Biochemical and Biophysical Research Communications 445 (2014) 132–137

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Genome-wide analysis of murine renal distal convoluted tubular cells for the target genes of mineralocorticoid receptor

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ARTICLE INFO

Article history:
Received 23 January 2014
Available online 1 February 2014

Keywords:
Mineralocorticoid receptor
Aldosterone
Distal convoluted tubules
ChIP-seq

ABSTRACT

Background and objective: Mineralocorticoid receptor (MR) is a member of nuclear receptor family proteins and contributes to fluid homeostasis in the kidney. Although aldosterone-MR pathway induces several gene expressions in the kidney, it is often unclear whether the gene expressions are accompanied by direct regulations of MR through its binding to the regulatory region of each gene. The purpose of this study is to identify the direct target genes of MR in a murine distal convoluted tubular epithelial cell-line (mDCT).

Methods: We analyzed the DNA samples of mDCT cells overexpressing 3xFLAG-hMR after treatment with 10^{-7} M aldosterone for 1 h by chromatin immunoprecipitation with deep-sequence (ChIP-seq) and mRNA of the cell-line with treatment of 10^{-7} M aldosterone for 3 h by microarray.

Results: 3xFLAG-hMR overexpressed in mDCT cells accumulated in the nucleus in response to 10^{-9} M aldosterone. Twenty-five genes were indicated as the candidate target genes of MR by ChIP-seq and microarray analyses. Five genes, Sgk1, Fkbp5, Rasl12, Tns1 and Tsc22d3 (Gilz), were validated as the direct target genes of MR by quantitative RT-qPCR and ChIP-qPCR. MR binding regions adjacent to Ctgf and Serpine1 were also validated.

Conclusions: We, for the first time, captured the genome-wide distribution of MR in mDCT cells and, furthermore, identified five MR target genes in the cell-line. These results will contribute to further studies on the mechanisms of kidney diseases.

1. Introduction

Mineralocorticoid receptor (MR) is a member of steroid-responsive nuclear receptor family and is evolutionarily conserved especially in terrestrial animals. Specific ligand of MR is aldosterone, which is secreted from adrenal cortex and contributes to fluid homeostasis through the activation of MR in the kidney.

MR is expressed in renal tubular epithelial cells of distal nephrons, which include distal convoluted tubules (DCT), connecting tubules, and collecting ducts (CD) [1]. Serum/glucocorticoid regulated kinase 1 (Sgk1), epithelial Na(+) channel subunit alpha (ENaCα), FK506 binding protein 5 (Fkbp5) and TSC22 domain family protein 3 (Tsc22d3/Gilz) are well-known target genes of MR in CD, while recent research investigations have shown that MR is widely expressed in in vivo and that the target genes of MR depend on cell-types [2]. Furthermore, MR activation has been reported to be associated with tissue inflammation and fibrosis through gene expressions of connective tissue growth factor (Ctgf) and serpin peptidase inhibitor clade E member 1 (Serpine1/PAI-1) in several animal models of kidney diseases [3]. Attention is now focused on the target genes of MR in each cell-type.

Abbreviations: MR, mineralocorticoid receptor; DCT, distal convoluted tubule; CD, collecting duct; ChIP-seq, chromatin-immunoprecipitation with deep-sequencing; MBS, MR-binding sequence.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.125
0006-291X/

Chromatin imunoprecipitation with deep-sequencing (ChIP-seq) has been used for the research on behaviors of various transcription factors. This technique makes it possible to detect the direct bindings of a transcription factor to DNA. Meanwhile, a genome-wide analysis of MR binding sites has been poorly performed to renal cell-types.

In this study, we performed ChIP-seq and microarray analyses of mDCT cells, which is a cell-line of DCT [4], to explore the genome-wide profile of MR-bindings and the direct target genes of MR in the cell-line.

2. Materials and methods

2.1. Cell culture

mDCT cells were incubated in DMEM low glucose (Sigma) with 5% FBS at 37 °C as previously described [5]. HEK293T cells were cultivated in DMEM high glucose (Sigma) with 10% FBS at 37 °C [6]. The conditionally immortalized murine podocyte cell line (MPC) [7] between passages 17 and 28 was maintained in RPMI 1640 (Sigma) supplemented with 10% FBS in the presence of 10 U/ml recombinant murine interferon-γ (Peprotech) at 33 °C. To differentiate MPC, the cells were plated on type I collagen dishes and cultured with 1% FBS in the absence of interferon-γ at 37 °C from Day 0. The concentration of FBS was reduced to 0.5% on Day 3 and medium change was done every 3 days. Experiments were done after Day 14.

2.2. Animals

All animal procedures were approved by the University of Tokyo Ethics Committee for Animal Experiments. Kidneys were collected from anesthesized adult C57BL/6 mice, snap-frozen in liquid N_2 and stored at −80 °C.

2.3. Plasmid DNA transfection

To overexpress 3xFLAG-tagged human MR, CSII-CMV-3xFLAG-hMR and X-tremeGene HP transfection reagent (Roche Diagnostic Systems) were dissolved in Opti-MEM in the ratio of 1:3 as shown in the manufacturer's instruction. After 15 min of incubation at room temperature, the mixture was delivered by drops to the medium. The experiments were done 48 h after the transfection.

2.4. Treatment with aldosterone and spironolactone

Aldosterone and MR blocker spironolactone were dissolved in 100% ethanol at a concentration of 10^{-2} M and stocked at −80 °C. The cells were incubated in charcoal-stripped medium overnight. Then the cells were exposed to 10^{-7} or 10^{-9} M aldosterone for 1 or 3 h as specified in each experiment. The same concentration of ethanol was used for control. 5×10^{-6} M Spironolactone was added 2 h prior to aldosterone treatment.

2.5. Real-time quantitative reverse transcription PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The synthesis of cDNA was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was determined by TaqMan or SYBR Green real-time quantitative reverse transcription PCR (RT-qPCR) using Step One Plus Real-Time PCR System (Applied Biosystems).

2.6. Primers

Primer sequences used in this study are listed in Supplementary Table 1.

2.7. Western blot analysis

Whole cell lysates were prepared using magnesium containing lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 25 mM NaF, 10 mM $MgCl_2$, 1 mM sodium orthovanadate, 1% IgepalCA-630, 10% glycerol and protease inhibitor cocktail Complete (Roche, Basel, Switzerland)). To prepare nuclear and cytosolic extracts, we used commercially available kits (BioVision). The same amounts of proteins were subjected to immunoblotting as previously described [6]. Primary antibodies were as follows: monoclonal mouse anti-FLAG M2 (1:1000, F1804 Sigma), monoclonal mouse anti-MR (clone 1D5, 1:1000, [8]), polyclonal rabbit anti-Actin (1:2000, Sigma) and monoclonal mouse anti-Nucleophosmin (NPM) (1:2000, Sigma). The signals were detected using ECL Prime or Advance Western blotting Detection Reagent. The images were analyzed using ImageQuant LAS 4000 mini (Fujifilm).

2.8. Microarray

mDCT cells with overexpression of 3xFLAG-hMR were incubated with 5×10^{-6} M spironolactone or vehicle for 2 h, and then with 10^{-7} M aldosterone or the same concentration of ethanol for 3 h. mRNA were extracted from the cells treated with ethanol/ethanol (group 1), ethanol/aldosterone (group 2) and spironolactone/aldosterone (group 3) and were analyzed by Affymetrix Mouse Genome 430 2.0 microarrays over 39000 transcripts (Affymetrix, Santa Clara, CA). Data were analyzed according to the minimum information about microarray experiment (MIAME) rule. We defined aldosterone-responsive genes as those upregulated more than 1.5 times in the presence of 10^{-7} M aldosterone for 3 h compared to the vehicle control, the increase of which by aldosterone was inhibited to less than 75% by pretreatment with 5×10^{-6} M spironolactone. The microarray data have been submitted at NCBI's Gene Expression Omnibus [GEO Accession number: GSE52686].

2.9. Chromatin immunoprecipitation (ChIP), deep-sequencing and ChIP-qPCR

More than 5×10^6 cells overexpressing 3xFLAG-hMR ("tagged" sample) or with vehicle transfection control ("non-tagged" sample) were incubated with 10^{-7} M aldosterone for 1 h, crosslinked with 1% formaldehyde for 10 min and prepared for ChIP as previously described [9]. Crosslinked cells were lysed and sonicated to shear the chromatin to the size of 150–500 bp. After picking up some samples as whole cell lysate (input), the rest of the lysate was divided into two tubes. The anti-FLAG mouse monoclonal M2 antibody or nonspecific mouse IgG (Sigma) was preincubated for 2 h with protein G Dynabeads (Dynal). Then, each divided lysate was incubated with the antibody-beads complex for more than 18 h at 4 °C. The samples were heated for 20 min at 65 °C and the eluates were incubated at 65 °C overnight to reverse crosslinks and then treated with RNaseA and then with proteinase K. The samples were purified by QIAquick PCR Purification Kit (Qiagen). The ChIP DNA and the input DNA were end-repaired, ligated to sequencing adapters and amplified using NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina (NEB). DNA libraries were then sequenced by HiSeq 2500 (Illumina) to generate single-end 51 bp reads. Sequenced reads of both the ChIP and the input DNA samples were aligned to the murine genome (UCSC mm9) using Bowtie ver. 0.12.5 (http://bowtie-bio.sourceforge.net/). Enrichment of the