Mineralocorticoid Receptor Mutations and a Severe Recessive Pseudohypoaldosteronism Type 1

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ABSTRACT

Pseudohypoaldosteronism type 1 (PHA1) is a rare genetic disease of mineralocorticoid resistance characterized by salt wasting and failure to thrive in infancy. Here we describe the first case of a newborn with severe recessive PHA1 caused by two heterozygous mutations in NR3C2, gene coding for the mineralocorticoid receptor (MR). Independent segregation of the mutations occurred in the family, with p.Ser166X being transmitted from the affected father and p.Trp806X from the asymptomatic mother. Whereas the truncated MR_{166X} protein was degraded, MR_{806X} was expressed both at the mRNA and protein level. Functional studies demonstrated that despite its inability to bind aldosterone, MR_{806X} had partial ligand-independent transcriptional activity. Partial nuclear localization of MR_{806X} in the absence of hormone was identified as a prerequisite to initiate transcription. This exceptional case broadens the spectrum of clinical phenotypes of PHA1 and demonstrates that minimal residual activity of MR is compatible with life. It also suggests that rare hypomorphic NR3C2 alleles may be more common than expected from the prevalence of detected PHA1 cases. This might prove relevant for patient’s care in neonatal salt losing disorders and may affect renal salt handling and blood pressure in the general population.


Type 1 pseudohypoaldosteronism (PHA1) is a rare genetic disease of mineralocorticoid resistance.† Patients present with neonatal salt wasting and failure to thrive, associated with hyponatremia, hyperkalemia, and metabolic acidosis, despite extremely high levels of plasma renin and aldosterone.‡ Generalized and renal forms of the disease have been described,§ these are distinguished at the clinical and genetic level.¶,|| Infants with generalized PHA1 present multi-organ salt loss from the kidney, colon, sweat and salivary glands.¶¶ This severe recessive form of PHA1 is caused by homozygous or compound heterozygous loss of function mutations in genes encoding the three subunits of the epithelial sodium channel ENaC.¶¶ Renal PHA1 is a mild, autosomal dominant form of mineralocorticoid resistance, with a phenotypic expression restricted to the kidney.¶† Patients can be asymptomatic or pres-
ent a salt-losing syndrome in the neonatal period; systematically, symptoms improve with age. To date, all cases of familial or sporadic renal PHA1 have been associated with heterozygous inactivating mutations in the NR3C2 gene coding for the mineralocorticoid receptor (MR) highlighting the importance of aldosterone and the MR for the maintenance of fluid and electrolyte homeostasis.

Here we report a unique pedigree, in which the index case (case 00_01, Figure 1A) was referred at age 2 and 3 mo with failure to thrive. He was the second child of a nonconsanguineous family and was born at full term from an uneventful pregnancy with normal birth weight [3970 g, +1.1 SD Score (SDS)] and birth length (53 cm, +0.8 SDS). Biochemical examination showed hyponatremia (133 mmol/L) and hyperkalemia (5.7 mmol/L). As rapid weight gain followed without treatment on both occasions, the child was discharged without further evaluation. At the age of 9 mo, he represented with failure to thrive (weight from -1 SDS to -3 SDS), dehydration and malnutrition (Body Mass Index -4 SDS, skinfold, sunken eyes and decreased muscle mass). Biochemical measures were highly suggestive of PHA1 (Table 1) and the diagnosis was confirmed by genetic testing of the NR3C2 gene, which showed a frameshift mutation in exon 2 (c.497_498delCT) leading to replacement of Ser166 by a stop codon (p.Ser166X) (Figure 1B). The infant was treated with salt supplementation (5 mEq/kg/d) and ion exchange resins, with rapid remission of symptoms. Currently, at the age of 4 yr, clinical and biochemical assessments are normal with a salt supplementation of 2 g/d.

Two years later, his younger sister (patient 00_04, Figure 1A) presented with an extremely severe phenotype (Table 1). She was born at full term with normal birth weight (3540 g, -0.1 SDS) and length (51 cm, -0.2 SDS). At day 5, her weight was 3240 g and she showed moderate hyperkalemia (5.8 mmol/L) and increased urinary sodium excretion (52 mmol/L). At the age of 8 d, she developed vomiting and severe dehydration with weakness and hypotonia. Her weight was 3060 g and she had profound hyponatremia, very marked hyperkalemia with altered ECG (peaked T waves and widening of the QRS complex), metabolic acidosis (alkaline reserve 13 mmol/L) and increased urinary sodium excretion (112 mmol/L) (Table 1). Plasma aldosterone and renin levels were extremely elevated, despite salt supplementation and ion exchange resins (Table 1). Normalization of electrolyte balance was particularly challenging because of unusually high salt needs: 30 mEq/kg/d given by nasogastric tube then by percutaneous endoscopic gastrostomy. Addition of indomethacin and fludrocortisone was unable to reduce her salt requirement. Currently, at 2 yr of age, 31 mEq/kg/d of sodium chloride is required to achieve a normal salt balance and the child has growth retardation (-1.9 SDS) and moderate psychomotor delay.

Although asymptomatic, the father (patient 01_10) and an older sister (patient 00_03) reported increased salt appetite, and the father had a history of poor weight gain during infancy. The family was screened for the p.Ser166X mutation, which was transmitted from the father (00_01) to the index-case (00_01) and the younger sister (00_04), while the mother (01–20), the half-brother (00_02), and the older sister (00_03) had a wild-type NR3C2 sequence at this position (Figure 1A). The parents then elected to participate in the clinical research protocol “PHACARV—Cardiovascular Evaluation of Adult PHA1 Patients” (PHRC P070139, NCT00646828). It appeared that both the father and the mother, who did not carry the p.Ser166X mutation, had a phenotype suggesting PHA1, i.e. increased plasma aldosterone levels with normal or low mean 24h blood pressure (BP) on ambulatory 24h monitoring and normal potassium levels (Table 1). This unexpected phenotype prompted us to sequence the entire NR3C2 coding sequence and intron-exon junctions in patient 01_20. A nonsense mutation was identified in exon 6 (c.2418G>A/p.Trp806X). Genetic screening for this second mutation in the pedigree revealed its transmission from the mother to the asymptomatic

Figure 1. Identification of NR3C2 mutations. A. Pedigree of the PHA1 family explored in this study. The index case is indicated by an arrow. B. Results of NR3C2 sequencing on lymphocytes’ DNA, showing the two nonsense mutations found in the different family members.
sister (00_03) and to the younger sister (00_04) who was thus compound heterozygous for both familial nonsense mutations (Figure 1B).

Given the severity of the phenotype and the therapeutic difficulty encountered, we estimated the residual MR function associated with the two NR3C2 mutations. Both alleles were expressed in lymphocytes from patients 01_10, 01_20 and 00_04, as demonstrated by the presence of both nonsense mutations on cDNA amplification products of exon 2 and exon 6 in the absence of emetine (Figure 2A), indicating that these mutations did not induce Nonsense-Mediated mRNA Decay. We tested whether mutations p.Ser166X and p.Trp806X induced MR protein degradation. Western blot analyses were performed on protein extracts from renal RCSV3 cells transiently transfected with mutant MR proteins. Compared with MRWT transfected cells expressing a 110 kD product, an additional approximately 90 kD protein was detectable in cells transfected with MR_{806X}, corresponding to the expected size for this truncated protein (Figure 2B). In contrast, no protein was detected at approximately 20 kD, the expected size of MR_{166X}, indicating that this mutant is probably degraded by the proteasome.

The MR is functionally organized in three domains, involved in DNA binding, ligand binding and transcriptional activation. As expected, truncation at the very beginning of the MR ligand binding pocket completely abolished hormone binding by MR_{806X} (Figure 3A). The ability of MR_{806X} to activate transcription was assessed by transient transfection in RCSV3, COS-7 and HEK293T cells. Aldosterone increased transactivation by the MRWT in a dose-dependent manner (ED_{50} of approximately 5 \times 10^{-11}M; Figure 3B and Fig. S1A). Surprisingly, MR_{806X} was constitutively active in RCSV3 (Figure 3B) and COS-7 (Fig. S1A) cells, possessing approximately 25% and 45% of transcriptional activity observed with the MRWT at 10^{-8}M aldosterone, respectively; transcriptional activity was lower (approximately 7%) in HEK293T cells (Fig. S1A). Similar results were observed in RCSV3 cells in the presence of cortisol, a physiologic MR ligand, with MR_{806X} having a constitutive activity of about approximately 25% of that observed with the MRWT in the presence of 10^{-7}M cortisol (Fig. S1A).

The mechanisms whereby MR_{806X} had acquired partial ligand-independent transcriptional activity, despite the complete loss of ligand binding and of the activation function (AF)-2 domain, required for ligand-dependent transcriptional activation were investigated. First, the intracellular localization of MR_{806X} was studied in COS-7 cells transiently transfected with eGFP_MRW or eGFP_MR_{806X}. In contrast to MRWT, which translocates from the cytoplasm to the nucleus in an aldosterone-dependent manner within 30 min (Figure 3C), MR_{806X} was both cytoplasmic and nuclear in the absence of hormone, with no further modification of intracellular localization upon hormone addition (Figure 3C). The ability of MR_{806X} to recruit transcriptional coactivators was studied using a mammalian two

### Table 1. Biological characteristics of members of the PHA1 family explored in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Plasma Na(^+) mmol/L</th>
<th>Plasma K(^-) mmol/L</th>
<th>24 h mean Urinary Na(^+) mmol/L</th>
<th>BP SBP/DBP, mmHg</th>
<th>Plasma Aldosterone nmol/L</th>
<th>Plasma Renin concentration pmol/L</th>
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<tbody>
<tr>
<td>01_10</td>
<td>140</td>
<td>3.9</td>
<td>1.9</td>
<td>10/167</td>
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<td></td>
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<tr>
<td>01_20</td>
<td>141</td>
<td>3.9</td>
<td>1.9</td>
<td>10/167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>00_01**</td>
<td>130</td>
<td>5.6</td>
<td>26</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>00_03</td>
<td>137</td>
<td>5.1</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>00_04$</td>
<td>117</td>
<td>10.5</td>
<td>112</td>
<td>—</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Normal values: 135–145 mmol/L for Plasma Na\(^+\); 3.5–5 mmol/L for Plasma K\(^-\); 130–140 mmol/L for 24 h mean Urinary Na\(^+\); 105–115 mmHg for BP SBP/DBP; 0.5–1.5 pmol/L for Plasma Aldosterone; 0.1–2.0 pmol/L for Plasma Renin concentration.

Child <1.5 Adult*< 0.5 Aldosterone < 0.9 Aldosterone < 0.5

*Normal values refer to normal laboratory values for plasma aldosterone and plasma renin. To convert plasma aldosterone from pmol/L to ng/dL, divide by 0.0277; to convert plasma renin from pmol/L to pg/mL, divide by 0.0237. **Biochemical data at diagnosis; BP = blood pressure; SBP = systolic BP; DBP = diastolic BP.
hybrid assay. Ligand-dependent recruitment of the steroid receptor coactivator 2 (SRC-2) was observed with the MRWT in all cell lines, whereas MR806X was unable to recruit SRC-2 even in the presence of aldosterone (Figure 3D and Fig. S1B). Thus, the partial nuclear localization of MR806X in the absence of hormone is a prerequisite for its ability to interact with DNA and activate transcription. Loss of the AF-2 domain17 diminishes the repertoire of transcriptional coactivators recruited to the MR806X; however, truncation of part of the ligand binding domain also eliminates some inhibitory functions associated with this domain, which are usually relieved by ligand binding, similar to what observed for the MR splice variant, MR

The functional consequences of the two mutations explain the phenotypic differences observed within the family, with carriers of the p.Ser166X mutation presenting clinical PHA1 due to haploinsufficiency, while carriers of p.Trp806X are asymptomatic. In patient 00_04, the combined MR defect leads to an extremely severe phenotype comparable to autosomal recessive generalized PHA1 due to ENaC mutations. In contrast to generalized PHA1, recessive PHA1 linked to MR mutations was not associated with airway disease6 or with cutaneous lesions due to excessive salt loss from sweat glands.19 Sweat test at the age of 3 yr was borderline (chloride concentration 55 mmol/L, with normal test <45 mmol/L, positive test >60 mmol/L), although salt supplementation may interfere with this test.20 Although MR is expressed in sweat glands, the absence of a cutaneous phenotype in patient 00_04 suggests that minimal MR function is sufficient to regulate sweat salt balance or alternatively, MR is dispensable for Na⁺ reabsorption in this tissue.

Given the low prevalence of PHA1 (approximately 10 cases out of 800,000 births/yr diagnosed in France, PHA1.NET, coordinator M.-C. Zennaro22), the estimated probability of a compound heterozygous mutation is in the order of 10⁻¹¹. Our findings suggest that heterozygous carriers of NR3C2 mutations may be more frequent in the general population than expected, due to the large phenotypic variability of PHA1. This might prove relevant for patient’s care with wider screening for PHA1 indicated in neonates with renal salt losing syndromes and/or failure to thrive. Importantly, the possible existence of rare hypomorphic NR3C2 alleles in the general population puts a new perspective on BP regulation and the development of hypertension, as the combined effects of rare independent mutations (including in genes whose products mediate or regulate renal salt reabsorption) are expected to account for a substantial fraction of individual BP variation.23 The 1000 genomes Project pilot data indicate that each person carries approximately 250 to 300 loss-of-function variants in annotated genes.24 For NR3C2, 24 variations in coding exons have been catalogued. 13 of them are nonsynonymous; bioinformatic predictions using the Alamut software (Interactive Biosoftware, Rouen, France) suggest that several variants are deleterious (data not shown). As for certain renal transporters, further studies will need to address if rare independent NR3C2 mutations in the general population could result in undiagnosed salt losing disorders in infancy and modulate the individual risk for developing hypertension.

Figure 2. Characterization of MR expression from mutant alleles. A. RT-PCR and sequencing of exon 2 and exons 5 to 6 of lymphocytes' mRNA of patients 01_10, 01_20 and 00_04. Lymphocytes were isolated from whole blood and incubated for 24 h in the absence (UNT) or presence of emetine (Emetine), an inhibitor of Nonsense-Mediated mRNA Decay. H₂O, negative PCR control without cDNA. B. Western blot analysis of wild type and mutant MR. Proteins from untransfected cells and cells transfected with plasmids coding for MRWT, MR806X and MR166X were processed for immunoblotting with anti-MR (upper panel) and β-actin (lower panel) antibodies. RSCV3 cells express limited amounts of endogenous MR corresponding to a approximately 110 kD band. Note the presence of a approximately 90 kD band corresponding to the truncated MR806X, but the absence of a approximately 20 kD fragment, corresponding to MR166X.
Figure 3. In vitro characterization of MR_{806X}. A. MR_{WT} and MR_{806X} were analyzed for their capacity to bind aldosterone. Specific aldosterone binding was determined after incubation of MR_{WT} or MR_{806X} with 10^{-8}M tritiated aldosterone with or without a 100-fold excess of unlabeled aldosterone. B. Transcriptional properties of MR_{806X}. Aldosterone dose-response curves showing the transcriptional activation by MR_{WT} and MR_{806X} in RCSV3 cells. Cells were transiently transfected with expression vectors of MR_{WT} or MR_{806X}, together with GRE2-luciferase reporter plasmid. Relative transcriptional induction is represented relative to the induction obtained for MR_{WT} stimulated with 10^{-8}M aldosterone. Results represent mean ± SEM of at least three independent experiments performed in triplicate. ***P < 0.001. Insets represent negative controls transfected with empty vector. C. Intracellular trafficking of wild type and mutant receptors in living cells. COS-7 cells were transiently transfected with chimeric receptor proteins eGFP-MR_{WT} or eGFP-MR_{806X} and grown in steroid-free medium. Intracellular localization of fluorescence proteins was then observed in the absence (no Aldo) or presence of 10^{-8}M aldosterone during 30 min. D. Recruitment of transcriptional co-regulator SRC-2 by MR_{WT} and MR_{806X} in mammalian two-hybrid assays. RCSV3 cells were transiently transfected with the fusion proteins VP16-MR_{WT} or VP16-MR_{806X} and the GAL4 DNA-binding domain fused to the N-term and the receptor interacting domain of SRC-2; cells were incubated in triplicate with increasing concentrations of aldosterone or vehicle. After harvesting the cells, the luciferase activities were measured and normalized by the values obtained by vehicle. The results are the means ± SEM of three independent experiments. ***P < 0.001. Insets represent negative controls transfected with empty vector.
CONCISE METHODS

NR3C2 Sequencing
For NR3C2 sequencing please see supplemental appendix.

Lymphocyte Purification, Culture and Treatments
Blood samples were collected and stored at 4 °C. Lymphocytes were purified by density gradient centrifugation on UNI-SEPmaxi columns (Novamed, Chicago, Illinois) following the manufacturer’s protocol. Lymphocytes were transferred into six well plates (Costar, Washington, DC) and cultured in a complete medium containing RPMI, 10% charcoal-stripped fetal calf serum, 100 UI/mL, penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, California) for 24h at 37 °C in a 5% CO2 humidified atmosphere, in the presence or absence of emetine, an inhibitor of Nonsense-Mediated mRNA Decay,13 a cellular mechanism of mRNA surveillance which prevents the expression of truncated or erroneous proteins.

RNA Isolation and RT-PCR Experiments
Total RNA was extracted from lymphocytes with a Qiagen RNeasy mini kit according to the manufacturer’s recommendations. After DNasel treatment (Invitrogen, Carlsbad, CA, USA), 500 ng of total RNA were retro-transcribed using Superscript II RT (Invitrogen) and random hexamers (Promega, Madison, WI, USA). For PCR, please see supplemental appendix.

Plasmids and Transactivation Assays
For plasmids and transactivation assays, please see supplemental appendix.

Mammalian Two-Hybrid Assays
For mammalian two-hybrid assays, please see supplemental appendix.

Intracellular Localization of MR
COS-7 cells were transiently transfected with lipofectamine with plasmids coding for chimeric eGFP_MRWT, and eGFP_MR406X proteins. Cells were seeded in Lab-Tek culture chambers at a density of 100,000 cells/well and cultured in steroid-free medium. After addition of 10 nM aldosterone, intracellular fluorescence was observed by confocal microscopy at constant temperature, pH and CO2 (Zeiss LSM 510 laser scanning confocal microscope (CarlZeiss, Thornwood, New York)).

Western Blot Analyses
Given the limited amount of biologic material available from patients, Western blot analyses were performed on protein extracts from renal RCSV3 cells transiently transfected with plasmids coding for mutant MR proteins MR166X and MR806X. Total protein extracts were prepared from cells lysed at 4 °C and sonicated (protein extraction buffer, Cell Signaling, Danvers, Massachusetts). Lysates were subfractionated by SDS–PAGE on 4 to 15% gradient acrylamide gel (Biorad, Hercules, California). Immunoblots were incubated overnight with the 1D5 antibody (Sigma Aldrich, St. Louis, Missouri) followed by a peroxidase-conjugated goat anti-mouse antibody. Proteins were visualized by an ECL+ detection kit (GE healthcare, Buckinghamshire, UK).

Aldosterone Binding Assays
For aldosterone binding assays, please see supplemental appendix.

Statistical Analyses
Differences between wild type and mutant MR in cell culture experiments were analyzed by one-way or two-way ANOVA with Bonferroni post hoc tests or with nonparametric Kruskall-Wallis test. In vitro results are shown as the mean ± SEM. Statistical analysis of all of the in vitro results were performed with Graph Pad Prism version 5 (GraphPad software Inc, San Diego, California).

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REFERENCES
5. Rippe FG: Clinical and molecular features of type 1 pseudohypoaldosteronism. Horm Res 72: 1–9, 2009


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MINERALOCORTICOID RECEPTOR MUTATIONS AND A SEVERE RECESSIVE PSEUDOHYPOALDOSTERONISM TYPE 1

Online supplement

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Supplementary Materials and Methods:

**NR3C2 sequencing**

Blood samples for genetic studies were taken after informed consent from the patients or their parents. DNA was prepared from peripheral blood leucocytes cells using salt-extraction. All NR3C2 (Accession number M16801.1, GenBank, http://www.ncbi.nlm.nih.gov/Genbank/) coding exons, the intron-exon flanking regions and two 5'-untranslated exons were amplified using 13 pairs of primers. For each PCR experiment, 100 ng of DNA were amplified in the presence of 1.5 mM MgCl2, 400 nM of each primer, 200 μM deoxynucleotide triphosphate, 1.25 U Taq DNA polymerase (Sigma-Aldrich, Saint-Louis, Mi, USA). Reaction parameters were as follows: 1 cycle of 5 min at 95°C, followed by 30-35 cycles at 95°C for 45 sec, 50-62°C for 45 sec and 72°C for 45 sec. PCR was concluded by 7 min at 72°C. Products were separated in 1.5% agarose gels and visualized by ethidium bromide staining. Before sequencing, the unincorporated dNTPs and excess primers were inactivated and degraded with exonuclease I and Antarctic alkaline phosphatase (New England Biolabs, Ipswich, MA, http://www.neb.com). Direct sequencing of PCR products was then performed using the ABI Prism Big Dye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA, http://www.appliedbiosystems.com) on an ABI Prism 3700 DNA Analyzer. All identified mutations were confirmed on a second PCR product and on a second DNA sample. DNA mutation numbering is based on cDNA sequence, where +1 corresponds to the A of the ATG translation initiation codon in the reference sequence. Mutation nomenclature follows the guidelines of the Human genome variation society (http://www.hgvs.org/mutnomen/).

Sequences of cDNAs extracted from patient’s lymphocytes were generated using primer sequences located in exon 2 and exon 4 and 7, respectively, on gel-purified RT-PCR
products (PCR clean-up Gel extraction, NucleoSpin® Extract II, Macherey Nagel, Hoerd, France).

**RT-PCR experiments**

For each PCR experiment, 100 ng of cDNA were amplified. NR3C2 primer sequences were as follows:

- Ex2_F: 5' - GAGCAGCAGAAACCAACAAGGAA
- Ex2_R: 5' - TCGAAGGGCTGGAAACAGAGCA
- Ex4_F: 5' - TCAGGATGCCATTATGGGGTAGTC
- Ex7_R: 5' - CAAATGCAGCCTGGCTTGTGAG

Products were separated on 2% agarose gels and visualized by ethidium bromide staining. Direct sequencing of gel-purified RT-PCR products (PCR clean-up Gel extraction, NucleoSpin® Extract II, Macherey Nagel, Hoerd, France) was then performed using the ABI Prism Big Dye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

**Plasmids and transactivation assays**

MR mutations p.Ser166X (c.497_498delCT) and Trp806X (c.2418G>A) were created by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) on the recombinant pcDNA3-hMR plasmid containing a 3 kb hMR XmaIII_AflIII fragment inserted into pcDNA3. The desired mutations were identified by direct sequencing and were then entirely sequenced to verify absence of random mutations. hMR fragments were subsequently excised with HindIII and NotI and subcloned into a new pcDNA3 expression vector. To follow intracellular localization of the mutant receptors, MRWT and MR806X cDNA were excised from pcDNA3-hMR with HindIII and ApaI and
cloned into the peGFPc1 vector (Clontech, Montain View, CA, USA). For mammalian two-hybrid assays, site-directed mutagenesis of p.Trp806X was realized directly on a recombinant pVP16-MRwt plasmid kindly provided by ME Rafestin-Oblin using the same procedure. Oligonucleotide sequences used for site directed mutagenesis are as follows:
MR p.Ser166X: 5’-GAGATCATTTATGTGACTCTGGAGCTCCGTG-3’
MR p.Trp806X: 5’-TACCCTAATCCAGTATTCTTGAATGTGTCTATCATCATTGCC-3’

Rabbit RCSV3 cells derived from kidney cortical collecting duct (kindly provided by Pr. P. Ronco, Hôpital Tenon, Paris) were grown in DMEM-HAM’s F12 supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 2 mM glutamine, 100 UI/ml penicillin, 100 µg/ml streptomycin, 20 mM Heps, 50 mM sodium selenate, 50 mM dexamethasone and 2% charcoal-stripped fetal calf serum. COS-7 cells were grown in DMEM supplemented with 100 UI/ml penicillin, 100 µg/ml streptomycin, 20 mM HEPES and 10% charcoal-stripped fetal calf serum. HEK293T cells were cultured in high-glucose-containing DMEM, 25 mM HEPES, 2X nonessential amino acids, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, supplemented with 10% heat-inactivated fetal calf serum. Four hours before transfection, the HEK293T cells were switched to the same medium supplemented with 10% charcoal-treated fetal calf serum.

Functional effects on the transcriptional activity of the receptors were investigated in cis-trans-cotransactivation assays. Cells were transfected using lipofectamine 2000 (Invitrogen) with 0.25 µg of plasmid containing either wild type (WT) or mutant MR, 0.625 µg of a GRE2_TATA_luc reporter plasmid and 0.25 µg of pSVβgal. Transfections in HEK293T cells were carried out using the calcium phosphate precipitation method. Cells were transfected with 2 µg of one of the receptor expression vectors (pcDNA3_MRwt, pcDNA3_MR806X and pcDNA3_MR1-733), 7 µg of GRE2_TATA_luc reporter plasmid and 1 µg of pSVβgal in 1× HEPES-buffered saline supplemented with 160 mM CaCl2. 12 hs after
transfection, the cells were rinsed with phosphate-buffered saline, trypsized, and replated in 24-well plates. The steroids to be tested were added to the cells 24h after seeding. After incubation for 24 h, cell extracts were assayed for luciferase and β-galactosidase activities using the Dual-Light® System and the Galacton-Plus® Substrate (Applied Biosystems). Results were standardized for transfection efficiency and expressed as the ratio of luciferase activity over β-galactosidase activity in arbitrary units. Aldosterone and cortisol were purchased from Sigma Aldrich.

For mammalian two-hybrid assays, HEK293T, COS-7 and RCSV3 cells were transfected with 3 µg of wild type or mutant VP16_MR fusion proteins, 3µg of pGAL_SRC-2 (N-term-RID), 3 µg of pg5luc; and 1µg of pcβgal using the calcium phosphate precipitation method on petri dishes. 12 hs after transfection, the cells were rinsed with phosphate-buffered saline, trypsized, and replated in 24-well plates. Aldosterone (10^{-10} to 10^{-8} M) was added to the cells 24h after seeding. After incubation for 24 h, cell extracts were assayed for luciferase and β-galactosidase activities using the Dual-Light® System as described above. Aldosterone and cortisol were purchased from Sigma Aldrich.

**Aldosterone binding assays**

In vitro transcription and translation of wild type or mutant MR proteins were accomplished using the TNT Quick Coupled Transcription/Translation system (Promega) following the manufacturer’s protocol in the presence of cold methionine. Reticulocyte lysate containing wild type or mutant MR was diluted 2-fold with TEGWD buffer (20 mM Tris-HCl, pH 7.4; 1 mM EDTA; 10% glycerol; 20 mM sodium tungstate; and 1 mM dithiothreitol) and incubated for 4 h at 4°C with 10 nM [3H] aldosterone (Amersham Pharmacia Biotech, Little Chalfont, UK; specific activity, 1.92 TBq/mmol) alone or in the presence of a 100-fold excess of unlabeled steroid to determine specific and nonspecific binding. Bound (B) and
unbound (U) steroids were separated by the dextran-charcoal method: 25 µl lysate were stirred for 5 min with 50 µl 4% Norit A, 0.4% Dextran-T70 in TEGWD buffer, and centrifuged at 4500 g for 5 min at 4° C. Bound steroid was measured by counting the radioactivity of the supernatant in a liquid scintillation spectrometer (LKB, Rockville, MD) after adding 5 ml OptiPhase HiSafe (counting efficiency ~50%).

References:

Figure S1: In vitro characterization of MR_{806X}
A. Transcriptional properties of MR_{806X}. Aldosterone dose-response curves showing the transcriptional activation by MR_{WT} and MR_{806X} in Cos-7 and HEK293T cells and cortisol dose-response curves showing the transcriptional activation by MR_{WT} and MR_{806X} in RCSV3 cells. Cells were transiently transfected with expression vectors of MR_{WT} or MR_{806X}, together with GRE2-luciferase reporter plasmid. Relative transcriptional induction is represented relative to the induction obtained for MR_{WT} stimulated with 10^{-8}M aldosterone or 10^{-7}M cortisol, as indicated. Results represent mean ± SEM of at least 3 independent experiments performed in triplicate. ***p<0.001. B. Recruitment of transcriptional co-regulator SRC-2 by MR_{WT} and MR_{806X} in mammalian two-hybrid assays. COS-7 and HEK293 cells were transiently transfected with the fusion proteins VP16-MR_{WT} or VP16-MR_{806X} and the GAL4 DNA-binding domain fused to the N-term and the receptor interacting domain of SRC-2; cells were incubated in triplicate with increasing concentrations of aldosterone or vehicle. After harvesting the cells, the luciferase activities were measured and normalized by the values obtained by vehicle. The results are the means +/- SEM of three independent experiments. ***p<0.001. Insets represent negative controls transfected with empty vector.