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Transcriptional regulation of the human GM3 synthase (hST3Gal V) gene during monocytic differentiation of HL-60 cells

Hee-Jung Choi[a,b], Tae-Wook Chung[b,c], Nam-Young Kang[a,b], Kyoung-Sook Kim[b], Young-Choon Lee[a], Cheorl-Ho Kim[b,c,*]

[a] *Faculty of Biotechnology, Dong-A University, Busan 604-714, South Korea*
[b] *National Research Laboratory for Glycobiology, MOST, Korean Government, Kyungju 780-714, South Korea*
[c] *Department of Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University, Sukjang-Dong 707, Kyungbuk 780-714, South Korea*

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Abstract **We studied the transcriptional regulation of human GM3 synthase (hST3Gal V) during monocytic differentiation of HL-60 cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Northern blot and reverse transcription polymerase chain reaction indicated that the induction of hST3Gal V by TPA is regulated at the transcriptional level. To elucidate the mechanism underlying the regulation of hST3Gal V gene expression during the differentiation of HL-60 cells induced by TPA, we characterized the promoter region of the hST3Gal V gene. Functional analysis of the 5′-flanking region of the hST3Gal V gene by the transient expression method showed that the −177 to −83 region, which contains a cAMP responsive element binding protein (CREB) binding site at −143, functions as the TPA-inducible promoter in HL-60 cells. In addition, gel shift assays and site-directed mutagenesis indicated that the CREB binding site at −143 is crucial for the TPA-induced expression of the hST3Gal V in HL-60 cells.**

Key words: GM3 synthase; Gene expression; HL-60 cell; Monocytic differentiation

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]. GM3 is the first and the simplest of the gangliosides and is known to play important roles in the modulation of cell growth through modified signal transduction and cell differentiation. GM3 inhibits tyrosine phosphorylation of the epidermal growth factor (EGF) receptor and EGF-dependent cell growth, independent of receptor–receptor interaction, whereas De-*N*-acetyl-GM3 enhances serine phosphorylation of the EGF receptor and stimulates cell proliferation [3]. GM3 induces monocytic differentiation of the human myeloid and monocytic leukemic cell lines HL-60 and U937 [4]. GM3 is synthesized by CMP-NeuAc:lactosylceramide α2,3-sialyltransferase (GM3 synthase, EC 2.4.99.9) which catalyzes the transfer of NeuAc from CMP-NeuAc to the non-reducing terminal galactose of lactosylceramide. GM3 synthase is a key regulatory enzyme for ganglioside biosynthesis [5] because it catalyzes the first committed step in the synthesis of nearly all gangliosides.

HL-60 cells are a promyelocytic cell line that upon 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment can differentiate into monocyte/macrophages [6,7]; hence they have been used extensively as a model system to examine the factors involved in monocyte differentiation. The amount of GM3 increases markedly with a concomitant increase of human GM3 synthase (hST3Gal V) activity during monocytic differentiation of HL-60 cells treated with TPA, which is a monocyte/macrophage differentiation inducer, but not with a granulocyte differentiation inducer retinoic acid [8–11]. Also, hST3Gal V activity is markedly elevated in a time-dependent manner during monocytic differentiation [9,10]. In addition, the increases of GM3 and hST3Gal V activity were not related to the differentiated lineage but to the specific action of TPA, i.e. activation of protein kinase C (PKC) [9]. These results suggested that PKC might specifically activate hST3Gal V, resulting in an increase in the content of GM3 during the differentiation of HL-60 cells induced by TPA, and that PKC activated by TPA might regulate the expression of the hST3Gal V gene at the transcriptional level. Therefore, the regulation of hST3Gal V may be important for the expression of GM3 in HL-60 cells.

Very recently, we isolated and functionally characterized the hST3Gal V gene promoter in human hepatoma HepG2 and neuroblastoma SK-N-MC cells [12]. To elucidate the molecular basis of hST3Gal V gene expression during the differentiation of HL-60 cells induced by TPA, in this study we functionally characterized the ability of the promoter region to direct up-regulation of reporter gene transcription in response to induced HL-60 cell differentiation. The present results clearly indicate that the cAMP responsive element binding protein (CREB) binding site of the hST3Gal V promoter

*Corresponding author. Fax: (82)-54-770 2281.
E-mail address: chkimbio@dongguk.ac.kr (C.-H. Kim).

Abbreviations: hST3Gal V, human GM3 synthase; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CREB, cAMP responsive element binding protein; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay

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plays a critical role in transcriptional regulation of the hST3Gal V gene during HL-60 cell differentiation.

2. Materials and methods

2.1. Materials and cell culture

TPA was purchased from Sigma Chemicals (St. Louis, MO, USA). The HL-60 cell line was maintained in RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin under 5% CO_2 at 37°C. To induce differentiation, HL-60 cells were cultured for different times in the presence of 30 nM TPA. Cell differentiation was monitored by adhesion to culture plates and cell morphology.

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

Total RNA was isolated from untreated and TPA-induced HL-60 cells using the Trizol reagent (Invitrogen, Life Technologies). RNA (2 μg) was subjected to reverse transcription with the random nonamers and Takara RNA PCR kit (Takara Shuzo, Shiba, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: hST3Gal V (1002 bp), 5′-TATACTACTGAAGAATGTGACATG-3′ (sense) and 5′-TGTTCAAAATTCACGATCAATGCC-3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense). The PCR products were separated by gel electrophoresis on 3% agarose containing ethidium bromide with 1×TAE buffer. After electrophoresis, the intensity of the bands obtained from RT-PCR result was estimated using *Total*Lab software of the Frog Gel Image Analysis System (CorebioSystem, Seoul, Korea). To assess the specificity of the amplification, the PCR product (1002 bp) for hST3Gal V was subcloned into pGEM-T vector (Promega, Madison, WI, USA) and sequenced and was found to be identical to the expected cDNA.

Northern blot analysis was performed by the same method as described previously [13], using the [α-^{32}P]dCTP-labeled hST3Gal V fragment (1002 bp obtained by RT-PCR) as a probe.

2.3. Preparation of reporter plasmids and mutagenesis

Reporter plasmids, pGL3-1600 and its derivatives (pGL3-83 to pGL3-1210) were prepared by insertion of the *Sac*I/*Bgl*II fragments of the plasmids constructed previously [12] into the corresponding sites of the promoterless and enhancerless luciferase vector pGL3-Basic (Promega). Mutation with base substitution at the CREB binding site was constructed using a QuikChange® II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol using the following oligonucleotide primers: CREB-L, 5′-GTCCTCGTGTTGTCAGACCCCGCCCACGCGCCCCT-3′ and CREB-R, 5′-CGGGGTCTGACAACACGAGGACGCGGGACGGCCAAT-3′ (mutated nucleotides underlined). The presence of mutation was verified by sequence analysis.

2.4. Transfection and luciferase assay

For the reporter analysis of the hST3Gal V promoter, transient transfection of HL-60 cells was carried out by electroporation. Briefly, the cultured cells were washed with phosphate-buffered saline and were then centrifuged at 800 rpm for 5 min at room temperature. The cells were washed in Puck's saline buffer containing 137 mM NaCl, 5.4 mM $MgCl_2$, 4.2 mM $NaHCO_3$ and 5.5 mM glucose before being suspended in permeabilization buffer (20 mM HEPES pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, pH 7.0) containing 10 μg of the luciferase reporter constructs and 10 μg of a cytomegalovirus-β-galactosidase vector (pCMVβ) as a transfection efficiency control. The 0.9 ml sample (2.5×10^6 cells) suspended in permeabilization buffer was placed in a cuvette. Electroporation was accomplished using a Bio-Rad Gene Pulser II at 500 μF and 300 V. To stimulate resealing of the cell after electroporation, the cells were incubated for 1 h at 37°C in 5% CO_2 in air and the cells were then resuspended in RPMI 1640 medium containing 10% fetal bovine serum and cultured for 12 h. TPA (30 nM) was added to induce cell differentiation at 12 h after transfection and cells were cultured for another 24 h. Cells were harvested and luciferase activity was measured using the dual luciferase reporter assay system kit (Promega) and Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland). Luciferase activity was normalized to β-galactosidase activity.

2.5. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from resting and TPA-induced HL-60 cells were prepared as described previously [13]. EMSAs were performed using a gel shift assay system kit (Promega) according to the manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus sequence for CREB (5′-TCCTCGTGACGTCAGACCCC-3′) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and used as probes for EMSA. Competition was performed using the unlabeled wild-type CREB or a mutant oligomer 5′-TCCTCGTGTTGTCAGACCCC-3′ (mut CREB) in 50-fold molar excess. Nuclear extract proteins (2 μg) were preincubated with the gel shift binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC)) for 10 min and then incubated with the labeled probe for 20 min at room temperature. For antibody supershift assays, the reactions were performed by preincubating nuclear extracts with anti-CREB antibody (Upstate Biotechnology, Lake Placid, NY, USA) at 4°C for 60 min. Each sample was electrophoresed in a 4% non-denaturing polyacrylamide gel in 0.5×TBE buffer at 250 V for 20 min. The gel was dried and subjected to autoradiography.

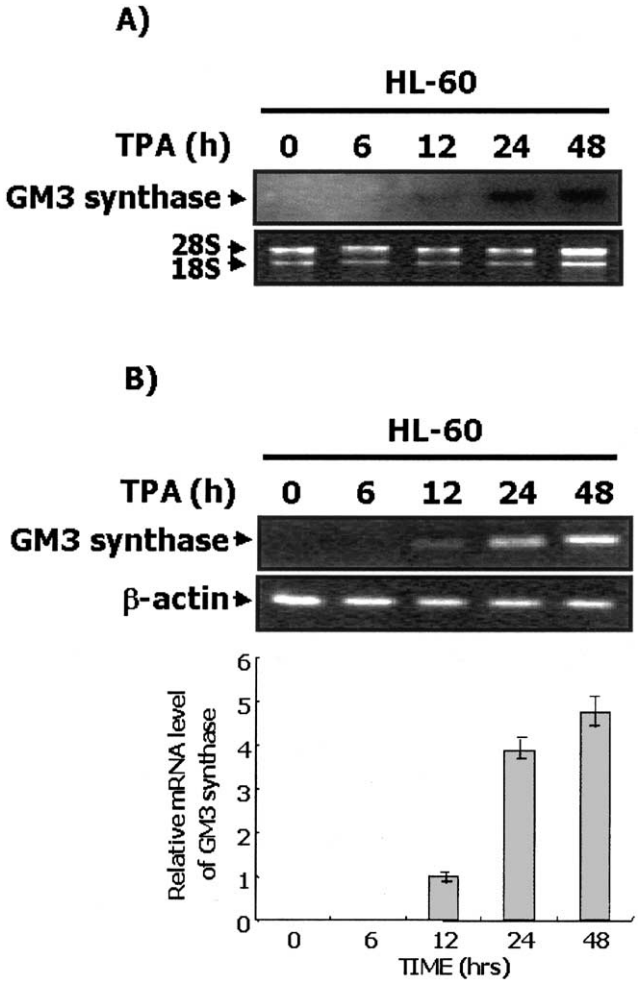

Fig. 1. Expression of hST3Gal V mRNA from HL-60 cells before and after TPA treatment. Total RNA from HL-60 cells was isolated after 0, 6, 12, 24 or 48 h of TPA treatment and hST3Gal V mRNA was detected by Northern blot analysis (A) and RT-PCR (B). The bar graphs represent the intensity of the bands obtained from RT-PCR by densitometry. The values are mean ± S.D. of three independent experiments.

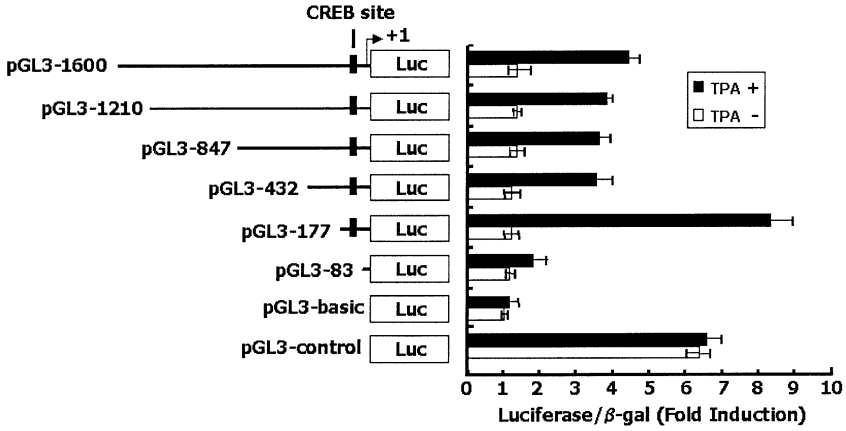

Fig. 2. Deletion analysis of hST3Gal V promoter in HL-60 cells before and after TPA treatment. A schematic representation of DNA constructions containing various lengths of the 5′-flanking region of hST3Gal V linked to the luciferase reporter gene is presented. The restriction sites are shown and the transcription start site is indicated as +1. pGL3-Basic without any promoter and enhancer was used as negative control. PGL3-Control with SV40 promoter and enhancer was used as positive control. Each construct was co-transfected into HL-60 cells with pCMVβ as the internal control. The transfected cells were incubated in the presence (solid bars) and absence (open bars) of 30 nM TPA for 24 h. Relative luciferase activity was normalized with β-galactosidase activity derived from pCMVβ. The values represent the mean ± S.D. for three independent experiments with triplicate measurements.

3. Results

3.1. Induction of hST3Gal V expression during HL-60 cell differentiation

To determine whether hST3Gal V gene expression is regulated during HL-60 cell differentiation, we analyzed the expression profile of hST3Gal V in HL-60 cells which were treated with 30 nM TPA for cellular differentiation. As shown in Fig. 1, Northern blots and RT-PCR showed that the induction of hST3Gal V mRNA became detectable 12 h after TPA treatment and increased up to 48 h. Morphological changes and surface attachment of HL-60 cells on the culture dish were also seen 12 h after TPA treatment and increased up to 48 h (data not shown). These results clearly show that the expression of hST3Gal V is stimulated during TPA-induced monocyte differentiation.

3.2. Determination of hST3Gal V promoter activity during TPA-induced cell differentiation

To characterize the region regulating the transcription activity of hST3Gal V during TPA-induced differentiation of HL-60 cells, we prepared luciferase constructs carrying 5′-deleted hST3Gal V promoters and transfected them into TPA-untreated HL-60 cells. Regulation of hST3Gal V promoter activity by TPA was examined. In TPA-uninduced cells, as shown in Fig. 2, deletion of nucleotides −1600 to −83 had no significant effect on transcriptional activity. In TPA-induced cells, however, transcriptional activities were significantly higher than in untreated control cells and gradual deletion of 5′ sequences from nucleotides −1600 to −177 resulted in an about four-fold increase in transcriptional activity compared with the promoterless and enhancerless control vector pGL3-Basic. The maximum activity was obtained

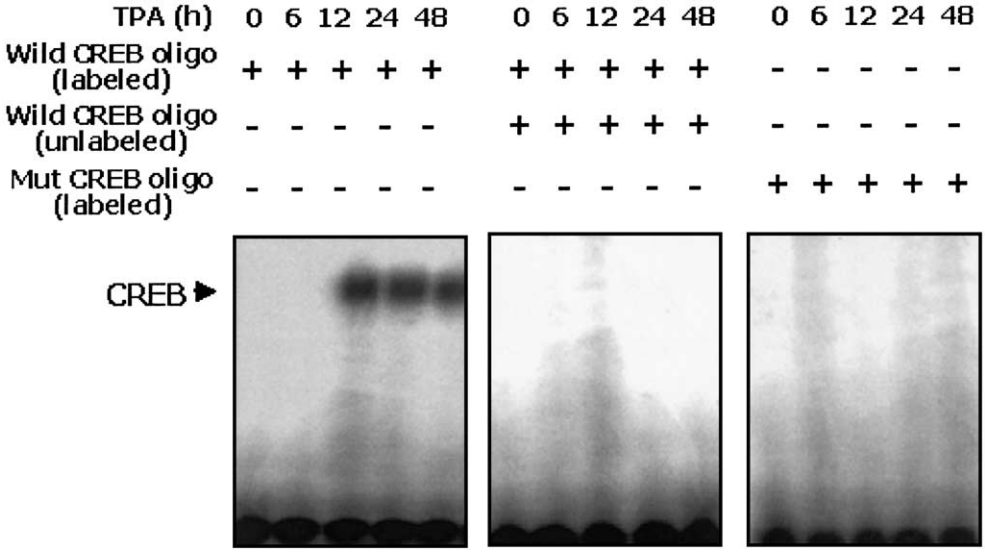

Fig. 3. EMSA with nuclear extracts and the CREB binding site sequence of the hST3Gal V promoter. Nuclear extracts isolated from HL-60 cells after 0, 6, 12, 24 or 48 h of TPA treatment were incubated with ^{32}P-labeled wild-type probe or unlabeled wild-type probe, or labeled mutant CREB probe. For competition experiments, a 50-fold molar excess of unlabeled wild-type CREB oligonucleotides was used. The DNA–protein complexes were analyzed on a 4% non-denaturing polyacrylamide gel.

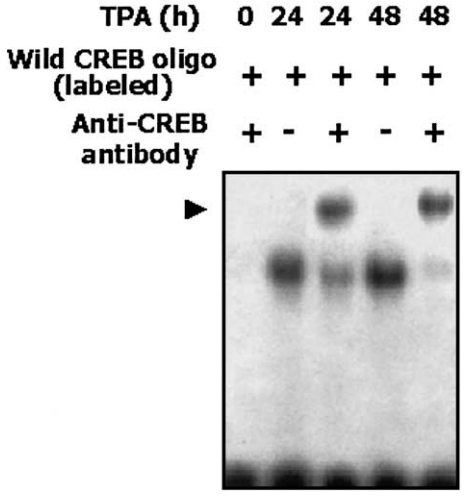

Fig. 4. Gel supershift analysis with nuclear extracts and anti-CREB antibody. Supershift analysis was performed in the presence of anti-CREB antibody (2 μg). The arrowhead indicates the shift of the complex by anti-CREB antibody.

with pGL3-177 and reached about eight-fold higher activity than pGL3-Basic. Further deletion to nucleotide −83 markedly reduced transcriptional activity to a level similar to that of the control vector pGL3-Basic. These results show that the region from nucleotides −177 to −83 functions as the TPA-inducible promoter in HL-60 cells.

3.3. *Identification of TPA-responsive element in nucleotides −177 to −83 region of the hST3al V promoter*

Our previous study [12] showed that this region from −177 to −83 is GC-rich (GC content 74%) and contains NFY, CREB, SP1, EGR3 and MZF1 binding sites. These binding sites are present in the inverted orientation except for CREB and MZF1. Moreover, the TransSignal Protein/DNA array with nuclear extract of HeLa cells suggested that of these sites only the consensus CREB binding site (TGACGTCA) at position −143 to −136 might be contributory to the hST3Gal V promoter activity [14].

To determine whether this site contributes to TPA-induced expression of hST3Gal V in HL-60 cell, we performed gel EMSA using a double-stranded ^{32}P-labeled oligo fragment (20 bp) containing the consensus sequence for CREB, and nuclear extract from TPA-treated HL-60 cells. As shown in Fig. 3, a single complex that binds to this fragment was detected, and the intensity of this complex increased significantly 12 h after TPA treatment and remained at a steady level for the next 48 h. This binding could be competed with a 50-fold molar excess of unlabeled oligo fragment itself, or mutant CREB oligo fragment. To clarify whether this band of DNA–protein complex contained CREB, we performed gel mobility shift analysis in the presence of anti-CREB antibody (Fig. 4). The incubation of nuclear extracts with anti-CREB antibody resulted in a supershift of the complex with a concomitant diminution of the retarded band (Fig. 4, lanes 3 and 5). These results indicated that the binding to 20 bp fragment is CREB-specific, and a complex contains CREB protein.

To further confirm that this CREB binding site plays an important role in TPA-induced expression of hST3Gal V in HL-60 cells, a CREB mutant (pGL3-177mtCREB) was generated which contains the exact same construct as wild-type pGL3-177 except that two nucleotides within the CREB binding site had been changed (from TGACGTCA to TG*TT*GTCA). It was clearly observed that this change abolished CREB binding to this CREB binding site, since the mutant CREB oligomer (containing TG*TT*GTCA) could not compete for the binding while the wild-type CREB oligomer (containing TGACGTCA) could (Fig. 3). In TPA-treated HL-60 cells, as expected, this mutation markedly reduced transcriptional activity to a level comparable to that exhibited by the promoterless and enhancerless control vector pGL3-Basic (Fig. 5). These combined results indicate that this CREB site is crucial for the TPA-induced expression of hST3Gal V, and that the CREB binding to this site is involved in the induction of hST3Gal V by TPA.

4. Discussion

Previous studies have shown that hST3Gal V activity and the amount of GM3 ganglioside markedly increase hST3Gal V in HL-60 cells treated with TPA, which is a well-known monocyte/macrophage differentiation inducer [8–11]. Those results have also suggested that the dramatic GM3 increase

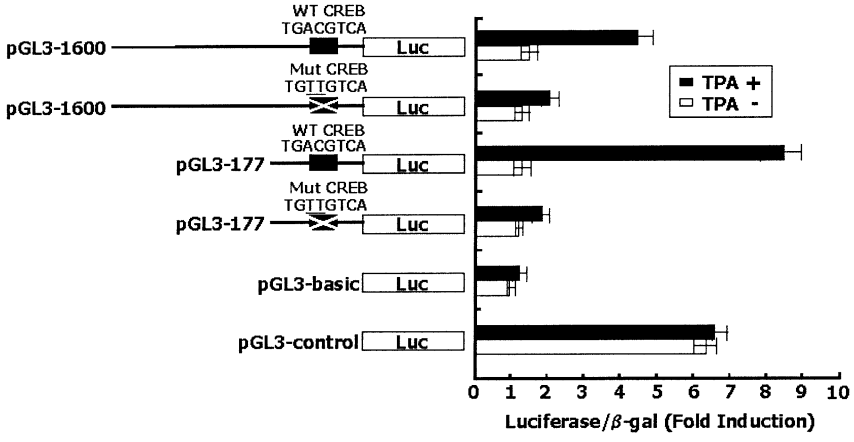

Fig. 5. Effect of mutation in the CREB binding site on hST3Gal V promoter activity. pGL3-Basic without any promoter and enhancer was used as negative control. PGL3-Control with SV40 promoter and enhancer was used as positive control. Each construct was co-transfected into HL-60 cells with pCMVβ as the internal control. The transfected cells were incubated in the presence (solid bars) and absence (open bars) of 30 nM TPA for 24 h. Relative luciferase activity was normalized with β-galactosidase activity derived from pCMVβ. The values represent the mean ± S.D. for three independent experiments with triplicate measurements.

is the consequence of the up-regulation of hST3Gal V. However, transcriptional regulation of hST3Gal V during TPA-induced monocyte differentiation of HL-60 cells has not been examined at the molecular levels. Here, we report that the expression of hST3Gal V is up-regulated during TPA-induced monocyte differentiation of HL-60 cells. TPA treatment increased hST3Gal V mRNA levels in a time-dependent fashion: the induction of hST3Gal V mRNA became detectable 12 h after TPA treatment and increased up to 48 h. The hST3Gal V activity also increased in a time-dependent manner upon TPA treatment (data not shown). The marked increases of hST3Gal V activity and GM3 levels in HL-60 cells have been also observed 12 h after TPA treatment and increased up to 48 h [9,10]. Therefore, these results clearly indicate that the relative levels of hST3Gal V mRNA and its protein are increased in a time-dependent fashion by TPA treatment in HL-60 cells.

Our results in this study show that the region between −177 and −83 in the hST3Gal V promoter functions as the core promoter essential for transcriptional activation of hST3Gal V in TPA-induced HL-60 cells. This region was found to be also needed for the enhancer activity of the hST3Gal V promoter in SK-N-MC and HepG2 cells [12]. Endogenous hST3Gal V gene expression was detected in both cells by RT-PCR [12], but not in TPA-uninduced HL-60 cells. This indicates that this region functions as the TPA-inducible promoter in HL-60 cells. Our previous study revealed the existence of several transcription factor binding sites such as NFY, CREB, SP1, EGR3 and MZF1 in this region [12]. A recent report has suggested that only the consensus CREB binding site (TGACGTCA) at position −143 to −136 in this region might contribute to hST3Gal V promoter activity [14]. Our present result by site-directed mutagenesis indicates that this CREB element mediates TPA-dependent up-regulation of hST3Gal V expression. By EMSA, we demonstrate that CREB binds to this site of the hST3Gal V promoter in a time-dependent manner by TPA induction, as shown by the increases of hST3Gal V expression, enzyme activity and GM3 levels. Thus, our data strongly suggest that CREB may play an import role during TPA-induced monocyte differentiation of HL-60 cells.

CREB is a transcription factor that is the target of a variety of signaling pathways mediating cell responses to extracellular stimuli, involving proliferation, differentiation, and adaptive responses of cell processes [15,16]. Several signal pathways, such as protein kinase A, PKC, Ca^{2+}/calmodulin kinases, stress-activated protein kinase/c-Jun N-terminal kinase, P38, and extracellular signal-regulated kinase mitogen-activated protein kinases (MAPKs), could activate CREB binding to CRE, and the capacity of CREB to activate transcription is regulated by phosphorylation at serine 133 [15,16]. A previous study has shown that the activation of PKC by TPA in human fibroblasts stimulates CREB phosphorylation and subsequent CREB-mediated gene transcription [17]. Other studies have also indicated that CREB phosphorylation in oligodendrocytes may be mediated by PKC and MAPK-dependent signal transduction pathways [18–20].

It has been suggested that PKC may specifically activate hST3Gal V, resulting in an increase in the content of GM3 ganglioside during the differentiation of HL-60 cells induced by TPA, and that PKC activated by TPA may regulate the expression of the hST3Gal V gene at the transcriptional level [9]. It is possible, therefore, that the expression of the hST3Gal V gene may be activated by PKC and MAPK-dependent signaling pathway. Further studies are scheduled to dissect these pathways for clarifying the up-regulation-associated mechanisms of hST3Gal V expression in TPA-induced HL-60 cells.

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