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CpG methylation prevents YY1-mediated transcriptional activation of the vimentin promoter

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ABSTRACT

Vimentin exhibits a complex pattern of tissue-specific and developmentally regulated expression, but the mechanisms underlying the complex transcriptional regulation remain poorly understood. Here we examined whether vimentin expression can be regulated by CpG methylation of the vimentin promoter. Two subclones of the rat C6 glioma cells were established with (C6vim+) and without (C6vim−) vimentin. Bisulfite genomic sequencing revealed that the vicinity of the transcription start site within the vimentin promoter is highly methylated in C6vim− cells but not in C6vim+ cells. Treatment of C6vim− cells with a demethylating agent, 5-aza-2′-deoxycytidine, restored vimentin expression, indicating that hypermethylation of the promoter region correlates with transcriptional silencing of the vimentin gene. Electrophoretic mobility shift assay (EMSA) and transient transfection experiments demonstrated that YY1 is a key transcriptional activator regulating vimentin expression and that CpG methylation is sufficient to prevent the binding of YY1 to the vimentin promoter. These data suggest that the inability of YY1 to access the hypermethylated promoter may be one of the mechanisms that mediate vimentin downregulation.
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1. Introduction

Vimentin is a component of the eukaryotic cytoskeleton belonging to the family of intermediate filament protein (IFP) [1]. Vimentin exhibits a complex pattern of gene expression during embryonic development and cell proliferation and in neoplasia [2,3]. In the embryo, vimentin expression is specific to the mesoderm, and maintained in tissues of mesenchymal origin in the adult organism [4]. During terminal differentiation, vimentin expression is turned off and replaced by distinct IFPs [1]. Vimentin is also observed in most cultured cells regardless of their embryonic origin [5]. Moreover, in tissue culture, vimentin mRNA synthesis can be induced by serum, fibroblast growth factor, and platelet-derived growth factor [6,7]. Although the biological functions of vimentin have not been well defined, vimentin expression can contribute to the augmentation of motility and invasiveness of some cancer tumors, and is a marker for the metastatic potential of many tumor cells [8,9]. Therefore, it is important to determine how the vimentin gene is selectively downregulated during terminal differentiation, while it remains expressed in most cultured cells, or is aberrantly reexpressed in metastatic cells.

To date, functional analysis of the vimentin promoter has identified multiple regulatory elements, some of which may contribute to the control of vimentin gene expression. Within these elements are a TATA-box and several positive regulatory elements including eight GC-boxes [10], NF-κB [11], and PEA3 [12]. The region further upstream consists of two AP-1 binding sites [13] and a unique transcriptional element termed an antisilencer element [14,15]. However, these elements can not account for the complex pattern of vimentin gene expression.

CpG methylation is a major epigenetic modification of genomes that is shown to be involved in the regulation of cell-specific gene expression [16]. It has been proposed that this modification may cause transcriptional repression by directly modulating transcription factor function or by triggering the formation of inactive chromatin [17,18]. To identify possible epigenetic mechanisms responsible for the repression of vimentin expression, we investigated the methylation status of the vimentin CpG island in the rat C6 glioma cells chosen as a model [19]. We found that the inaccessibility of YY1 (Yin-Yang 1) to the CpG-methylated promoter may be one of the mechanisms that mediate vimentin downregulation.

Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; ChIP, Chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFP, intermediate filament protein; YY1, Yin-Yang 1.

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2. Materials and methods

2.1. Cell culture and treatment with 5-aza-2′-deoxycytidine (5-aza-dC)

Rat C6 glioma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were treated with 2 μM 5-aza-dC (Sigma) for 1–4 days.

2.2. Plasmids

A DNA fragment of the rat vimentin promoter (−828 to −20) was subcloned into the pGL3-Enhancer vector (Promega) to generate pGL3-vim. Expression vectors for myc-tagged rat YY1 were made from pCR-YY1 [20].

2.3. Northern blot analysis

Total RNA was isolated using ISOGEN (Wako). For Northern blotting, 10 μg of total RNA was separated in a 1% agarose gel and transferred to Hybond-N + nylon membranes (GE Healthcare). Hybridization and detection were performed with a digoxigenin-labeled vimentin cDNA probe (Roche Diagnostics) as described previously [21].

2.4. Western blot analysis

Western blotting was performed using monoclonal antibodies against vimentin, β-tubulin, or myc (Santa Cruz Biochemicals).

2.5. Immunofluorescence analysis

C6 cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and treated with anti-vimentin. Antibody binding was visualized with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) for immunofluorescence microscopy.

2.6. Bisulfite modification and sequencing analysis

Bisulfite modification of genomic DNA and PCR were performed as described elsewhere [22]. The bisulfite-treated DNA was PCR-amplified and sequenced using pairs of primers (Supplementary Table S1). The conditions for the PCR were as follows: 1 cycle of 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 48 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

2.7. Electrophoretic mobility shift assay (EMSA)

A nuclear protein extract was prepared from C6 cells as described previously [23]. Complimentary oligonucleotide probes (Supplementary Table S1) were annealed and end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase. Binding reactions were carried out for 10 min at room temperature using 2 μg of nuclear protein extract in buffer containing 10 mM HEPES (pH7.9), 1 mM dithiothreitol, 5 mM $MgCl_2$, 60 mM KCl, 0.05% Nonidet P-40, 200 ng poly(dI-dC), 10% glycerol, and 50 μg/ml bovine serum albumin, in a total volume of 10 μl. The methylated or unmethylated competitors and the YY1 consensus oligonucleotide were added at a 200-fold molar excess. Samples were resolved in 6% polyacrylamide gels in 0.5× Tris–glycine buffer. In some reactions, antibodies against YY1 and NF-κB (Santa Cruz Biochemicals) or normal rabbit serum were added prior to incubation with the probe.

2.8. Transfection and luciferase assay

Luciferase reporter assays were carried out in a 24-well plate as described previously [23]. For each well, 50 ng of reporter construct was cotransfected with 50 ng of *Renilla* luciferase plasmid, pRL-TK (Promega), using Effectene (Qiagen). Luciferase activities were determined using a Dual–Luciferase Reporter Assay System (Promega). For *in vitro* methylation assay, a vimentin promoter construct was incubated overnight with *Sss*I methylase (New England Biolabs) in the presence (methylated) or absence (mock-methylated) of 1 mM S-adenosylmethionine.

2.9. Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as described previously [23]. DNA precipitated with anti-YY1 or nonimmune immunoglobulin (IgG) was PCR-amplified using pairs of primers (Supplementary Table S1).

3. Results

3.1. Isolation and characterization of C6 subclones

The rat C6 glioma cell line is known to comprise a mixture of cells with and without vimentin IFP networks [19]. Both vimentin-positive and -negative clones (C6vim+ and C6vim−) were obtained by limiting dilution cloning (Fig. 1A). Northern and Western blotting demonstrated that vimentin expression is regulated at the mRNA and protein levels in these subclones (Fig. 1D and E). To assess whether the lack of vimentin expression in C6vim− cells could be a result of the lack of appropriate transcription factors, transient transfection experiments were performed with the vimentin-promoter construct pGL3-vim. Unexpectedly, the reporter activity in C6vim− cells was greater than that in C6vim+ cells (Fig. 1B), indicating that the regulatory machinery is intact in C6vim− cells and that some other mechanisms might be involved in vimentin gene silencing.

3.2. Involvement of CpG methylation in silencing of vimentin expression

It is well known that CpG methylation in the promoter region often represses gene expression. Therefore, CpG methylation in both C6 subclones was initially analyzed by Southern blotting with methylation-sensitive enzymes *Hha*I and *Hpa*II (Supplementary Fig. S1). DNA from C6vim− cells was relatively resistant to *Hha*I and *Hpa*II digestions, indicating that heavily methylated regions are present in the vimentin promoter. To further examine whether CpG methylation can repress vimentin gene expression, C6vim− cells were treated with DNA methyltransferase inhibitor 5-aza-dC. Vimentin mRNA and protein were easily detected within 48 h, and maximal levels were observed between 72 and 96 h (Fig. 1D and E), indicating that demethylation induced by 5-aza-dC reactivated vimentin gene expression. Moreover, the promoter activity of the *Sss*I-modified reporter construct pGL3-vim was completely inhibited (Fig. 1C). These results suggest that CpG methylation is involved in silencing of the vimentin gene expression.

3.3. Characterization of the methylation status of the vimentin CpG island

Bisulfite genomic sequencing was used to determine the methylation status of the vimentin CpG island in both subclones. Many CpG sites in the regions C and D are partially or fully methylated in C6vim− cells, whereas they are largely unmethylated in C6vim+ cells (Fig. 2). In the other regions (A, B, and E), similar methylation

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