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ScienceDirect
Biochemical and Biophysical Research Communications 353 (2007) 1017–1022
BBRC
www.elsevier.com/locate/ybbrc

The role of CpG methylation in cell type-specific expression of the aquaporin-5 gene

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Received 12 December 2006
Available online 26 December 2006

Abstract

Aquaporin-5 (AQP5) is expressed in a cell type-specific manner. Here, we show that the AQP5 gene is regulated by CpG methylation. The AQP5 promoter containing a putative CpG island was highly methylated in NIH-3T3 or freshly isolated alveolar epithelial cells, correlating with the repression of this gene in these cells. In contrast, the AQP5 promoter was hypo-methylated in MLE-12 or cultured alveolar epithelial cells, which express high levels of AQP5. Repression of AQP5 transcription in NIH-3T3 cells could be relieved with 5-azacytidine, and *in vitro* methylation of the AQP5 promoter resulted in inhibition of transcription of the reporter gene in MLE-12 cells. Chromatin immunoprecipitation assays showed that endogenous Sp1 bound to the hypo-methylated, but not highly methylated, AQP5 promoter region. These results demonstrate that the hypo-methylated state of the AQP5 promoter leading to increased Sp1 binding may play a role in regulation of cell type-specific expression of the AQP5 gene.

Keywords: Aquaporin-5; Cell type-specific expression; DNA methylation; Sp1 transcription factor

Aquaporins (AQPs) are water channel proteins that allow the rapid movement of water across the plasma membrane in response to osmotic/hydrostatic pressure gradients [1]. AQPs are found in tissues such as kidney, salivary gland, and lung where the rapid and regulated transport of fluid is necessary. Expression of AQPs is regulated in a cell- or tissue-specific manner. AQP5 is a member of the AQP family that is selectively expressed in the apical membrane of serous cells in submucosal glands [2], and in alveolar type I cells in the lungs [3]. Targeted deletion of AQP5 in mice results in significantly reduced fluid secretion from submucosal glands [4], and a 10-fold decrease in osmotic water permeability of the alveolar-capillary barrier in distal lung [5], suggesting the importance of AQP5 for water homeostasis in the lung. However, despite its indicated key role in the lung, the molecular mechanisms that underlie the regulation of cell type-specific expression of the AQP5 gene have not yet been elucidated.

Methylation of the cytosine residue in the sequence 5′-CpG-3′ is an epigenetic modification that is involved in the establishment and maintenance of cell type-specific gene expression [6,7]. Methylation patterns of distinct cell- or tissue-specific genes among tissues or cells are unequivocally different. It has been reported that several genes with Sp1/Sp3-regulated promoters are regulated by CpG methylation [8–10]. In addition, we have previously reported that the rat AQP5 core promoter region contains three Sp1/Sp3 binding elements (SBEs) and at least one of these SBEs is critical for AQP5 basal promoter activity [11], raising the possibility that CpG methylation at the SBE may play a role in the regulation of cell type-specific expression of AQP5. In this study, therefore, we investigated the correlation between CpG methylation and AQP5 expression.

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.126

Here, we demonstrated that the AQP5 gene is regulated by CpG methylation, and that the hypo-methylated state of the AQP5 promoter and increased Sp1-DNA binding may play an important role in the regulation of cell type-specific expression of AQP5.

Materials and methods

Cell culture and reagent treatment. MLE-12 (immortalized murine lung epithelial) cells and NIH-3T3 (immortalized murine fibroblast) cells were cultured as described previously [12]. For 5-azacytidine (5-AC, Sigma, St. Louis, MO) treatment, 5-AC or vehicle (0.1% DMSO) was added to the cells and incubated for 3 or 5 days, changing the medium every 24 h.

Isolation and culture of rat alveolar epithelial cells. Alveolar epithelial cells were isolated from pathogen-free male Wistar rats (180–200 g) as described previously [13]. Briefly, trypsin was used to dissociate the cells from lung tissue, and the resultant cell suspension was incubated on rat IgG-coated plastic Petri dishes for 60 min to remove non-epithelial cells. The isolated epithelial cells were then suspended at a concentration 1×10^6 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin and 20 μg/ml gentamycin at 37 °C in a humidified 5% CO_2, and 95% air atmosphere. This procedure routinely yielded about 1.0×10^7 cells/rat. The cells were dispensed onto plastic culture dishes at a density of 2×10^5 cells/cm^2 and cultured in 5% CO_2/95% air at 37 °C. In all experiments, non-adherent cells were removed from the dishes by washing with DMEM after 24 h of cultivation.

Total RNA preparation and semi-quantitative RT-PCR. Cells were harvested and total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcriptase (RT)-PCR was performed with 0.5 μg of total RNA using a RNA PCR™ kit (AMV) Ver.3.0 (TaKaRa, Shiga, Japan) according to the manufacturer's instructions: 1 cycle at 42 °C for 60 min, 99 °C for 5 min, and 5 °C for 5 min for reverse transcription; 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min for PCR. The oligonucleotide primers used in the PCR amplifications are as follows: AQP5, 5′-GGCCACATCAATCC AGCCATTA-3′ (sense) and 5′-GGCTGGGTTCATGGAACAGCC-3′ (antisense); GAPDH, 5′-ACCATCTTCCAGGAGCGAGA-3′ (sense) and 5′-CAGTCTTCTGGGTGGCAGTG-3′ (antisense).

Western blotting. Western blotting experiments were performed as described previously [12].

Sodium bisulfite DNA sequencing. Genomic DNA was isolated using the Wizard® SV Genomic DNA purification system (Promega, Madison, WI) according to the manufacturer's recommendations. Five micrograms of genomic DNA in 50 μl dH_2O were denatured with 5.5 μl of 3 M NaOH at 37 °C for 20 min. Fifteen microliters of freshly prepared 0.4 M hydroquinone and 540 μl of freshly prepared 3.8 M sodium bisulfite (pH 5.0) were then added and mixed. Amplification was performed in iCycler (Bio-Rad, Tokyo, Japan, 5 cycles of 95 °C for 3 min and 55 °C for 57 min). The bisulfite-treated DNA was isolated using Wizard® Minipreps DNA purification Resin/Minicolumns (Promega). The DNA was eluted with 100 μl of warm dH_2O. Eleven microliters of 3 M NaOH was then added and samples were incubated at 37 °C for 20 min. The DNA was ethanol precipitated and resuspended in 50 μl of water. PCR amplification was performed using High Fidelity AccuPrime™ *Taq* DNA polymerase (Invitrogen), and a 5-μl aliquot of the DNA solution was then amplified in a nested PCR. The oligonucleotide primers used in PCR amplifications were as follows: Mouse, 5′-GTTTAAGGAGGAGAAAAGGGGAAGG TTG-3′ (1st sense) and 5′-AAAAACAACTAAACACACCTCCTTC-3′ (1st antisense); 5′-GTYGGGTTAGTTTATATTGT-3′ (2nd sense) and 5′-AACCTTAAAAACCCRATAAC-3′ (2nd antisense); Rat, 5′-GATTT TTAGAGTTTTGTGGGAG-3′ (1st sense) and 5′-CTTAAAAAAAACA AAAAAACACACCTCC-3′ (1st antisense); 5′-GATTTTTAGAGTTTT G TGGGAG-3′ (2nd sense) and 5′-AACCTTAAAAACCCRATAAC-3′ (2nd antisense). Products from the second round of the nested PCR amplification were sub-cloned into the pCR®2.1 vector (Invitrogen), and clones were sequenced. The frequency of CpG methylation at each CpG site was calculated.

In vitro methylation of reporter plasmid. The rat AQP5 promoter (−160/+69) in pGL4.17 (Promega), which contains a *firefly* luciferase reporter gene, was *in vitro* methylated with *Hpa*II or *Sss*I methylase (New England Biolabs, Ipswich, MA) in the presence of 160 μM *S*-adenosylmethionine. The procedure for mock methylation is identical to that for DNA methylation except that no *S*-adenosylmethionine was included. Complete methylation of the promoter region was confirmed by methylation-sensitive restriction enzyme digestion.

Transient transfection and luciferase assay. Transient transfections of DNA plasmids were performed with TransFast™ reagent (Promega) according to the manufacturer's recommendations. Briefly, cells cultured in 12-well plates were incubated with DNA-transfection reagent mixture (ratio: 1 μg DNA/3 μl reagent) at 80–90% confluency. Twenty-four hours after transfection, cells were harvested for detection of luciferase activity. Luciferase activity was measured by a luminometer (Lumat LB9507, EG&G Berthtold) using Dual luciferase assay kits (Promega). Co-transfection with pGL4.74 (*hRluc*/TK, Promega), which expresses *Renilla* luciferase, was performed to normalize for transfection efficiency.

Chromatin immunoprecipitation (ChIP). Cells were fixed with 1% formaldehyde at 37 °C for 10 min, and the cross-linking reaction was stopped by addition of 125 mM glycine for 10 min. After washing twice with ice-cold PBS, cells were scraped and collected by centrifugation. Collected cells were resuspended in SDS lysis buffer [50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 1% SDS, and 1% (v/v) protease inhibitor cocktail] and incubated on ice for 20 min. The lysates were then sonicated for 5 cycles of 30 s each, resting on ice for 1 min between cycles, on a bioruptor UCD-250 (Cosmo Bio, Tokyo, Japan) with a setting of output power of 250 W. After sonication, the samples were centrifuged and the supernatants were diluted 10-fold in ChIP dilution buffer [50 mM Tris–HCl (pH 8.0), 167 mM NaCl, 1.1% Triton X-100, 0.11% deoxycholate, and 1% (v/v) protease inhibitor cocktail] and pre-cleared with salmon sperm DNA/protein G agarose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) slurry for 4 h at 4 °C. Cross-linked chromatin was incubated with 4 μg of anti-Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Immuno-complexes were collected by immunoprecipitation with salmon sperm DNA/protein G agarose slurry for 3 h at 4 °C. The beads were washed sequentially in the following buffers: low salt wash buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% deoxycholate]; high salt wash buffer [50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% deoxycholate]; LiCl wash buffer [10 mM Tris–HCl (pH 8.0), 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40, and 0.5% deoxycholate]; and Tris-EDTA buffer. Immuno-complexes were extracted from the beads with ChIP elution buffer [10 mM Tris–HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, and 0.5% SDS]. Cross-linking was reversed by incubation at 65 °C for 6–12 h. The eluants were digested with proteinase K at 55 °C for 1 h, and then further subjected to phenol/chloroform extraction. The DNA was purified by ethanol precipitation. PCR amplification was performed with LA *Taq*® polymerase (TaKaRa) in GC buffer II: 1 cycle at 94 °C for 2 min and 45 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s. The oligonucleotide primers used in PCR amplifications are as follows: 5′-AACAGCGCTGAGCGCACGATG-3′ (sense) and 5′-TGGTAGCGG CGGGCGTCTGTCTG-3′ (antisense). PCR products were analyzed on a 2% agarose gel.

Results

To investigate whether AQP5 expression is regulated by CpG methylation, MLE-12 and NIH-3T3 cells were treated with a concentration range of 5-azacytidine (5-AC) which induced demethylation of DNA but was not toxic to the cells. Semi-quantitative RT-PCR showed that AQP5 was endogenously and highly expressed in MLE-12, but not NIH-3T3 cells (Fig. 1A and C). In NIH-3T3