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Sphingosine Kinase 1/S1P Pathway Involvement in the GDNF-Induced GAP43 Transcription

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) is important for the development and maintenance of dopamine neurons (Lin et al. [1993] Science 260: 1130–1132). GDNF is neuroprotective in animal models of Parkinson disease, where dopamine neurons show selective degeneration. We previously reported GDNF-induced SPHK1 gene expression in a neuroblastoma cell line, TGW (Murakami et al. [2007] J Neurochem 102: 1585–1594). In the present study, we focused on the regulatory mechanism of GAP43 (GDNF-induced neuronal phenotype) transcription to further elucidate physiological roles of GDNF-induced SPHK1 expression and activity. Stable wild-type (SPHK1-WT) but not dominant-negative SPHK1 (SPHK1-DN) overexpression increased both control- and GDNF-induced GAP43 expression. SPHK1-WT cells showed enhanced GDNF-induced sphingosine 1-phosphate (S1P) secretion compared with mock- and SPHK1-DN cells. Exogenous S1P also increased GAP43 expression. In TGW cells, PD98059, a MEK inhibitor, but not SB203580 (a p38 MAPK inhibitor) and LY294002 (a PI3K inhibitor) inhibited GDNF-induced GAP43 expression, suggesting the MEK/ERK pathway has a major role in GDNF-induced GAP43 transcription. A G-protein-coupled receptor inhibitor, pertussis toxin, and $S1P_1$ and $S1P_3$ receptor antagonists (VPC23019 and CAY10444) also inhibited ERK activation. Moreover, both S1P1 and S1P3 were serine-phosphorylated by GDNF, suggesting their activated states. C/EBPβ transcription factor was induced by GDNF, and DNA pull-down and chromatin immunoprecipitation assays revealed the C/EBP binding site between −131 bp and −98 bp from the first exon of GAP43. Taken together, our results showed that in TGW cells, GDNF increased SPHK1 transcription, leading to the production and secretion of S1P. Through MEK/ERK pathway, S1P stimulates GAP43 transcription with increased binding of C/EBPβ to the 5′-promoter. J. Cell. Biochem. 112: 3449–3458, 2011.

KEY WORDS: GDNF; TGW CELLS; SPHK1; S1P RECEPTOR; MEK/ERK PATHWAY; C/EBPβ; GAP43 TRANSCRIPTION

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite involved in many cellular processes including proliferation, survival, migration, and differentiation [Taha et al., 2004; Maceyka et al., 2009]. It has been shown that the balance of S1P/ceramide determines the cell's fate and has been proposed as the sphingolipid rheostat model [Spiegel and Milstien, 2003]. Cellular S1P level is tightly regulated by the balance between its synthesis and degradation. Sphingosine kinases (SPHK), enzymes that produce S1P, are the essential elements in the regulation of S1P levels. SPHK1 activity is associated with cell survival and

Abbreviations used: SPHK1, sphingosine kinase 1; S1P, sphingosine 1-phosphate; GDNF, glial cell line-derived neurotrophic factor; GAP43, growth associated protein 43; ChIP, chromatin immunoprecipitation; C/EBP, CCAAT/enhancer binding protein.

Additional Supporting Information may be found in the online version of this article.

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proliferation [Hait et al., 2006]. To date, although the activation processes of SPHK1 have been extensively studied, the physiological roles of SPHK1 and the downstream pathways in neuronal differentiation processes remain to be elucidated.

Neurotrophic factor-induced signaling is essential for the survival and phenotypic expression of neuronal cells. After binding with GPI-anchored coreceptors called glial cell line-derived neurotrophic factor (GDNF) family alpha (GFRα), GDNF activates the RET receptor tyrosine kinase leading to the activation of multiple downstream signaling pathways [Ichihara et al., 2004]. Disruption of GDNF-RET signaling in mice causes dysgenesis and lack of enteric neurons which is the animal model of Hirschsprung's disease [Ledda et al., 2007]. Recently, we reported that GDNF-induced SPHK1 gene expression through the cell surface GDNF receptor, RET, and also suggested the involvement of SPHK1 in GDNF-induced differentiation [Murakami et al., 2007]. Similarly, another neurotrophic factor, NGF, induced SPHK1 transcription through increased transcription factor Sp1 [Sobue et al., 2005]. Thus, the SPHK/S1P signaling pathways induced by neurotrophic factors play important roles for the neural differentiation. Elucidation of these mechanisms might also be helpful for the understanding of the clinical neurodegenerative diseases.

In the current study, we focused on GAP43 expression, which plays a role in axonal outgrowth or growth cone formation in response to the extracellular as well as intracellular signals [Pfenninger et al., 1991]. We attempted to elucidate the signaling pathways between SPHK1 and GAP43 transcription by identifying responsible transcription factors. By examining stable wild-type (SPHK1-WT) and dominant-negative SPHK1 (SPHK1-DN) transfectants, we found that the SPHK1 level is important for GAP43 expression. Furthermore, the present study demonstrated for the first time the sequential change starting from S1P secretion through GDNF-induced SPHK1 activity, followed by the activated MEK/ERK pathway leading to increased/activated transcription factor, C/EBPβ, which stimulates GAP43 transcription. Based on the close relationship between SPHK1/S1P pathway and GDNF-induced GAP43 transcription, the significance of this SPHK1/S1P pathway of GDNF-induced neuronal differentiation was then discussed.

MATERIALS AND METHODS

CELL CULTURE, REAGENTS

A human neuroblastoma cell line, TGW, was cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. A GDNF was purchased from PeproTech EC (London, UK). PD98059, SB203580, and LY294002 were purchased from Calbiochem (La Jolla, CA). S1P, pertussis toxin, VPC23019, and CAY10444 were from Biomol (Plymouth Meeting, PA), Seikagaku Biobusiness Corporation (Tokyo, Japan), Avanti Polar Lipids (Alabaster, AL), and Cayman Chemical (Ann Arbor, MI), respectively. siRNA targeting SPHK1 and scrambled siRNA were purchased from Sigma Genosys (Hokkaido, Japan) and their sequences were according to the previous report [Shu et al., 2002].

EXPRESSION VECTORS

Human WT and DN (G82D) SPHK1 expression vectors (pcDNA3/hSPHK1-WT and pcDNA3/hSPHK1-DN) were the kind gifts of Dr. S.M. Pitson (Hanson Centre for Cancer Research, Adelaide, SA, Australia) [Pitson et al., 2000]. The expression vectors of pcDNA3.1/C/EBPα and pcDNA3.1/C/EBPβ were the kind gifts of Dr. H. Hirai (Kyoto Prefectural University of Medicine, Kyoto, Japan). NeuroD1 and NeuroD6 cDNA were cloned with the PCR-based method using cDNA of TGW cells as a template. For cloning of NeuroD1 (GenBank ID: NM_002500) cDNA, the following primer set was used. Sense: 5′-GGGAAGCTTAGGAAATCGAAACATGACCAAATCGTACAG-3′ (underline denotes *Hin*dIII site), antisense: 5′-GGGGAATTCAAT-GGTGAAACTGGCGTGCCTCTAA-3′ (double underline means *Eco*RI site). PCR product digested by *Hin*dIII and *Eco*RI and pcDNA3.1 (+) vector digested by same enzymes were ligated together.

For cloning of Nex1/MATH-2 (NeuroD6; GenBank ID: NM_022728) cDNA, the following primer set was used. Sense: 5′-GGGGAATTCAGAACCATGTTAACACTACCGTT-3′ (double underline denotes *Eco*RI site), antisense: 5′-GGGCTCGAGTCATTAAT-TATGAAAAACTGCATT-3′ (thick line shows *Xho*I site). PCR product digested by *Eco*RI and *Xho*I, and pcDNA3.1 (+) vector digested by the same enzymes were ligated together. After confirming DNA sequences, these expression vectors were used for further experiments.

ESTABLISHMENT OF WILD-TYPE AND DOMINANT NEGATIVE SPHK1 STABLE TRANSFECTANTS

To establish stable transfectants of SPHK1-WT and SPHK1-DN, DNA transfection was performed by the calcium phosphate precipitation method. Twenty micrograms of pcDNA3/hSPHK1-WT or pcDNA3/hSPHK1-DN expression vector was transfected to TGW cells. G418 selection was started 2 days after transfection at the concentration of 400 μg/ml. After limiting dilution, established clones were checked by Western blotting using anti-human SPHK1 antibody (ABGENT, San Diego, CA). Subclones showing the highest expression of SPHK1-WT or SPHK1-DN were used for further experiments.

SPHK ENZYME ACTIVITY

SPHK1 and SPHK2 enzyme activities were measured separately as described previously [Nemoto et al., 2009]. Assay was performed in triplicate. The activity was calculated as pmol/min/mg proteins, and the relative enzyme activity of control TGW cells was defined as 1.0.

SPHINGOSINE 1-PHOSPHATE MEASUREMENT

Secreted S1P was measured by a metabolic labeling method. Briefly, TGW cells were plated in triplicate at the confluence of 70–80%. One day after plating, L-[3-^{14}C]-serine (185 KBq/ml) was simultaneously added to the culture medium, and incubated for 24 h. After ^{14}C-serine labeling, cells were washed with PBS and transferred to serum-free medium with or without GDNF (20 ng/ml), and then incubated for another 24 h. After 24-h incubation, cell supernatant and cell pellets were collected. Collected supernatant (1 ml/each) and cell pellets (resuspended in 100 μl of PBS) were added at 1/10th the volume of a solution containing 1 N HCl, $CHCl_3$/MeOH/conc. HCl (100:200:1 v/v/v), and then $CHCl_3$ (0.4 ml for cell pellets and 1.2 ml for supernatant) and an equal volume of 1 M KCl were added. After centrifugation, the lipids recovered in a lower phase were applied on silica gel thin layer plate (Merck & Co., Whitehouse Station, NJ), and were developed with an authentic S1P in 1-butanol/acetic acid/

H_2O (94:1:5 v/v/v). The quantification of ^{14}C-labeled S1P was performed with BAS 2500 systems (Fuji Film Co., Tokyo, Japan). The relative cellular S1P and S1P in culture medium were calculated based on the respective protein level. Data of TGW cells without GDNF treatment were regarded as 1.0.

WESTERN BLOTTING

Western blotting was performed with anti-GAP43 (Novus Biologicals, Littleton, CO), anti-C/EBPα, anti-C/EBPβ, anti-NeuroD1, anti-p-ERK, anti-ERK, anti-$S1P_3$ antibodies (Santa Cruz Biotechnology Inc., CA), anti-S1P1 antibody (Affinity BioReagent, Golden, CO), anti-Nex1/MATH-2 (NeuroD6) antibody (Abcam, Cambridge, UK), and anti-β-actin antibody (Cytoskeleton Inc., Denver, CO). Immobilon™ Western Chemiluminescent HRP Substrate (Millipore Co., Billerica, MA) was used for detection.

DETECTION OF PHOSPHO $S1P_1$ AND $S1P_3$

To detect phosphorylated S1P1 and S1P3, immunoprecipitation was performed followed by Western blotting. Before GDNF treatment, TGW cells were washed with PBS, and incubated with serum-free medium for overnight. After being kept overnight in the serum-starved condition, TGW cells were treated with or without GDNF for 30 min, then washed with TBS (25 mM Tris–HCl pH 7.5, 150 mM NaCl), and dissolved in TEN-modified buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, protease inhibitor). Lysates were incubated with protein A agarose beads (Santa Cruz) linked with anti-S1P1 (Affinity BioReagent) or anti-S1P3 antibody (Santa Cruz) at 4°C overnight. Collected beads were washed three times with TEN-modified buffer containing BSA (1 mg/ml), and TBS containing the protease inhibitor, respectively. The immune complexes were released from the beads by boiling in an SDS-sample buffer for 5 min. Immunoprecipitates were analyzed by Western blotting using anti-phosphoserine antibody (Assay Designs, Ann Arbor, MI).

SEMI-QUANTITATIVE RT-PCR

Semi-quantitative RT-PCR was performed according to the method described previously [Nakade et al., 2003]. The first-strand cDNA was prepared with 2 μg of RNA using the Super Script III First-Strand System (Invitrogen, Carlsbad, CA). In the preliminary experiments, the relative amounts of cDNA and the range of PCR cycles that permitted the linear amplification of GAP43 and GAPDH were determined. The primers were as follows: GAP43 sense: 5′-CTGCTGCTGTCACTGCTG-3′, antisense: 5′-TTGGTTTCATCTA-CAGCTTCTA-3′, GAPDH sense: 5′-CAGGAGCGAGATCCCTCCAA-3′, antisense: 5′-CCCCCTGCAAATGAGCCC-3′. The PCR conditions for GAP43 and GAPDH were 98°C for 10 s and 60°C for 30 s, followed by 68°C for 30 s. The number of cycles for GAP43 were 28, 30, and 32, and those for GAPDH were 20, 22, and 24, respectively. Using the NIH image (version 6), each band was surveyed for its relative band intensity. Within the range of the linear PCR amplification, the relative expression of the *GAP43* mRNA expression level was evaluated by calculating the band intensity ratio of GAP43/GAPDH. To detect *S1P1∼5* mRNA, the following primer sets were used: S1P1, sense: 5′-CTGCTTGAGCGAGG-CTGCGG-3′, antisense: 5′-GAGCTGCGGTGGGCCTTGAC-3′, S1P2, sense: 5′-GCCTAGCCAGTTCTGAAAGC-3′, antisense: 5′-GCAAT-GAGCACCAGAAGGTT-3′, S1P3, sense: 5′-CAGCCGACGGAG-GAGCCCTT-3′, antisense: 5′-TGCTCCCGCAGGGTCTCGTT-3′, $S1P_4$, sense: 5′-TGGTGCTGGAGAACTTGCTGGTGC-3′, antisense: 5′-GCAGTGAAGAGCAGGCTGAAGGTGGA-3′, $S1P_5$, sense: 5′-CAC-GACCAGGGCGCAGACCT-3′, antisense: 5′-CACACCGCCAGGCA-CACCAC-3′.

RAPID AMPLIFICATION OF 5′cDNA ENDS (5′-RACE) OF HUMAN GAP43

RNA ligase-mediated rapid amplification of 5′-cDNA ends (5′-RACE) was performed with a Gene Racer kit (Invitrogen) according to the instruction manual to determine the transcription initiation point of GAP43 in TGW cells. The reverse GAP43 gene-specific primer and reverse GAP43 gene-specific nested primer were 5′-GCTTCAGCCTCAGCAGCTTGGAC-3′, and 5′-CTGAATTTTGGTTG-CGGCCT-3′, respectively. Cloned PCR products were analyzed for their DNA sequences, and the 5′-transcription initiation point was determined.

CLONING OF 5′-PROMOTER OF HUMAN GAP43

According to the DNA sequence of 5′-promoter region of human GAP43 (DBTSS ID:NM_002045, http://dbtss.hgc.jp/), the following primer sets were prepared for PCR. Sense: 5′-GGGACGCGTA-CATTCTCTGCCTCCTCCCTTC-3′, antisense: 5′-GGGAGATCTAGCA-CAGCATGGTTGTCTCGTC-3′ (double and single underlines denote *Mlu*I site and *Bgl*II site, respectively). PCR product digested by *Mlu*I and *Bgl*II and pGL3 basic vector (Promega) digested by the same enzymes were ligated. This construct was named −1,777 bp/pGL3. Deletion mutants were prepared as follows. To prepare −1717 bp promoter/pGL3, −1,777 bp/pGL3 was digested by *Mlu*I and *Bsu*36I, and then this construct was blunted and self-ligated. To prepare −765 bp/pGL3, −1,777 bp/pGL3 was digested by *Hin*dIII, and blunted. The product was further digested by *Bgl*II. This digested construct and pGL3 basic vector digested by *Sma*I and *Bgl*II were ligated together. To prepare −338 bp/pGL3, −1,777−bp/pGL3 was digested by *Xba*I, blunted, and then digested by *Bgl*II. This construct and pGL3 basic vector digested by *Sma*I and *Bgl*II were ligated. To prepare −6 bp/pGL3, the following primer sets were used. Sense: 5′-GGGACGCGTAGCGAGCAGAAAAGAGGTGGAG-3′, antisense: 5′-TCTTTATGTTTTTGGCGTCTTCCA-3′ (double underline denotes *Mlu*I site). PCR product digested by *Mlu*I and *Bgl*II, and pGL3 basic vector digested by the same enzymes were ligated. To circumvent putative transcriptional factor binding sites, further deletion mutants were prepared by the PCR-based method and digested by restriction enzymes.

Sense primers were as follows. For −177 bp promoter, 5′-GGG-ACGCGTAGCACCAGAGAGAGAGGGAG-3′: for −131 bp promoter. 5-GGGACGCGTAGCGAGCATGTGCGATGA-3′: for −98 bp promoter, 5-GGGACGCGTCTTACAGTTGCTGCTAACTGCC-3′: for −75 bp promoter, 5-GGGACGCGTTGGTGTGTGTGAGGGAGAGA-3′ (double underline denotes *Mlu*I site). Antisense primer was the same as that of −6 bp/pGL3. Both PCR product and pGL3 basic vector were digested by *Mlu*I and *Bgl*II, then ligated.

Introduction of mutation to E-box and C/EBP binding site were performed by PCR amplification using WT −131 bp/pGL3 promoter construct as a template.

To introduce the mutation to the DNA sequence of E-box present between −131 and −98 bp as shown in Figure 3, the following primer sets were used: sense: 5′-GGGACGCGTAGCGAGAATGTGC-GATGAGCAATAGCTGTGGACC-3′, antisense: 5′-GGGCCTTTCTTT-ATGTTTTTGGCGTCTTC-3′ (double underline denotes *Mlu*I site and single underline shows mutated base). To introduce the mutation to the C/EBP binding site, the following sense primer was used: sense: 5′-GGGACGCGTAGCGAGCATGTGCCACGAACCCTAGCTGTGGA-CCTTACAGTTGCTGCTAACTGC-3′ (double and single underlines denote *Mlu*I site and mutation sites of C/EBP family binding site, respectively). Antisense primer was the same as that used to introduce the mutation of E-box.

LUCIFERASE ASSAY

DNA transfection was performed using Lipofectamine™ 2000 (Invitrogen). Luciferase and β-galactosidase activities were measured according to the method described previously [Sobue et al., 2005]. After DNA transfection for 4 h, cells were transferred to serum-free medium with or without 20 ng/ml GDNF for another 24 h. The relative reporter activity was demonstrated as Luc/β-gal.

CHROMATIN IMMUNOPRECIPITATION ASSAY

This assay (ChIP assay) was performed as described previously [Sobue et al., 2005]. TGW cells with or without GDNF treatment were cross-linked by formaldehyde. For immunoprecipitation, normal IgG, anti-C/EBPα, and anti-C/EBPβ antibody (0.4 μg each/sample) were added to the lysate and incubated at 4°C overnight with constant rotation. Immune complexes were extracted, and cross-linking was reversed by heating at 65°C overnight. The eluate was purified by High Pure PCR cleanup micro kit (Roche Applied Science, Mannheim, Germany). The *GAP43* promoter region was amplified by PCR using the following primer set. Sense: 5′-AGCGAGCATGTGCGATGA-3′, antisense: 5′-AGCACAGCATGGT-TGTCTCGTC-3′.

DNA PULL-DOWN ASSAY USING BIOTIN-LABELED PROBE

DNA pull-down assay was performed as described previously [Murakami et al., 2007]. Nuclear extracts (100 μg) from TGW cells with or without GDNF treatment for 24 h were mixed with 60 pmol of biotin-labeled double-strand probe and 15 μg of Poly dI − dC in DNAP (DNA affinity precipitation) buffer (20 mM HEPES–KOH pH 7.9, 80 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 10% w/v glycerol, 0.1% Triton X-100, protease inhibitor) for 60 min on ice. Sequences used are shown in the Figure legends. The probe–protein complexes were collected by adding 50 μl of Dynabeads M-280 streptavidin (Invitrogen Dynal AS, Oslo, Norway) to the mixture, and rotated at 4°C for 30 min. Beads were then washed three times with 500 μl of DNAP buffer. The complex was dissolved in SDS sample buffer and heated at 95°C for 5 min. Protein components were identified by Western blotting using anti-C/EBPα and C/EBPβ antibody (Santa Cruz Biotechnology).

STATISTICAL ANALYSIS

Results were expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test or one-way factorial analysis of variance and multiple comparison test (Fisher's method) using Stat view ver5 (SAS Institute Inc., Cary, NC).

RESULTS

CHARACTERIZATION OF WILD-TYPE AND DOMINANT-NEGATIVE SPHK1 EXPRESSED TGW CELLS

We established stable TGW cells overexpressing SPHK1-WT or SPHK1-DN (Fig. 1A). SPHK1-WT cells demonstrated a remarkable increase in SPHK1 enzyme activity, whereas the SPHK1 activity in SPHK1-DN cells was as low as the mock-transfected cells. In contrast to SPHK1 activity, SPHK2 activity did not change significantly by the GDNF treatment (data not shown). In mock and SPHK-WT cells, S1P levels in the culture medium also increased in a GDNF-dependent manner (Fig. 1B). SPHK1-WT cells exhibited higher secreted S1P compared with that of Mock cells, whereas SPHK1-DN did not secrete more S1P in response to GDNF. However, some discrepancies were observed between SPHK1 enzyme activity and secreted S1P level using our present assay method.

In mock cells, GDNF stimulated the GAP43 expression. In SPHK1-DN cells, GDNF-dependent GAP43 expression was nearly abolished, whereas constitutively high expression levels were observed in SPHK1-WT cells (Fig. 1C). The *GAP43* mRNA level in SPHK1-WT cells was also higher than that in mock cells, whereas SPHK1-DN cells did not exhibit the GDNF-induced increase (Fig. 1D). The absence of a significant difference between SPHK1-WT with or without GDNF is thought to be due to the high GAP-43 mRNA level of control SPHK1-WT cells.

S1P RECEPTORS AND CELLULAR SIGNALING PATHWAY LEADING TO GAP43 EXPRESSION

RT-PCR analysis showed that TGW cells express S1P receptors including *S1P*$_1$, *S1P*$_2$, *S1P*$_3$ (Fig. 2A). $S1P_2$ receptor signaling has been reported to be mainly involved in the retraction of neurite and inhibition of their outgrowth through Rho [Donati and Bruni, 2006], which is a rather reciprocal effect of the GDNF-induced phenotypes in this cell line. Therefore, we focused on $S1P_1$ and $S1P_3$ receptors, which were also detected by Western blotting. Figure 2B demonstrates that phosphorylated-serine of $S1P_1$ and $S1P_3$, activated from of S1P receptors, increased by the GDNF treatment. These results together revealed the involvement of $S1P_1$ and $S1P_3$ receptor signaling in GDNF-induced GAP43 expression. PD98059 (MEK inhibitor), but not SB203580 (p38 MAPK inhibitor) and LY294002 (PI3K inhibitor), inhibited the GDNF-induced GAP43 expression, suggesting that the MEK/ERK pathway is the major signaling pathway to increase GAP43 expression (Fig. 2C). Effective inhibition by SB203580 and LY294002 of p38 MAPK and PI3K/AKT pathway, respectively, was confirmed in the preliminary experiments (Supplementary Fig. 1).

Pertussis toxin (a G-protein-coupled receptor inhibitor), VPC23019 ($S1P_{1/3}$ receptor antagonist), and CAY10444 ($S1P_3$ receptor antagonist) suppressed GDNF-induced GAP43 expression. Exogenous S1P (1 μg/ml) also increased GAP43 expression

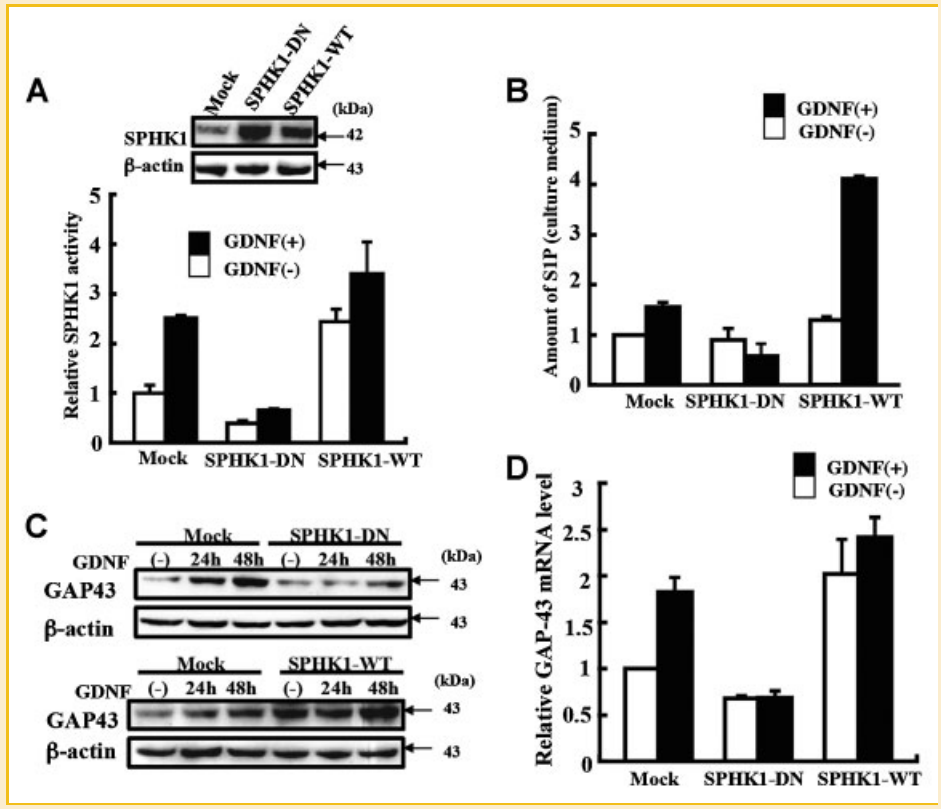

Fig. 1. Increased GDNF-induced GAP43 expression in SPHK1-overexpressed TGW cells. A: Establishment of SPHK wild-type (SPHK1-WT) and dominant-negative (SPHK1-DN)-TGW cells. Upper part shows Western blotting of Mock-, SPHK1-DN-, and SPHK1-WT-TGW cells by anti-SPHK1 antibody. Lower part illustrates SPHK1 enzyme activity. Mock-, SPHK1-DN-, and SPHK1-WT-TGW cells were measured for their respective SPHK1 enzyme activities with or without GDNF treatment (20 ng/ml) for 24 h. Mock cells without GDNF treatment were regarded as 1.0. B: S1P secreted into culture medium was examined as described in Materials and Methods Section. Mock-, SPHK1-DN-, and SPHK1-WT-TGW cells were cultured in triplicate with or without GDNF treatment for 24 h, and their culture medium was collected. Mean ± SD of S1P level in culture medium is shown. Data on mock cells without GDNF treatment were regarded as 1.0. C: Western blotting of GAP43 and β-actin of Mock-, SPHK1-DN-, and SPHK1-WT-TGW cells was performed. D: *GAP43* mRNA of cells treated with or without GDNF for 24 h was measured by semi-quantitative RT-PCR described in Materials and Methods Section. The relative *GAP43* mRNA level was expressed as *GAP43/GAPDH* shown by the bar graph. Data of GDNF (−) mock cells were defined as 1.0. The asterisk denotes $P < 0.01$. N.S. means not statistically significant.

(Fig. 2D). Considering that GDNF increased S1P secretion (Fig. 1B), these results suggest that S1P is involved in GAP43 expression by an inside-out signaling mechanism. Intriguingly, PTX, VPC23019, and CAY10444 also inhibited ERK activation (Fig. 2E).

5′-PROMOTER ANALYSIS OF GAP43 GENE

We cloned and inserted 1.7 kb of the 5′ promoter region of GAP43 gene, or the various truncated forms into a reporter vector, and measured the luciferase activity. Figure 3A illustrates the results using TGW cells, suggesting that GDNF-responsive elements were located between −131 and −98 bp from the first exon. A previous report suggests that Nex1/MATH-2 (NeuroD6) plays a key role in GAP43 expression of a rat pheochromocytoma cell line, PC12 [Uittenbogaard et al., 2003]. Neuro D family has also been reported to bind E-box of DNA sequence. Furthermore, the E-box and C/EBP binding sites were identified in this 5′-promoter region (Fig. 3A inlet). Among the candidate promoter binding factors, C/EBPβ but not C/EBPα NeuroD1 and NeuroD6, was increased by GDNF (Fig. 3B). Therefore, we overexpressed C/EBPβ in TGW cells, which increased the luciferase activity as high as the level observed by GDNF stimulation, whereas NeuroD6 overexpression did not increase the promoter activity remarkably (Fig. 3C). Introduction of mutation into the C/EBP binding site but not to E-box decreased GDNF-induced promoter activity (Fig. 3D).

DNA PULL-DOWN AND CHIP ANALYSES

Based on the promoter analysis described above, we focused on C/EBP protein and the C/EBP binding site in GDNF-induced GAP-43 transcription. ChIP assay revealed that C/EBPβ but not C/EBPα binds to this region after GDNF treatment (Fig. 4A). DNA pull-down assay clearly demonstrated direct binding of C/EBPβ but not C/EBPα (data not shown), to this region, and showed that mutation of C/EBP binding site inhibited C/EBPβ binding (Fig. 4B).

EFFECTS OF C/EBPS AND NEUROD FAMILY OVEREXPRESSION

In Figure 5A, we showed that transient expression of C/EBPβ but not C/EBPα, NeuroD1, and NeuroD6, induced GAP43 expression similar to that with GDNF treatment. C/EBPβ was increased with GDNF (Fig. 3B), and this increase was significantly inhibited by PD98059 (MEK inhibitor), but not by SB203580 (p38 MAPK inhibitor) or

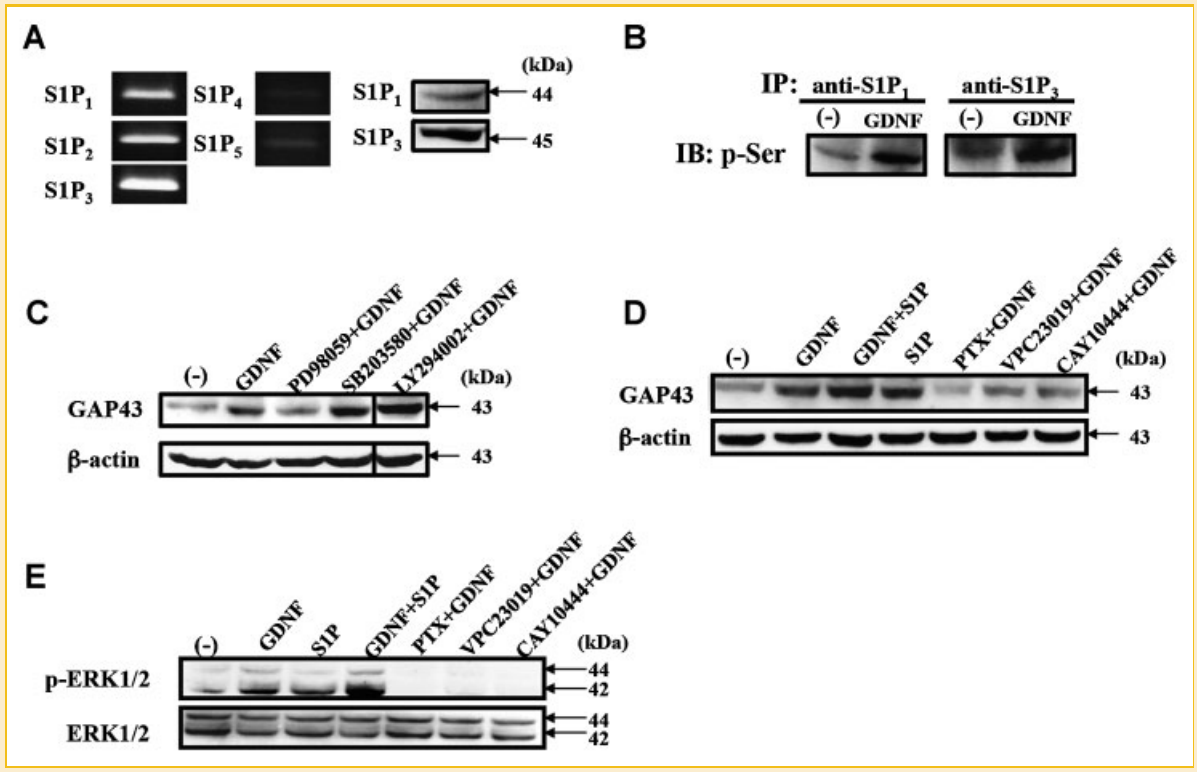

Fig. 2. Involvement of S1P receptors in GDNF-induced GAP43 expression. A: S1P receptor isoforms were examined by both RT-PCR method (left and middle part) and Western blotting using anti-$S1P_1$ and anti-$S1P_3$ antibody (right part). RT-PCR was performed using the respective primer sets described in Materials and Methods Section. B: Effects of GDNF on serine-phosphorylated $S1P_1$ and $S1P_3$ are demonstrated. Serine-phosphorylated (activated) $S1P_1$ and $S1P_3$ were examined by immunoprecipitation by anti-$S1P_1$ or anti-$S1P_3$, followed by anti-phosphoserine antibody as described in Materials and Methods Section. C: Inhibitors of signaling pathway (50 μM of PD98059, MEK inhibitor; 5 μM of SB203580, p38 MAPK inhibitor; 10 μM of LY294002, PI3K inhibitor) were added 2 h before GDNF treatment. Western blotting of GAP43 was performed. D: Effects of S1P receptor inhibitors including PTX (100 ng/ml, general G-protein-coupled receptor inhibitor) VPC23019 (5 μM, $S1P_{1/3}$ antagonist) and CAY10444 (5 μM, $S1P_3$ antagonist) were examined using GDNF-treated TGW cells. PTX, VPC23019, and CAY10444 were added 12, 2, and 2 h, respectively, before GDNF treatment. S1P (1 μg/ml) was added to some samples instead of GDNF. GAP43 and β-actin were analyzed by Western blotting. E: Based on data shown in Figure 2C, effects of PTX, VPC23019, and CAY10444 on activated ERK1/2 were also analyzed using both anti-phospho ERK1/2 and anti-ERK1/2 antibody, respectively.

LY294002 (PI3K inhibitor; Fig. 5B). Furthermore, an siRNA targeting *SPHK1* inhibited GDNF-induced C/EBPβ expression (Fig. 5C), suggesting that the SPHK1 and S1P pathways were important for GDNF-induced C/EBPβ expression.

DISCUSSION

GDNF/RET signaling is important for the formation of the enteric nervous system. RET mutation is involved in Hirschsprung's disease [Gershon, 2010]. GDNF is also important for the development and maintenance of dopamine neurons [Lin et al., 1993]. Selective degeneration of dopamine neurons has been observed in Parkinson disease, and GDNF has been shown to be neuroprotective in animal models of Parkinson disease. Thus, GDNF signaling pathways have attracted keen attention as the target of therapy [Glavaski-Joksimovic et al., 2010]. We previously reported that GDNF induced *SPHK1* gene expression [Murakami et al., 2007]. It has recently been reported that GDNF induced SPHK1 activation and that S1P plays both a role in survival and as a differentiation factor of retinal neurons [Miranda et al., 2009]. These results suggested that GDNF-induced signaling pathways might involve SPHK1 and S1P functions.

The sphingolipid rheostat model has been proposed according to which cellular sphingolipid metabolites, and especially the ratio of ceramide/S1P, determine the cell's fate. Recently, the LC/MS-MS system has made it possible to measure a small amount of S1P. Using this technique, secretion of S1P into the culture medium has been reported in an in vitro culture model [Chen et al., 2004; Serrano-Sanchez et al., 2008], indicating that not only cellular sphingolipids but also secreted ones are important [Takabe et al., 2008].

The binding of S1P with the respective S1P receptor can evoke a unique cellular response [Verzijl et al., 2010]. As an example of S1P/S1P receptor signaling leading to the target gene transcription, it has been reported that S1P regulates the expression of the liver receptor homolog-1 through S1P-induced changes of transcription factors in a human breast cancer cell line, MCF-7 [Hadizadeh et al., 2008]. In our study, S1P secretion was increased with GDNF treatment through SPHK1 but not SPHK2 activation, suggesting the different roles of two SPHK isoforms in GDNF signal transduction (Fig. 1b and data not shown).

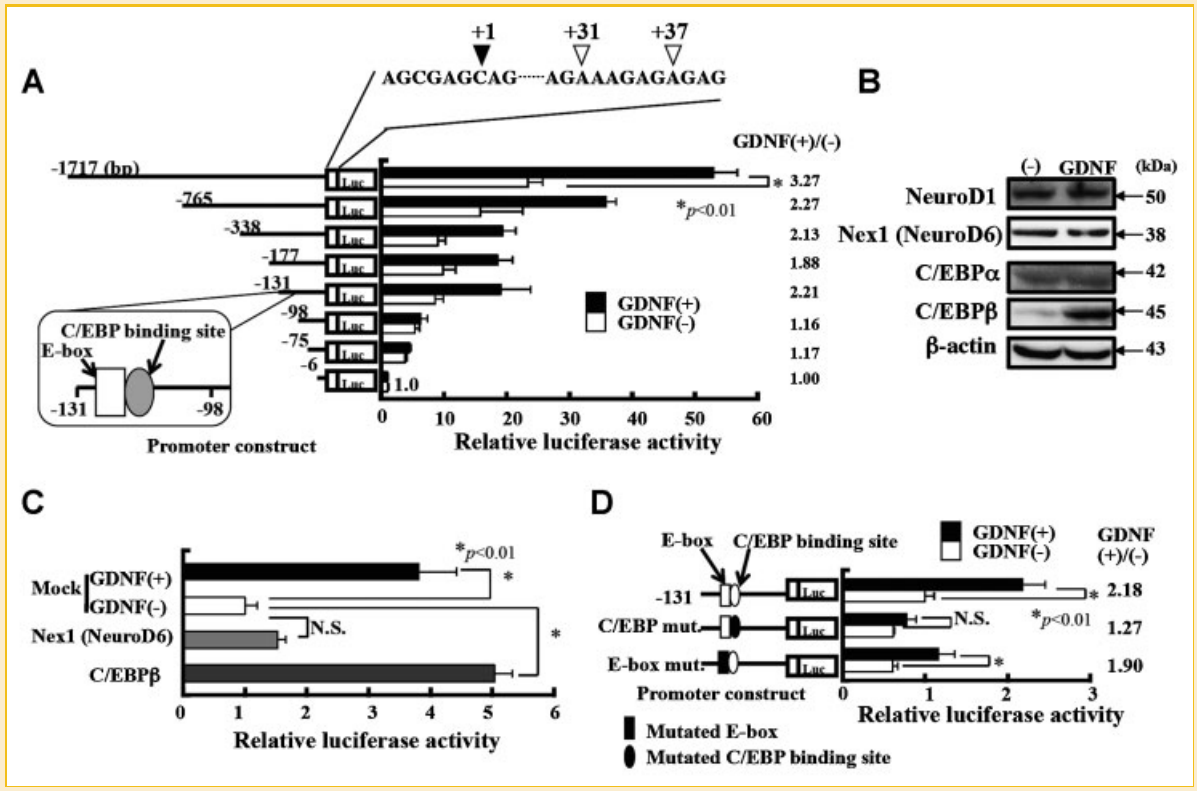

Fig. 3. Promoter analysis of the 5′-promoter of GAP43. A: Using various lengths of GAP43 promoters, luciferase reporter assay was performed using TGW cells as described in Materials and Methods Section. Relative promoter activity was calculated as luciferase activity/β-gal activity. Luciferase activity of −6 bp Luc of control (GDNF (−)) TGW cells was determined as 1.0. Mean ± SD of three independent experiments is indicated. A solid triangle (attached with C position) denotes the conventional transcription start site available through the database (GenBank ID: NM_002045), whereas open triangles attached to A positions mean transcription start sites of TGW cells with our 5′-RACE experiments described in Materials and Methods Section. TGW cells were used for assay. GDNF(+)/(−) ratio was illustrated at right. Inset denotes localization of C/EBP binding site and an E-box between −131 and −98 bp of the 5′ promoter. B: Western blotting of NeuroD1, Nex1/MATH-2 (NeuroD6), C/EBPα, and C/EBPβ of TGW cells with or without GDNF treatment for 24 h. Long exposure was needed to detect weak C/EBPα expression of TGW cells. C: Either NeuroD6 or C/EBPβ was transiently overexpressed in TGW cells and after 24 h, GAP43 promoter analysis was performed using −131 bp/luc. Mock-TGW cells transfected with −131 bp/luc and β-gal expression vector were also treated with or without GDNF for 24 h, and their promoter activities were measured. D: Mutation was introduced to either E-box or C/EBP binding site as shown at left. Solid box denotes mutated E-box, whereas solid circle means C/EBP binding site mutation. TGW cells were used for this assay. GDNF(+)/(−) ratio was illustrated at right.

We adopted metabolic labeling followed by TLC analysis for the measurement of secreted S1P, because our LC/MS-MS system could not accurately detect a small amount of secreted S1P. In Figure 1B, a discrepancy between SPHK1 enzyme activity and secreted S1P was observed, possibly due to the sensitivity problem of the TLC method we used. In addition, the localization/activation of SPHK1 enzyme after GDNF treatment and the regulatory mechanism of S1P secretion through various S1P transporters [Kawahara et al., 2009; Kim et al., 2009] might affect total S1P secretion. Further experiments are needed especially to improve the detection limit of our LC/MS-MS system. However, exogenous S1P can mimic GDNF function in GAP43 expression, and S1P receptor activation and downstream signaling in GAP43 expression were clearly shown in GDNF-induced TGW cells (Fig. 2B,D). These results strongly support our hypothesis that secreted S1P plays an important role in GAP43 transcription. Moreover, estradiol-induced export of S1P from human breast cancer cell line, MCF-7, has been reported [Takabe et al., 2010]. Their data also exhibited discordance between overexpressed SPHK1 activity and secreted S1P. Activation of SPHK1 and production of S1P at the plasma membrane are not sufficient for its release.

Experiments using SPHK1 stable transformants demonstrated that increased SPHK1 expression/activity enhanced GAP43 protein and mRNA levels. SPHK1-WT showed high GAP43 expression even without GDNF treatment, and did not exhibit significant increase after GDNF treatment (Fig. 1C,D). Our study demonstrated that the MEK/ERK pathway is a major one in GDNF-induced GAP43 expression in TGW cells (Fig. 2D,E). Crosstalk between neurotrophin and sphingolipid signaling has been reported [Coelho et al., 2010]. However, we focused on the relationship between SPHK1/S1P signaling and GAP-43 transcription. Therefore, it remained to be determined whether or not RET and S1P receptors directly interact in the cell membrane of TGW cells.

Promoter analysis revealed the importance of the C/EBP binding site located between −131 and −98 bp (Fig. 3A). Introduction of mutation to the C/EBP binding site but not E-box in the reporter assay supports the major role of the C/EBP binding site in GAP43 transcription of TGW cells (Fig. 3D). We also examined GAP43

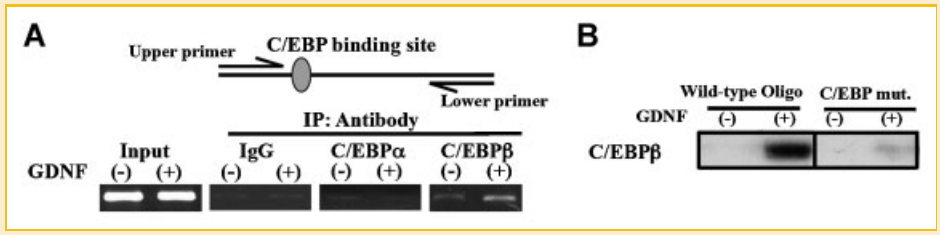

Fig. 4. ChIP assay and DNA pull-down assay A: ChIP assay was performed as described in Materials and Methods Section using primer set covering E-box and C/EBP binding site (shown in the Figure). Antibody used for immunoprecipitation was illustrated as IP: Antibody. B: Result of DNA pull-down assay was shown. Nuclear extract was prepared from TGW cells treated with or without GDNF for 24 h. Wild-type and C/EBP mutated oligo were used as the binding probes. Anti-C/EBPβ antibody was used for detection of bound protein with respective oligo probe. Wild-type Oligo; 5′-AGCGAGCATGTGCGATGAGCAATAGCTGTGGACCTTACAGTTGCTGCTA-3′, C/EBP mut; 5′-AGCGAGCATGTGC-CACGAACCCTAGCTGTGGACCTTACAGTTGCTGCTA-3′ (underline denotes mutation of C/EBP binding site).

promoter analysis using SPHK1-WT and SPHK-DN cells. It was shown that SPHK1-WT cells possessed enhanced GAP43 promoter activity regardless of GDNF treatment whereas SPHK1-DN exhibited decreased activity (data not shown), supporting results of Figure 3a.

Reportedly, overexpression of C/EBPβ in Neuro2A cells induces neuronal differentiation including neurite formation [Cortes-Canteli et al., 2002]. The involvement of C/EBPβ transcription factor in GAP-43 transcription of TGW cells was proved by the over-expression experiment and GDNF induced increase of C/EBPβ protein (Fig. 3B,C). The 5′-promoter of rat GAP-43 has been extensively analyzed using a rat pheochromocytoma cell line, PC12 [Uittenbogaard et al., 2003]. In PC12 cells, the NeuroD family, especially Nex1/MATH-2 (NeuroD6), reportedly has a major role in GAP43 gene regulation. However, in our case, Neuro D6 over-expression did not increase GAP43 promoter activity so much (Fig. 3C). Thus, it is less likely that NeuroD6 plays a major role in GAP43 transcription of TGW cells, although we could not completely overlook its contribution as the modulator of endogenous GAP43 expression (Fig. 3D). DNA pull-down and ChIP assay (Fig. 4) demonstrated the binding of C/EBPβ to this GAP43 promoter region with GDNF treatment.

GDNF/RET activates various downstream signaling including Jun/ATF2, Ras/ERK, and PI3K/Akt pathways [Kodama et al., 2005; Parsadanian et al., 2006; He et al., 2008]. The MAPK/C/EBP signaling cascade has been implicated in the commitment of progenitor cells to a neuronal fate, and MEK-mediated C/EBP phosphorylation is essential for neuronal fate determination [Menard et al., 2002]. A mutation study of the MEK/ERK-induced phosphorylated site of C/EBPβ strongly suggests the signaling pathway starting from NGF, NGF receptor, and MEK/ERK to C/EBPβ activation [Sterneck and Johnson, 1998]. Similarly, BDNF/TrkB receptor signaling has been reported to induce the early immediate gene transcription by recruiting C/EBPα and C/EBPβ to its promoter [Calella et al., 2007]. Our present findings together with these previous reports suggest the possibility that heterogeneous factors including NGF, BDNF, and GDNF, utilize the similar signaling pathway leading to the C/EBP activation followed by specific neuronal gene expression. Results shown in Figure 5 revealed the close relationship between MEK pathway and SPHK1 activity on this C/EBPβ expression.

The present study revealed for the first time that GDNF stimulates S1P secretion through SPHK1 expression, and secreted S1P

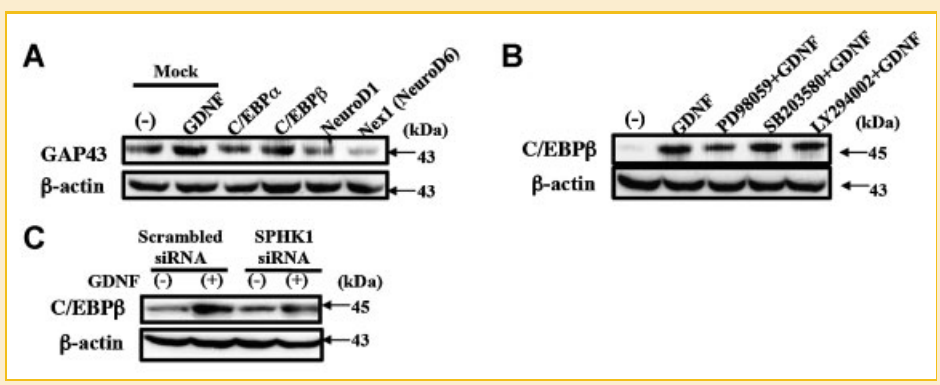

Fig. 5. The relationship between GDNF, C/EBPβ, and GAP43 and the effect of SPHK1 siRNA on C/EBPβ protein level. A: Overexpression effects of C/EBPα/β and NeuroD family proteins. TGW cells were transiently transfected with C/EBPα, C/EBPβ, NeuroD1, and Nex1/MATH-2 (NeuroD6), respectively. GAP43 protein level was examined 24 h after respective expression vector transfection. B: Effect of GDNF on C/EBPβ protein level. Western blotting of C/EBPβ and β-actin of TGW cells treated with or without GDNF was performed. Effects of various inhibitors of signaling pathway (Fig. 2C) were also examined. C: TGW cells were treated with siRNA of SPHK1 or scrambled siRNA as described in Materials and Methods Section. After transfecting each siRNA, cells were incubated with or without GDNF for 24 h. C/EBPβ and β-actin protein were examined by Western blotting.

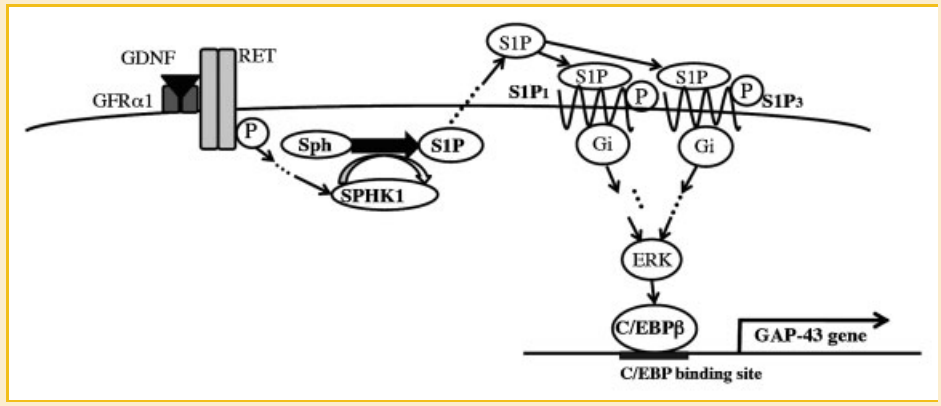

Fig. 6. Schematic illustration of GDNF-induced GAP43 transcription through activated SPHK1/S1P pathway.

transmits signals through S1P receptor to the MEK/ERK pathway leading to the increased/activated transcription factor, C/EBPβ, which then stimulates GAP43 transcription. A schematic illustration of our interpretation was shown in Figure 6. Our present data elucidate a novel aspect of SPHK1/S1P signaling in GDNF-induced neuronal differentiation which may well pave the way for future translational research of neurodegenerative diseases.

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