, more likely, may indicate the presence of several isoforms corresponding to various posttranslational modifications of the synthesized protein. Nevertheless, this molecular mass is in accordance with the 107-kDa value calculated from the deduced amino acid sequence of hMR (10). No detectable immunoreactive protein was seen in wild-type-infected (lane 9) or mock-infected (lane 10) Sf9 cells.

Based on the loaded material and the intensities of both autoradiographic and immunoblotting signals, the amount of expressed hMR appears to increase 5- to 10-fold between 24 and 48 hr postinfection. This is in accordance with the results of the time-course binding studies. However, the amount of expressed hMR determined by aldosterone binding is probably underestimated (≈10^5 receptors per cell) because of the strong intensity of the signal observed on the Western blot. It seems likely that only a portion of the expressed receptor is able to bind hormone, as already reported for other steroid receptors produced in other expression systems (17, 35). This suggests that steroid binding by hMR requires posttranslational modifications. Some maturation factors present in Sf9 cells might become limiting due to alteration of cellular function by viral infection.

Physicochemical Characterization and Subunit Composition of MR. We analyzed the sedimentation behavior and subunit
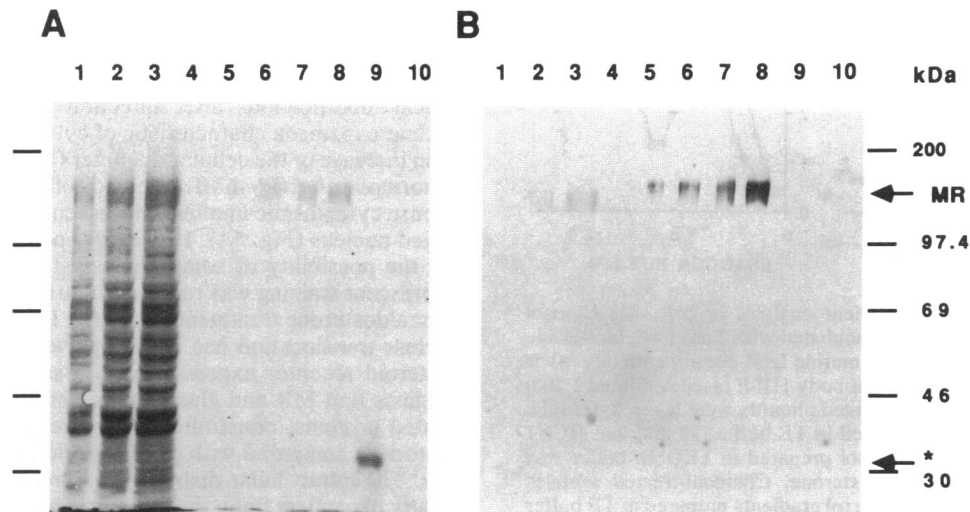

FIG. 2. Metabolic labeling and immunoblotting of recombinant MR. Cell lysate proteins were subjected to SDS/7.5% PAGE and transferred to a BA85 nitrocellulose membrane (Schleicher & Schuell). At 24 hr (lanes 1–3) or 48 hr (lanes 4–10) after infection, [^{35}S]methionine-labeled Sf9 cells that were infected with AcNPV-hMR virus (lanes 1–8) or the wild-type virus (lane 9) or were mock-infected (lane 10) were lysed by boiling in SDS sample buffer for 3 min. The samples loaded corresponded to the following numbers of cells (× 10^{-5}): 2.4 (lane 1); 4.8 (lane 2); 7.2 (lane 3); 0.24 (lane 4); 0.48 (lane 5); 0.72 (lane 6); 0.96 (lane 7); 1.2 (lane 8); 2.4 (lanes 9 and 10). The star indicates the position of polyhedrin. The Rainbow protein molecular size markers (Amersham) were myosin (200 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa). (*A*) Autoradiography of ^{35}S-labeled proteins. (*B*) Western blotting with the A4 polyclonal antibody (1:250 dilution).

composition of the expressed MR by using highly specific antibodies directed against both the steroid-binding receptor subunit and the associated hsp90. When the cytosol was prepared in buffer containing tungstate, MR was recovered as a single specific peak at 9.60 ± 0.18 S (mean ± SEM, $n = 12$) (Fig. 3A). The monoclonal anti-idiotypic antibody H10E, which recognizes the steroid-binding domain of MR (26), was able to completely inhibit [³H]aldosterone binding to the untransformed receptor (Fig. 3A). The immunoreactivity of H10E for the recombinant hMR extends the data obtained in transfected COS cells (27) and indicates a proper folding of the steroid-binding domain of the protein expressed in insect cells. In the presence of molybdate (Fig. 3B), MR sedimented at 8.50 ± 0.24 S ($n = 5$), a value significantly lower than that of tungstate-stabilized MR, suggesting that the apparent subunit composition of recombinant MR is dependent upon the experimental conditions. Indeed, a number of studies have reported the association of several proteins besides hsp90 with the untransformed state of steroid receptors (5, 6). The nature of the oxyanion used to stabilize the oligomeric

structure of MR may modulate the interaction between these proteins.

When analyzed in gradients containing 0.4 M KCl, molybdate-stabilized MR sedimented at 4.2 S, indicating a complete receptor transformation that could not be obtained with the tungstate-stabilized MR (Fig. 3B). All attempts to induce *in vitro* transformation of the recombinant MR by heat or salt treatment led to an almost complete loss of steroid binding activity. Our experimental protocol (i.e., 0.4 M KCl/glycerol gradient ultracentrifugation) was the only procedure allowing receptor transformation without loss of steroid binding, probably because the glycerol decreased the hormone dissociation rate. After incubation with A4 antibody, the expressed MR was shifted to the 11S region of the gradient, whereas an unrelated antiserum did not have any significant effect (Fig. 3C). This clearly indicates that the anti-peptide antibody A4 specifically recognizes the native form of hMR in its hetero-oligomeric structure as well as the denatured form of the receptor as evidenced on Western blots. Previous work showed that the untransformed form of chicken MR was associated with hsp90 (3). The presence of hsp90 in the recombinant MR structure was investigated using the polyclonal anti-peptide antibody B174, which is directed against hsp90. Fig. 3D depicts the shift of the 9S form of the receptor into the 11S region after incubation with B174, providing evidence that the MR expressed in the baculovirus system interacts with the insect equivalent of mammalian hsp90. The physicochemical characteristics of the hMR reported in this paper are virtually identical to those of MR from various aldosterone target tissues (2, 3). It should be interesting to check the effects of aldosterone antagonists on the recombinant MR structure and to know whether or not they dissociate rapidly and facilitate hsp90 release as reported in other models (9, 36).

Intracellular Distribution of hMR. Very little information is available concerning the intracellular localization of MR. On rat or rabbit kidney histological sections, immunoreactive MR appears to be both cytoplasmic and nuclear. The intracellular compartmentation was not modified after adrenalectomy or aldosterone treatment (37, 38). It was therefore of interest to study the cellular distribution of the expressed hMR in Sf9 cells. We applied an indirect immunofluorescence method using the A4 antibody. The validity of this approach was strengthened by the specificity of A4 antibody, which recognized a single band in Western blots (see Fig. 2B). It is noteworthy that Sf9 cells undergo important morphological modifications after infection by baculoviruses. A nuclear expansion characteristic of cytopathic effects results in an increase of the cellular diameter (Fig. 4). In the absence of hormone, AcNPV-hMR-infected cells clearly displayed an intense cytoplasmic immunofluorescence surrounding an enlarged nucleus (Fig. 4A). However, one cannot entirely rule out the possibility of some nuclear staining. The immunofluorescent staining was readily redistributed over the nuclei after aldosterone treatment (Fig. 4B). This hormone-induced nuclear translocation has been reported for the glucocorticosteroid receptor expressed in the same system (24) and suggests that MR and glucocorticosteroid receptor, closely related proteins, constitute a separate group of steroid receptors as compared with estrogen and progesterone receptors. The intracellular distribution of receptors seems to be greatly dependent upon the biological material and the methodology employed (39) and may therefore explain in part the discrepancy observed between insect cells and whole mammalian kidney (37, 38). No immunofluorescence was observed when cells were stained either with a nonimmune rabbit antiserum as primary antibody or with fluorescein-conjugated goat anti-rabbit IgG antibody alone (data not shown). In addition, the specificity of the immunostaining was further strengthened by the negative results obtained
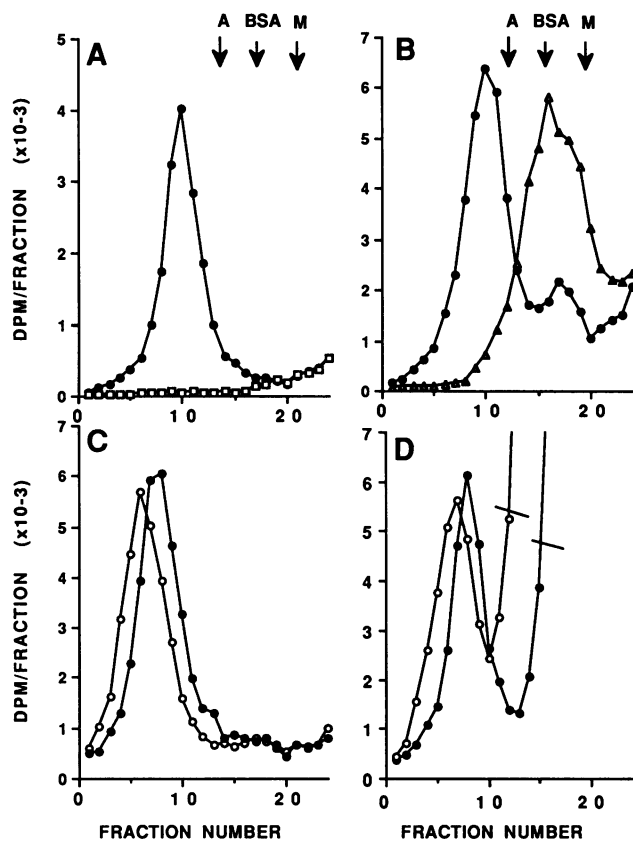

FIG. 3. Sedimentation gradient analysis of MR. (A) Cytosol prepared in TEGW buffer was incubated with 2 nM [³H]aldosterone in the presence of a nonimmune murine IgG1 control antibody (●) or the monoclonal anti-idiotypic antibody H10E (ascites diluted 1:100) (□) for 4 hr at 4°C. Charcoal-treated aliquots were layered onto the top of glycerol gradients prepared in TE buffer (20 mM Tris·HCl/1 mM EDTA, pH 7.4). (B) Cytosol prepared in TEGMo buffer was incubated with 5 nM [³H]aldosterone. Charcoal-treated samples were loaded onto the top of glycerol gradients prepared in TE buffer (●) or in TE containing 0.4 M KCl (▲). (C) After charcoal treatment, tungstate-stabilized MR labeled with [³H]aldosterone was incubated for 3 hr at 4°C with 50 μl of either A4 polyclonal antibody (○) or nonimmune rabbit antiserum (●). (D) [³H]Aldosterone-labeled MR, prepared in TEGW, was incubated with 10 μl of either B174 antibody (○) or nonimmune rabbit antiserum (●) for 3 hr at 4°C. Gradients were ultracentrifuged in a Beckman SW60 rotor for 18 hr at 50,000 rpm at 4°C. External standards were myoglobin (M, 2 S), bovine serum albumin (BSA, 4.6 S), and aldolase (A, 7.9 S).
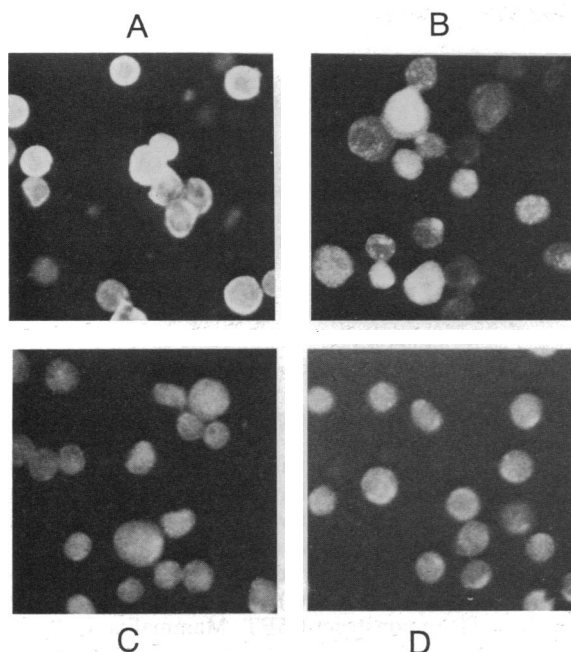
FIG. 4. Immunolocalization of MR in Sf9 cells. The distribution of expressed hMR in Sf9 cells was examined by indirect immunofluorescence staining with A4 polyclonal antibody (1:500). Immunofluorescence micrographs of infected Sf9 cells are shown. At 40 hr postinfection AcNPV-hMR-infected cells were incubated in the absence (A) or presence (B) of 10 nM aldosterone for 90 min. Sf9 cells infected with wild-type virus (C) or mock-infected (D) are presented as controls. (×400.)

with AcNPV-infected (Fig. 4C) or mock-infected (Fig. 4D) cells. Preliminary studies indicate that the antimineralocorticoid compound ZK91587 may partially inhibit the nuclear translocation of hMR induced by aldosterone (data not shown). Further experiments are needed to evaluate this antagonistic effect.

In conclusion, the baculovirus expression system is capable of producing substantial amounts of hMR whose tested biochemical and cytochemical properties are indistinguishable from those of authentic MR. Until now, purification of soluble receptor was not feasible due to the lack of a rich source of receptors or an appropriate expression system. The large-scale production of recombinant receptor, together with a suitable purification procedure by affinity chromatography (40) or immunoaffinity, should allow one to obtain sufficient amounts of MR for detailed structural and functional studies. This system should facilitate our understanding on MR function, in particular the crucial transformation step and the MR interaction with response elements and the transcriptional machinery responsible for regulation of gene expression.

Note. Just before submission of this manuscript, G. Litwack sent us a paper (41) reporting the expression of the hMR in the baculovirus system. The results are in general agreement with our observations.

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