Human mineralocorticoid receptor A and B protein forms produced by alternative translation sites display different transcriptional activities

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Abstract

Objective: Aldosterone binds the mineralocorticoid receptor (MR) and is involved in the regulation of ionic transport, mainly sodium retention. MR is encoded by one single gene transcribed into various messengers that are thought to be translated into a unique 107 kDa protein. The aim of this study was to identify and characterize translation initiation variants of the human MR protein.

Methods: For this purpose we analyzed the extreme N-terminal fragment of various MR species, and confronted the predicted pattern of expression with in vitro translation results. Transactivation functions of the identified MR forms obtained by targeted mutagenesis were evaluated by transient transfection assays with different promoter reporter genes.

Results: Two major forms of human MR (hMR), named MRA and MRB, generated by alternative initiation of translation from start methionine 1 and 15 respectively, were predicted. Their expected expressions were confirmed by in vitro transcription/translation studies. Interestingly, each form of MR displayed reduced transactivation capacities with wild type (WT) = A + B > MRA > MRB suggesting that the extreme N-terminal fragment of MR has an effect on the transcriptional properties of the receptor.

Conclusions: Altogether these data indicate that MR is expressed, through alternative translation initiation, as distinct protein variants which possess different functional properties. These MR forms could tightly dictate the modulation of aldosterone responsiveness in various tissues or in pathophysiological situations.

European Journal of Endocrinology 150 585–590

Introduction

In the nuclear receptor family, mineralocorticoid (MR) and glucocorticoid (GR) receptors are two closely related steroid receptors sharing common features, in terms of modular molecular structures, signaling pathways and overlapping regulated target genes (1, 2). They are structurally organized into conserved functional domains carrying specific properties with a ligand-binding domain (LBD) responsible for hormone selectivity, a DNA-binding domain (DBD) interacting with responsive elements of target-gene promoters, and a long specific N-terminal domain (NTD) which is involved in the modulation of the transcriptional properties of the receptor. Since the recent sequencing of complex genomes and the discovery of fewer genes than expected, the dogma of one gene encoding one protein has been further re-evaluated. For steroid receptors and as exemplified by GR, which is encoded by a single gene, the mechanisms involved in creating protein diversity include alternative splicing of exons leading to distinct protein forms such as human GRα (hGRα) (3) and hGRβ (4). On the other hand, utilization of multiple translation initiation sites in the GR mRNA coding sequence has also been reported for the generation of protein isoforms referred to as GRA and GRB (5). All these hGR protein variants display divergent functional properties. hGRβ possesses a dominant negative effect on hGRα functions, which has been proposed as a factor involved in glucocorticoid insensitivity in human pathophysiology, whereas GRA and GRB have distinct transcriptional properties.

With regard to MR, which is also encoded by a single gene, only alternative splicing leading to several mRNA isoforms has been described to date. Among them, alternative transcription of 5′ untranslated first exons gives rise to hMRα and hMRβ mRNA isoforms translated into a unique protein due to the location of the initiation start site in exon 2 (6). In contrast, both the Δ5,6 splice hMR variant (7) and the 10bp deletion of the rat MR (8) produce truncated LBD. Finally, a 12bp insertion MR variant codes for a protein with no functional difference (9).
We investigated novel mechanisms creating MR protein diversity by studying translational variants, and identified two hMR forms, named hMRA and hMRB, produced by differential ribosomal scanning of the hMR mRNA. They display distinct transactivation properties that may account for variability in tissue-specific aldosterone responsiveness and may potentially be involved in pathophysiology.

Material and methods

In vitro transcription/translation

In vitro translated [\(^{15}\)S]-methionine (Amersham)-labeled proteins were obtained in reticulocyte lysates by a Tnt-T7 Quick Coupled Transcription/Translation kit (Promega) using 2 \(\mu\)g pcDNA3 hMR or 1–602 plasmids (10). Proteins were diluted into 2 \(\times\) Laemmli loading buffer, boiled, separated onto 7.5% acrylamide SDS-PAGE and then autoradiographed.

Targeted mutagenesis of hMR

Met (ATG) to Thr (ACG) substitutions were obtained by single T to C point mutation of start codons using pcDNA3-hMR as a matrix for PCR-based targeted mutagenesis performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolle, CA, USA). Sense primers used to generate single MR forms were 5'-CCGGGCCAGGCGGGACGGAGACCAAAGGCTACC-3' for M1T producing the MRB form only and 5'-CTGAAGGT-CTAGATACGGAAAGACGGTGGG-3' for M1ST producing the MRA form only and their reverse complementary associated primers. Mutated plasmids were sequenced to verify correct mutation and HindIII and XhoI digested for subcloning at the same sites into a new pcDNA3 vector.

Cell culture and transient transfection

R.C.SV3 rabbit kidney tubule cell line (11) was cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 medium supplemented with 2% charcoal-stripped fetal calf serum, 20 mmol/l Hepes, 2 mmol/l glutamine, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin, 5 \(\mu\)g/ml bovine insulin, 5 \(\mu\)g/ml transferrin, 50 mmol/l sodium selenite, 50 mmol/l dexamethasone in a saturated 5% \(\text{CO}_2/95\%\) air atmosphere at 37°C. All products for cell culture were purchased from Invitrogen. For transient transfections, cells were seeded into 2 \(\times\) 10^5 cells per well into 6-well dishes in dexamethasone-free medium for 6 h. Plasmids used for transfection experiments were hMR wild type (WT) or hMRA or hMRB cloned into pcDNA3, pGL3-GRE2-TATA-Luc or pF31-MMTV-Luc as reporter gene constructions and pSV-\(\beta\)-Gal to normalize transfection efficiencies. Plasmids were transfected in cells according to the classical phosphate-calcium precipitation method and incubated for 18 h in steroid-free medium. After a 24 h aldosterone incubation period, cells were rinsed twice with cold PBS and recovered into 250 \(\mu\)l lysis buffer (25 mmol/l glycylic-acid, pH 7.8, 1 mmol/l EDTA, 8 mmol/l magnesium sulfate, 1 mmol/l dithiothreitol (DTT), 1% Triton X100, 15% glycerol) at 4°C. Supernatants were used for enzymatic \(\beta\)-galactosidase and luciferase assays (100 \(\mu\)l each) as previously described (12). Luciferase activities were normalized to \(\beta\)-galactosidase activities and expressed as a percentage of relative transcriptional activity compared with control.

Results

Identification of Kozak consensus sites in MR sequence

In order to investigate whether MR is subject to alternative utilization of translation initiation sites in its extreme N-terminal part, as recently described for GRs, we analyzed the human, rat and mouse MR nucleotide cDNA sequences. This revealed that the NTD possesses multiple translation initiation consensus sites, of which only two could be considered as strong start sites according to the original definition of the consensus proposed by Kozak (13, 14). Figure 1 shows that these two strong consensus Kozak sites are perfectly conserved among mammalian species and correspond to methionine 1 and methionine 15 in MR protein sequences. Interestingly, these first 15 amino acids of MRs are between 93 and 100% identical in MRs of all known mammalian species. Furthermore, when examined against the recently sequenced teleost fish MR cDNA (15), the homology remains close to 60%. This represents an extremely high score for homology considering the phylogenetic distance between fish and mammalian species, and strongly supports important conserved functions for this fragment of the NTD. In the teleost fish, Met 15 is replaced by Thr preventing translation from this start site. However, it should be noticed that another strong Kozak consensus site is present at Met 26, downstream in the sequence of the lsh MR (Fig. 1) and may also serve as an initiation start site.

We next examined the expression of hMR protein by in vitro transcription and translation of the hMR cDNA using reticulocyte lysates to compare the predicted pattern (Fig. 2A) with experimental observations (Fig. 2B). Figure 2B (left panel) presents the protein profile obtained for the full-length hMR with a 107 kDa band as the most abundant form. Under optimized electrophoresis conditions, an additional prominent band of an apparently lower molecular weight of approximately 105 kDa was detected. Numerous smaller bands were also resolved with lower intensity, which all strictly corresponded to predicted alternative forms according to the position of the six weak AUG start codons in the hMR sequence (Fig. 2A). It is unlikely that these
bands were the result of a degradation process since experiments performed in the presence of a protease-inhibitor cocktail did not impede the appearance of the 105 kDa or other translational products (data not shown). Additional evidence against degradation as a source of these lower bands was the utilization of different constructs of the NTD as templates for MR translation. With the entire 1–602 NTD of hMR (Fig. 2B, right panel) or its 1–167 fragment (data not shown), patterns of expressed proteins revealed the presence of the two expected translational variants at their predicted sizes (Fig. 2A). Taken together these data

Figure 1 5′ end of cDNA (upper) and N-terminal protein (lower) sequence alignments of MR. Theoretical translation initiation sites of MR from various mammals are boxed and compared with the Kozak consensus defined as A/GxxATGG sequence (bold characters) (14). ATG codons corresponding to Met 1 and Met 15 for mammalian species, and Met 26 for teleost fish are potential translation start sites for MRA and MRB forms respectively.

Figure 2 Predicted (A) and experimental (B) hMR protein expression profiles. (A) The open reading frame (ORF) of hMR mRNA is depicted in the inset (small bars are AUG codons). The two strong Kozak consensus sites (M1 and M15) are boxed whereas the six weak Kozak sites (M43, M105, M109, M128, M453, M461) are unboxed. The theoretical molecular weights (in kDa) of the different forms of the full-length or the 1-602 hMR are reported below. (B) In vitro protein expression profiles of full-length hMR and its 1-602 NTD obtained by transcription/translation assays were resolved onto 7.5% SDS-PAGE.
suggest that the hMR is expressed as two major products of 107 and 105.4 kDa which will be referred to as MRA and MRB forms respectively.

**Transcriptional properties of MRA and MRB forms versus wild-type MR**

We generated, by PCR targeted mutagenesis substitution of methionine 15 and methionine 1 into threonines, the cDNA coding sequences of both human MRA and MRB respectively. After sequencing and *in vitro* transcription/translation to check the correct mutation and expression of the proteins, we tested their transactivation properties. For this purpose, MRA or MRB expression vectors were transiently transfected into RC.SV3 renal cells, and compared with wild-type hMR which is expressed as both A and B forms. Their transcriptional functions were assessed on two promoters, the mouse mammary tumor virus (MMTV) promoter (Fig. 3, left panel) and a GRE2-TATA promoter containing two palindromic consensus glucocorticoid response elements (Fig. 3, right panel), driving luciferase gene expression. Aldosterone dose–response experiments showed that both A and B forms displayed the same half-maximal effective concentration (EC50) as the wild-type MR at approximately $5 \times 10^{-11}$ mol/l on MMTV and GRE2. In contrast, maximal transactivation of MRA and MRB forms was lower than that of the wild-type receptor in both promoter contexts with, in decreasing order, WT > MRA > MRB. Comparison of results obtained at $10^{-8}$ mol/l aldosterone concentration revealed that MRA and MRB forms were both significantly less effective than the wild type in transactivating MMTV (MRA, 15% inhibition, $P < 0.03$, $n = 11$; MRB, 27% inhibition, $P < 0.001$, $n = 12$) and GRE2 (MRA, 20% inhibition, $P < 0.01$, $n = 6$; MRB, 30% inhibition, $P < 0.001$, $n = 6$) promoters. These data indicate that alternative translational MRA and MRB variants exhibit reduced transcriptional activities irrespective of the promoter context. Furthermore, MRB acts as a weaker transactivator compared with MRA suggesting that the first 15 amino acids play an important role in transactivation capacity of MR.

**Role of the extreme N-terminal structure in MR transactivation properties**

To explain the observed differences in transcriptional properties of MR forms, we investigated secondary structures of the extreme N-termini of MRA and MRB by computer predictive studies (Network Protein Sequence Analysis (www.pbil.ibcp.fr/NPSA)). Figure 4 shows that MRA presents a very well-organized structure composed of an $\alpha$-helix structure spanning from amino acid 13 to 27, followed by a short $\beta$-strand. In contrast, the deletion of the first 15 amino acids in MRB results in the truncation of this $\alpha$-helix. These findings were compared with those obtained for GRA and GRB forms, initiated at Met 1 and Met 27 respectively (5). The N-terminus of the GRA form is also structured into an $\alpha$-helix, which is lacking in the GRB form. We hypothesize that deletion of the first amino acids of MR and GR leads to important alteration of the folding of their NTD which presumably modulates the functional properties of the full-length receptor.

**Discussion**

This study describes, for the first time, MR protein variants that were not generated by alternative splicing of mRNA, or point mutations in the MR gene, but resulted from the utilization of alternative initiation AUG sites located in the extreme NTD of MR protein. Two major forms, named MRA and MRB, of 107 and 105.4 kDa molecular weight are initiated at Met 1 and Met 15 of hMR protein respectively. These results might explain the smaller MR species purified by gel filtration.

**Figure 3** Transcriptional properties of MR protein variants. Transient transfections of RC.SV3 cells were performed with 0.1 $\mu$g of wild-type, MRA, or MRB plasmids, 0.5 $\mu$g reporter gene (MMTV or GRE2) and 1 $\mu$g $\beta$-Gal plasmid to normalize transfection efficiencies. Transfected cells were incubated with increasing aldosterone concentrations (from $10^{-11}$ to $10^{-7}$ mmol/l) for 24h in steroid-free medium. Results were expressed as a percentage of control, arbitrarily set at 100% for the wild-type MR treated with $10^{-8}$ mol/l aldosterone. Results are means ± s.d. of three independent determinations (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus wild-type MR).
highly conserved through evolution, even being found
known MRs reveal that this 15-amino-acid segment is
sequence alignments of the extreme NTD of various
those expressed in the hippocampus (16). Interestingly,
chromatography from the rat kidney compared with

Figure 4 Predictive secondary structures of the extreme N-terminal fragments of MR and GR forms. Computer analysis was performed using the Network Protein Sequence Analysis (www.pbil.ibcp.fr/NPSA). Tall black bars, $\alpha$-helix; short grey bars, $\beta$-strand.

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