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Original Article

Cordycepin-mediated transcriptional regulation of human GD3 synthase (*hST8Sia I*) in human neuroblastoma SK-N-BE(2)-C cells

Ji-Sue Baik[1†], Kyoung-Sook Kim[1†], Hyung-In Moon[1], Hyun-Kyu An[1], Shin-Ji Park[1], Cheorl-Ho Kim[2], and Young-Choon Lee[1*]

[1]College of Natural Resources and Life Science, Dong-A University, Busan 604-714, South Korea
[2]Molecular and Cellular Glycobiology Unit, Department of Biological Sciences, SungKyunKwan University, Kyunggi-Do 440-746, South Korea
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +82-51-200-7591; Fax: +82-51-200-6536; E-mail: yclee@dau.ac.kr

In the present study, we firstly found that cordycepin elevated the gene expression of the human GD3 synthase (*hST8Sia I*) in human neuroblastoma SK-N-BE(2)-C cells. To elucidate the mechanism underlying the upregulation of *hST8Sia I* gene expression in cordycepin-treated SK-N-BE(2)-C cells, functional characterization of the promoter region of the *hST8Sia I* gene was performed. Analysis of promoter activity using varying lengths of 5′-flanking region showed a dramatic increase by cordycepin in the −1146 to −646 region, which contains putative binding sites for transcription factors c-Ets-1, CREB, AP-1, and NF-κB. Site-directed mutagenesis for these binding sites and chromatin immunoprecipitation assay revealed that the NF-κB binding site at −731 to −722 is essential for the cordycepin-induced expression of the *hST8Sia I* in SK-N-BE(2)-C cells. Moreover, the *hST8Sia I* expression induced by cordycepin was significantly repressed by pyrrolidinedithiocarbamate, an inhibitor of NF-κB. These results suggested that cordycepin induces upregulation of *hST8Sia I* gene expression through NF-κB activation in SK-N-BE(2)-C cells.

Keywords cordycepin; human GD3 synthase; SK-N-BE(2)-C cell; transcription factor

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Introduction

Cordycepin (3′-deoxyadenosine) is the main component of the parasitic fungus *Cordyceps militaris*, an ingredient of traditional Chinese medicine and has been reported to have remarkable anti-cancer activity by several mechanisms including inhibition of cell proliferation [1,2], induction of apoptosis [3–5], and inhibition of cell migration and invasiveness [6,7]. Previous studies have also shown that cordycepin has various biological effects, such as anti-inflammation, inactive mRNA polyadenylation [8–10], and reinforcement of the immune system [11]. It has been reported that cordycepin inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced matrix metalloproteinase (MMP)-9 expression by suppressing AP-1 activation via mitogen-activated protein kinases signaling pathway in MCF-7 human breast cancer cells [12] and UVB-induced MMP expression by repressing the NF-κB pathway in human dermal fibroblasts [13]. It has also been reported that cordycepin suppresses tumor necrosis factor-α-induced invasion, migration, and MMP-9 expression by reducing the transcriptional activity of NF-κB and AP-1 in human bladder cancer cells [7].

Gangliosides, sialic acid-containing glycosphingolipids, mainly exist in the outer leaflets of vertebrate plasma membranes and play important roles in multiple biological processes, such as cell–cell interaction, adhesion, cell differentiation, growth control, oncogenic transformation, and receptor function [14,15]. Among the various gangliosides, GD3 is a structurally simple ganglioside that is markedly overexpressed in human melanoma and neuroblastoma cells, playing a key role in tumor progression [16,17]. GD3 is synthesized by GD3 synthase (ST8Sia I) known as CMP-NeuAc:GM3 α2,8-sialyltransferase and GD3 expression is generally regulated at the transcriptional level of *ST8Sia I* gene [17–20].

Although there have been a few reports on the effects of cordycepin on transcriptional regulation of *MMP-9* gene in human cancer cells [12,13], the effect of cordycepin on the gene expression of human sialyltransferases responsible for ganglioside expression has not yet been studied.

Therefore, the present study was undertaken to investigate whether cordycepin regulates gene expression of human sialyltransferases related to ganglioside biosynthesis in human neuroblastoma cells. We have found for the first time that the mRNA expression of *hST8Sia I* was induced by cordycepin in human neuroblastoma SK-N-BE(2)-C cells. In this study,

furthermore, to investigate the molecular basis of *hST8Sia I* gene expression induced by cordycepin, the promoter region to direct upregulation of reporter gene transcription in response to cordycepin was functionally characterized.

Materials and Methods

Cell cultures

The human neuroblastoma cell line SK-N-BE(2)-C, obtained from American Type Culture Collection (Manassas, USA) was maintained at 37°C in a 5% CO_2 incubator and cultured in Dulbecco's modified Eagle's medium (WelGENE Co., Daegu, Korea) containing 1 mM sodium pyruvate and 1× MEM non-essential amino acids, supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, USA). For the treatment of cordycepin (Sigma, St Louis, USA), cells were starved in serum-free medium for 12 h. The starved cells were induced by 150 μM cordycepin for various time periods.

Cell viability assay

Cell viability assay was performed as described previously [19–21]. The amount of formazan salt was determined by measuring the optical density at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, USA). Cell viability was quantified as a percentage compared with the control.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen; Carlsbad, USA) and first-strand cDNA was synthesized using RNA to EcoDry™ Premix (Oligo dT) kit (Clontech 639543; Clontech, Mountain View, USA). The synthesized cDNA was amplified by polymerase chain reaction (PCR) with specific *hST8Sia I* and *β-actin* primers, as described previously [18–21]. PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide.

Quantitative real-time PCR analysis

Total cellular RNAs and single-stranded cDNAs were prepared from cells as described above.

Real-time quantitative reverse transcription-PCR was performed using a CFX96™ real-time system with SYBR Premix (Bio-Rad). The primers were: *hST8Sia I* (D26360.1) sense, 5′-CAGAGCCATCTTTGAGGGTTTA-3′; antisense, 5′-CTTTCCAATGCTACGCAGAAAG-3′ and human *β-actin* (NM_001101.3) sense, 5′-ACCCACTCCTCCACC TTTGAC-3′; antisense, 5′-CCTGTTGCTGTAGCCAAATT CG-3′. The transcript copy number of the *hST8Sia I* gene was normalized to the *β-actin* transcript copy number for each sample. Relative quantitation was performed using CFX Manager v2.1 software (Bio-Rad). Real-time PCR amplification of the *hST8Sia I* and *β-actin* genes was carried out for 50 cycles of 95°C for 10 s, 56°C for 15 s, and 72°C for 15 s.

Transfection and luciferase assay

The luciferase reporter plasmids used herein, namely pGL3-2646/-646 and its derivatives (pGL3-1146/-646 to pGL3-2246/-646) with base substitutions at the CREB, AP-1, c-Ets-1, NF-κB binding sites, have been described elsewhere [18–21]. Transient transfection and luciferase assays were performed as previously described [20,21]. SK-N-BE(2)-C cells were transiently co-transfected with 0.5 μg of the indicated reporter plasmid and 50 ng of the control *Renilla* luciferase vector pRL-TK (Promega, Madison, USA), using 1 μl Lipofectamine 2000 (Invitrogen). After incubation for 3 h, transfection medium was replaced by normal medium without cordycepin and cultured for 18 h. Then, the medium was changed to serum-free medium and incubated for 12 h. After being cultured for an additional 6 h in serum-free medium containing 150 μM cordycepin, cells were collected and treated with passive lysis buffer (Promega). Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, and a GloMax™ 20/20 luminometer (Promega). Firefly luciferase activity of the reporter plasmid was normalized to *Renilla* luciferase activity and expressed as a fold induction over the empty pGL3-Basic vector, used as a negative control. Independent triplicate experiments were performed for each plasmid.

Western blot analysis

Whole-cell pellets were solubilized with a RIPA buffer (Pierce, Rockford, USA) containing protease inhibitor mix (GE Healthcare, Piscataway, USA). Protein concentrations were measured using the Bradford assay. Aliquots of cellular proteins (20 μg per lane) were electrophoresed on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare). The membrane was blocked in 5% skim milk for 1 h and then incubated with specific antibody (H-76) for hST8Sia I (Santa Cruz Biotechnology, Santa Cruz, USA) for 12 h. The signals of bound antibody were visualized using the ECL chemiluminescence system (GE Healthcare) with horseradish peroxidase-complexed anti-rabbit and anti-mouse IgG antibody. Equal loading was confirmed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Millipore, Milford, USA).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP kit (Upstate Biotechnology, New York, USA) following the manufacturer's protocol. Immunoprecipitation using 4 μg of NF-κB (Santa Cruz

Biotechnology) and IgG antibodies (Sigma) and PCR analysis using primers flanking the NF-κB binding sites on the *hST8Sia I* promoter were carried out as described previously [21].

Flow cytometry analysis

SK-N-(BE)2-C cells were detached with trypsin/ethylenediaminetetraacetic acid and washed twice with cold Dulbecco's phosphate-buffered saline. Cells were incubated for 1 h at 4°C with a GD3 monoclonal antibody (mouse IgM, Kappa-chain, clone: GMR19; Seigakagu, Tokyo, Japan) diluted 1 : 50 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After washing with PBS, cells were incubated for 1 h at 4°C with secondary fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG/M/A mix antibody (Sigma) diluted 1 : 100 in PBS containing 1% BSA. Staining without the primary anti-GD3 antibody served as a negative control. A total of 1×10^4 labeled cells were analyzed using a Beckman-Coulter Cytomics FC500 flow cytometer and CXP software (Beckman-Coulter, Miami, USA).

Results

Effect of cordycepin on cell proliferation

Before the investigation into the regulatory effect of cordycepin on *hST8Sia I* expression, we first examined the cytotoxicity of cordycepin in SK-N-BE(2)-C cells using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. Relative cell viability was determined by the amount of MTT converted into formazan salt. SK-N-BE(2)-C cells were treated with cordycepin with various concentrations for 24 h. As shown in **Fig. 1**, cell viability at 150 μM was about 67%, but cordycepin at the concentration of more than 200 μM significantly inhibited the cell proliferation (<50%).

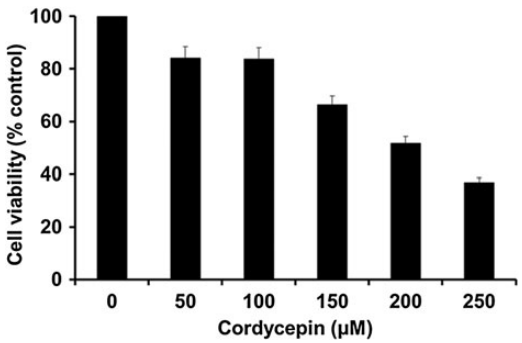

Figure 1. Effect of cordycepin on viability of SK-N-BE(2)-C cells The cytotoxic effects of cordycepin on SK-N-BE(2)-C cells were evaluated by MTT assay. Cells were treated with various concentrations of cordycepin (0–250 μM) for 24 h, and their absorbance at 490 nm was measured on an ELISA reader. The results are expressed as percentages of cell proliferation in the control (0 μM cordycepin) and represented as the mean ± SD of three independent experiments.

Effect of cordycepin on *hST8Sia I* expression in SK-N-BE(2)-C cells

Initial experiments were designed to determine whether cordycepin modulates the expression of human sialyltransferase genes responsible for the ganglioside biosynthesis in SK-N-BE(2)-C cells. After cells were treated with varying doses of cordycepin for varying periods of time, we investigated the specific expressions of sialyltransferase genes including *ST3Gal II*, *ST3Gal V*, *ST8Sia I*, and *ST8Sia V* by reverse transcription-PCR (RT-PCR) and did not find marked changes of gene expression patterns, except for *hST8Sia I* (data not shown). RT-PCR and quantitative real-time PCR results showed that mRNA levels of *hST8Sia I* were markedly increased at 150 μM cordycepin treatment and then continued in a dose-dependent manner (**Fig. 2A,B**). In addition, the induction of *hST8Sia I* mRNA was remarkably increased at 150 μM cordycepin treatment for 3 h and then continued for 24 h after cordycepin treatment (**Fig. 2C,D**). These results clearly showed that the expression of *hST8Sia I* was induced by cordycepin.

To investigate whether an increase of *hST8Sia I* mRNA levels by cordycepin treatment leads to induction of increased protein levels of hST8Sia I, we performed western blot analysis using the specific antibody for hST8Sia I. As shown in **Fig. 2E**, protein levels of hST8Sia I were remarkably increased at 150 μM cordycepin treatment for 3 h and then continued for 24 h after cordycepin treatment. These results indicated that the expression of hST8Sia I is induced by cordycepin at both transcriptional and translational levels.

Effect of cordycepin on ganglioside GD3 expression in SK-N-BE(2)-C cells

To investigate whether the induction of hST8Sia I expression by cordycepin increases the cellular expression level of ganglioside GD3 synthesized by hST8Sia I, we performed ganglioside GD3 analysis by flow cytometry (fluorescence-activated cell sorting (FACS)) using GD3 monoclonal antibody and FITC-conjugated anti-mouse IgG/M/A mixture as secondary antibody. As shown in **Fig. 3**, FACS analysis clearly demonstrated the shift in GD3 binding, indicating that the levels of cellular GD3 were significantly increased in cordycepin-treated SK-N-BE(2)-C cells, compared with cordycepin-untreated cells.

Analysis of transcriptional activity of *hST8Sia I* promoter by cordycepin in SK-N-BE(2)-C cells

Because the levels of *hST8Sia I* mRNA were significantly increased in SK-N-BE(2)-C cells stimulated with cordycepin (**Fig. 2A**), the transcriptional activity of the *hST8Sia I* promoter using luciferase reporter gene assay system has been checked in order to analyze whether the transcriptional activity of hST8Sia I is regulated in cordycepin-induced

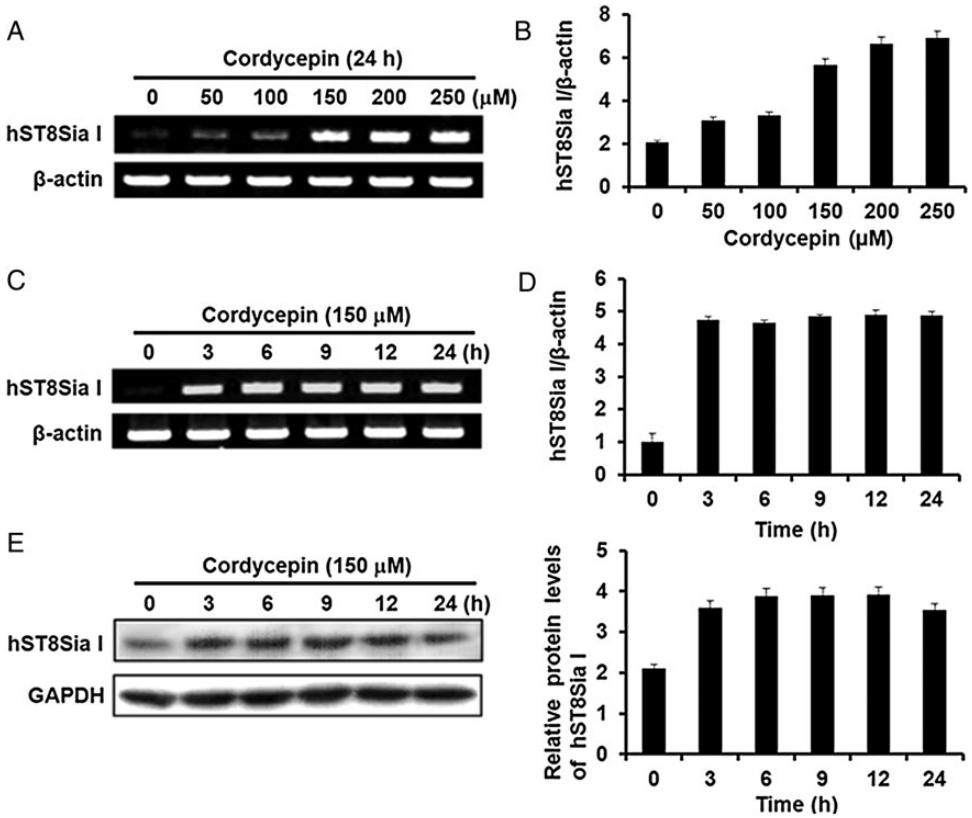

Figure 2. Effect of cordycepin on levels of hST8Sia I expression Total RNA from SK-N-BE(2)-C cells was isolated after treatment with various concentration of cordycepin for 24 h (A and B) or after treatment with 150 μM cordycepin for different time points (C and D) and *hST8Sia I* mRNA was detected by RT-PCR. As an internal control, parallel reactions were performed to measure levels of the housekeeping gene *β-actin*. (B and D) Human *ST8Sia I* mRNA expression was analyzed by quantitative real-time PCR. The transcript copy number of *hST8Sia I* was normalized to the *β-actin* transcript copy number for each sample. Experiments were repeated five times to ascertain reproducibility of results. The error bar indicates standard error. (E) Equal amounts of cell lysates (20 μg) were separated on SDS–polyacrylamide gels and transferred to PVDF membrane. Membrane was probed with specific antibody (H-76) for hST8Sia I. GAPDH was used as an internal control.

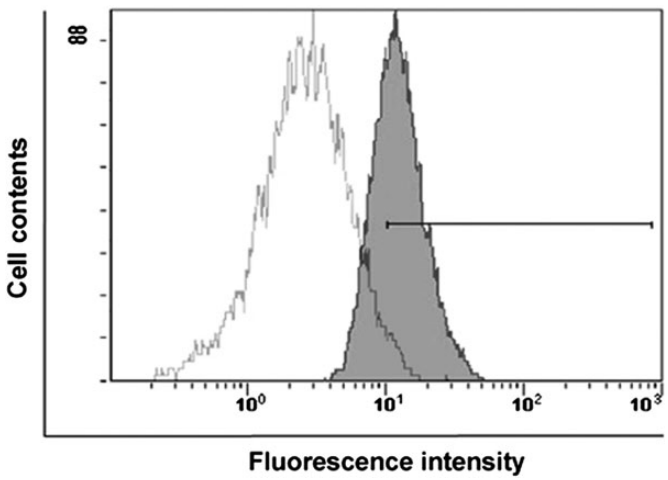

Figure 3. Flow cytometric analysis of ganglioside GD3 expression in SK-N-BE(2)-C cells treated with cordycepin After 150 μM cordycepin treatment for 48 h, SK-N-BE(2)-C cells were stained with anti-GD3 mAb and secondary FITC-conjugated antibody. The shift in anti-GD3 binding is shown in histogram form. The increase in binding to cordycepin-treated cells (gray shaded area) is exhibited in comparison with untreated cells (open area).

SK-N-BE(2)-C cells. As shown in **Fig. 4A**, cells harboring the pGL3-1146/-646 construct showed a marked increase in luciferase activity after cordycepin treatment, about 2.5-fold higher than untreated cells. On the contrary, cordycepin stimulation did not induce the significant increase of the luciferase activity in cells expressing other promoter constructs and the pGL3-basic (negative control). These results clearly suggested that the region containing nucleotides −1146 to −646 functions as the cordycepin-inducible promoter of *hST8Sia I* in SK-N-BE(2)-C cells.

Identification of cordycepin-responsive element in nucleotide −1146 to −646 region of *hST8Sia I* promoter

Our previous studies have demonstrated that the region from −1146 to −646 contains putative binding sites for transcription factors such as c-Ets-1, AP-1, CREB, and NF-κB binding sites [18–21]. To determine whether these binding sites contributed to cordycepin-induced expression of *hST8Sia I* in SK-N-BE(2)-C cells, four mutants (pGL3-1146/-646mtCREB, mtAP-1, mtNF-κB, and mtc-Ets-1) were

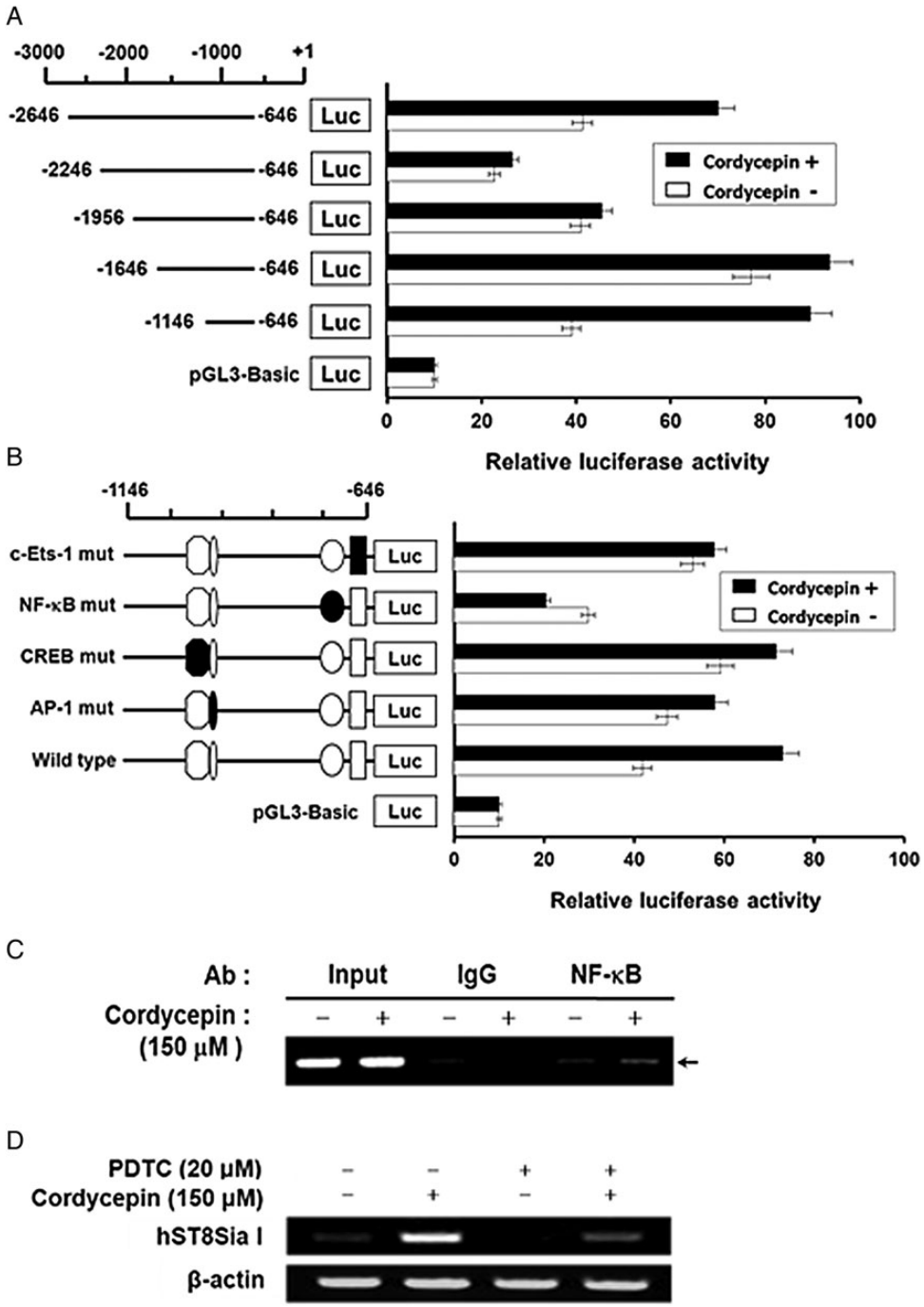

Figure 4. Analysis of *hST8Sia I* promoter activity in SK-N-BE(2)-C cells stimulated by cordycepin The schematic diagrams represent DNA constructs (A) containing various lengths of the wild-type *hST8Sia I* promoter, or constructs (B) with mutants c-Ets-1, AP-1, CREB, and NF-κB sequences in the 5′-flanking region, upstream of a luciferase reporter gene; the transcription start site is designated +1. The pGL3-basic construct, which did not contain a promoter or an enhancer, was used as a negative control. Each construct was transfected into SK-N-BE(2)-C cells, with pRL-TK co-transfected as an internal control. The transfected cells were incubated in the presence (solid bar) or absence (open bar) of 150 μM cordycepin for 12 h. Relative firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and all firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values were represented as the mean ± SD of three independent experiments with triplicate measurements. (C) PCR amplification in the −1146 and −646 region of the *hST8Sia I* promoter on immunoprecipitated chromatin obtained from SK-N-BE(2)-C cells treated with or without cordycepin. The input (10-fold diluted) represents the positive control. (D) Cells were incubated with 20 μM of NF-κB inhibitor, PDTC, for 1 h, and then co-incubated with 150 μM cordycepin for 24 h. *hST8Sia I* mRNA was detected by RT-PCR. *β-Actin* was used as an internal control.

used, which contained exactly the same construct as wild-type pGL3-1146/-646 except that the combined nucleotides within these binding sites had been changed [18–21]. Luciferase constructs with substituted mutations (**Fig. 4B**) were transfected into SK-N-BE(2)-C cells and luciferase assays were performed. The luciferase activity of each construct was

compared with that of pGL3-basic or wild-type (pGL3−1146/-646) used as negative or positive control, respectively. In cordycepin-treated cells, pGL3-1146/-646mtNF-κB of four constructed mutations remarkably decreased transcriptional activity to more than 3 fold of pGL3-1146/-646wt, whereas the activities of the pGL3-1146/-646mtCREB, mtAP-1, and mtc-Ets-1 constructs were not reduced (**Fig. 4B**). These results indicated that this NF-κB site is indispensable for the cordycepin-induced expression of hST8Sia I.

Based on these results, we performed ChIP assay to confirm the binding of NF-κB to this site of *hST8Sia I* promoter in SK-N-BE(2)-C cells. An amplification of the *hST8Sia I* promoter regions was obtained in the presence of NF-κB specific antibody and IgG. As shown in **Fig. 4C**, only NF-κB has the specific amplification and DNA−protein complex was observed in SK-N-BE(2)-C cells untreated with cordycepin to regulate the expression of *hST8Sia I* gene. There was no detectable binding in a control assay with cordycepin non-treatment or IgG. Moreover, the cordycepin-induced expression of *hST8Sia I* was significantly repressed by pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB (**Fig. 4D**). These results indicated that *hST8Sia I* gene expression was modulated by the interaction between NF-κB and NF-κB elements at nucleotide positions −731 and −722.

Discussion

In previous studies, we have shown that valproic acid, a simple branched-chain fatty acid, induced transcriptional activation of *hST8Sia I* in SK-N-BE(2)-C [20], whereas triptolide, a diterpenoid triepoxide, downregulated *hST8Sia I* gene expression in SK-MEL-2 human melanoma cells [21]. In the present study, we also demonstrated for the first time that cordycepin upregulated *hST8Sia I* expression in human neuroblastoma cells. Moreover, cordycepin elicited a significant dose-dependent increase of *hST8Sia I* mRNA level: we detected a markedly induced *hST8Sia I* mRNA signal at 150 μM treatment of cordycepin, and the signal increased up to the 250 μM. In addition, as evidenced by western blot analysis, protein levels of hST8Sia I was also increased after 3 h of stimulation with 150 μM cordycepin and then continued for 24 h. This increase was coincident with the time-dependent increase in *hST8Sia I* mRNA level, suggesting that cordycepin-induced upregulation of *hST8Sia I* at both transcriptional and translational levels. As a downstream consequence of this cordycepin treatment, the ganglioside GD3 levels in SK-N-BE(2)-C cells were remarkably increased, as demonstrated by FACS analysis using GD3 monoclonal antibody, and our results suggested that this GD3 induction occurred in a close temporal relation to the cordycepin-induced increase of *hST8Sia I* gene expression. We also identified that the promoter region of the *hST8Sia I* gene contains cordycepin-responsive element(s), which supported the idea that the induction of *hST8Sia I* gene expression would lead to an active hST8Sia I production directing GD3 formation eventually in response to cordycepin.

In this study, we also clarified a part of the transcriptional regulation mechanism that underlies the induction of *hST8Sia I* gene expression in response to cordycepin. In order to investigate cordycepin-responsive elements involved in the enhanced expression of the *hST8Sia I* gene in SK-N-BE(2)-C cells, we firstly tried to identify the region within the *hST8Sia I* promoter that was crucial for cordycepin-induced gene expression. We isolated the region between −1146 and −646 as the core promoter for transcriptional activation of *hST8Sia I* in cordycepin-induced SK-N-BE(2)-C cells. Our previous studies showed four kinds of transcription factor binding sites (c-Ets-1, AP-1, CREB, and NF-κB) in this region [18−21]. We have demonstrated that only the NF-κB binding site at positions −731 to −722 in this region contributes to *hST8Sia I* promoter activity in Fas-induced Jurkat T cells [18], human melanoma SK-MEL-2 cells [19], triptolide-induced SK-MEL-2 cells [20], and valproic acid-stimulated SK-N-BE(2)-C cells [21]. In agreement with these findings, our present site-directed mutagenesis and ChIP analysis demonstrated that binding to this NF-κB element mediated cordycepin-dependent upregulation of *hST8Sia I* gene expression. And NF-κB is a crucial transcription factor that controls the expression of numerous genes involved in immune and inflammatory responses, proliferation, apoptosis, and oncogenesis [22,23].

Although cordycepin inhibits MMP expression by repressing the NF-κB pathway in human dermal fibroblasts [13] and human bladder cancer cells [7], NF-κB-mediated gene expression by cordycepin stimulation in human neuroblastoma cells have been not reported. Therefore, it is important to elucidate which signaling pathways in response to cordycepin are upstream of this NF-κB-mediated expression of the *hST8Sia I* gene. Further study is required to clarify the precise mechanisms involved in the cordycepin-mediated activation of NF-κB leading to a transcriptional upregulation of *hST8Sia I* gene SK-N-BE(2)-C cells.

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