Glycobiology vol. 16 no. 5 pp. 375–389, 2006
doi:10.1093/glycob/cwj087
Advance Access publication on February 15, 2006

Transcriptional regulation of the human GD3 synthase gene expression in Fas-induced Jurkat T cells: a critical role of transcription factor NF-κB in regulated expression

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Received on August 1, 2005; revised on December 27, 2005; accepted on February 3, 2006

The transcriptional regulation mechanisms involved in the up-regulation of Fas-induced GD3 synthase gene have not yet been elucidated. 5′-Rapid amplification of cDNA end (5′-RACE) using mRNA prepared from Fas-induced Jurkat T cells revealed the presence of multiple transcription start sites of human GD3 synthase gene, and the 5′-end analysis of the longest of its product showed that transcription started from 650 nucleotides upstream of the translational initiation site. Promoter analyses of the 5′-flanking region of the human GD3 synthase gene using luciferase gene reporter system showed strong promoter activity in Fas-induced Jurkat T cells. Deletion study revealed that the region from –1146 to –646 (A of the translational start ATG as position +1) was indispensable for the Fas response. This region lacks apparent TATA and CAAT boxes but contains putative binding sites for transcription factors c-Ets-1, cAMP-responsive element-binding (CREB) protein, activating protein 1 (AP-1), and NF-κB. Base-substitution experiment showed that only the NF-κB-binding site of putative binding sites is required for the maximal expression induced by Fas. Both DNase I footprint and electrophoretic mobility shift assays with the nuclear extract of Fas-induced Jurkat T cells revealed that NF-κB was bound specifically to the probe being mediated by its binding site in the promoter sequence. Taken together, these results indicate that NF-κB plays an essential role in the transcriptional activity of human GD3 synthase gene in Fas-induced Jurkat T cells. In addition, the translocation of NF-κB-binding protein to nucleus by Fas activation is also crucial for the increased expression of the GD3 synthase gene in Fas-activated Jurkat T cells.

Key words: Fas-induced Jurkat T cell/GD3 synthase/NF-κB/transcriptional regulation

Introduction

Gangliosides, the sialic acid (*N*-acetylneuraminic 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 (Svennerholm, 1980). They play important roles in a large variety of biological processes such as cell–cell interaction, adhesion, cell differentiation, growth control, and receptor function (Hakomori and Igarashi, 1993; Varki, 1993). These gangliosides have been utilized as tumor-associated antigens for diagnosis and treatment, and they have been implicated as modulators of signal transduction (Hakomori, 2002). Among these gangliosides, the disialoganglioside GD3, which is weakly expressed in most normal tissues, but highly expressed during development and in pathological conditions such as cancer, neurodegenerative disorders, and atherosclerosis, is responsible for diverse events including proliferation, differentiation, and apoptosis (Malisan and Testi, 2002). In particular, GD3 has recently attracted considerable attention because of its emerging role in apoptotic signaling pathway as an apoptotic effector.

Recent studies have demonstrated that GD3, which is synthesized from monoganglioside GM3 by the Golgi-resident GD3 synthase (ST8Sia I), directly targets mitochondria, inducing the loss of mitochondrial transmembrane potential and ensuing release of cytochrome *c* and activation of caspases, which contributes to apoptotic program (Kristal and Brown, 1999; Scorrano *et al*., 1999; Garcia-Ruiz *et al*., 2000; Rippo *et al*., 2000). Furthermore, the significance of GD3 and GD3 synthase in the process of Fas-induced apoptosis has been highlighted by recent studies that demonstrate that the intracellular accumulation of GD3 contributes to mitochondrial damage, a crucial event during the apoptotic program (De Maria *et al*., 1997, 1998; Malisan and Testi, 2002). In addition, it was reported that GD3 contributes to Fas-mediated apoptosis via association with the cytoskeletal protein ezrin (Giammarioli *et al*., 2001).

Fas (CD95/APO-1) is a member of the tumor necrosis factor (TNF) receptor superfamily and is currently recognized as the principal cell-surface receptor involved in the transduction of signals that induce apoptosis in lymphocytes and in a variety of tumor cells (Nagata and Golstein, 1995). Fas-mediated apoptosis is triggered following engagement of the Fas receptor by a specific ligand (FasL) expressed on activated cytotoxic T cells (Nagata and Golstein, 1995) and by specific anti-Fas monoclonal antibodies

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(mAb). The interaction between Fas and FasL induces receptor trimerization, death domain-mediated recruitment of the Fas-associated death domain (FADD) adaptor protein, and binding and activation of caspase-8 to FADD through their death-effector domain, thereby initiating the caspase cascade responsible for the apoptotic process (Muzio *et al.*, 1996; Krammer, 2000).

Previous studies have shown that GD3 rapidly accumulates upon Fas triggering through cross-linking by anti-Fas mAb CH11 or ceramide exposure and directly induces apoptosis in human tumor lymphoid and myeloid cell lines. GD3 accumulation requires ceramides produced by the action of acidic sphingomyelinase (De Maria *et al.*, 1997, 1998). Moreover, the enforced expression of the human GD3 synthase was sufficient to trigger cell death. Prevention of endogenous GD3 accumulation, by suppressing human GD3 synthase expression through the use of specific antisense oligodeoxynucleotides, substantially blocked Fas- and ceramide-induced apoptosis. Thus ceramide conversion into GD3 is a crucial step in Fas- and ceramide-induced apoptosis. GD3 rapidly induces dissipation of the inner mitochondrial transmembrane potential and stimulates a burst of reactive oxygen species (ROS) from mitochondria. Recent report also showed that activation of Fas receptor is entirely responsible for the increase in GD3 levels and GD3 synthase mRNA in cultured cerebellar granule cells, which contributes to the development of apoptosis by trophic deprivation (Castiglione *et al.*, 2004). Despite increasing understanding of the important physiological roles for GD3 and GD3 synthase in Fas-induced apoptotic process, little is yet known about how the GD3 synthase expression responsible for GD3 formation in Fas-induced apoptosis is regulated.

Using the human acute leukemia cell line Jurkat T, which is well characterized and has been used extensively to establish the molecular components and the epistasis of the Fas-mediated apoptosis (Delneste *et al.*, 1996; Totpal *et al.*, 1996; Cuvillier *et al.*, 1998; Holmström *et al.*, 1998; Whitekus *et al.*, 1999), in this study, we investigated the mechanism controlling enhanced expression of GD3 synthase gene in response to Fas signaling. This report is the first that we are aware of to demonstrate the regulatory molecular mechanism of the GD3 synthase gene expression in Fas-induced cells, and it demonstrates that human GD3 synthase gene expression is up-regulated especially through NF-κB-dependent activation in Fas-induced Jurkat T cells.

Results

The increase in GD3 synthase gene expression and induction of intracellular GD3 levels from Fas-induced Jurkat T cells

To determine whether Fas stimulation induces the up-regulation of human GD3 synthase gene and the increase of its product, ganglioside GD3 in Jurkat T cells, we treated Jurkat T cells with anti-Fas CH11, Fas agonist antibody. As shown in Figure 1, reverse transcription polymerase chain reaction (RT–PCR) and northern blots showed that the expression of human GD3 synthase mRNA represented remarkable increase in quantity, which peaked at 5 min after the anti-Fas CH11 treatment and then significantly

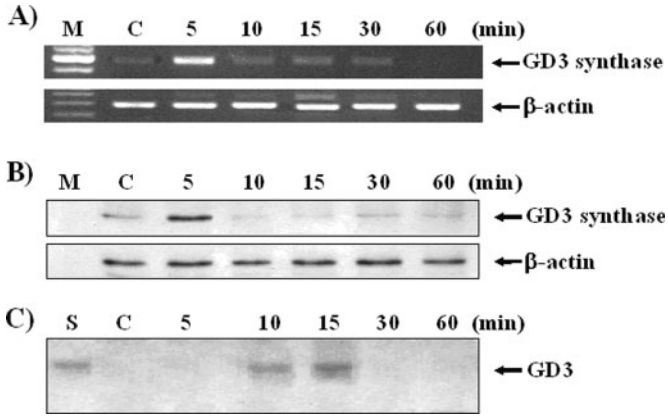

Fig. 1. The increase of human GD3 synthase gene expression and induction of intracellular GD3 levels from Fas-stimulated Jurkat T cells. (**A**) Total RNA from Jurkat T cells was isolated after 0, 5, 10, 15, 30, or 60 min of anti-Fas mAb CH11 treatment and human GD3 synthase mRNA was detected by reverse transcription polymerase chain reaction (RT–PCR) and β-actin was included as an internal control. (**B**) Total RNA from Jurkat T cells was isolated after 0, 5, 10, 15, 30, or 60 min of anti-Fas mAb CH11 treatment and human GD3 synthase mRNA was detected by northern blot and β-actin was included as an internal control. (**C**) Glycolipids from Jurkat T cells were isolated after 0, 5, 10, 15, 30, or 60 min of anti-Fas mAb CH11 treatment. After separation of gangliosides on high-performance thin-layer chromatography (HPTLC), ganglioside GD3 was detected by HPTLC immunostaining with 4F6 anti-GD3 monoclonal antibody.

decreased (Figure 1A and B). Whether an increased level of total cellular GD3 followed human GD3 synthase mRNA induction was next studied by high-performance thin-layer chromatography (HPTLC) immunostaining using the anti-GD3 mAb. It was revealed that GD3 levels also represented remarkable increase in quantity by Fas stimulation, which peaked at 15 min after the anti-Fas CH11 treatment and then rapidly decreased (Figure 1C), indicating that GD3 was transiently accumulated within 15 min after the anti-Fas CH11, just as reported previously (De Maria *et al.*, 1997). Consistent with the effect of Fas induction on human GD3 synthase mRNA expression, Fas activation dramatically increased the expression of GD3 in Jurkat T cells.

Identification of the transcription start site of Fas-induced human GD3 synthase gene

To determine the transcriptional mechanisms underlying the regulation of human GD3 synthase mRNA expression in response to Fas in Jurkat T cells, we sought to identify and to characterize the promoter sequences of human GD3 synthase gene. As the first step, which is necessary to analyze the promoter activity of 5′-flanking region of human GD3 synthase gene, as shown in Figure 2A, we sought to determine the transcription start site of the human GD3 synthase gene from mRNA prepared from Fas-induced Jurkat T cells by the same 5′-rapid amplification of cDNA end (5′-RACE) method as we reported previously (Kim *et al.*, 2003). The secondary nested polymerase chain reaction (PCR) produced several discrete bands, indicating the existence of multiple mRNA forms (Figure 2B), as have been identified in other human sialyltransferase genes (Harduin-Lepers *et al.*, 2001). Among them, two products (about 1.0 kb

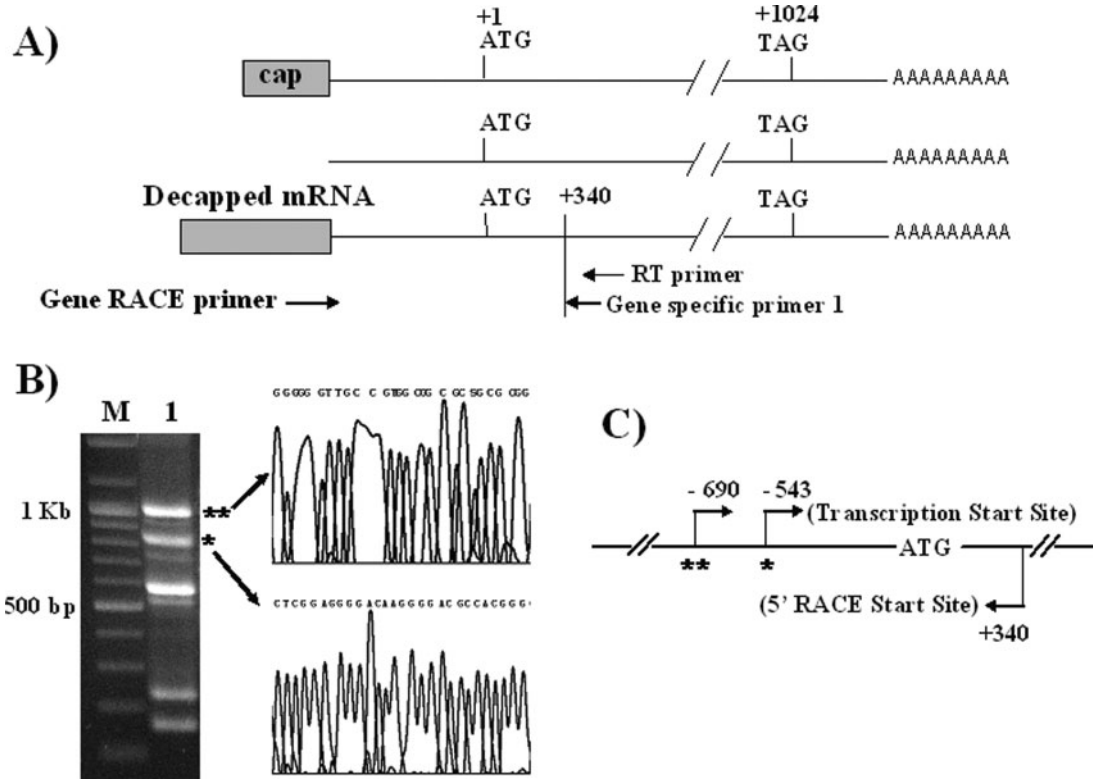

Fig. 2. Identification of the transcription start site in the 5′-flanking region of Fas-induced human GD3 synthase gene by rapid amplification of cDNA end (RACE) polymerase chain reaction (PCR). (**A**) mRNA from Jurkat T cells was prepared after 5 min of anti-Fas mAb CH11 treatment. Reverse transcriptase reaction was performed using RT primer and PCR was performed with gene RACE primer and gene- specific primer 1 with 5′-RACE strategy. (**B**) Sequencing and agarose gel (2%) analysis of the 5′-RACE nested PCR product. Lane 2 indicates the final products amplified by 5′-RACE nested PCR for the determination of transcription starts of the human GD3 synthase gene after 5 min of anti-Fas mAb CH11 treatment. (**C**) Transcription initiation sites of Fas-induced human GD3 synthase gene. Asterisks represent each of nested PCR bands or transcription start sites from 5′-RACE.

and 0.84 kb), which are supposed to be longer cDNA than that of human GD3 synthase amplified by 5′-RACE from human melanoma cell line SK-MEL-28 (Furukawa *et al.*, 2003), were subcloned and sequenced. From the sequence analysis of these products, the main start sites from 1.0 kb product were found at –650, –648, and –646 nucleotides (Figure 3), whereas those from 0.84 kb product were mapped at –499, –497, –493, and –490 nucleotides. These sequences included a 482-bp segment at their 3′-ends that exactly matched the sequence in the 5′-untranslated region of the previously described human GD3 synthase cDNA (Sasaki *et al.*, 1994). In addition, the 1.0-kb product extended 167 bp further in the 5′ direction than the 5′-RACE product reported previously (Furukawa *et al.*, 2003). These results confirm that these 5′-RACE products obtained from Fas-induced Jurkat T cells did indeed represent the 5′-flanking region of the human GD3 synthase gene. Based on this finding, a search of GenBank™ human genomic sequences resulted in the identification of a genomic sequence upstream of the transcription start site of human GD3 synthase gene.

Cloning and sequence analysis of the 5′-flanking region of human GD3 synthase gene

We identified a human genomic sequence (GenBank™ accession number NT_009714, at nucleotides 15096192–15263337) derived from 12p.12.1-p11.2 containing the human GD3 synthase cDNA sequence. The genomic sequence completely matched the coding region of human GD3 synthase gene and 5′-untranslated region obtained from 5′-RACE. To determine whether this sequence included the promoter region of the human GD3 synthase, three fragments (+1 to 2000, –499 to –2499, and –646 to –2646) were amplified from human genomic DNA by long and accurate PCR (LA-PCR) and cloned into a promoterless and enhancerless pGL3-Basic vector. To predict the transcription factor binding sites on 5′-flanking region, we used the conserved promoter region of human, mouse, and rat. We obtained 3.1-kb upstream regions (promoter region) from transcription start site of GD3 synthase. Mouse and rat GD3 synthase promoter regions are homologous to 5′-flanking region of human (identifiable by BLAST). Conserved regions of human, mouse, and rat GD3 promoter region were predicted by using CLUSTALW multiple alignment program. The conserved promoter region is analyzed using MATCH in TRANSFAC 8.0. MATCH is a weight matrix-based tool for searching putative transcription factor binding sites in DNA sequences. MATCH is closely interconnected and is distributed together with the TRANSFAC database. Therefore, the analysis revealed that this region lacks canonical TATA and CAAT boxes but contains several putative Fas-activated transcription factor-binding sites near or upstream of the putative transcription start site, including c-Myb, Elk-1, GATA-1, c-Ets-1, cAMP-responsive

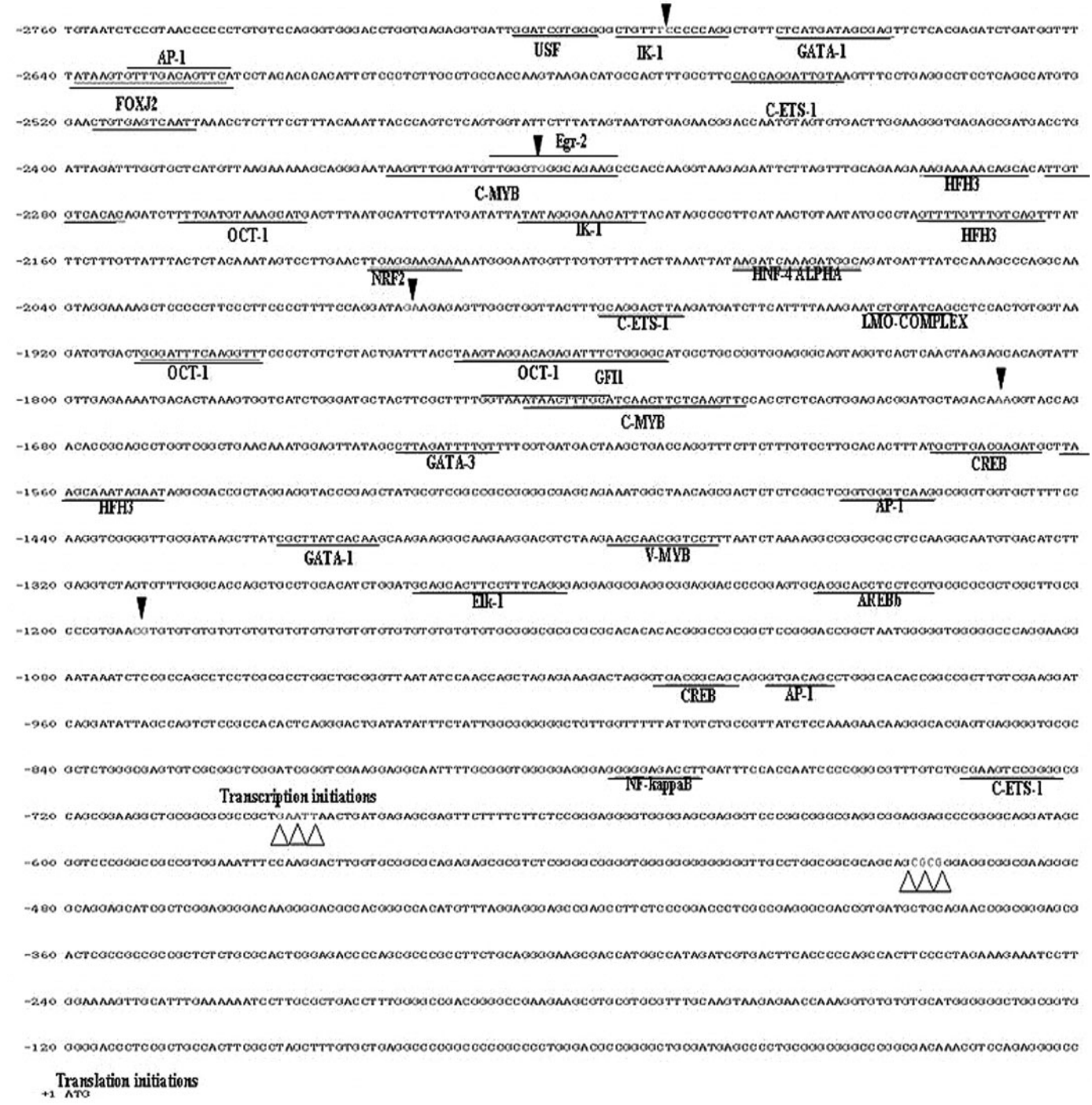

Fig. 3. Nucleotide sequence of the 5′-flanking region of the Fas-induced human GD3 synthase gene. The numbering of the nucleotides begins with the A of translation initiation codon (ATG) as +1. The open arrowheads indicate the transcription initiation sites determined by 5′-RACE of Figure 2A. The putative transcription factor binding sites are shown by under (sense) or upper (antisense) lines. For the deletion of promoter activity, the start point of each construction is indicated by filled arrowheads.

element-binding (CREB) protein, activating protein 1 (AP-1), NF-κB (Figure 3). However, it should be noted that these putative sites were not found in sequence analysis of 5′-flanking region with the Matlnspector v2.2 program (core similarity 1.0, matrix similarity 0.95) using TRANSFAC 4.0 matrices (Furukawa *et al.*, 2003).

Functional analysis of the 5′-flanking region of the human GD3 synthase gene in Fas-induced Jurkat T cells

To determine whether or not the 5′-flanking sequence of the human GD3 synthase gene contains a Fas-responsive promoter, we constructed three kinds of reporter plasmids (–2000 ~ +1/pGL3, –2646 ~ –646/pGL, and –2499 ~ –499/pGL3)

each containing 5′-flanking region (–2000 ~ +1, –2646 ~ –646, –2499 ~ –499) of the human GD3 synthase gene fused to the promoterless and enhancerless luciferase gene in pGL3-Basic. The three constructed reporter plasmids and pGL3-Basic plasmid as a negative control were transfected into the Fas-uninduced Jurkat T cells, and regulation of the human GD3 synthase promoter activity by Fas was examined. As shown in Figure 4A, the –2646 ~ –646/pGL3 showed a remarkable increase of promoter activity in Fas-induced Jurkat cells, which was about 2-fold higher than in Fas-uninduced Jurkat cells. However, this significant response to Fas was not observed in the other constructs, –2000 ~ +1/pGL3 and –2499 ~ –499/pGL3, although they exhibited a high level of promoter activity as

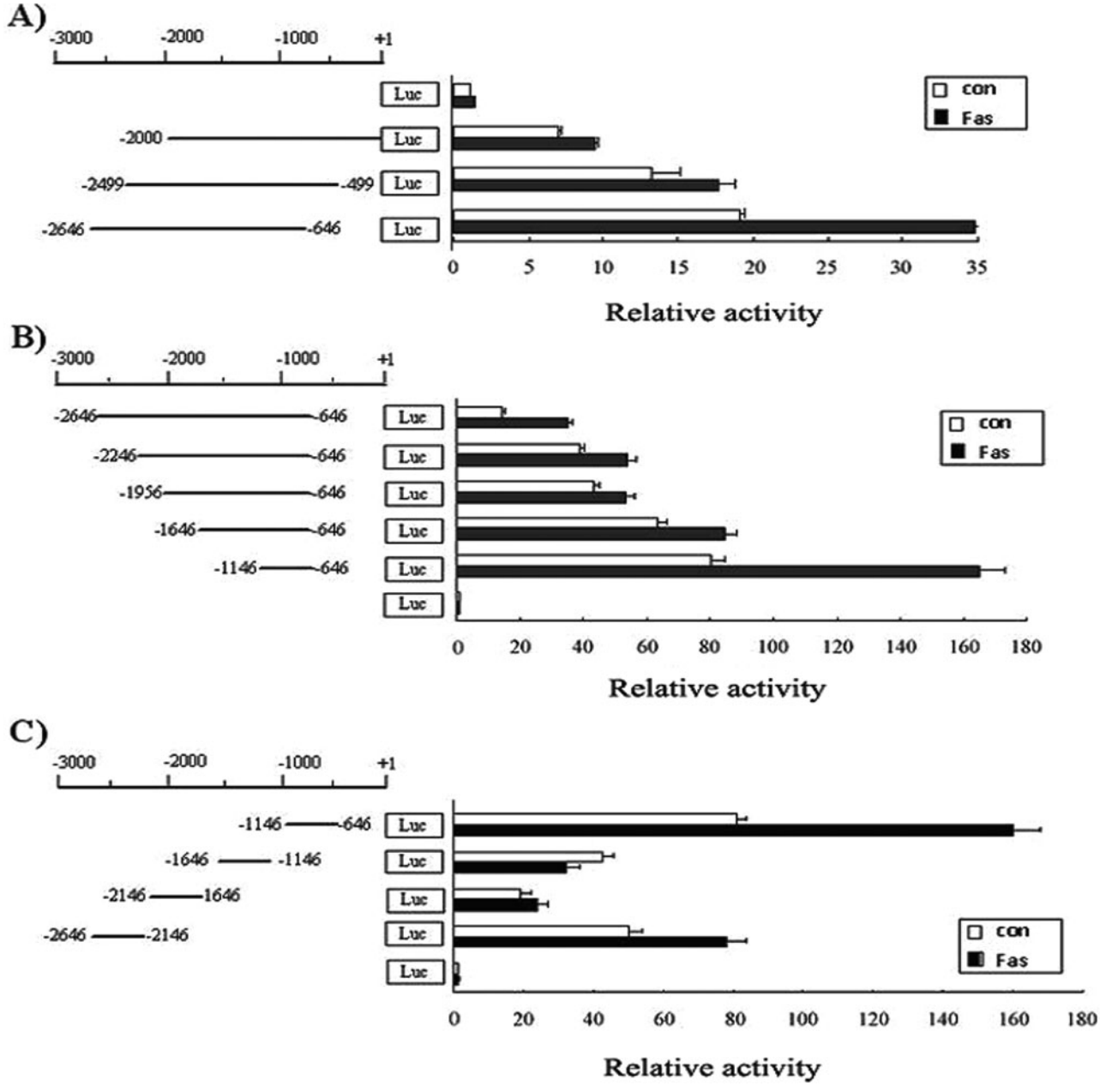

Fig. 4. Deletion analysis of human GD3 synthase gene promoter in Jurkat T cells before and after Fas stimulation. (**A**) A schematic representation of DNA constructions containing three equal lengths from different starts of the 5′-flanking region of human GD3 synthase gene linked to the luciferase reporter gene is presented. The length sizes are shown and the translation start site is indicated as +1. pGL3-Basic without any promoter and enhancer was used as a negative control. pGL3-control with SV40 promoter and enhancer was used as a positive control. Each construct was co-transfected into Jurkat T cells with pCMV as the internal control. The transfected cells were incubated in the presence (solid bars) and absence (open bars) of anti-Fas mAb CH11 (300 ng/ml) for 5 min. Relative luciferase activity was normalized with β-galactosidase activity derived from pCMV. The values represent the mean ± SD for three independent experiments with triplicate measurements. (**B**) A schematic representation of DNA constructions containing various lengths of the –2646 to –646 promoter region regulating the human GD3 synthase gene by Fas in Jurkat T cells is presented. (**C**) A schematic representation of DNA constructions containing same length deletions of the –2646 to –646 promoter region regulating the human GD3 synthase gene by Fas in Jurkat T cells is presented.

compared with that of the pGL3-Basic vector in Fas-uninduced Jurkat T cells. These results clearly suggest that the region between nucleotides –2646 and –646 was important for the expression of human GD3 synthase gene in response to Fas in Jurkat T cells.

Based on this finding, to analyze the minimal Fas-responsive region controlling the maximal promoter activity of the human GD3 synthase in Jurkat T cells, we constructed four additional reporter plasmids (–1146 ~ –646/pGL3 to –2246 ~ –646/pGL3) containing progressive 5′ deletions between –2646 and –646 and transfected into Fas-uninduced Jurkat T cells, and changes in the promoter activities by Fas stimulation were analyzed. As shown in Figure 4B, deletion of nucleotides –2646 ~ –1146 resulted in significantly increased promoter activity as compared with that of the pGL3-Basic vector in human Jurkat T cells treated for 5 min with anti-Fas CH11, which was more than 2-fold increase in promoter activity. Although sequential deletions of the region between nucleotides –2646 and –1646 had no significant effect on promoter activity, further deletion from –1646 to –1146 increased the promoter activity in Fas-induced Jurkat T cells, suggesting the presence of a negative regulatory element in this region. The maximum activity was obtained with –1146 ~ –646/pGL3 and reached 160-fold higher activity than pGL3-Basic in Fas-induced Jurkat T cells. These results show that the region between nucleotides –1146 and –646 functions as the Fas-inducible promoter in Jurkat T cells treated for 5 min with anti-Fas CH11.

Furthermore, based on this result, to determine whether the region from nucleotides –1146 to –646 has Fas-responsive elements in Jurkat T cells, we also prepared three additional reporter plasmids containing simultaneous deletions from both 5′- and 3′-ends of the human GD3 synthase gene promoters and transfected into Fas-uninduced Jurkat T cells. Then, promoter activity of the human GD3 synthase gene in response to Fas was determined. As shown in Figure 4C, the promoter activity with –2146 ~ –1646/pGL3 was very low in Jurkat T cells with or without Fas stimulation. Deletion in the region from –2146 to –646 resulted in an increase of the promoter activity, whereas deletion in the region from –1646 to –646 markedly reduced the promoter activity in Jurkat T cells with or without Fas induction. In Fas-induced Jurkat T cells, however, the most marked decrease was associated with loss of sequence between –1146 and –646. These results suggest that potential Fas-responsive regulatory elements exist within the –1146 to –646 and –2646 to –2146 and that potential negative regulatory elements exist within the –2146 to –1646. Taken together, these results clearly indicate that the region between –1146 and –646 functions as the Fas-responsive core promoter region essential for the transcriptional activation of the human GD3 synthase gene in Jurkat T cells. This also suggests that promoter elements located between nucleotide positions –1146 and –646 are dominantly working for enhanced expression of the human GD3 synthase gene by Fas in this cell line. As shown in Figure 3, this region from –1146 to –646 contains putative binding sites such as c-Ets-1, AP-1, CREB, and NF-κB.

Identification of nuclear factor-interacting elements in the core promoter of the human GD3 synthase gene controlling by Fas

The transient transfection results indicated that the region from –1146 to –646 contains several potential elements such as CREB, AP-1, NF-κB, and c-Ets-1 that regulate human GD3 synthase expression by Fas in Jurkat T cells. To identify the Fas-effective sites for interaction between DNA sequences and nuclear proteins involved in transcriptional regulation, DNase I footprint analyses were performed using Fas-induced Jurkat nuclear extract and DNA fragments that span the functional –1146 ~ –646 region of the human GD3 synthase gene. Two different probes, which contain the region from–1146 to –905 bp (Figure 5A) and from –905 to –646 bp (Figure 5B), were used in this experiment; each of them was repeated four times with independently prepared Fas-induced Jurkat nuclear extracts. As shown in Figure 5A, two protected regions, FP1 (footprint) 1 in the –966 to –957 bp and FP2 in the –955 to –947 bp

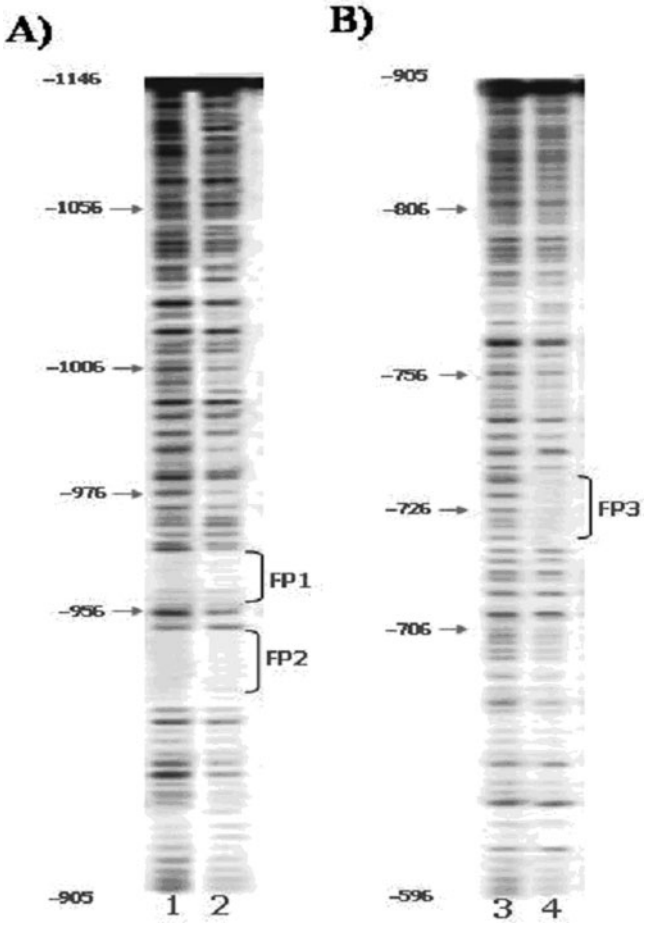

Fig. 5. DNase I footprinting analysis of the Fas-responsive promoter region of the human GD3 synthase gene. 5′-end labeled probes of –1146/–905 (**A**) and –905/–646 (**B**) were used. These probes were incubated with Jurkat nuclear extracts (lane 1 and lane 3) and Fas-induced Jurkat nuclear extracts (lane 2 and lane 4). Treatment time of anti-Fas mAb CH11 was limited at 5 min. Undigested 5′-end labeled nucleotides are presented with arrows as a size marker. The protected regions, FP1, FP2, and FP3, are indicated by square brackets.

regions, in Jurkat T cells with or without Fas treatment were observed in the –1146 ~ –905 fragment of the functional –1146 ~ –646 region. As shown in Figure 5B, however, one protected region, FP3 in the –731 to –722 region, in Fas-induced Jurkat T cells in comparison with Fas-uninduced control cells was detected in the –905 ~ –646 fragment of the same region. These results indicate that footprint analysis of the region –1146 ~ –905 (Figure 5A) revealed two protected areas, –966 ~ –957, and –955 ~ –947, which encompass CREB and AP-1 core motif in Jurkat T cells with or without Fas stimulation. However, footprint analysis of the sequence –905 ~ –646 (Figure 5B) showed one protected region: the –731 ~ –722, which corresponds to an NF-κB site in stimulating the Fas into the Jurkat T cells differently in control cells. The binding sites located within proximal promoter region of the human GD3 synthase gene that include perfect matches with putative regulatory elements for CREB (consensus sequence motif: 5′-TGACGTCA-3′), AP-1 (sequence consensus motif: 5′-TGACG-3′and 5′-TGACA-3′) and NF-κB (sequence consensus motif: 5′-GGGGA-3′) as well as additional consensus binding motif, c-Ets-1 (sequence consensus motif: 5′-TCCGG-3′), as shown in Figure 3. The results suggest that, although the region from –1146 to –646 contains several potential elements that regulate the human GD3 synthase gene expression, Fas-responsive transcription factor responsible for the up-regulation of human GD3 synthase gene is shown by only one binding site, NF-κB, existing in the core promoter region of human GD3 synthase gene.

Determination of the Fas-responsive binding sites in nucleotides –1146 ~ –646 region of the human GD3 synthase promoter

From the DNA footprinting data, to determine whether these binding sites contribute to transcriptional regulation of human GD3 synthase gene in Fas-induced Jurkat T cells, electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts of Jurkat T cells with or without Fas induction for 5 min. To further characterize the binding sites for CREB, AP-1, NF-κB, and c-Ets-1 motifs found within the –1146 ~ –646 region of human GD3 synthase promoter, four synthetic double-stranded oligonucleotides (Table I) were ^{32}P-labeled and subjected to EMSA (Figure 6). The mobility results obtained from four independent experiments demonstrated the formation of protein–DNA complex for CREB, AP-1, NF-κB, and c-Ets-1 motifs after incubation with Fas-induced Jurkat nuclear extract and control nuclear extract in comparison with incubation in the absence of nuclear extract with or without Fas treatment. All of the complexes were abolished by competition experiments when the Fas-induced nuclear extract was pre-incubated with an unlabeled homologous oligonucleotide, thus demonstrating the specificity of the interactions between the nuclear proteins and the CREB, AP-1, NF-κB, and c-Ets-1 motifs of the –1146 ~ –646 region sequence. As shown in Figure 6, a CREB probe (–981 to –950) formed two DNA–protein complexes with Jurkat nuclear proteins by Fas or not (Figure 6A, lanes 2 and 3). AP-1 probe (–961 to –932) also formed two DNA–protein complexes with Jurkat nuclear proteins by Fas or not (Figure 6B, lanes 2 and 3). These results indicate that putative transcription factors of CREB and AP-1 were turned out not Fas-responsive elements but constitutive elements involved in human GD3 synthase gene expression (Figure 6A and B, lane 2). Similarly, as shown in Figure 3, different transcription factor remaining in the –1146 ~ –646 region, c-Ets-1 was proved to be irrelevant to the human GD3 synthase gene expression in Jurkat T cells with or without Fas induction (Figure 6D). Interestingly, as shown in Figure 6C, NF-κB probe (–740 to –711) formed two DNA–protein complexes with Fas-uninduced Jurkat nuclear proteins (Figure 6, lane 2) but three DNA–protein complexes with Fas-induced Jurkat nuclear proteins (Figure 6C, lane 3). Complex formation completely disappeared by competition with a 50-fold molar excess of unlabeled probe (Figure 6C, lane 4), but not by the same molar excess of unlabeled NF-κB mutant oligonucleotide (Figure 6C, lane 5). Furthermore, to clarify whether this band of DNA–protein complex contains NF-κB, we performed gel mobility super shift analysis in the presence of anti-NF-κB antibody against RelA (p65) (Figure 6E). The incubation of nuclear extracts with anti-NF-κB antibody resulted in a supershift of the complex with a concomitant diminution of the retarded band (Figure 6E, lanes 3 and 5). These results indicated that the binding to 30-bp fragment is NF-κB specific and a complex contains NF-κB protein. The nuclear protein, NF-κB, also interacts with the Fas-responsive NF-κB elements at nucleotide positions –740 and –711 and thereby modulates the Fas-activated transcription of human GD3 synthase gene expression in Fas-induced Jurkat T cells.

The NF-κB regulates the core promoter of human GD3 synthase gene in Fas-induced Jurkat T cells

From the DNase I footprinting and EMSA analysis, four nuclear protein binding sites were identified between –1146 and –646 bp. To further confirm whether these binding sites play an important role in Fas-induced expression of human GD3 synthase gene in Jurkat T cells, four mutants (pGL3–1146/–646mtCREB, mtAP-1, mtNF-κB, and mtc-Ets-1) were generated, which contain the same construct as wild-type pGL3–1146/–646, except that combined nucleotides within these binding sites had been changed. The mutated oligonucleotides used in the mutagenesis by PCR are described in Table I. A series of substituted mutations of luciferase constructs (Figure 7) were transfected into Fas-uninduced Jurkat T cells, and luciferase assays by Fas induction were carried out. The activity of each construct was compared with that of pGL3–1146/–646wt in response to Fas induction. In Fas-induced Jurkat T cells, as expected, pGL3–1146/–646mtNF-κB of four constructed mutations markedly reduced transcriptional activity to 47% of pGL3–1146/–646wt, whereas the activities of the pGL3–1146/–646mtCREB, mtAP-1, and mtc-Ets-1 constructs were not decreased (Figure 7). These combined results indicate that this NF-κB site is crucial for the Fas-induced expression of human GD3 synthase gene and that the NF-κB binding to this site is involved in the induction of human GD3 synthase gene expression by Fas activation.

Table I. Primer sequences used in this study

Primer	Sequence	Strand	Position
P-2646S	5′-ATGAGCTCTTCCCCCAGGCTGTTCTCATGA-3′	Sense	–2646 to –2624
P-2499S	5′-ATGAGCTCTCCTGAGGCCTCCTCAGCCATG-3′	Sense	–2499 to –2477
P-2246S	5′-ATGAGCTCCAGCACATTGTGTCACACAGATC-3′	Sense	–2246 to –2223
P-2146S	5′-ATGAGCTCGTAATATGCCCTAGTTTTGTTTGTC-3′	Sense	–2146 to –2121
P-2146A	5′-GTCTCGAGCAGTTATGAAGGGGCTATGTAAATG-3′	Antisense	–2171 to –2146
P-2000S	5′-ATGAGCTCGGCAAGTAGGAAAAGCTCCCCC-3′	Sense	–2000 to –1978
P-1646S	5′-ATGAGCTCAAAGGTACCAGACACCGCAGCC-3′	Sense	–1646 to –1625
P-1646A	5′-GTCTCGAGTGTCTAGCATCCGTCTCCACTG-3′	Antisense	–1668 to –1646
P-1146S	5′-ATGAGCTCCGTGTGTGTGTGTGTGTGTGTG-3′	Sense	–1146 to –1122
P-1146A	5′-GTCTCGAGGTTCACGGGCGCAAGCGAGCGC-3′	Antisense	–1168 to –1146
P-646A	5′-GTCTCGAGTCAGCGGCGCGCCGCAGCCTTC-3′	Antisense	–668 to –646
P-499A	5′-GTCTCGAGGACGCGCTCTCTGCGCCGCACC-3′	Antisense	–521 to –499
P+1A	5′-GTCTCGAGTCGCAGCCCCGGCGTCCCAGGG-3′	Antisense	–22 to +1
MuCREB-s	5′-TAGGGT***GAGCTC***AGCAGGGTGACAGCCTGGGCAC-3′	Sense	–971 to –938
MuCREB-a	5′-CCCTGCT***GAGTCT***ACCCTAGTCTTTCTCTAGCTGGT-3′	Antisense	–988 to –953
MuAP-1-s	5′-GCAGG***GAGCTC***GCCTGGGCACACCGGCCGCTTGTC-3′	Sense	–968 to –924
MuAP-1-a	5′-GCCCAGGC***GAGCTC***CCTGCTGCCGTCACCCTAGTCT-3′	Antisense	–975 to –940
MuNF-κB-s	5′-AGGGAG***GAGCTC***ACCTTGATTTCCACCAATCCCC-3′	Sense	–737 to –704
MuNF-κB-a	5′-AAATCAAGGT***GAGCTC***CTCCCTCCCCCACCCGCA-3′	Antisense	–749 to –716
MucEts-1-s	5′-GTCTGCGAAG***GAGCTC***GCGCAGCGGAAGGCTGCGG-3′	Sense	–695 to –661
MucEts-1-a	5′-GCTGCGC***GAGCTC***CTTCGCAGACAAACGCCCGGGGA-3′	Antisense	–708 to –673
CREB-s	5′-AGAGAAAGACTAGGGTGACGGCAGCAGGGT-3′	Sense	–981 to –950
CREB-a	5′-ACCCTGCTGCTGCCGTCACCCTAGTCTTTCTCT-3′	Antisense	–981 to –950
AP-1-s	5′-GCAGCAGGGTGACAGCCTGGGCACACCGGC-3′	Sense	–961 to –932
AP-1-a	5′-GCCGGTGTGCCCAGGCTGTCACCCTGCTGC-3′	Antisense	–961 to –932
NF-κB-s	5′-GGGAGGGAGGGGGAGACCTTGATTTCCACC-3′	Sense	–740 to –711
NF-κB-a	5′-GGTGGAAATCAAGGTCTCCCCCTCCCTCCC-3′	Antisense	–740 to –711
c-Ets-1-s	5′-TTTGTCTGCGAAGTCCGGGGCGCAGCGGAA-3′	Sense	–698 to –669
c-Ets-1-a	5′-TTCCGCTGCGCCCCGGACTTCGCAGACAAA-3′	Antisense	–698 to –669
MtCREB	5′-AGAGAAAGACTAGGGTGA**TTT**CAGCAGGGT-3′	Sense	–981 to –950
MtAP-1	5′-GCAGCAGGGTG**GGG**GCCTGGGCACACCGGC-3′	Sense	–961 to –932
MtNF-κB	5′-GGGAGGGAGG**GAAA**AGACCTTGATTTCCACC-3′	Sense	–740 to –711
Mtc-Ets-1	5′-TTTGTCTGCGAAGT**AA**GGGGCGCAGCGGAA–3′	Sense	–698 to –669

Primers P-2646S to P+1A were used for the isolation of 5′-flanking region of the human GD3 synthase gene and for the construction of the deletion mutants. These contain SacI and XhoI sites in sense and antisense primers, respectively. The mutated nucleotides in the oligonucleotides for mutation are in boldface and italic type. The sequences corresponding to putative transcription factor binding sites are underlined. The primers used for site-directed mutagenesis include the restriction enzyme SacI site (GAGCTC). The number indicates the nucleotide positions relative to translation start site (+1) of human GD3 synthase gene.

Expression change of nuclear protein activated by Fas in the –1146 ~ –646 region of the human GD3 synthase promoter

To further investigate the change in nuclear binding protein levels activated by Fas in the Jurkat T cells, we performed a time course analysis of their changes by western blot with the samples divided into cytosolic and nuclear fractions. In cytosolic fraction, as shown in Figure 6F, phosphorylation of the c-Ets-1 was increased at 5 min after treating the anti-Fas CH11 and then decreased at 10 min up to 60 min, whereas phosphorylation of nuclear binding proteins CREB, AP-1, NF-κB1 (p50), and RelA (p65) subunit of NF-κB by Fas stimulation was unchanged in Jurkat T cells. In nuclear faction, phosphorylation of the p65 was apparently increased at 5 min and then decreased at 10 min up to 60 min after treating the anti-Fas CH11, which differs from that in the cytosolic fraction, whereas the rest of nuclear binding proteins showed the same results as the cytosolic fraction (Figure 6G). This result indicates that only RelA (p65) subunit of NF-κB translocated to the nucleus by Fas stimulation, with maximum nuclear localization occurring 5 min after anti-Fas CH11 treatment. Together with the DNase I footprinting and EMSA analyses, this also suggests that the nuclear accumulation of Rel (p65) subunit of NF-κB may cause an increase in NF-κB-dependent transcription of human GD3 synthase gene.

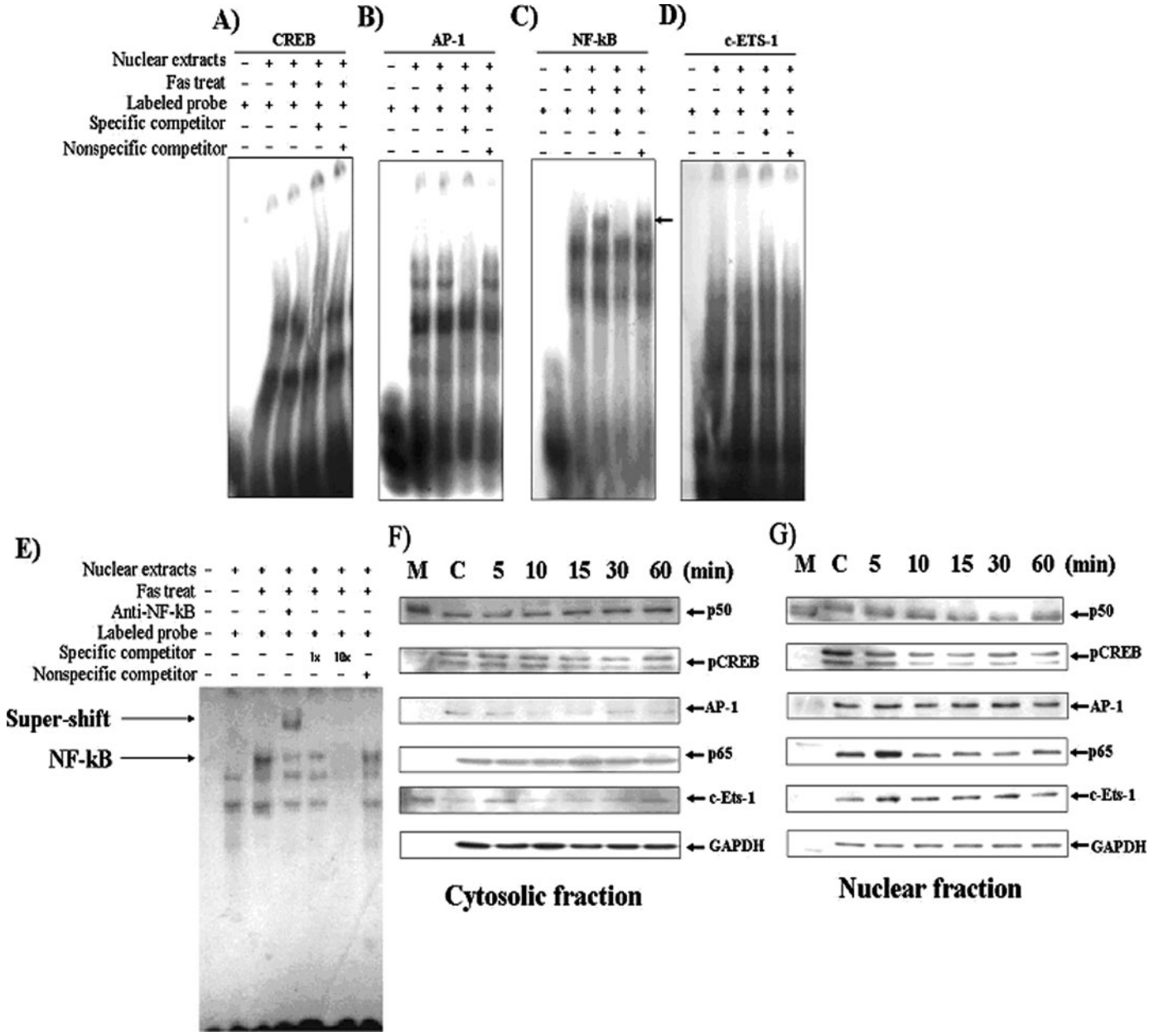

Fig. 6. Electrophoretic mobility shift assay (EMSA), gel supershift analysis of the –1190 to –690 region of the human GD3 promoter, and detection level of Jurkat fractional proteins activated by Fas. Nuclear extracts isolated from Jurkat T cells after 5 min of anti-Fas mAb CH11 treatment were incubated with ^{32}P-labeled four kinds of wild-type probes or unlabeled wild-type probes, or labeled mutant CREB (**A**), mutant AP-1 (**B**), mutant NF-κB (**C**), and c-Ets-1 (**D**) probes. For competition experiments, a 10-fold molar excess of four kinds of unlabeled wild-type oligonucleotides was used. The arrow indicates binding of Fas-induced Jurkat nuclear proteins and labeled wild-type NF-κB (**C**) oligonucleotides. The DNA–protein complexes were analyzed on a 4% non-denaturing polyacrylamide gel. (**E**) Anti-NF-κB (p65) antibody (2 μg) was preincubated with the reaction mixture at 4°C for 60 min prior to the addition of the labeled probes. The RelA (p65) of NF-κB subunit (lane2-lane7) was used as probes in supershift EMSA. The upper arrow indicates the shift of the complex by anti-NF-κB antibody. The cytosolic protein fraction (**F**) and the nuclear protein fraction (**G**) from Jurkat T cells were isolated after 0, 5, 10, 15, 30, or 60 min of anti-Fas mAb CH11 treatment. The fractional protein lysates from these cells were prepared by different methods. The expression or phosphorylation change for the isolated fractional proteins was detected by western blot analysis using antibodies recognizing expression or phosphorylation forms of CREB, AP-1, p50, and p65 of NF-κB subunits and c-Ets-1. GAPDH was included as an internal control.

Discussion

In this study, we have shown that the increases in GD3 level and GD3 synthase mRNA are induced by Fas stimulation in Jurkat T cells, a finding which is consistent with the previous reports (De Maria *et al.*, 1997; Castiglione *et al.*, 2004). Furthermore, we now demonstrate that GD3 synthase gene expression and GD3 formation in Fas-activated Jurkat T cells are rapidly induced (peaking at 5 and 15 min, respectively). This observation is in agreement with the early wave of GD3 accumulation, which is an early signaling event, detectable and confined within the first 15–20 min from Fas cross-linking in cells undergoing apoptosis (De Maria *et al.*,

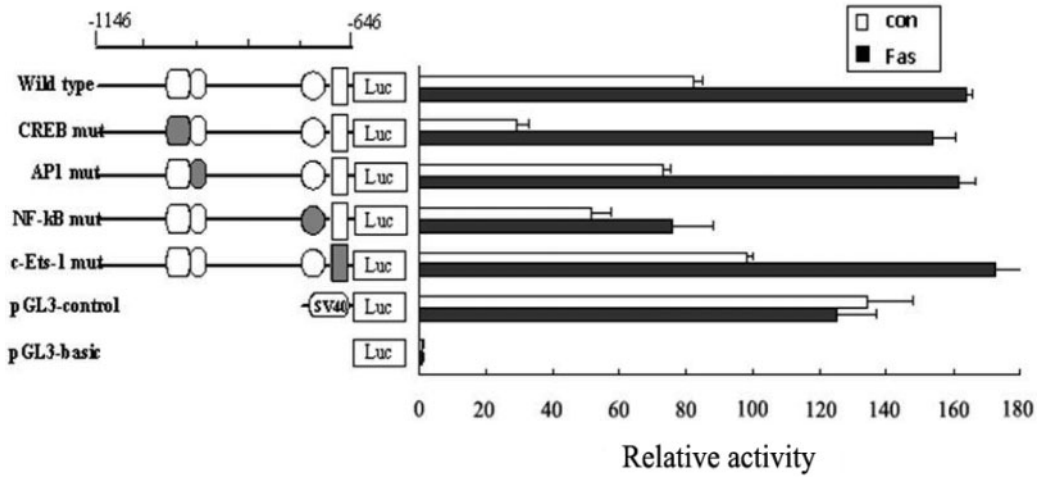

Fig. 7. Mutation promoter assay for the transcription factor binding sites in the Fas-responsive promoter region. pGL3-Basic without any promoter and enhancer was used as a negative control. pGL3-control with SV40 promoter and enhancer was used as a positive control. Each construct was co-transfected into Jurkat T cells with pCMV as the internal control. The transfected cells were incubated in the presence (solid bars) and absence (open bars) of anti-Fas mAb CH11 (300 ng/ml) for 5 min. Relative luciferase activity was normalized with β-galactosidase activity derived from pCMV. The values represent the mean ± SD for three independent experiments with triplicate measurements. The mutation mark of promoter construction is indicated by closed form or opened form (wild-type).

1997). Therefore, these results suggest that the Fas-induced increase in GD3 synthase gene expression appears in close temporal relation to the elevation of GD3 level and that Fas-responsive element(s) exists in the promoter region of the GD3 synthase gene. This also supports that the induction of GD3 synthase would finally direct the GD3 formation in response to Fas.

In this work, we focused on the transcriptional regulation of human GD3 synthase gene in response to Fas signaling and therefore undertook experiments to identify the promoter sequence of the human GD3 synthase gene. Our first step was to define the 5′-end of the human GD3 synthase mRNA obtained from Fas-induced Jurkat T cells, because the human GD3 synthase gene from human melanoma cell line SK-MEL-28 as well as other human sialyltransferase genes (Harduin-Lepers *et al.*, 2001) bears multiple potential transcription start sites (Furukawa *et al.*, 2003). The 5′-RACE analysis, using mRNA isolated from Fas-induced Jurkat T cells, showed the existence of multiple mRNA forms (Figure 2B), as have been identified in other human sialyltransferase genes such as *hST3Gal II* (Taniguchi, Morishima *et al.*, 2003), *hST3Gal IV* (Taniguchi, Hioki *et al.*, 2003), *hST3Gal V* (Kim *et al.*, 2001), *hST3Gal VI* (Taniguchi *et al.*, 2001), *hST6Gal I* (Aasheim *et al.*, 1993; Lo and Lau, 1999; Taniguchi *et al.*, 2000), and *hST6GalNAc IV* (Kim *et al.*, 2003). These multiple mRNA forms, differing only in their 5-untranslated region, are expressed in tissue- and cell type-specific manners. This mRNA heterogeneity has been attributed, at least in part, to transcription from a number of physically distinct promoter regions that govern and regulate their expression in specific cell types, which suggests that these promoters may respond to different physiological signals and stimuli in different cell types. In addition, the sequence analyses of the 5′-flanking region of these human sialyltransferase genes also reveal the heterogeneous transcription start sites and the absence of the canonical TATA and CCAAT boxes coupled with the presence of several GC boxes. These features are found in housekeeping genes that usually contain several transcription start sites spread over a fairly large region and several Sp1-binding sites (Smale and Baltimore, 1989; Smale, 1997). Similarly, we have also found in this study that the 5′-flanking region of human GD3 synthase gene obtained from Fas-induced Jurkat T cells included at least five distinct transcriptional start sites (about 160, 240, 570, 840, and 1000 bp 5′-RACE products, Figure 2B). The 5′-terminus of the predominant 5′-RACE product was mapped at –646 bp from the translation initiation site and was located 167 bp upstream of the major site identified by 5′-RACE from human melanoma cell line SK-MEL-28 (Furukawa *et al.*, 2003). Interestingly, unlike other human sialyltransferase genes, neither TATA and CAAT boxes nor a typical Sp1-binding site related to GC boxes was at the site appropriate to the transcription start site of the human GD3 synthase gene in Fas-stimulated Jurkat T cells, which suggests the regulatory function of human GD3 synthase promoter, which is not constitutively active but is rather regulated during differentiation and development, as other promoters with similar structural characteristics (Smale and Baltimore, 1989; Smale, 1997).

Despite the absence of typical TATA and CCAAT homologies, analysis of the 2.0-kb nucleotide sequence of the 5′-flanking region revealed that it contained a number of putative regulatory *cis*-acting elements. Furthermore, several sequences corresponding to binding sites of lymphoid-specific transcription factors Ets, NF-κB, GATA, and AP-1 were identified (Figure 3). Of particular interest were the consensus motifs for transcription factors NF-κB, CREB, and AP-1 that are known to be activated in Fas-stimulated cells (Barnhart *et al.*, 2004). In fact, we demonstrated that our cloned 5′-flanking region (–2646 ~ –646/pGL3) of the human GD3 synthase gene contained a functional

promoter that was highly activated by Fas stimulation in Jurkat T cells and that the region between –1146 and –646 functions as the core promoter essential for transcriptional activation of GD3 synthase gene in Fas-induced Jurkat T cells, as revealed by the deletion mutant analysis. In this region, there are four regulatory elements c-Ets-1 (–689 to –680), NF-κB (–731 to –722), AP-1 (–955 to –947), and CREB (–966 to –957). It is important to characterize the nuclear factors in this region that might mediate the transcriptional activation of the human GD3 synthase gene in Fas-induced Jurkat T cells. With the combination analysis using DNase I footprinting, EMSA, and supershift, we have identified NF-κB element located at nucleotide positions –731 to –722 as a functional site for the human GD3 synthase gene expression in response to Fas. Moreover, mutation of this NF-κB-binding site led to a significant loss of responsiveness to Fas, but other binding sites did not (Figure 7). These results indicate that NF-κB element at nucleotide positions –731 to –722 is the most functional for the transcription of human GD3 synthase gene in Fas-induced Jurkat T cell. However, relative to reporter activity in Fas-uninduced control cells, the NF-κB mutant did not completely block Fas inducibility, which suggests that other elements such as CREB, AP-1, and c-Ets-1 and/or other factors not identified thus far likely contribute to the regulation of the human GD3 synthase gene in Fas-activated Jurkat T cells. In fact, footprinting and EMSA clearly showed that CREB and AP-1 specifically bound to their binding sites, regardless of Fas activation, in the region between –1146 and –646 (Figures 5 and 6). Furthermore, luciferase assay with –1146 ~ –646/pGL3 reporter plasmid displayed the promoter activity reaching 80-fold higher activity than pGL3-Basic even in Fas-uninduced Jurkat T cells. These findings suggest that these act as necessary activators for the basal transcriptional activity of GD3 synthase gene, which may contribute to constitutive activation of the human GD3 synthase promoter.

NF-κB belongs to the Rel family of transcription factors that regulate genes involved in immune and inflammatory responses, cell-cycle progression, apoptosis, and oncogenesis (Baeuerle and Baltimore, 1996; Chen *et al*., 2001; Ghosh and Karin, 2002). In mammals, the Rel/NF-κB family forming homodimers and heterodimers includes p50/p105, p52, p100, RelA (p65), c-Rel, and Rel B. In unstimulated cells, NF-κB exists in the cytoplasm as an inactive dimer bound to IκBα. Upon appropriate stimulation, IκBα is phosphorylated by the IKKα or IKKβ kinase at specific serines, which then allows IκBα to undergo proteolysis through the proteosome pathway with the subsequent NF-κB translocation to the nucleus (Sizemore *et al*., 2002). These dimers then bind to specific DNA consensus sequences (κB motifs) in promoters and thus regulate the expression of a number of genes, depending on the cell type and the stimulus, such as mitogens, cytokines, reactive oxygen intermediates, lipopolysaccharides, and viral infection.

Although the vast majority of reports have documented NF-κB as an antiapoptotic molecule, in recent years, numerous studies have demonstrated the role of NF-κB in the induction of apoptosis (Lin *et al*., 1999; Chen *et al*., 2001, 2003; Ravi *et al*., 2001; Ghosh and Karin, 2002). Thus, it has become clear that NF-κB can play both an antiapoptotic role and a proapoptotic role, depending on the stimulus utilized and the cell type involved. For example, NF-κB has been shown to inhibit TNF-α-mediated apoptosis in Jurkat T cells, in primary rat and human fibroblasts, and in MCF-7 breast carcinoma cell lines (Van Antwerd *et al*., 1996; Liu *et al*., 1996), whereas previous studies have revealed that NF-κB with AP-1 promotes stress-induced apoptosis via FasL expression following activation by DNA-damaging agents in Jurkat T cells (Kasibhatla *et al*., 1998) and that NF-κB enhances the apoptosis of T-cell hybridomas through the induction of T-cell activator-mediated FasL expression (Lin *et al*., 1999). In addition, overexpression of NF-κB gene in Jurkat T cells provided a protective effect against Fas-mediated apoptosis (Dudley *et al*., 1999), whereas the activation of NF-κB by Fas stimulation induced apoptosis in human CEM-C7 T cells by signaling pathways that are distinct from those induced by TNF-α (Packham *et al*., 1997). Moreover, previous studies have shown that Fas stimulation resulted in the activation of NF-κB DNA binding in human bladder carcinoma T24 cell resistant to Fas-mediated apoptosis, although no such activation was observed in Jurkat T cells (Ponton *et al*., 1996). Contrary to this observation, in this study, we have clearly demonstrated that Fas stimulation with anti-Fas mAb leads to the activation of NF-κB and NF-κB-dependent gene expression in Jurkat T cells. This discrepancy may be explained by the difference in experimental conditions: to trigger the Fas signal, in our experiment, Jurkat T cells were incubated for 5 min with 300 ng/ml of anti-Fas mAb, whereas in the other experiments Jurkat T cells were incubated for 1 h with 50 ng/ml of anti-Fas mAb. Taken together, these results strongly suggest that NF-κB functions as a complex and cell-specific regulator of apoptosis and that Fas triggering can cause the activation of NF-κB DNA binding in a cell type-dependent manner.

NF-κB activates the transcription of vast number of genes, some of which encode for proteins that function as inducers of apoptosis (Lin *et al*., 1999; Pahl, 1999; Chen *et al*., 2001). NF-κB also enhances the gene expressions of Fas and FasL (Matsui *et al*., 1998; Hsu *et al*., 1999; Lin *et al*., 1999; Ouazz *et al*., 1999). In addition, previous work has shown that Fas gene expression was induced through RelA (p65) activation of NF-κB (Ouazz *et al*., 1999), which suggests that activation of specific NF-κB subunits is necessary for the enhanced expression of these proapoptotic agents. In this study, we have also found that NF-κB activates the enhanced expression of the human GD3 synthase gene in Fas-induced Jurkat T cells, and our supershift experiments have demonstrated that RelA (p65) activation is necessary for the increased promoter activity of human GD3 synthase gene. It is well established that the most critical step involved in NF-κB activation and in the modulation of its transcriptional activity is the translocation of NF-κB dimmer (mostly p50/p65 complex) to nucleus, because without entering the nucleus NF-κB cannot regulate transcription (Baeuerle and Baltimore, 1988, 1996; Chen *et al*., 2001; Ghosh and Karin, 2002). In this study, we have also revealed that Fas stimulation caused translocation of RelA (p65) protein to nucleus at 5 min after treatment with anti-Fas CH11 (Figure 7). Taken together, these findings suggest that the rapid activation of NF-κB and its

translocation to nucleus in Fas-induced Jurkat T cells is readily reconciled with the rapid human GD3 synthase gene expression and increase of GD3 level in these cells. This may support the previous report (Clement and Stamenkovic, 1994) that apoptosis mediated by Fas signal occurs within 15 min in some cell types such as MC40-Fas cell.

How Fas stimulation activates NF-κB is unclear, although a previous study suggested that Fas signaling activates NF-κB through the canonical pathway of the activation of the IκB kinase (IKK) complex and degradation of IκBα (Barnhart *et al.*, 2004). Although most of the studies so far addressed the effects of Fas on NF-κB activation, the mechanism by which the activated NF-κB triggers the downstream apoptotic machinery and the functional consequence of this activation remain unclear. In this study, the results obtained from EMSA and mutation analyses provide strong evidence that in Fas-induced Jurkat T cells NF-κB activated by Fas signaling directly binds to its binding site in the promoter region and up-regulates human GD3 synthase gene expression, which subsequently induces intracellular accumulation of GD3, leading to GD3-mediated apoptosis. This is consistent with previous reports that NF-κB acts directly to FasL transcription when activated by DNA-damaging agents (Kasibhatla *et al.*, 1998) and that NF-κB directly binds to Fas L-κB1 site and enhances FasL gene expression (Matsui *et al.*, 1998). Furthermore, in this study, we have demonstrated that one functional consequence of NF-κB activation by Fas signaling is promoting the GD3-mediated apoptosis by intracellular GD3 accumulation.

On the other hand, previous studies have shown that exogenous gangliosides blocked the activation of NF-κB and subsequent NF-κB-dependent expression of interleukin-2 (IL-2) and interferon-γ (IFN-γ) genes in activated T cells (Irani *et al.*, 1996; Uzzo *et al.*, 1999). In addition, a recent study has demonstrated that in GD3-treated rat hepatocytes and HepG2 cells, exogenous GD3 prevented the nuclear translocation of NF-κB, despite IκBα degradation, resulting in blocking the activation of NF-κB and subsequent NF-κB-dependent expression of antiapoptotic genes induced by TNF-α, which induce TNF-α-stimulated apoptosis of cells (Colell *et al.*, 2001). Considering the differences in experimental conditions, the type of cell used, and the dose of GD3, it is conceivable that NF-κB is differentially regulated by GD3, though the mechanisms by which gangliosides including GD3 inhibit the activation of NF-κB-binding activity is not defined. Furthermore, these results have revealed that GD3 acts in a late step in the pathway of NF-κB activation.

On the basis of these previous observations and our present data, although post-translational mechanism of GD3 synthase and transport system of GD3 to mitochondria remains to be elucidated, we would hypothesize the following pathway to explain GD3-mediated apoptosis via NF-κB-dependent gene expression of GD3 synthase by Fas signaling. In early stage, the rapid activation of NF-κB and its translocation to nucleus by Fas stimulation leads to the increased expression of GD3 synthase, which in turn induces the early and transient formation of GD3. In late stage, the GD3 accumulated in cells directly targets mitochondria, which then induce the loss of mitochondrial transmembrane potential and subsequent release of cytochrome *c* and activation of caspases, which contributes to apoptotic program. Simultaneously, the accumulated GD3 blocks the nuclear translocation of NF-κB, preventing the ability of NF-κB to induce antiapoptotic genes, which eventually enhance GD3-mediated apoptosis. In the present study, we demonstrate, for the first time, that Fas rapidly induces the enhanced expression of the human GD3 synthase gene and subsequent accumulation of ganglioside GD3 levels through the NF-κB activation in Jurkat T cells.

Materials and Methods

Cell cultures

The anti-Fas mAb clone CH11 was purchased from Upstate Biotechnology (Lake Placid, NY). Human Jurkat T cells were cultured in RPMI-1640 supplemented with 1 mM sodium pyruvate and 1× minimum essential medium (MEM) nonessential amino acids at 37°C in a 5% CO_2 incubator. Medium was supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% (v/v) fetal bovine serum (Gibco BRL, Life Technologies, Bethesda, MD). To induce the increased expression of human GD3 synthase gene by Fas triggering, Jurkat T cells were cultured for different times in the presence of 300 ng/ml of anti-Fas CH11.

RT–PCR and northern blot analysis

Total RNA was isolated from Jurkat T cells treated with or without anti-human Fas mAb CH11 using Trizol reagent (Invitrogen, Bethesda, MD). Two micrograms of RNA was subjected to reverse transcription with random nonamers, utilizing Takara RNA PCR kit (Takara Bio, Shiga, 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× Tris-acetate-EDTA buffer. To assess the specificity of the amplification, the PCR product of human GD3 synthase was subcloned into pGEM-T Easy vector (Promega, Madison, WI) and then sequenced. These genes were found to be identical to the expected human GD3 synthase cDNA. Northern blot analysis was performed by the same method as described previously (Kim *et al.*, 2003), using the [α-^{32}P]dCTP-labeled PCR fragments as a probe.

HPTLC immunostaining

Assessment of intracellular GD3 levels was performed as described previously (Copani *et al.*, 2002). Cultured cells were washed twice with ice-cold phosphate-buffered saline (PBS), and cells were scraped from the dishes and homogenized. Gangliosides were extracted according to the method of Svennerholm and Fredman (1980) and analyzed by HPTLC using analytical precoated silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). All plates were first activated by heating to 100°C for 30 min. Samples were

spotted onto plates with a Hamilton syringe in chloroform–methanol–0.25% KCl (5:4:1, v/v/v). Authentic GD3 (Wako Chemical, Osaka, Japan) was used as standard. GD3 was immunodetected by using the 4F6 anti-GD3 mAb (1:100; CovalAb, Lyon, France). The plates were incubated for 1 h at room temperature with the primary antibody, washed twice with PBS–Tween 20, and then incubated for 45 min at room temperature with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:200; Sigma, St. Louis, MO). The GD3 bands were identified by enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). The bands were scanned and quantified by densitometric analysis using TotalLab software (BioSystematica, Devon, UK) of Frog Gel Image Analysis System (CorebioSystem, Seoul, Korea).

5′-RACE

Amplification of the 5′-end of human GD3 synthase was performed with the 5′-RACE kit (Invitrogen) according to the manufacturer's instructions, using 5 μg of mRNAs prepared from Jurkat T cells treated for 5 min with anti-human Fas CH11. The gene-specific primer GD3RT (5′-CACAGCCA CTCTTCTT-3′, complementary to nucleotides 442–457) was used for initial reverse transcription. After synthesis of the first-strand cDNA, an anchor primer provided by Abridged and the gene-specific primer GSP1 (5′-CACCATTTCCCACC ACCGCGCATT-3′, complementary to nucleotides 410–433) were used in the first PCR. The second PCR was performed with an Abridged (Bethesda, MD) universal amplification primer and the gene-specific primer GSP2 (5′-TTGCCT GTGGGAAGAGAGAGTAAGTTG-3′, complementary to nucleotides 359–381). The PCR products were subcloned into pGEM-T Easy vector (Promega) and sequenced.

Cloning of the 5′-flanking region of the human ST8Sia I *gene*

Using the Human Genome Resources of the National Center for Biotechnology Information, National Library of Medicine, and National Institutes of Health, three kinds of fragments of the 5′-flanking sequence (– 2000 to +1, –2499 to –499, and –2646 to –646, when the translation start site was numbered as +1) of the human *ST8Sia I* gene were obtained by LA-PCR amplification with LA-*Taq* polymerase (Takara Bio). Primers containing SacI and XhoI sites (Table I) were synthesized on the basis of the human genomic sequence (GenBank™ accession number NT_009714). LA-PCR was performed using human genomic DNA (Clontech, Palo Alto, CA) as template under the following conditions: 94°C for 1 min, then 30 cycles of 98°C for 20 s and 68°C for 3 min, with a final elongation of 72°C for 10 min. PCR products were subcloned into pGEM-T Easy vector (Promega) to give pTHP2-1 (for –2000 to +1), pTHP2-2 (for –2499 to –499), and pTHP2-3 (for –2646 to –646). PCR products were sequenced in both directions by cloning convenient restriction fragments into pUC119 or using primers designed from a known sequence.

Construction of reporter plasmids and mutagenesis

To identify the Fas-activated minimal promoter sequence in the 5′-flanking region of the human GD3 synthase gene, three reporter plasmids (–2000/pGL3, –2646 ~ –646/pGL3, and –2499 ~ –499/pGL3) were constructed by subcloning the SacI/XhoI fragments of the pTHP2-1, pTHP2-2, and pTHP2-3, respectively, into the pGL3-Basic luciferase expression vector which lacked a promoter and enhancer (Promega). Human GD3 synthase promoter fragments containing varying lengths of 5′-flanking sequence, such as –2646 ~ –646/pGL3-related derivatives (–1146 ~ –646/pGL3 to –2246 ~ –646/pGL3) and –1146 ~ –646/pGL3-related different derivatives (–1646 ~ –1146/pGL3, –2146 ~ –1646/pGL3, and –2646 ~ –2146/pGL3), were generated by LA-PCR with sense and antisense primers containing SacI and XhoI sites, respectively (Table I), using pTHP2-2 described above as template. The PCR fragments were subcloned into pGEM-T Easy vector (Promega) and sequenced. Each fragment obtained by digestion with SacI and XhoI was inserted into the corresponding sites of the pGL3-Basic vector, which was used as a negative control. The pGL3 control with SV40 promoter and enhancer was used as a positive control. Mutations with base substitution at the CREB, AP-1, c-Ets-1, and NF-κB-binding sites were constructed using a QuikChange® II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol, using the oligonucleotide primers shown in Table I. The presence of mutation was verified by sequence analysis.

Transient transfection and reporter assay

For the reporter analysis of human ST8Sia I promoter in response to Fas treatment, the constructed plasmids (1.5 μg) and control vectors (1.5 μg) were transfected into the Jurkat T cells at 70% confluence by SuperFectant transfection protocol (Qiagen, Valencia, CA). Jurkat T cells were cultured in RPMI-1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% (v/v) fetal bovine serum (Gibco BRL, Life Technologies) at 37°C under 5% CO_2. 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 pCMV (Clontech). The cells were resuspended in RPMI-1640 medium containing 10% fetal bovine serum 48 h after transfection and then treated with anti-Fas CH11 (300 ng/ml) for 5 min. Lysates were prepared by four cycles of freezing and thawing of the harvested cells followed by centrifugation. 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 and expressed as a fold induction over pGL3-Basic.

DNase I footprint assay

To identify the Fas-activated transcription factor-binding sites, footprint assay was carried out by Core Footprinting System (Promega) according to the manufacturer's instructions. The plasmid (–1146 ~ –646/pGL3) was digested with KpnI/XhoI to generate a 502-bp fragment of the human GD3 synthase promoter prior to 5′-end labeling with [γ-^{32}P] dATP after dephosphorylation of 5′-ends. Then, this labeled double-stranded fragment was further digested with SacI and subsequently purified. A DNase footprinting assay was performed by incubating ^{32}P-labeled probe (10,000 cpm) with 2 μl of either bovine serum albumin (BSA) (control) or nuclear extract from Jurkat T cells treated for 5 min with anti-Fas CH11 (300 ng/ml) on ice for 10 min. To all samples were added

50 μl of Ca^{2+}/Mg^{2+} solution at room temperature, and samples were incubated for 1 min at room temperature. After incubation, the reaction in the samples was terminated by adding 90 μl of stop solution. Then, all samples were phenol extracted, ethanol precipitated, and loaded on a 6% acrylamide and 7 M urea sequencing gel. A sequencing ladder was obtained by loading a sample of the probe that had been treated with formic acid and piperidine to cleave at G and A residues.

EMSA and supershift assay

EMSA was performed using gel shift assay system kit (Promega) according to the manufacturer's instructions. Nuclear extracts of Jurkat T cells treated for 5 min with anti-Fas CH11 (300 ng/ml) were prepared as described previously (Kang *et al*., 2004); the protein concentrations of the extracts were determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Double-stranded oligonucleotides synthesized using sets of oligonucleotides (Table I) 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. And anti-NF-κB (p65) antibody recognizing the DNA-binding domain of human NF-κB p65 (C-20) X TransCruz was preincubated with nuclear extract prior to the addition of the labeled probes. The preincubation with anti-NF-κB (p65) antibody resulted in a supershift of the complex.

Western blot analysis

Jurkat T cells with anti-Fas CH11 (300 ng/ml) were homogenized for 5 min in a sample buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 3 mM NaN_3, 0.57 mM phenyl methyl sulfonyl fluoride, 0.15 μM aprotinin, and 1% Triton X-100. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad). Lysates and nuclear extracted proteins were prepared by four cycles of freezing and thawing of the harvested cells followed by centrifugation. Twenty micrograms of six samples of total cell lysates were size fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes using the Hoefer electrotransfer system (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated with CREB (Upstate Biotechnology), NF-κBp65, and p50 (SantaCruz, Santa Cruz, CA), c-Ets-1 (SantaCruz), AP-1 (SantaCruz), and GAPDH antibodies (Chemicon, Temecula, CA). Detection was performed using a secondary horseradish peroxidase-linked anti-mouse antibody and anti-rabbit chemiluminescence system (Amersham Biosciences).

Acknowledgments

This work was in part supported by the National Research Laboratory Program (M10203000024-02J0000-01300) and Glycomics Research Project from the Ministry of Science and Technology, Korea. N-Y Kang received fellowship from the Brain Busan 21 Project.

Conflict of interest statement

None declared.

Abbreviations

AP-1, activating protein 1; CREB, cAMP-responsive element-binding protein; EMSA, electrophoretic mobility shift assay; HPTLC, high-performance thin-layer chromatography; LA-PCR, long and accurate PCR; mAb, monoclonal antibody; NF-κB, nuclear factor kappa B; pCMV, cytomegalovirus-β-galactosidase vector; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end; RT–PCR, reverse transcription polymerase chain reaction.

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