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Purification and characterization of recombinant human Mineralocorticoid Receptor

Colin D. Clyne¹, Ching-Yi Chang², Rachid Safi², Peter J. Fuller¹, Donald P. McDonnell², and Morag J. Young^{1,*}

¹Prince Henry's Institute of Medical Research, Clayton, Melbourne, Victoria, Australia, 3168

²Dept Pharmacology and Cancer Biology, Duke University, Durham, NC, United States, 27710

Abstract

The mineralocorticoid receptor (MR) plays a critical role in the maintenance of electrolyte homeostasis and blood pressure *via* direct effects on the distal nephron and the cardiovascular system. The MR also has an important role in the pathology of cardiovascular disease, particularly heart failure, and is therefore an attractive therapeutic target. However, renal side effects limit its use in the clinic. Previous studies of MR molecular pharmacology have been performed on its isolated ligand-binding domain (LBD); however, current evidence suggests that nuclear receptor LBDs behave differently in isolation, than in the context of the full-length receptor. To date, technical issues have precluded production of full-length MR, thereby preventing molecular and structural studies of the MR LBD in its natural context. Here, we describe expression and purification of full-length human MR (hMR). hMR was expressed in Sf9 insect cells with an N-terminal biotinylated (bt)- tag, and stabilised by addition of ligand. bt-hMR exhibited ligand-binding and transactivation properties similar to that of the native protein. Affinity purification using an avidin matrix yielded ~120µg MR protein from 0.5lt Sf9 culture, and the receptor was purified bound to either aldosterone or cortisol. Recombinant hMR had a molecular weight of 110-130kDa, bound an MR DNA response element *in vitro* and interacted with a known coregulator, PGC-1α, in GST pull-down assays, indicating its functional activity. Availability of this reagent will now enable analysis of MR structure and ligand interactions in the context of the full-length receptor, a prerequisite for future development of ligand-selective MR antagonists for the treatment of cardiovascular disease.

Keywords

Mineralocorticoid receptor; Protein Purification; Baculoviral expression

Introduction

The mineralocorticoid receptor (MR, NR3C2) is the largest member of the steroid receptor family of transcription factors (Arriza et al. 1987). It is best known for its role in the maintenance of electrolyte homeostasis and blood pressure through direct effects on the distal nephron (reviewed in (Fuller and Young 2005)). This is exemplified by activating mutations

* Corresponding author: Morag Young, Prince Henry's Institute, PO Box 5152, Clayton, Victoria, AUSTRALIA, Tel: +61 3 9594 4286, Fax: +61 3 9594 6125, Email: E-mail: morag.young@princehenrys.org.

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within the MR which manifest with severe early-onset hypertension (Geller et al. 2000). In the last 10-15 years a role for the MR in the cardiovascular system has also been described. In particular, several clinical trials (RALES, EPHESUS and 4E) have demonstrated that MR blockade by spironolactone or eplerenone can significantly improve patient outcomes in terms of both mortality and morbidity in severe heart failure (Pitt et al. 2003a;Pitt et al. 2003b;Pitt et al. 1999). However, broad use of these antagonists for cardiovascular disease has been limited by side effects associated with receptor non-selectivity (spironolactone) or off-target effects (inhibition of renal MR leading to hypokalemia). The identification of tissue- and ligand-selective MR antagonists would therefore be of considerable benefit.

Despite this, the MR remains the least well understood member of the steroid receptor family. This has largely been due to difficulties in producing large quantities of pure, biologically active full-length receptor for structural and biochemical analyses. The isolated MR ligand-binding domain (LBD) has been successfully purified and crystallised (Bledsoe et al. 2005;Fagart et al. 2005;Huyet et al. 2007;Li et al. 2005), yielding valuable information on structure: function relationships and determinants of ligand-binding specificity. However, it is now clear that for other steroid receptors (for example the estrogen receptor), the conformation adopted by the ligand-binding pocket in the context of the full-length receptor differs significantly from that adopted in the context of the isolated LBD (Bapat and Frail 2003). This is of particular relevance for the MR, since the MR exhibits an N/C-terminal interaction that subtly alters its pharmacology and that may contribute to ligand-specific transcriptional events (Rogerson and Fuller 2003). For these reasons, interrogation of MR structure and ligand interactions in the context of the full-length receptor would appear to be crucial for future drug development.

To date full-length recombinant MR has resisted purification. This is a consequence both of its inherent *in vitro* instability (Galigniana 1996) and lack of a suitable host in which to express the active receptor at high level. Bacterial systems, whilst suitable for expression of isolated MR domains, have not yielded full-length receptor due to problems with solubility and aggregation. While the full-length MR has been successfully expressed in fission yeast (Bureik et al. 2005), its ligand binding properties in this system do not mirror those of the native receptor. The most promising approach has been to use baculovirus to express the MR in *Spodoptera frugiperda* (Sf9) insect cells (Alnemri et al. 1991;Binart et al. 1991). Baculovirus-expressed MR displays high-affinity aldosterone and corticosteroid ligand binding specificity similar to that of the native receptor (Binart et al. 1991). However, less than 0.5% of the total expressed MR is soluble, presumably because the majority of protein forming insoluble nuclear aggregates as the over-expressed protein exceeds the limiting amounts of insect cell heat-shock proteins and other chaperones required for MR stability (Alnemri and Litwack 1993). These limiting amounts of soluble MR are not sufficient to permit purification of the protein.

Here, we describe a method for the rapid purification of large quantities of biologically active full-length human MR (hMR). By expressing the hMR in Sf9 cells fused to a biotinylated tag, and stabilising the receptor with ligand, we used affinity chromatography to isolate purified hMR under native conditions in amounts sufficient for use in functional and structural studies such as epitope mapping by peptide phage display.

Materials and Methods

Plasmids

Full length (1-984) human MR was amplified from pRShMR (a gift from Professor R. M. Evans), by two rounds of PCR introducing appropriate restriction sites (5': *AscI*, 3': *ApaI*) and protease cleavage (enterokinase) sites. The primers used were as follows (all 5'-3'; protease and restriction sites *italicized*): round 1 sense: *gatgacgatgataagatggagaccaaaggctaccac*; round

1 antisense: gatcgggccctcacttcggtggaagta; round 2 sense: gatcggcgcccgatgacgatgataagatg; round 2 antisense: gatcgggccctcacttcggtggaagta. The PCR product was cloned into pGEM-T-Easy (Promega Corp., Madison, WI) and sequenced to confirm PCR fidelity. The MR cDNA was subcloned (using *AscI*, and *ApaI*) into the bacmid shuttle vector pDW464 (Duffy et al. 1998), to create pDW464hMR, which encodes an N-terminal fusion between the biotin acceptor peptide (BAP) and the MR. The cDNA encoding BAP-tagged MR was subcloned from pDW464hMR into pcDNA3 (Invitrogen, Carlsbad, CA) by first digesting pDW464hMR with *BsiW1*, blunting with Klenow enzyme, digesting with *ApaI* and ligating into *EcoRV* / *ApaI*-digested pcDNA3. To make the biotin ligase (*BirA*) expression construct, the *BamHI* – *HinDIII* fragment of pDW464 was subcloned into pcDNA3.1-(Invitrogen). pDEST15-PGC-1 α ₆₋₂₂₅ encodes a fusion between GST and the acidic activation domain (amino acids 6-255) of human PGC-1 α and was a gift from Dr. L. Grasfeder, Dept. Pharmacology and Cancer Biology, Duke University, NC. The MMTV-luciferase reporter (MMTV-luc) and the β -galactosidase expression vector (pCMV β) have been described previously (Mettu et al. 2007).

Cell culture, transfection and reporter assays

HepG2 human hepatoma cells were maintained in MEM (Invitrogen) supplemented with 8% fetal bovine serum (HyClone Laboratories, Logan, UT), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen) in a humidified 37°C incubator with 5% CO₂. Cells were seeded in phenol red-free MEM (Invitrogen), supplemented with charcoal stripped fetal bovine serum and additives as before, onto 24-well cell culture plates at 50% confluence. Cells were transfected 24h later using (per triplicate well) 1500ng MMTV-luc reporter, 100ng of hMR expression construct (wild-type or BAP-tagged), 100ng pcDNA3-BirA, 100ng pCMV β -gal and 1200ng of pBluescript using 2 μ l per well Lipofectin Reagent (Invitrogen) following the manufacturer's instructions. Steroid treatments were added 24h later in MEM/charcoal-stripped media and transactivation responses determined the following day using standard luciferase/ β -gal assays.

Generation of recombinant baculovirus and expression of hMR in Sf9 cells

Recombinant hMR-expressing baculovirus particles were generated by recombining pDW464hMR with baculovirus DNA *in vivo*, using the Bac-to-Bac® baculovirus expression system (Invitrogen) following the manufacturer's recommended procedure. Sf9 cells were grown as suspension culture in HyQ SFX Insect medium (Hyclone) at 27°C to a density of 2 $\times$ 10⁶ cells / ml and infected with recombinant baculovirus at a MOI of 0.5. 24h later, ligands were added as indicated and the cells incubated for a further 24h before being harvested by centrifugation and stored at -80°C.

Affinity purification of hMR

Sf9 cell pellets derived from 500ml culture were resuspended in 40 ml hypotonic buffer (10 mM Tris-HCl, pH 8.0; 10 mM NaCl; 1.5 mM MgCl₂; 10 mM β -mercaptoethanol; 1 μ M ligand; 80 μ l Protease Inhibitor Cocktail Set III (Calbiochem)) and incubated on ice for 15 min. Nuclei were released by homogenisation using a Dounce tissue grinder (25 strokes, "tight" pestle) and collected by centrifugation (7,000g, 10 mins, 4°C). The nuclear pellet was resuspended in 15 ml nuclear-extract buffer (10 mM Tris-HCl, pH 8.0; 0.3 M NaCl; 1.5 mM MgCl₂; 10 mM β -mercaptoethanol; 50 mM β -glycerophosphate; 50 mM NaF; 1 μ M ligand, 20 μ l protease inhibitor cocktail set III) and incubated with gentle agitation for 30 min at 4°C. The nuclear extract was clarified by centrifugation at 50,000g for 30 minutes at 4°C.

hMR was purified from Sf9 nuclear extracts using SoftLink Soft Release Avidin Resin (Promega). The affinity matrix was equilibrated in 5 vol of 0.1M NaPO₄ (pH 7.0), and non-reversible biotin binding sites blocked according to the manufacturer's instructions. 250 μ l resin

was added to 15ml Sf9 nuclear extract and incubated with rotation for 2h at 4°C. Samples were centrifuged for 2 min at 2000g and the supernatant (flow-through fraction) was discarded. The resin was washed twice with 40 volumes of wash buffer 1 (20mM Tris-HCl, pH 8.0; 100mM KCl; 10mM β -mercaptoethanol; 10% glycerol; 1 μ M ligand) and once with 40 volumes of wash buffer 2 (20mM Tris-HCl, pH 8.0; 1M KCl; 10mM β -mercaptoethanol; 10% glycerol; 1 μ M ligand). hMR was recovered in 3 successive fractions by incubation with 0.5ml elution buffer (50mM Tris-HCl, pH 8.0; 200mM NaCl; 4mM DTT; 10% glycerol; 1mM CHAPS; 5mM Biotin; 1 μ M ligand) for 10 mins at 4°C.

SDS-PAGE and Western blotting—Protein samples (total Sf9 cell lysates or purified protein fractions) were separated on 10% polyacrylamide gels and either visualised directly using Coomassie brilliant blue G-250 or silver staining, or electoblotted onto PVDF membranes (2h at 200mA). Membranes were blocked with 5% low-fat milk in PBST (PBS containing 0.1% Tween20) for 1h at room temperature. To detect the biotinylated MR tag, membranes were incubated with streptavidin-HRP conjugate (1:10,000; GE Healthcare, Piscataway, NJ) for 1h at room temperature. To detect the MR protein, membranes were incubated with anti-MR antibody (antibody rMR1–18 6G1 (Gomez-Sanchez et al. 2006) kindly provided by Professor Celso Gomez-Sanchez, University of Mississippi Medical Center) for 1h at room temperature (1:25,000 dilution) followed by anti-mouse IgG-HRP conjugate (Sigma, 1:20,000) for 30 min at room temperature. Membranes were then washed and developed using the Western Lighting Chemiluminescence Reagent (Perkin Elmer Life Sciences, Boston, MA).

Radioligand binding assay

Sf9 cells were cultured in 96-well dishes (100,000 cells / well) and infected with hMR baculovirus as described above. After 24h, aldosterone was added to 1 μ M and the incubation continued for a further 24h. Cells were washed three times (each for 30 min) with 1ml HyQ SFX Insect medium, and incubated with [1,2,4,6,7-³H]dexamethasone (2 nM final concentration in HyQ SFX medium) in the presence or absence of various unlabeled compounds at the concentrations indicated, for 1h at room temperature. Cells were then washed three times with ice-cold PBS (300 μ l /well) and the cell-associated radioactivity recovered in 100 μ l of 1N NaOH, and quantified by scintillation counting.

DNA binding assay

Costar 96-well plates were coated with 20 μ g / well neutravidin (Pierce Biotechnology, Rockford, IL) in 100 μ l of 0.1M NaHCO₃, pH 8.5 for 2h at room temperature. Wells were blocked with 150 μ l 2% milk in 0.1M NaHCO₃, pH 8.5 for 1 hr at room temperature and washed 5 times with 300 μ l PBST. Wells were coated with (or without) double-stranded biotinylated DNA (2pmol in 100 μ l PBST) containing a consensus GR response element for 1 hr at room temperature, and any remaining neutravidin sites were blocked by adding 0.1mM biotin for 1 hr at room temperature. Wells were washed 5 times with 300 μ l PBST, and incubated with 0.5 μ g of purified hMR (in 100 μ l PBST $\pm$ the indicated ligands) overnight at 4°C. The plate was washed 5 times with PBST, and 50 μ l of 1X SDS sample loading buffer added per well. The plate was heated for 10 min at 95°C and samples analyzed by SDS-PAGE and Western blotting.

Co-regulator recruitment assay

Recruitment of PGC-1 α to hMR was determined by GST pull-down assay. pDEST15-PGC-1 α ₆₋₂₂₅ (encoding a fusion between GST and PGC-1 α amino acids 6-225) and pGEX-5X-1 (encoding GST alone) were transformed into *E. Coli* BL21 cells, and GST fusion proteins purified from 200 ml bacterial culture using Glutathione Sepharose 4B resin (GE Healthcare) according to the manufacturer's instructions. Recombinant protein yield was

determined by SDS-PAGE. 2µg of GST- PGC-1 α ₆₋₂₅₅ or GST alone were incubated with 0.5µg hMR and 50µl glutathione sepharose in 0.5ml lysis buffer (20mM TRIS-HCl, pH 8.0; 0.5mM Nonidet NP-40; 100mM NaCl; 6 mM MgCl₂; 1 mM EDTA; 1 mM dithiothreitol; 8% glycerol) in the presence of the ligands shown, overnight at 4°C with rotation. The sepharose beads were washed 4 times with 1ml lysis buffer, resuspended in 50µl SDS lysis buffer, denatured at 95°C for 5 min, and a 10µl aliquot analysed by SDS-PAGE followed by Western blotting.

Results and Discussion

Expression of hMR in Sf9 cells

Our strategy for hMR purification involved expressing the receptor in Sf9 insect cells with an N-terminal affinity tag containing a biotinylation sequence (biotin acceptor peptide, BAP) for the *E.coli* biotin holoenzyme synthetase (BirA) (Schatz 1993). Co-expression of the BAP-tagged hMR and BirA in Sf9 cells produces a biotinylated receptor that can then be purified using streptavidin affinity resin. We first confirmed that addition of the BAP tag does not interfere with hMR transcriptional activity. Figure 1 shows that the abilities of wild-type and biotinylated BAP-tagged hMR to transactivate an MMTV-luciferase reporter in response to a range of agonists were indistinguishable. The BAP-tagged hMR cDNA was then subcloned into the baculovirus expression vector pDW464, baculoviral particles generated and used to infect Sf9 cells. Initial experiments indicated that only a relatively narrow range of multiplicities of infection (MOI, ~0.25-0.5) produced stable expression of soluble hMR in Sf9 cells; higher MOI resulted in significant degradation of full-length hMR (not shown), presumably reflecting exhaustion of endogenous Sf9 proteins required for hMR stability (Alnemri and Litwack 1993). In the absence of ligand, whole cell lysates derived from infection of Sf9 cells produced a single immunoreactive band of approximately 110kDa that was recognised both by streptavidin-HRP (to detect the biotin tag) and by an anti-MR antibody (Figure 2A, lanes 3 and 4). The antibody was raised against a peptide corresponding to MR amino acids 1-18 (Gomez-Sanchez et al. 2006), suggesting that the addition of the N-terminal tag does not alter the tertiary structure of the receptor in this region. When Sf9 cells were cultured in the presence of aldosterone, the amount of soluble hMR in Sf9 cell lysates was significantly enhanced, and a shift to a higher molecular weight doublet species was apparent (Figure 2A, lanes 5 and 6). Enhanced expression of this high molecular weight doublet form of the receptor was also observed when cells were cultured in the presence of other agonist (cortisol and DOC) ligands; however, in the presence of antagonist (progesterone), the majority of hMR was represented by the lower molecular weight doublet band (Fig 2A, lanes 11 and 12). A similar ligand-dependent upwards shift in the mobility of the progesterone receptor (PR) on reducing SDS-PAGE has previously been reported (Lange et al. 2000), and shown to result from phosphorylation of PR on multiple serine residues. These data suggest that ligand binding stabilises hMR in Sf9 cells, possibly through conformational changes and / or phosphorylation events.

Although full-length hMR has previously been expressed in yeast, its ligand binding properties in this system do not mirror those of the native receptor (Bureik et al. 2005). We therefore determined the ligand binding profile of baculoviral-expressed hMR. Sf9 cells infected with the hMR virus bound [³H]-dexamethasone (Figure 2B) whereas mock-infected cells showed no specific binding (not shown). [³H]-dexamethasone binding was dose-dependently inhibited by a variety of unlabelled steroids at nanomolar concentrations; the rank order of potency (progesterone ≥ DOC ≥ aldosterone ≥ corticosterone ≥ spironolactone ≥ cortisol ≫ 17β-estradiol) was similar to that of the endogenous receptor (Sutanto and de Kloet 1991). Note that 17β-estradiol has previously been shown to have weak affinity for the MR (Arriza et al. 1987). Therefore, Sf9 cells are a suitable host from which to purify recombinant hMR.

Purification and characterisation of full-length hMR

hMR was purified from medium-scale (500ml) Sf9 cultures using Promega “Soft-link” streptavidin resin. We were unable to purify significant quantities of hMR in the absence of ligand, or from whole-cell extracts (not shown); instead, the nuclear extract fraction of Sf9 cells cultured in the presence of aldosterone or cortisol was used for affinity purification (Figure 3). hMR was eluted in three fractions and was ~85% pure as determined by Coomassie staining (Figure 3A). When resolved by low percentage SDS-PAGE and silver staining, hMR did not migrate as a single band, but as a heterogeneous population of species with apparent molecular weight 110-130kDa (Figure 3B). Western blot confirmed that the N-terminal MR antibody recognised a population of proteins in this same size range. This heterogeneity likely reflects the presence of multiple post-translationally modified forms of the receptor, a phenomenon that has been observed previously for insect cell-expressed hMR (Binart et al. 1991). hMR is subject to a range of post-transcriptional modifications including phosphorylation, acetylation, sumoylation and ubiquitination (Pascual-Le and Lombes 2005; Viengchareun et al. 2007). Sf9 cell-expressed proteins also have the potential for glycosylation, although removal of N-linked oligosaccharides using PNGase F did not alter the mobility of hMR (not shown). Regardless, we obtained approximately 120µg of purified hMR from 500ml Sf9 cells, and purified the receptor bound to aldosterone or cortisol (Figure 3B), as well as to progesterone and deoxycorticosterone (not shown).

To determine the functional activity of recombinant hMR, DNA- and coregulator-binding assays were performed. hMR purified in the presence of aldosterone or cortisol bound to a double-stranded glucocorticoid receptor response element (GRE)-containing oligonucleotide that had been immobilised on a 96-well plate (Figure 4A). In contrast, no hMR binding was detected in wells that did not contain the response element. We also tested the ability of hMR to interact with PGC-1α, a known MR coactivator (Knutti et al. 2000), by GST pull-down assay. Full length hMR interacted strongly with an N-terminal fragment of PGC-1α (amino acids 6-225, Figure 4B) fused to GST; however, no interaction with GST alone was observed. Thus, recombinant hMR is functional with regard to both DNA and coregulator binding.

In conclusion, we describe a rapid method for expressing and purifying full length hMR using Sf9 cells and a biotinylated-tag to facilitate affinity purification. Stabilizing the receptor by culturing cells in the presence of ligand allowed large amounts of functional hMR, bound to different ligands, to be obtained. Studies on other receptor have shown that ligand-binding pocket conformation is dependent upon the context, i.e. full-length receptor or the ligand-binding domain alone, suggesting that similar analysis of MR structure, ligand and protein interactions in the context of the full-length receptor is crucial for the development of ligand-selective MR antagonists. This method therefore provides an important tool for the development of novel MR-targeted treatments for cardiovascular disease.

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Figure 1. Transcriptional activities of wild type and biotinylated BAP-tagged hMR

HepG2 cells were transfected with vectors encoding either wt-hMR (■) or the bt-hMR (▼) an expression plasmid encoding the BirA enzyme, an MR-responsive MMTV-luciferase reporter gene, and a β -galactosidase expression construct. 24h later, cells were incubated with the indicated concentrations of (A) aldosterone; (B) cortisol; (C) DOC; and (D) corticosterone. Luciferase activity was normalized to that of β -galactosidase, and is expressed as a percentage of the maximal activity of wt-hMR.

Figure 2. Expression of bt-hMR in Sf9 cells

(A) Sf9 cells were infected with bt-hMR baculovirus at an MOI of 0.5. Next day, cells were incubated with the ligands indicated for 24h. Soluble cell extracts were separated by SDS-PAGE, transferred to PVDF membranes and the bt-hMR detected using (upper panel) streptavidin HRP to detect the biotin tag, or (lower panel) Western blot using an antibody raised against MR amino acids 1-18. Duplicate samples are shown. (B) Ligand binding characteristics of baculovirus-expressed bt-hMR: Sf9 cells expressing bt-hMR were incubated with $[1,2,4,6,7\text{-}^3\text{H}]$ dexamethasone (2nM) in the presence of various concentrations of: (▪) aldosterone; (▲) cortisol; (▼) DOC; (◆) spironolactone; (●) progesterone; (□) 17β -estradiol

or (∇) corticosterone. Specifically-bound radioactivity was quantified by scintillation counting.

Figure 3. Affinity purification of bt-hMR in the presence of different ligands

(A) bt-hMR was purified from nuclear extracts of bt-hMR baculovirus-infected Sf9 cells in the presence of 1 μ M aldosterone: (T) total nuclear extract fraction before purification; (FT) flow-through fraction; (W1-W3) wash fractions; (E1-E3) elution fractions. 0.5 μ l of each fraction was loaded in lanes 1-3 and 10 μ l loaded in all other lanes. The 10% SDS PAGE gel was stained with Coomassie Blue. (B) bt-hMR was purified from Sf9 cells cultured in the presence of (A) aldosterone and (F) cortisol (1 μ M). The bt-hMR was resolved on a 6% SDS-PAGE gel and detected by (left panel) silver staining, 100ng bt-hMR per lane or (right panel) Western blot, 100pg per lane).

A **B** **Figure 4. DNA and coregulator binding activity of purified bt-hMR**

(A) Costar 96-well plates were coated with (or without) a biotinylated double-stranded oligonucleotide containing a consensus GRE. bt-hMR was added (500ng protein per well) in the presence of (A) aldosterone or (F) cortisol (10nM). Plates were incubated overnight at 4° C, washed, and specifically-bound protein detected by Western Blot. (B) bt-hMR interacts with PGC-1α *in vitro*: GST pull-down was performed using 500ng bt-hMR in the presence of (A) aldosterone and (F) cortisol, and either GST alone or GST- PGC-1α₆₋₂₅₅ (both 2μg). Captured bt-hMR was detected by Western blotting. Addition of equal amounts of GST fusion proteins were confirmed by staining the PDVF membrane with MemCode.