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Involvement of membrane protein GDE2 in retinoic acid-induced neurite formation in Neuro2A cells

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Abstract **We show that a glycerophosphodiester phosphodiesterase homolog, GDE2, is widely expressed in brain tissues including primary neurons, and that the expression of GDE2 in neuroblastoma Neuro2A cells is significantly upregulated during neuronal differentiation by retinoic acid (RA) treatment. Stable expression of GDE2 resulted in neurite formation in the absence of RA, and GDE2 accumulated at the regions of perinuclear and growth cones in Neuro2A cells. Furthermore, a loss-of-function of GDE2 in Neuro2A cells by RNAi blocked RA-induced neurite formation. These results demonstrate that GDE2 expression during neuronal differentiation plays an important role for growing neurites.**

Keywords: Glycerophosphodiester phosphodiesterase; GDE2; Retinoic acid; Neuro2A cells

1. Introduction

Escherichia coli (*E. coli*) glycerophosphodiester phosphodiesterases (GP-PDEs), *GlpQ* and *UgpQ*, are periplasmic and cytosolic proteins, which play an important role in the hydrolysis of deacylated glycerophospholipids to glycerol phosphate and alcohol, which are utilized as a major source of carbon and phosphate in *E. coli* [1,2]. In contrast, two novel mammalian GP-PDEs, GDE1/MIR16 and GDE3, were recently identified, and considered to be involved in several physiological functions [3,4]. GDE1/MIR16 was identified by yeast two-hybrid screening as a protein interacting with RGS16, a regulator of G protein signaling [3]. A recent study has shown that GDE1 is an integral membrane-bound glycoprotein that selectively hydrolyzes glycerophosphoinositol (GroPIns) [5]. In addition, Zheng et al. demonstrated that GroPIns phosphodiesterase activity in HEK293T cells can be regulated by stimulation of G protein-coupled α/β-adrenergic and lysophospholipid receptors [5]. Also, GDE3 was recently identified to be differentially expressed during specific stages of differentiation in mouse osteoblast-like MC3T3-E1 cells using a differential display method [4]. GDE3 encodes a protein with seven putative transmembrane regions and an extracellular loop containing a GP-PDE domain. We demonstrated that GDE3 protein accumulates at the cell periphery, and that overexpression of GDE3 changes transfected cells from a spread to a rounded form [4]. Moreover, endogenous GDE3 was co-localized with actin filament in MC3T3-E1 cells, suggesting that GDE3 is involved in the morphological change of cells accompanying modification of the cytoskeleton. Thus, mammalian GP-PDEs are considered to be involved in numerous physiological functions including signal transduction and cytoskeletal regulation.

Very recently, six mammalian GP-PDEs have been virtually cloned by an approach using bioinformatics [5,6]. In particular, we showed that GDE2 contained 607 amino acids with seven putative transmembrane regions, and that it was 43.7% identical to GDE3 at the amino acid level. Although GDE3 mRNA is restrictedly expressed in bone tissue and in the spleen, GDE2 mRNA was highly expressed in mouse brain, suggesting that GDE2 might have distinct roles in the nervous system. The physiological function of the nervous system is tightly associated with the highly specific pattern of connections formed between neurons. The specificity of these connections requires neurite extension toward their targets guided by the growth cone [7–9]. Ever since we showed that GDE3 protein was localized at the cell periphery and played a critical role for morphological change of cells, we have been particularly interested in the biological functions of mammalian GP-PDEs in neurons. These observations led us to hypothesize that GDE2 might play critical roles for neurite formation and/or neurite retraction. Neuro2A cells are neuroblastoma established from the mouse spinal cord, and are widely used for studies on neurite growth that is induced by retinoic acid (RA) treatment. Although several molecules have been reported to be involved in retinoid-induced neuronal differentiation of Neuro2A cells [10–12], our understanding of the mechanisms involved in the control of neuronal differentiation of Neuro2A cells remains limited. In this study, we demonstrated that the expression of GDE2 was dramatically upregulated during neuronal differentiation in response to RA, and that GDE2 played a critical role for neurite outgrowth of Neuro2A cells.

2. Materials and methods

2.1. Materials

Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). Murine neuroblastoma Neuro2A cells were obtained from Health Science Research Resources

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Abbreviations: GP-PDE, glycerophosphodiester phosphodiesterase; RA, retinoic acid; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

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Bank (Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Invitrogen. Antibodies specific for ERK1 and ERK2 were products of Santa Cruz Biotechnology. Anti-phospho-ERK1/2 antibody was obtained from Cell Signaling. Anti-GDE2 antibody was generated as we previously described [6].

2.2. Cell culture and transfection

Neuro2A cells were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were seeded at 3.0×10^4 and incubated for 24 h in a 90 mm-diameter plastic Petri dishes. Then induction of neuritogenesis was performed as previously described [13,14]. Briefly, differentiation was initiated by administration of 20 μM RA (Nacalai Tesque, Japan) in DMEM supplemented with 2% FCS. The open reading frame of GDE2 was digested with *Bam*HI, followed by blunting with T4 DNA polymerase. pEGFP-N1 (Clontech) was digested with *Xho*I, followed by blunting with T4 DNA polymerase. The resultant two DNA fragments were digested with *Kpn*I and ligated, generating pEGFP-GDE2. Neuro2A cells were transiently transfected with pEGFP-GDE2 using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's instructions. After transfection into Neuro2A cells, the cells were treated with 500 μg/ml of G418 for 14 days. After G418-resistant colonies were isolated, independent colonies were re-seeded in the growth medium, respectively. The morphology of each cell line was observed under a microscope, and photographs were taken at 100× magnification. To quantitatively evaluate neuritogenesis, the 80 differentiated cells were counted in five randomly chosen fields of each dish. Cells bearing neurites at least 1.5-fold longer than the soma diameter were regarded as being differentiated. pGFP-GDE2 was digested with both *Eco*RI and *Not*I, and insert fragment was ligated to *Eco*RI and *Not*I sites of the pMX retrovirus vector. High titer retroviruses harboring GDE2-GFP were produced in Phoenix 293 cells, and used to infect Neuro2A cells, as reported previously [15]. After Neuro2A cells were grown on glass slides with chamber polystyrene vessels in DMEM containing 10% FCS, they were directly fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Subsequently, the cells were washed for 10 min in PBS with 0.1% Tween 20, treated with rhodamine phalloidin (Molecular Probes) for 1 h, and visualized by fluorescence microscopy.

Primary cortical neurons and primary glial cells from mouse fetuses were isolated as we described previously [16]. Expression of GDE2 mRNA in these cultures was examined using RT-PCR with primers 5′-CCGCCATGCTGGCGCTCATCTC-3′ and 5′-AGGTCTCAGCTTCTCCGGGATT-3′.

2.3. Small Interfering RNAs (siRNAs)

Duplex siRNAs with a two nucleotide overhang at the 3′-end of the sequence were designed at iGENE Therapeutics and synthesized at Hokkaido System Science Co., Ltd. The target sequence was as follows: GDE2, CCUGCAUCAUGGAGAAAAAAGACCU. Neuro2A cells were transfected with siRNAs in a final concentration of 20 nM and pEGFP-N1 using LipofectAMINE 2000. At 8 h post-transfection, differentiation was initiated by administration of 20 μM of RA. The morphology of the cells was observed under a microscope, and photographs were taken at 100× magnification. To quantitatively evaluate neuritogenesis, neurite lengths of eighty GFP-positive cells in five randomly chosen fields of each dish were measured.

2.4. Western blot analysis

Neuro2A cells were washed with ice-cold PBS, and scraped in ice-cold RIPA buffer (10 mM Tris–HCl, pH 7.4, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA). The homogenates were centrifuged at $10000 \times g$ for 10 min. Protein concentration of the supernatant was determined using the Bio-Rad protein assay kit (Bio-Rad) with BSA as a standard. Ten micrograms (protein equivalents) of the supernatant were subjected to SDS–PAGE and transferred to an Immobilon P filter (Millipore). The filter was blocked for 18 h at 4 °C by soaking in 4% non-fat dried milk (Nacalai Tesque,) in PBS and was incubated for 18 h at 4 °C with anti-GDE2 antibody (diluted 1:1000). Signals were detected using horseradish peroxidase-conjugated anti-rabbit IgG and the enhanced chemiluminescence system (Amersham Bioscience).

2.5. Northern blot analysis

Total RNAs were isolated using ISOGEN (Nippon gene, Toyama, Japan), and fractionated in a 1% agarose gel containing 0.66 M formaldehyde and 0.02 M MOPS (pH 7.0). Fractionated RNAs were transferred onto a nylon filter by capillary blotting and then cross-linked by ultraviolet irradiation. ^{32}P-labeled cDNA fragments encoding mouse GDE2, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and human β-actin were used as probes for Northern blotting hybridization. Hybridization was performed in 6× SSC, 0.5% SDS,

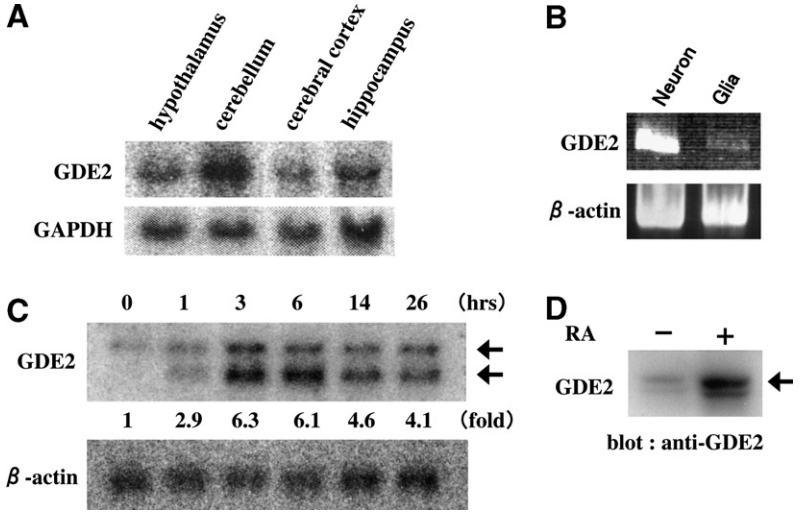

Fig. 1. Expression of GDE2 mRNA in a variety of brain regions and neuronal cells. (A) Ten μg of total RNAs from brain regions were subjected to Northern blot analysis. Expression of GDE2 mRNA was observed in adult mouse brain regions, including the cerebral cortex, cerebellum, hippocampus, and hypothalamus. (B) Expression of GDE2 mRNA in primary neurons (Neuron) and glia cells (Glia). GDE2 mRNA was particularly abundant in primary neuronal cells. (C) Neuronal differentiation of Neuro2A cells was initiated by administration of 20 μM RA. 10 μg of total RNA from Neuro2A cells cultured at the indicated time was subjected to Northern blot analysis. The total densities of the GDE2 mRNA bands were normalized by that of the β-actin. Results are expressed as the fold-increase in GDE2 mRNA relative to Neuro2A cells in the absence of RA. (D) GDE2 protein is upregulated during differentiation of Neuro2A cells. Membrane fraction of Neuro2A cells were subjected to SDS–PAGE. The gels were analyzed by Western blot with anti-GDE2 antibody. Expression of GDE2 protein is upregulated during differentiation of Neuro2A cells.

5× Denhardt's solution, and 100 μg/ml salmon sperm DNA at 65 °C for 16 h with the probe. The membrane was washed with 0.1× SSC and 0.5% SDS at 65 °C for 1 h and exposed to an imaging plate.

3. Results

3.1. Expression of GDE2 mRNA in mouse brain and Neuro2A cells

Northern blot analyses were performed using total RNAs from mouse brain and Neuro2A cells, and the GDE2 cDNA fragments as probes. A 3.7 kb GDE2 mRNA was widely expressed in the adult mouse brain, including the hippocampus, cerebellum, cerebral cortex, and hypothalamus (Fig. 1A). Furthermore, to identify the cell types in which GDE2 mRNA is expressed, we isolated primary cortical neurons and primary glial cells from mouse fetuses. The expression of GDE2 mRNA in these cultures was examined using RT-PCR with primers specific to GDE2 cDNA, showing that GDE2 mRNA was particularly abundant in primary neuronal cells (Fig. 1B). Mouse neuroblastoma Neuro2A cells have served as a useful model system for the study of neuronal differentiation and morphology. When Neuro2A cells are exposed to RA for 1–2 days, they acquire many features of sympathetic neurons, such as an outgrowth of neurites. To investigate the role of GDE2 in neuronal cell morphology, we examined whether RA influences the GDE2 mRNA expression in Neuro2A cells. Total RNA was extracted from Neuro2A cells cultured in the presence of 20 μM of RA for 0, 1, 2, 6, 14, and 26 h. Fig. 1C showed that treatment of Neuro2A cells with RA resulted in a significant increase in the GDE2 mRNA level, and that GDE2 mRNA level peaked at 3–6 h and decreased thereafter. Two discrete GDE2 mRNA species (3.7 and 2.9 kb) were observed in Northern blot analyses. We have previously isolated two different sized cDNAs corresponding to mouse GDE2 mRNA (data not shown), revealing the presence of two different species of mouse GDE2 mRNA. The long transcript of GDE2 was dominant in mouse brain tissues. When using GDE3 cDNA as a probe, no hybridization signal was observed with and without RA treatment (data not shown). Western blot analysis showed that the GDE2 protein level was upregulated at 24 h in the membrane fraction of Neuro2A cells (Fig. 1D).

Next, we were interested in the mechanism underlying the upregulation of GDE2 mRNA expression in Neuro2A cells. A previous report showed that differentiation and neuritogenesis of Neuro2A cells were induced by exogenously added ganglioside GM1 [17]. A previous report by Prinetti et al. [18] demonstrated that c-src protein was accumulated in a glycosphingolipid-enriched microdomain of Neuro2A cells by GM1 treatment, suggesting that the molecular mechanism of neuronal outgrowth is different for treatment with RA vs. GM1. To determine whether the upregulation of GDE2 mRNA expression is dependent on neuronal differentiation per se, we examined the effect of exogenously added GM1 on GDE2 mRNA expression. In this experiment, GM1 was shown to be able to induce neurite formation (data not shown), but showed

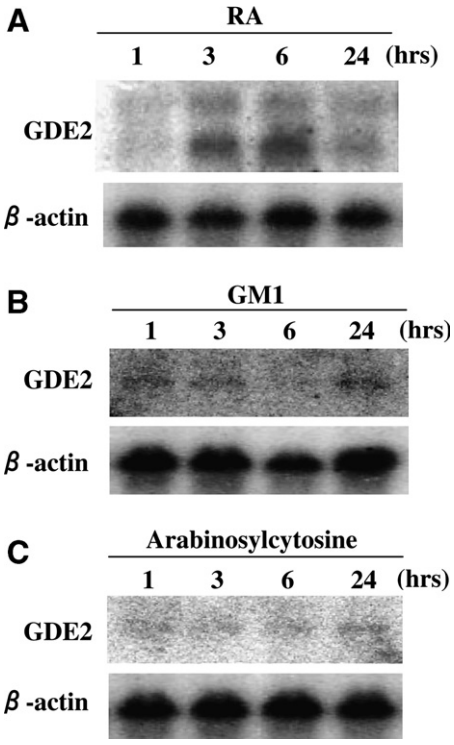

Fig. 2. Expression of GDE2 mRNA in Neuro2A cells. Differentiation and neuritogenesis of Neuro2A cells were induced by administration of 20 μM of RA (A), 10 μM of ganglioside GM1 (B), or 10 μM of cytosine arabinoside (C). 10 μg of total RNA from Neuro2A cells cultured at the indicated time was subjected to Northern blot analysis.

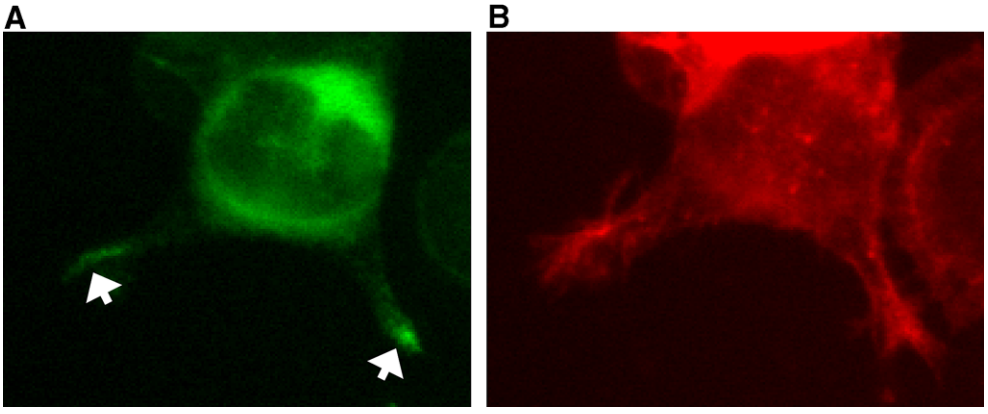

Fig. 3. Intracellular localization of GDE2 protein in Neuro2A cells. Retroviruses harboring GDE2 with green fluorescent protein fused to the C terminus (GDE2-GFP) were used to infect Neuro2A cells (A). Actin filaments were stained with rhodamine phalloidin (B), followed by analysis using fluorescence microscopy. GDE2-GFP accumulated at the regions of perinuclear and growth cones (arrows) in Neuro2A cells.

no effect on GDE2 expression in Neuro2A cells (Fig. 2B). GDE2 was again induced as expected in the control experiment with RA (Fig. 2A). Moreover, because RA-primed neural differentiation of Neuro2A cells was performed in a medium with low serum, we could not exclude the possibility that GDE2 mRNA expression is upregulated by growth arrest of Neuro2A cells. However, arabinosylcytosine (Fig. 2C) or cultivation without serum (data not shown) did not influence GDE2 mRNA expression, indicating that the increased expression of GDE2 mRNA in Neuro2A cells was dependent not on growth inhibition but on RA. In order to address the intracellular localization of GDE2 protein, we infected Neuro2A cells with retroviruses harboring GFP-fused GDE2. Analysis using fluorescence microscopy after visualization of actin filaments with rhodamine phalloidin demonstrated that GDE2-fused GFP was concentrated at the perinuclear region and also observed at growth cones (Fig. 3). Wild-type GFP was distributed evenly over the entire cytoplasm in Neuro2A cells when transfected with plasmid DNA encoding the wild-type GFP (data not shown).

3.2. Constitutive expression of GDE2 dramatically promotes neuronal outgrowth of Neuro2A cells

Because both GDE2 mRNA and protein were expressed during RA-induced differentiation of Neuro2A cells, we

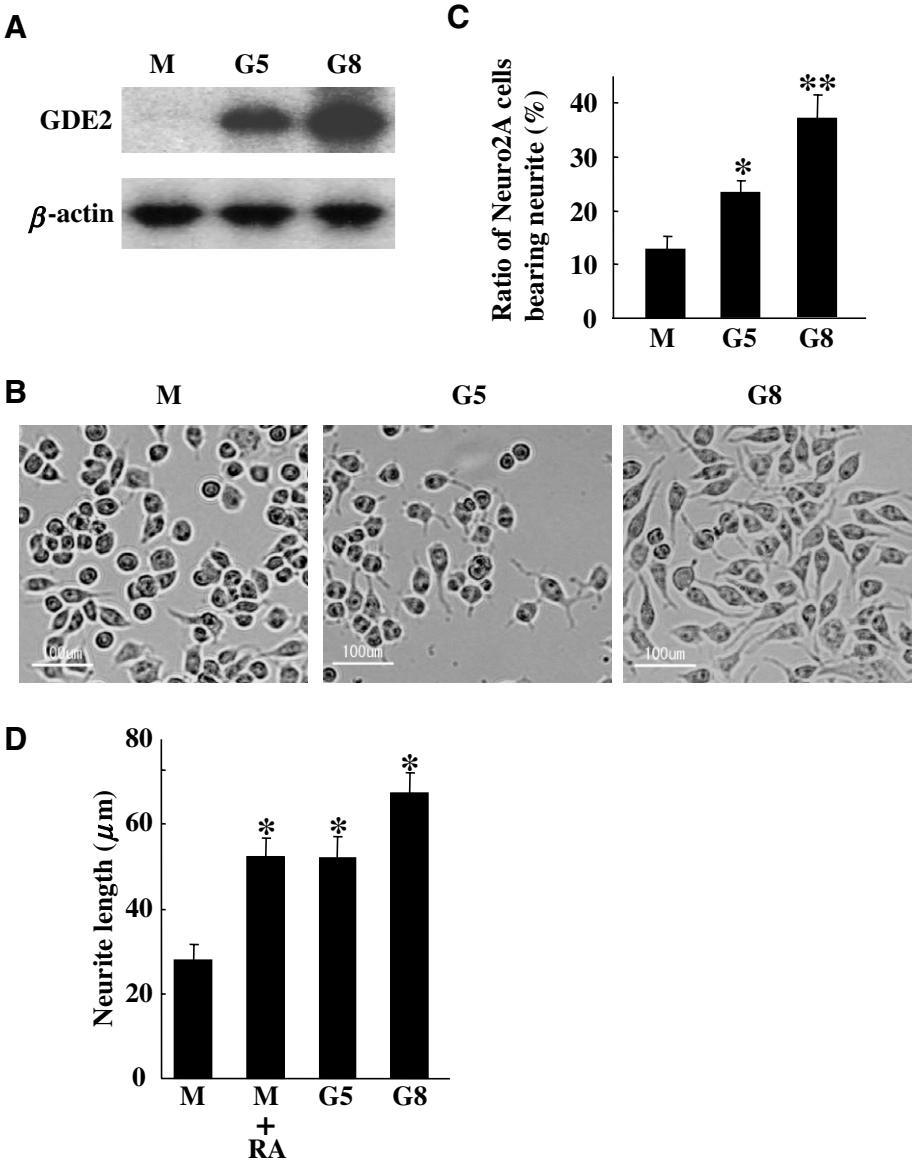

Fig. 4. Overexpression of GDE2 promotes neuronal outgrowth. After transfection with pGFP-GDE2 into Neuro2A cells, Neuro2A cells stably expressing the full-length protein were constructed as described in Section 2. (A) 10 μg of total RNA from two positive clones (G5 and G8) and empty vector-transfected cells (M) cultured in the absence of RA were subjected to Northern blot analysis. (B) The morphology of each cell line cultured in the absence of RA was observed under a microscope, and photographs were taken at 100× magnification. (C) Cells bearing neurites at least 1.5-fold longer than the soma diameter were regarded as being differentiated cells as described in Section 2. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with mock cells (M). (D) Neurite lengths were measured in each cell in randomly chosen fields of each dish. In this experiment, neurite lengths were measured in each mock cell treated with RA for 24 h (M + RA). $^{*}P < 0.05$ compared with those of mock cells. Values are expressed as means ± S.E. Statistical significance was determined by unpaired Student's *t* test.

investigated whether Neuro2A gene expression could alter the program of neuronal differentiation. These cells were stably transfected with the full-length coding region of GDE2 or with the mock vector. As shown in Fig. 4A, a significant elevation of GDE2 mRNA was observed in two stably transfected clones, G5 and G8, in the absence of retinoic acid, but not in the mock clone M. After neurite lengths were measured in each cell in randomly chosen fields of each clone, cell number bearing neurites at least 1.5-fold longer than the soma diameter was counted. Constitutive expression of GDE2 increased the number of cells bearing neurites in the absence of RA (with 1.8- and 2.8-fold increases, respectively) and also increased neurite lengths (with 1.9- and 2.4-fold increases, respectively), compared with the mock M clone (Fig. 4B–D). These results were consistent with the expression level of GDE2.

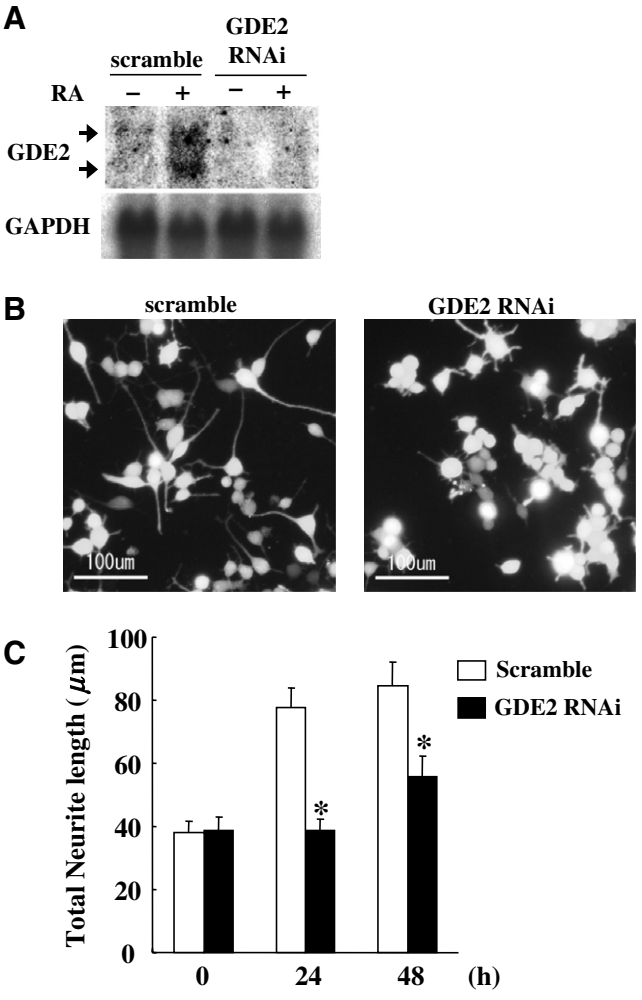

Fig. 5. Effects of GDE2 siRNA on neurite formation of Neuro2A cells. Neuro2A cells were transfected with scrambled siRNA (scramble) or GDE2 siRNA (GDE2 siRNA) with pEGFP and then stimulated with 20 μM of RA. After treatment with RA for 24 h, 10 μg of total RNA was subjected to Northern blot analysis. (B) The morphology of these cells cultured in the presence of RA for 48 h was observed under a microscope, and photographs were taken at 100× magnification. (C) After treatment with RA for 24 or 48 h, neurite lengths were measured in each GFP-positive cell in randomly chosen fields of each dish. $^{*}P < 0.05$ compared with those of cells transfected with scramble siRNA. Values are expressed as means ± S.E. Statistical significance was determined by unpaired Student's t test.

3.3. Effects of GDE2 siRNA on neurite formation of Neuro2A cells

To investigate roles of GDE2 in neurite formation, we finally tried to examine the effects of GDE2 siRNA on RA-induced neuronal outgrowth of Neuro2A cells. These cells were transfected with GDE2 siRNA or scrambled siRNA (scramble) together with a plasmid carrying GFP cDNA and then stimulated with RA. After treatment with 20 μM of RA for 24 h or 48 h, neurite lengths were measured in each GFP-positive cell in randomly chosen fields of each dish. GDE2 RNAi gene silencing, which resulted in a strong reduction of mRNA expression (Fig. 5A), significantly reduced the extent of neurite outgrowth in Neuro2A cells after RA treatment for 24 h and 48 h (by 50.4% and 34.2%, respectively) (Fig. 5B and C). Previous work by Singh et al. has shown that a MAP kinase pathway including ERK1/2 was activated during RA-induced neuronal differentiation of neuroblastoma SH-SY5Y cells [19]. Although the current study showed that RA could promote ERK1/2 activation in Neuro2A cells (Fig. 6A), knockdown of GDE2 in Neuro2A cells by RNAi did not influence ERK1/2 activation by RA, as shown in Fig. 6B.

4. Discussion

RA is well known to play critical roles in nervous system development, including neuronal patterning and neurite outgrowth [20]. In particular, RA is demonstrated to induce neurite outgrowth from spinal cord, dorsal root ganglia neurons, and neuroblastoma cells [13,21,22]. One mechanism whereby RA affects neurite extension was previously shown to be by regulating the expression of neurotrophin receptors including

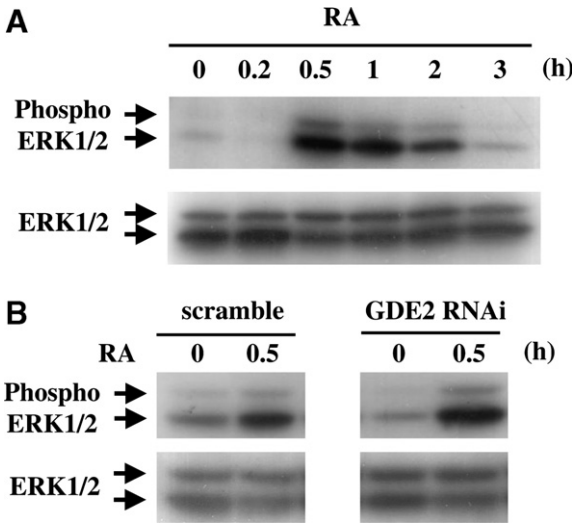

Fig. 6. Effect of GDE2 siRNA on the activation of ERK induced by RA in Neuro2A cells. (A) Total lysates from Neuro2A cells were prepared after stimulation with RA for the indicated time, and subjected to Western blot analyses using antibody specific for phosphorylated form of ERK1/2 (upper panel). The same membrane was re-blotted with anti-ERK1/2 antibody (lower panel). (B) Neuro2A cells were transfected with scrambled siRNA (scramble) or GDE2 siRNA (GDE2 siRNA), and then stimulated with 20 μM of RA. Cell lysates from Neuro2A cells were prepared after stimulation with RA for the indicated time, and subjected to Western blot analyses as described above.

TrkA and $p75^{NTR}$ [22,23], whereas recent works have shown that a number of mRNAs are identified to be rapidly induced by RA in SH-SY5Y and Neuro2A cells [24,25]. Merrill et al. have reported that expression of 14 genes is rapidly upregulated by RA treatment in SH-SY5Y cells, and that mRNAs encoding candidate neurite regulating factors including ephrin B2 and a novel Rho GTPase-activating protein family member are induced by RA [24]. An interesting recent report has revealed that 38 genes change their expression rapidly after RA treatment in Neuro2A cells [25]. Among them, the expression pattern of 6 genes, including *Gse1* and *Garn14*, is similar to that of GDE2, suggesting that they might collaborate with GDE2 to trigger neurite outgrowth.

GDE2 has been virtually cloned by an approach using bioinformatics as a membrane protein with an extracellular loop containing a GP-PDE domain [5,6]. A recent work by Zheng et al. demonstrated that GDE1 could selectively hydrolyze GroPIns, indicating that GDE1 might play physiological roles through the hydrolysis of GroPIns and/or some of its phospholyrated derivatives such as glycerophosphoinositol 4-phosphate (GroPIns4P) [5]. GroPIns and GroPIns4P are water-soluble phosphoinositide metabolites and were originally reported to be associated with the expression of oncogenic Ras [26]. Mancini et al. showed the remarkable effects of GroPIns4P on the reorganization of actin cytoskeleton in Swiss 3T3 cells, demonstrating that exogenously added GroPIns4P potently induces the formation of membrane ruffles and stress fibers via the activation of two small G-proteins, Rac and Rho [27]. Additionally, we previously demonstrated that GDE3 was co-localized with actin filaments and that GDE3 induced morphological change in cells when overexpressed [4]. Following the proposal that a rearrangement of actin filaments in growth cones is essential for neuronal outgrowth and polarity [28,29], it was further suggested that GP-PDEs are involved in the morphological change of neuronal cells through actin remodeling by regulation of GroPIns and/or GroPIns4P levels. Previous reports have implicated the Rho family of small G-proteins, Rho, Rac and Cdc42 in the regulation of neurite outgrowth and patterning. Importantly, several studies have shown that Rac1 and Cdc42 have a significant role in neuronal differentiation and neurite outgrowth [30,31]. These observations suggest that a mechanism whereby GDE2 plays a role for neurite extension might be by regulating the Rac1 and/or Cdc42 signaling via the regulation of GroPIns/GroPIns4P level.

On the other hand, non-genomic actions of RA have been recently proposed. RA has been shown, in some cases, to rapidly activate kinase signaling cascades. Singh et al. have shown that tissue transglutaminase mediates activation of the MAP kinase pathway including ERK1/2, JNK1 and p38 kinases during RA-induced neuronal differentiation of SH-SY5Y cells [19]. Additionally, a recent work by Evangelopoulos et al. has demonstrated that ERK1/2 is activated in Neuro2A cells in response to serum withdrawal [32]. In our study, RA was shown to promote ERK1/2 activation in Neuro2A cells. Knockdown of GDE2 in Neuro2A cells by RNAi did not influence ERK1/2 activation by RA, indicating that the physiological action of GDE2 is not dependent on ERK1/2 signaling.

During the course of the current study, Rao et al. have demonstrated that GDE2 is necessary to drive spinal motor neuron differentiation in vivo [33]. Although information about the control of neurite extension of Neuro2A cells remains unclear at the molecular level, the current study demonstrated a critical role of GDE2 for retinoid-induced neuronal outgrowth. Further investigations are needed to resolve the molecular mechanism of GDE2-mediated neuronal outgrowth.

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