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'- GAGCAGCAGAACCAACAAGGAA

Ex2_R: 5'- TCGAAGGGCTGGAAACAGAGCA

Ex4_F: 5'- TCAGGATGCCATTATGGGGTAGTC

Ex7_R: 5'- CAAATGCAGCCTGGCTTTTGAG

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_AflII 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, MR_{WT} and MR_{806X} 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'-GAGATCATTTATGTGACTCTGGGAGCTCCGTG-3'

MR p.Trp806X: 5'-TACCCTAATCCAGTATTCTTGAATGTGTCTATCATCATTTGCC-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 Hepes, 50 nM sodium selenate, 50 nM 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 [1] and 0.25 µg of pSVβgal. Transfections in HEK293T cells 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_TATAluc reporter plasmid and 1 µg of pSVβgal in 1× HEPES-buffered saline supplemented with 160 mM $CaCl_2$. 12 hs after

transfection, the cells were rinsed with phosphate-buffered saline, trypsinized, 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, trypsinized, 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:

1. Asselin-Labat ML, Biola-Vidamment A, Kerbrat S, Lombes M, Bertoglio J, Pallardy M. FoxO3 mediates antagonistic effects of glucocorticoids and interleukin-2 on glucocorticoid-induced leucine zipper expression. Mol Endocrinol 2005;19:1752-64.

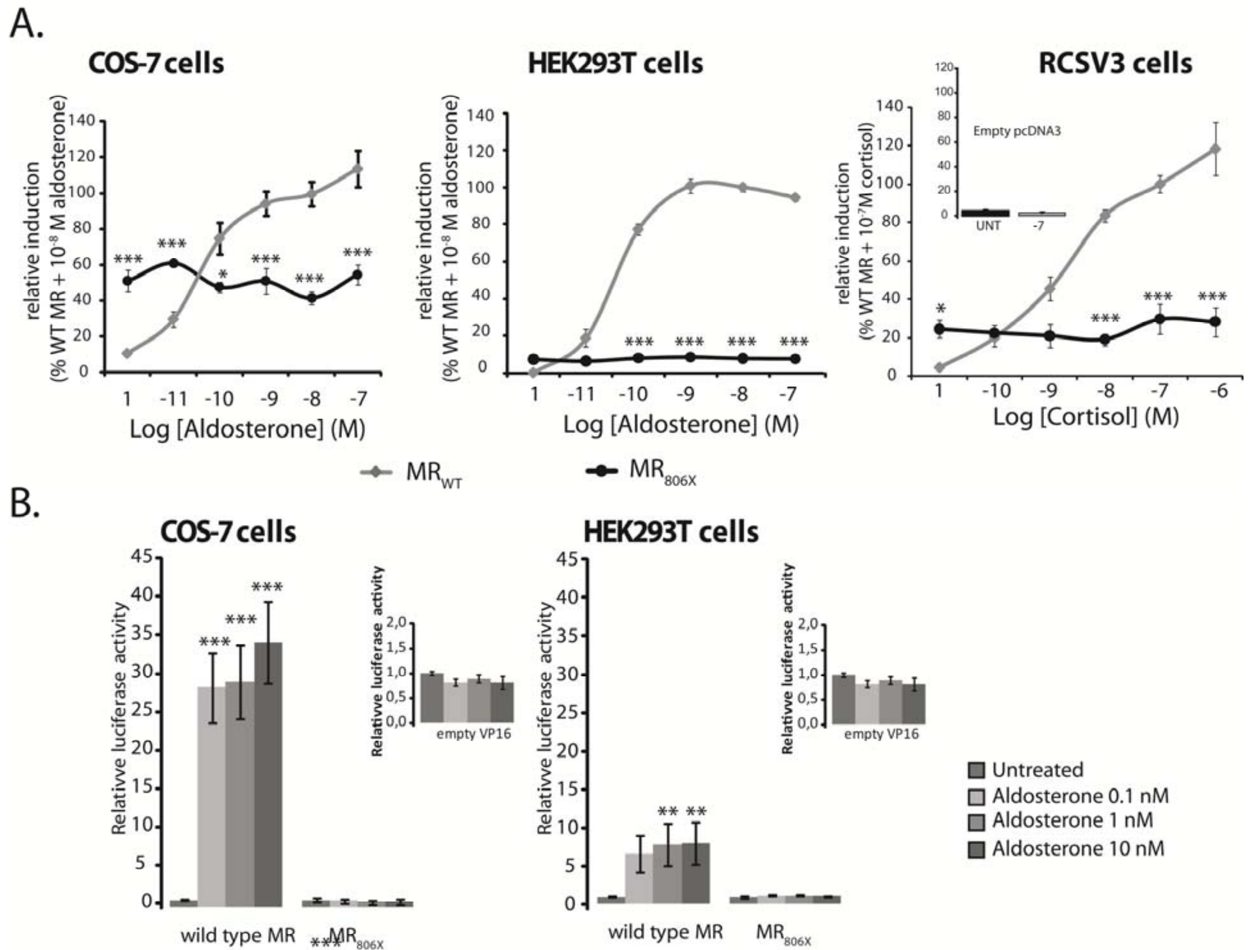

Figure S1: In vitro characterization of MR_{806X}

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.