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Expression of the human CMP-NeuAc:GM3 α2,8-sialyltransferase (GD3 synthase) gene through the NF-κB activation in human melanoma SK-MEL-2 cells

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Abstract

To elucidate the mechanism underlying the regulation of human GD3 synthase gene expression in human melanoma SK-MEL-2 cells, we identified the promoter region of the human GD3 synthase gene. The 5′-rapid amplification of cDNA end (5′-RACE) using mRNA prepared from SK-MEL-2 cells revealed the presence of multiple transcription start sites of human GD3 synthase gene. Promoter analyses of the 5′-flanking region of the human GD3 synthase gene using luciferase gene reporter system showed the strong promoter activity in SK-MEL-2 cells. Deletion study revealed that the region as the core promoter from −1146 to −646 (A of the translational start ATG as position +1) was indispensable for endogenous expression of human GD3 synthase gene. This region lacks apparent TATA and CAAT boxes but contains putative binding sites for transcription factors c-Ets-1, CREB, AP-1 and NF-κB. Electrophoretic mobility shift assays using specific competitors, chromatin immunoprecipitation assay and site-directed mutagenesis demonstrated that only NF-κB element in this region is required for the promoter activity in SK-MEL-2 cells. These results indicate that NF-κB plays an essential role in the transcriptional activity of human GD3 synthase gene essential for GD3 synthesis in SK-MEL-2 cells.
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1. Introduction

Gangliosides, the sialic acid (NeuAc)-containing glycosphingolipids, are found on the outer leaflet of the plasma membrane of vertebrate cells and are particularly abundant in the central nervous system [1]. They play important roles in a large variety of biological processes, such as cell–cell interaction, adhesion, cell differentiation, growth control and receptor function [2,3]. In addition, they have been studied as molecules characteristically expressed in brain tissues of various mammals [1–3] and also as tumor markers of neuroectoderm-derived malignant cells [4] such as melanomas [5,6] and neuroblastomas [7].

Gangliosides, especially GD3, are highly expressed in human melanoma tissues and melanoma cell lines [8,9]. Although GD3 is a relatively minor species among gangliosides present in the adult brain [10], it is a major ganglioside in early stages of the development of fetal rat brain [11]. Furthermore, GD3 appears in activated human T lymphocytes [12] as well as in T cell acute lymphoblastic leukemia cells [13]. In particular, GD3 and GD2 have been considered to be important molecules not only for the tumor markers but also as targets of antibody therapy [7,14,15]. GD3 synthase is a key enzyme for the synthesis of whole b-series gangliosides including GD2 in addition to GD3 itself [16]. In general GD3 expression appears to be regulated at the transcriptional level of GD3 synthase gene [17,18]. To understand

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transcriptional regulation mechanism for GD3 synthase gene expression in these biologically important events, it is very important to characterize the promoter function of GD3 synthase gene. GD3 synthase promoters have been characterized for the rat [19] and mouse gene [20]. Especially, regulatory mechanisms for GD3 expression in human melanoma cells are of quite importance, since GD3 is well known as a human melanoma-specific antigen [8,21]. Although isolation and functional analysis of the promoter region of human GD3 synthase gene from melanoma cells has been reported [22], the transcriptional regulation mechanism for melanoma-specific expression of human GD3 synthase gene has not yet been studied in detail.

In this study, the promoter region to direct up-regulation of human GD3 synthase gene transcription in human melanoma SK-MEL-2 cells was functionally characterized. The present results clearly indicate that the NF-κB binding site of the human GD3 synthase promoter plays a critical role in transcriptional regulation of human GD3 synthase expression necessary for GD3 synthesis highly expressed in melanoma.

2. Materials and methods

2.1. Cell cultures

The human melanoma SK-MEL-2 cells obtained form American Type Culture Collection (Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) (WelGENE Co., Daegu, Korea). Human Jurkat T cells were cultured in RPMI 1640 supplemented with 1 mM sodium pyruvate and 1× MEM nonessential amino acids. Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (WelGENE Co., Daegu, Korea), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO_2.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis

Total RNA was isolated from SK-MEL-2 cells using Trizol reagent (Invitrogen, USA). Two micrograms of RNA was subjected to reverse transcription with random nonamers utilizing Takara RNA PCR kit (Takara, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: GD3 synthase (460 bp), 5′-TGTGGTCCAGAAAGACATTTGTGGACA-3′ (sense) and 5′-TGGAGTGAGGTATCTTCACATGGGTCC-3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense). The PCR products were separated by gel electrophoresis on 2% agarose containing ethidium bromide with 1× TAE buffer. To assess the specificity of the amplification, the PCR product for GD3 synthase was subcloned into pGEM-T vector (Promega, USA) and then sequenced. These genes were found to be identical to the expected cDNA. Northern blot analysis was performed by the same method as described previously [23], using the [α-^{32}P] dCTP-labeled fragments of GD3 synthase as a probe.

2.3. Rapid amplification of the 5′ cDNA ends (RACE)

Amplification of the 5′-end of GD3 synthase was performed with the 5′-RACE kit (Invitrogen) according to the manufacturer's instructions, using 5 μg of mRNAs from SK-MEL-2 cells. The gene-specific primer GD3RT (5′-CACAGCCACTCTTCTT-3′, complementary to nucleotides 397–412) was used for initial reverse transcription. After synthesis of the first strand cDNA, an Abridged anchor primer provided by the company and the gene-specific primer GSP1 (5′-CACCATTTCCCACCACCGCGCATT-3′, complementary to nucleotides 365–388) were used in the first PCR. The second PCR was performed by gradient PCR with various temperature conditions, 61 °C, 64 °C, and 67 °C using an Abridged universal amplification primer and the gene-specific primer GSP2 (5′-TTGCCTGTGGGAAGAGAGAGTAAGTTG-3′, complementary to nucleotides 314–336). The PCR products obtained at 67 °C were subcloned into pGEM-T Easy vector (Promega) and sequenced.

2.4. Preparation of reporter plasmids

To identify the minimal promoter sequence in the 5′-flanking region of the GD3 synthase gene, luciferase reporter plasmids [23], Group I; −2000/pGL3, −2646 to −646/pGL3, −2499 to −499/pGL3, Group II; −2646 to −646/pGL3 and its derivatives ([−1146 to −646/pGL3] to [−2246 to −646/pGL3]) and Group III; −2646 to −646/pGL3 and its different derivatives (−646 to −1146/pGL3, −1146 to −1646/pGL3, −1646 to −2146/pGL3 and −2146 to −2646/pGL3), were used. The promoterless and enhancerless luciferase vector pGL3-Basic and the pGL3-Control with SV40 promoter and enhancer (Promega, Madison, WI) were used as negative and positive controls, respectively. Mutant plasmids [23] with base substitution at the CREB, AP-1, c-Ets-1, NF-κB binding sites were also used to identify the transcription factor-binding sites contributing to transcriptional regulation of human GD3 synthase gene in SK-MEL-2 cells.

2.5. Transient transfection and reporter assay

For the reporter analysis of GD3 synthase promoter, the constructed plasmids (1.5 μg) and control vectors (1.5 μg) were transfected into the SK-MEL-2 human melanoma cells and human Jurkat T cells at 70% confluence by SuperFectant transfection protocol (Qiagen, Germany). SK-MEL-2 and Jurkat T cells were cultured in DMEM and RPMI 1640 media, respectively, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal bovine serum (WelGENE Co., Daegu, Korea) at under 5% CO_2 at 37 °C. Each transfection experiment was repeated at least twice, yielding reproducible results. To normalize for the efficiency of transfection, these cells were simultaneously co-transfected with 1 μg of pCMVβ (Clontech, Palo, CA). The cells were resuspended in DMEM medium containing 10% fetal bovine serum 48 h after transfections. Lysates were prepared by four cycles of freezing and thawing of the harvested cells followed by centrifugation. Luciferase activity was measured using the luciferase assay system kit (Promega, Madison, WI) and Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland). Luciferase activity was normalized to β-galactosidase activity and expressed as a fold induction over pGL3-Basic.

2.6. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIp) assay

EMSA was performed using gel shift assay system kit (Promega) according to the manufacturer's instructions. Nuclear extracts of SK-MEL-2 cells were prepared as described previously [24], the protein concentrations of the extracts were determined using Bio-Rad protein assay kit. Double-stranded oligonucleotides synthesized using for sets of oligonucleotides [23] encompassing their binding sites for the transcription factors NF-κB, c-Ets-1, AP-1 and CREB were end labeled with [γ-^{32}P] dATP using T4 polynucleotide kinase and used as probes for EMSA. ChIP assay was performed using the chromatin immunoprecipitation assay kit (Upstate Biotechnology) following the manufacturer's protocol. Briefly, SK-MEL-2 cells (1×10^7 cells for one assay) were cross-linked in 1% formaldehyde at room temperature for 10 min to cross-link proteins and DNAs, followed by sonication to shear the DNAs to an average size of 200–1000 bp. Immunoprecipitation was carried out using 4 μg of CREB (Cell signaling), ETS-1(Santa Cruz Biotechnology), AP-1 (Santa Cruz Biotechnology), NF-κB (Santa Cruz Biotechnology) antibodies. After reversal of cross-linking, the DNA fragments were purified by phenol extraction and ethanol precipitation, followed by PCR analysis using primers flanking the NF-κB, c-Ets-1, AP-1 and CREB binding sites on the GD3 promoter: C-ETS-1–5′:ACCAATCCCCGGGCGTTT (forward), C-ETS-1–3′: GCCGCACCAAGTCCTTGGA (reverse), CREB-5′: CGTGTGTGTGTGTGTGTGTGTGTG (forward), CREB-3′: CCGGTGTGCCCAGGCTGT (reverse), AP-1–5′: GACTAGGGTGACGGCAGCAGG (forward) AP-1–3′: CCCCCACCCGCAAAATTG (reverse), NF-κB-5′: CTCCGCCACACTCAGGGACT (forward), NF-κB-3′: ACAAACGCCCGGGGATTG (reverse).

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