Overexpressed GM1 Suppresses Nerve Growth Factor (NGF) Signals by Modulating the Intracellular Localization of NGF Receptors and Membrane Fluidity in PC12 Cells*

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Ganglioside GM1 has been considered to have a neurotrophic factor-like activity. To analyze the effects of endogenously generated GM1, the rat pheochromocytoma cell line PC12 was transfected with the GM1/GD1b/GA1 synthase gene and showed increased expression levels of GM1. To our surprise, GM1-transfectant cells (GM1* cells) showed no neurite formation after stimulation with nerve growth factor (NGF). Autophosphorylation of NGF receptor TrkA and activation of ERK1/2 after NGF treatment were scarcely detected in GM1* cells. Binding of 125I-NGF to PC12 cells was almost equivalent between GM1* cells and controls. However, dimer formation of TrkA upon NGF treatment was markedly suppressed in GM1* cells in both cross-linking analysis with Bis(sulfosuccinimidyl)suberate 3 and 125I-NGF binding assay. The sucrose density gradient fractionation of the cell lysate revealed that TrkA primarily located in the lipid raft fraction moved to the non-raft fraction in GM1* cells. p75NTR and Ras also moved from the raft to non-raft fraction in GM1* cells, whereas flotillin and GM1 persistently resided in the lipid raft. TrkA kinase activity was differentially regulated when GM1 was added to the kinase assay system in vitro, suggesting suppressive/enhancing effects of GM1 on NGF signals based on the concentration. Measurement of fluorescence recovery after photobleaching revealed that the membrane fluidity was reduced in GM1* cells. These results suggested that overexpressed GM1 suppresses the differentiation signals mediated by NGF/TrkA by modulating the properties of the lipid raft and the intracellular localization of NGF receptors and relevant signaling molecules.

Gangliosides, sialic acid-containing glycosphingolipids, are thought to play important roles in the development and function of the nervous system, because they accumulate in brain tissues of vertebrates, and their profiles of carbohydrate moiety of gangliosides are determined basically by the combination of activated glycosyltransferase genes (3). Among complex gangliosides, GM1 has been most rigorously studied, because it is one of the major gangliosides in vertebrate brain (2) and shows specific binding with the cholera toxin B subunit resulting in important biological events such as cAMP response (4). Since GM1 synthase cDNA was isolated by us (5), the mRNA expression of the gene has been directly examined, and a high expression level in the rat fetal brain has been demonstrated.

Recently, membrane microdomains such as glycolipid-enriched microdomains, detergent-insoluble microdomains, or lipid rafts have been thought of as sites for signal transduction as well as for endocytosis and cholesterol turnover on the cell membrane (6). They are enriched in cholesterol, glycosphosphatidylinositol-anchored proteins, sphingomyelin, glycosphingolipids, and various signaling molecules such as growth factor receptors, G-proteins, and Src family tyrosine kinases (7). Although ganglioside GM1 has been used as a mere marker of rafts, we demonstrated that GM1 might regulate the signal magnitude of PDGF/PDGFR by altering the intracellular localization of PDGFR receptor (R) in Swiss3T3 cells (8). There have also been a number of reports indicating the effects of glycosphingolipids on the growth/differentiation signals (9). These reports suggested that GM1 might affect the structure/function of lipid rafts in neuronal cells, resulting in the regulation of differentiation/proliferation signals.

A rat pheochromocytoma cell line PC12 has been widely used as a differentiation model of neuronal cells, because they show neurite extension after nerve growth factor (NGF) stimulation (10). The effects of NGF are mediated at least in part by TrkA (11), the high affinity NGF receptor containing a tyrosine kinase activity. Mutoh et al. (12) demonstrated that GM1 binds to TrkA tightly on the surface of PC12 cells and enhances the effects of NGF (12). Exogenous GM1 enhanced the TrkA phosphorylation and neurite formation when added with a low concentration of NGF. Furthermore, it could rescue PC12 cells

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**Abbreviations used: GM1, Galβ1,3GalNAcβ1,4(NeuAcα2,3)-Galβ1,4Glc-ceramide; GA1, NeuAcα2,3Galβ1,4Glc-ceramide; GT1b, NeuAcα2,3Galβ1,4GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc-ceramide; NGF, nerve growth factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; ERK(s), extracellular signal-regulated kinase(s); MEK, MAPK/ERK kinase; FRAP, fluorescence recovery after photobleaching; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; CTB, cholera toxin B subunit; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BS3, Bis(sulfosuccinimidyl)suberate; Mes, 2-morpholinoethanesulfonic acid; GEM, glycolipid-enriched microdomain(s).**

**Ganglioside nomenclature is based on that of Svennerholm (53).**
from apoptotic death induced by serum deprivation (13). These results seem to reflect the neurotrophic nature of GM1. However, it is not clear whether endogenously generated GM1 plays the same roles as exogenous GM1.

In the present study, we established stable transfected lines of PC12 with the GM1/GD1b/GA1 synthase cDNA, which we had isolated previously (5), and analyzed the response to NGF. To dissect the role of GM1-producing cells, we assessed the cell response to NGF-induced differentiation and in the activation of signaling molecules were found in GM1+ cells. We also demonstrated dramatic changes in the intracellular localization of NGF receptors and relevant molecules, i.e. the majority of TrkA, p75NTR, and Ras moved from the raft to the non-raft fraction in GM1+ cells, whereas the raft markers such as GM1 and flotillin persistently stayed in the raft fraction. These results suggested that GM1 plays critical roles in the regulation of the physiological nature of lipid raft and of the bio-signals to determine the cell fates.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse 2.5S NGF was obtained from Alomone Laboratory (Jerusalem, Israel). Anti-NGF receptor TrkA antibodies (C-14, rabbit IgG) and an anti-cathepsin D antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TrkA (rabbit IgG) was from Upstate biotechnology (Lake Placid, NY). Anti-p75 (rabbit IgG) was from Promega (Madison, WI). Anti-flotillin (rabbit IgG) and anti-phosphotyrosine monoclonal antibodies (mAb) (PY20) were from BD Transduction Laboratories (Lexington, KY). Anti-ERK1/2 rabbit IgG, anti-phospho-ERK1/2 rabbit IgG, and anti-rabbit IgG conjugated with horseradish peroxidase were from New England Biolabs (Beverly, MA). An anti-Ras mAb was from Seikagaku Co. (Tokyo, Japan). Cholera toxin B subunit (CTB) conjugated with biotin was from LIST Biological Laboratories (Campbell, CA). CTB-Alexa555 was from Molecular Probe (Eugene, OR). 14-3-3 NGF (~1500 C/mmol) was from Amersham Biosciences. Protein A-Sepharose Plus Fast Flow was from Amersham Biosciences. Immobilon-P Transfer Membrane was from Millipore (Bedford, MA). Anti-phospho-TrkA (New England Biolabs) and anti-extracellular domain of TrkA antibodies were obtained from Upstate Biotechnology (Lake Placid, NY).

Recombinant DNA—Human β1,3-galactosyltransferase cDNA clone pM1T-9 (5) was digested with Xhol-BamHI and inserted into the Xhol-BamHI site of pMIKneo to obtain pMIKneo/M1T-9. pMIKneo is a mammalian expression vector with the SRα promoter and was generously presented by Dr. K. Maruyama (Tokyo Medical and Dental University).

Cell Culture and Transfection—PC12 cells were maintained in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum, at 37°C in a humidified atmosphere containing 5% CO2 as described previously (14). PC12 cells used for cDNA transfection were plated in a 60-mm plastic tissue culture plate (Falcon) at a density of 7 × 105 cells/ml. The plasmid pMIKneo/M1T-9 (4 µg) was transfected into cells with LipofectAMINE (Invitrogen, Rockville, MD) according to the manufacturer's instructions. Stable transfected cells were selected in the presence of 250 µg/ml G418 (Invitrogen) and maintained continuously in the presence of G418 (200 µg/ml).

Neurite Outgrowth Assay—PC12 cells and the transfected cells were seeded at 1 × 104 cells/well in a type I collagen-coated 48-well culture plate (Falcon) and were either treated with 100 ng/ml 2.5S NGF (Alomone Laboratories) in serum-free medium or left untreated in the serum-containing medium. Neurite-bearing cells were counted on the following 4 days. To analyze the effect of exogenous GM1 on neurite outgrowth, PC12 cells were treated with either serum-free medium or medium containing 100 ng/ml NGF, 5 ng/ml NGF, 50 µg/ml GM1 (Sigma), or 5 ng/ml NGF with 50 µg/ml GM1 (preincubated with 50 µg/ml GM1 for 12 h at 37°C), then treated with 5 ng/ml NGF). The percentage of cells bearing neurites decreased with time. After being washed twice with ice-cold PBS, the cells were scraped in 1 ml of 0.5 mM sodium carbonate buffer, pH 11.0. The cells were homogenized sequentially using a loose fitting Dounce homogenizer (n times), a Polytron tissue grinder (three 10-s bursts), and a sonicator (three 20-s bursts). All procedures were carried out at 4°C. The homogenate (1 ml) was then adjusted to 1 M NaCl, 1 M MgCl2, 10 mM EGTA) for 4°C for 1 h. The cross-linking reaction was terminated by adding 1 mM Tris-HCl (pH 7.4) to a final concentration of 0.1 M. Then, the cells were washed twice in Tris-buffered saline and lysed in the lysis buffer as described above. TrkA was immunoprecipitated, and immunoprecipitates were separated on a 7% gel with an acrylamide/bisacrylamide ratio of 200:1. Samples were electroblotted onto membranes and immunoblotted with an anti-Trk antibody (B-3).

Isolation of Raft Fraction—Microdomain rafts were prepared using a non-detergent extraction method essentially as described by Song et al. (15). Cells were plated at a density of 2 × 105/15-cm dish and cultured up to 90% confluency, and five dishes of cells were used for each treatment. The cells were homogenized sequentially using a loose fitting Dounce homogenizer (n times), a Polytron tissue grinder (three 10-s bursts), and a sonicator (three 20-s bursts). All procedures were carried out at 4°C. The homogenate (1 ml) was then adjusted to 1 M NaCl, 1 M MgCl2, 10 mM EGTA) for 4°C for 1 h. The cross-linking reaction was terminated by adding 1 mM Tris-HCl (pH 7.4) to a final concentration of 0.1 M. Then, the cells were washed twice in Tris-buffered saline and lysed in the lysis buffer as described above. TrkA was immunoprecipitated, and immunoprecipitates were separated on a 7% gel with an acrylamide/bisacrylamide ratio of 200:1. Samples were electroblotted onto membranes and immunoblotted with an anti-Trk antibody (B-3).
containing 0.25 M sodium carbonate. The samples were centrifuged at 20,000 g for 16 h at 4 °C. From the top of the gradient, 0.5 ml of each fraction was collected to yield 10 fractions. The components in each fraction were concentrated by centrifugation at 100,000 g for 2 h at 4 °C in MNE buffer, and precipitates were resolved in the lysis buffer and used for immunoprecipitation or Western immunoblotting.

NGF-TrkA Cross-linking—Cells were resuspended in a binding buffer (Hanks’ containing 1 mg/ml each of glucose and bovine serum albumin) at 5 x 10^6 cells/ml and incubated at 4 °C for 1 h with 1 nM 125I-NGF (2750 dpm). To correct the nonspecific binding, unlabeled NGF (1 nM) was added during the binding. To cross-link NGF with TrkA, BS3 was added to a final concentration of 1 mM to the reaction mixture, and the mixture was incubated at 4 °C for 1 h. After the cross-linking reaction was terminated, cells were washed and lysed in the lysis buffer. The lysates were used for immunoprecipitation with an anti-Trk antibody, and the immunoprecipitates were separated using a 7% gel as described above. 125I-NGF-bound proteins were detected with autoradiography using an imaging plate and BAS2000 Bioimage Analyzer™ (Fujifilm, Tokyo).

NGF Binding Assay—Cells were resuspended in a binding buffer at 1 x 10^5 cells/0.2 ml and incubated at 37 °C for 45 min with various concentrations (5 pM to 10 nM) of 125I-NGF. The reaction mixture was overlaid onto 5% bovine serum albumin in 0.32 M sucrose and was centrifuged at 10,000 x g for 1 min at 4 °C. The supernatants were collected, and the tips of tubes containing the cell pellets were cut off. A 1,000-fold excess of unlabeled NGF was used to assess the nonspecific binding. The radioactivity was counted in a γ-counter, and the results were analyzed on the basis of Scatchard plot.

In Vitro Kinase Assay—The PC12 lysate was used for immunoprecipitation with an anti-TrkA antibody as described above. Immunoprecipitates were washed twice with the lysis buffer and twice with the Tris-buffered saline containing a phosphatase inhibitor (100 μM Na₃VO₄). Immunoprecipitates were resuspended in a tyrosine kinase assay buffer (50 mM HEPES, pH 7.4, 20 mM MnCl₂, 5 mM MgCl₂, 1 mM dithiothreitol, 100 μM Na₃VO₄, 5 μCi/sample [γ-32P]ATP) and then incubated for 10 min at 30 °C in the presence of various concentrations of GM1. The kinase reaction was terminated by adding Laemmli sample buffer followed by boiling. Relative kinase activity was measured by scanning the bands in the autoradiogram as described above. The effects of GM3 or GT1b were also examined for comparison.

Immunofluorescence Study—Cells were cultured on a poly-L-lysine-coated coverslip, and cells were fixed with 4% paraformaldehyde in PBS. Then, cells were treated with CTB-Alexa555 (10 μg/ml) in PBS for 1 h at 4 °C. The fixed cells were analyzed with a confocal laser microscope Fluoview FV500™ (Olympus, Tokyo).

Rho Kinase Assay—To determine the alteration in the activation levels of Rho, activated Rho was isolated and analyzed with the method...
of Ren et al. (16) using a Rho Activation Assay kit (Cytoskeleton, Denver, CO) according to the manufacturer's instruction. Cells were plated at a density of 6 × 10⁶/10 cm dish. After being washed, cells were lysed with lysis buffer, and the cell lysates were centrifuged at 10,000 × g for 5 min at 4 °C, and the supernatants were incubated with Rho-tekin-RBD beads under rotation for 1 h at 4 °C. The beads were washed three times with the lysis buffer, and the precipitates were solubilized in a Laemmli sample buffer, heated for 3 min at 98 °C, and then separated in SDS-PAGE. Bound Rho proteins were detected by Western immunoblotting using an anti-Rho polyclonal antibody in the kit.

Measurement of the Membrane Fluidity by Fluorescence Recovery after Photobleaching—Cells were cultured on a glass bottom dish coated with poly-D-lysine, and the medium was replaced with a standard external solution (10 mM Hepes, 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose) prewarmed at 37 °C. A fluorescent probe, DiI (DiIC₁₈, D-282, Molecular probes, Eugene OR) was added to the standard external solution at 2.5 μg/ml and incubated for 10 min, to be incorporated into the cell membranes. FRAP analyses were performed with a confocal laser scanning microscope (Olympus) equipped with a stage heater at 37 °C. A small area of the labeled membrane was photobleached by full laser power (100%), resulting in the immediate reduction of the intensity of fluorescence. A selected area (3-μm square) on the labeled cell membrane was photobleached for 8–15 s by a 488-nm laser beam. Then, the fluorescence recovery in the photobleached area was immediately recorded using a time-lapse option of the system until the fluorescence was recovered at a plateau level. Relative recovery in fluorescence intensity was calculated for 60 s after photobleaching and analyzed by Microsoft Excel. FRAP was calculated by a ratio of post-bleach intensity/pre-bleach intensity, and the membrane fluidity was evaluated by comparing the percentage of FRAP to a plateau as follows. Fluorescence intensities at a pre-photobleach state and the post-photobleach intensities at a plateau were expressed as average values of 10 scans. In each experiment, the value obtained at pre-photobleach was taken as 100% and that at just after photobleach was taken as 0% to compensate for the differences among experiments. The half-maximal recovery of fluorescence (t½) was also calculated on the basis of the duration (t) of fluorescence recovery reaching a plateau.

RESULTS

Expression of Gangliosides in GM1 Synthase Gene Transfectant Cells (GM1⁺ Cells)—After the transfection of PC12 cells with a GM1 synthase gene expression vector (pMIKneo/MIT-9), or pMIKneo, two transfectant lines (M1 and M3) and two vector controls (V1 and V2) were established, respectively. Expression profiles of gangliosides were examined by flow cytometry (Fig. 1A). The mean fluorescence values of individual gangliosides obtained by subtracting those of corresponding controls are shown in Fig. 1B. Among the gangliosides examined, expression levels of GM1 and fucosyl-GM1 markedly increased in the transfectants (M3 and M6). Expression levels of other gangliosides showed no apparent change compared with those in the vector control lines (V1 and V2) except that GT1b expression level reduced after the transfection. To compare the distribution pattern of GM1 on the cell membrane, we stained the cultured cells with CTB-Alexa555. Whereas GM1 in vector control cells showed a weak and patched distribution, those in the vector control lines (V1 and V2) except that GT1b expression level reduced after the transfection. To compare the distribution pattern of GM1 on the cell membrane, we stained the cultured cells with CTB-Alexa555. Whereas GM1 in vector control cells showed a weak and patched distribution, those in the GM1⁺ cells showed very strong staining and a uniform and thick distribution throughout the plasma membrane (Fig. 1C).

No Neurite Extension with NGF in PC12 Cells Transfected with GM1 Synthase cDNA—To examine the effects of GM1 expressed endogenously on NGF-induced differentiation, the GM1⁺ cells and vector controls were cultured with or without 50 ng/ml NGF for 5 days. Fig. 2 shows the morphological change in controls and GM1⁺ cells after 5 days of NGF treatment. As reported previously, cell proliferation was reduced.
Reduced phosphorylation of TrkA and ERK1/2 after NGF treatment in the transfectant cells. A, time courses of the phosphorylation levels of TrkA in the vector controls (V1 and V2) and the GM1\(^+\) cells (M3 and M6) were analyzed. Cells were treated with NGF (50 ng/ml) for the times indicated. TrkA was immunoprecipitated with an anti-Trk antibody (C-14), and immunoblotting was carried out using PY-20 (upper) or anti-TrkA antibody (bottom). B, activation of ERK1/2. Cells were treated as described in A and were then lysed with Nonidet P-40 lysis buffer, and total lysates were separated by 12% SDS-PAGE. Western blotting was carried out using an anti-phosphorylated ERK1/2 antibody (upper) or an anti-ERK1/2 (lower). A similar experiment was repeated, and essentially similar results were obtained. C, GM1 synthase gene transfectants were treated with GT1b (50 μM) for 4 h, and the cell surface GT1b was detected with flow cytometry (left). Then, phosphorylation of ERK1/2 after NGF treatment was examined as described above (right). Note that there are no differences between M3 and M6 in B and those in C.
FIG. 4. NGF binding was equivalent between the controls and GM1" cells. Using gradually diluted 125I-labeled NGF, NGF binding to the vector controls and GM1" cells were investigated. Two vector control cells and two GM1" cells were analyzed. Nonspecific binding was obtained by adding 1000-fold excess amounts of unlabeled NGF, and the values were used for the subtraction. Scatchard plot analysis was performed, and the results of individual samples are presented. Insets are the binding curves. Note that there is no significant difference in the binding kinetics between the controls and GM1" cells. B_max values calculated from the high affinity binding curves are expressed with the unit (×10^2 pmol/10^5 cells) in the individual figures.

FIG. 5. Reduced dimerization of TrkA with NGF treatment in GM1 synthase gene transfectant cells. The cell surface proteins were cross-linked with BS3 under NGF treatment as described under “Experimental Procedures.” After cross-linking, TrkA was immunoprecipitated and detected by immunoblotting using an anti-TrkA antibody. The results were reproducible in three independent experiments. The arrow represents the TrkA monomer; arrowhead, TrkA dimer.
control cells and the GM1\(^+\) cells, suggesting that overexpressed GM1 did not disturb the NGF binding itself.

**Reduced Dimerization of TrkA with NGF Treatment in the GM1\(^+\) Cells**—The GM1\(^+\) cells showed neither neurite extension nor signal activation after NGF treatment. Then, TrkA dimerization after NGF treatment was investigated. The cell surface proteins were cross-linked with BS3 under the NGF treatment as described under “Experimental Procedures.” After cross-linking, TrkA was immunoprecipitated from the cell lysates, and separated in a 7% gel followed by immunoblotting using anti-TrkA antibody (B-3). The immunoblotting showed high molecular mass bands (about 300 kDa) corresponding to TrkA dimer at 5 min after NGF treatment in control cells. On the other hand, the dimer bands of TrkA were scarcely found in the GM1\(^+\) cells (Fig. 5).

**Reduced Complex Formation between TrkA Dimer and NGF in the Transfectants**—Based on the reduction in the phosphorylation and dimerization of TrkA in the GM1\(^+\) cells, we questioned whether NGF binding to TrkA is attenuated by overexpression of GM1. The simple binding assay showed no differences as shown in Fig. 4. Then, the dimer formation was markedly reduced in GM1\(^+\) cells (Fig. 5). Therefore, we examined NGF binding to TrkA monomer and dimer with cross-linking experiment. Cells were incubated with\(^{125}\)I-NGF (1 nM) at 4°C for 1 h, and then with a cross-linker BS3 for 1 h. TrkA was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Radioactive bands, corresponding to \(^{125}\)I-NGF-bound TrkA monomer (~150 kDa) and \(^{125}\)I-NGF-bound dimer (~300 kDa), were found in vector control cells (Fig. 6A).

![Fig. 6. Reduced dimerization and binding of dimers with NGF in the transfectant cells as shown with ligand-receptor crosslinking.](image-url)

**Fig. 6.** Reduced dimerization and binding of dimers with NGF in the transfectant cells as shown with ligand-receptor crosslinking. Cells were incubated with \(^{125}\)I-NGF (1 nM) at 4°C for 1 h and then with a cross-linker BS3 for 1 h. TrkA was immunoprecipitated and analyzed. A, bands represent TrkA bound with \(^{125}\)I-NGF. TrkA monomer (arrow) and dimer (arrowhead) were found in vector control cells (lanes 1 and 3), whereas mainly monomer form of TrkA was detected in GM1\(^+\) cells. B, an evidence for the binding specificity. When binding was carried out in the presence of a 1000-fold excess of unlabeled NGF (indicated with “+”), the bands completely disappeared, suggesting that NGF specifically bound to TrkA.

**Fig. 7.** Alteration in the flotation in a sucrose gradient of NGF receptors in the GM1\(^+\) cells. Cells were lysed under detergent-free conditions, and the extracts were fractionated. Fractions were subjected to immunoblotting analysis using the antibodies against the proteins indicated. GM1 was detected with CTB. A, the results of vector control cells. B, the results of GM1\(^+\) cells. Note an increased ratio of TrkA and P75\(^{NTR}\) in non-raft fractions (6–10) in the GM1\(^+\) cells.
In contrast, dimer bands were hardly detected in GM1+ cells, whereas the band intensities of the monomer were equivalent with those in the controls. These findings indicated that the monomer form of TrkA in the GM1+ cells binds with NGF at the similar level with that in the control cells, although the transfectant cells could not form TrkA dimer or could not bind with NGF, if present. Taken together with the results in Fig. 5, the former case appeared more likely. When binding was carried out in the presence of a 1000-fold excess of unlabeled NGF (Fig. 6B), all bands completely disappeared, suggesting that NGF specifically bound to TrkA.

**Alteration in the Flotation in a Sucrose Gradient of NGF Receptors in GM1 Synthase Gene Transfectant Cells**—Because GM1 has been considered to be localized in lipid rafts, overexpression of GM1 may affect the nature of rafts. Then, we examined the effects of GM1 overexpression on the intracellular localization of NGF receptors (TrkA and p75NTR) by isolating lipid rafts with a detergent-free method. Ten fractions from the discontinuous sucrose gradient were prepared and analyzed for distribution of p75NTR, TrkA, and raft markers, such as flotillin. The distribution of Ras as well as GM1 was also analyzed. Immunoblot analysis showed that most of flotillin, Ras, and GM1 were found in fractions 3 and 4 containing the raft fraction (Fig. 7A). The majority of TrkA and p75NTR were also detected in this raft fraction. These results indicated that PC12 cells contained the lipid raft in buoyant density, GM1 content, and protein constituents. To our surprise, the distribution pattern of TrkA and p75NTR dramatically changed in the GM1+ cells (Fig. 7B). The main portions of TrkA and p75NTR were detected in the non-raft fraction comprising fractions 6–10, suggesting that the distribution of NGF receptors are strongly affected with the expression levels of GM1, probably based on the modification of the properties of GEM rafts. The Ras protein also moved in part from the raft to the non-raft fraction, whereas GM1 and flotillin consistently existed in the raft fraction.

**GM1 Differentially Regulates TrkA Tyrosine Kinase**—To analyze direct effects of GM1 on the TrkA kinase activity, immunoprecipitated TrkA was used for the in vitro kinase assay in the presence of various concentrations of GM1. As shown in Fig. 8A, low concentrations of GM1 rather enhanced the kinase activity, whereas relatively high concentrations of GM1 (>500 μM) suppressed the kinase in a dose-dependent manner. To examine the specificity of GM1 in the enhancing effect on the TrkA kinase activity, GM3 or GT1b was also added to the kinase assay system at various concentrations (Fig. 8B). Consequently, as shown in Fig. 8B, clearly neither GM3 nor GT1b enhanced TrkA activity at any concentration as GM1 did.

**Rho Is Not Modulated in the GM1 Synthase Gene Transfectants**—Because RhoA has been shown to be involved in the regulation of neuronal differentiation and neurite outgrowth (17), overexpression of GM1 might suppress the neurite extension by modulating the Rho activity. Then, we examined the activation level of Rho in the GM1 synthase gene-transfectant cells. Activated Rho was not detected in either the vector controls or the GM1+ cells, although LPA could induce an activated Rho band (data not shown). Therefore, it was not likely that GM1 affected the Rho kinase activity in the transfectant cells.

**High Expression of GM1 Resulted in the Reduction of Membrane Fluidity**—Because the GM1+ cells showed reduced dimerization of the TrkA receptor (Figs. 5 and 6), and an altered intracellular localization of TrkA receptors (Fig. 7), we thought that overexpression of GM1 might affect the membrane fluidity. Then, we examined the effects of overexpression of GM1 on the plasma membrane fluidity in live cells using a FRAP system as described under “Experimental Procedures.” To examine the membrane fluidity in living cells with FRAP experiments, we stained cell surface GM1 by using CTB-Allexa555. As shown in Fig. 1C, GM1+ cells were stained uniformly and strongly, and vector control cells showed patched distribution with low intensity. Furthermore, CTB-Alexa555 rapidly underwent endocytosis within 5–10 min, causing troubles to compare FRAP between these two cell types. In contrast, cell surface membranes were uniformly and moderately stained with DiI in both the vector controls and GM1+ cells. Therefore, we used DiI instead of CTB-Alexa555 for cell surface labeling in FRAP experiments. Cells were loaded with 2.5 μg/ml DiI for 10 min, then photobleach was applied to plasma membrane and the fluorescence recovery in these bleached regions was monitored. Fluorescence of DiI was markedly lost after photobleaching, and recovery was observed for ~60 s after photobleaching. Although the fluorescence recovery of membrane in vector control cells was 41.37%, the recovery in the GM1+ cells was 24.8% (Fig. 9B). The t1/2 values of fluorescence recovery were 7.27 ± 1.9 s and 9.36 ± 2.8 s for vector control and GM1 synthase gene transfectants, respectively (Fig. 9C).
These data suggested that overexpression of GM1 reduced the membrane fluidity and might result in the interference with the dimerization and phosphorylation of TrkA.

**DISCUSSION**

The characteristic expression patterns of gangliosides in the nervous tissues during development have suggested that they play important roles in the neurogenesis of vertebrates (18). Glycosyltransferase genes responsible for the synthesis of gangliosides also showed corresponding expression patterns during brain development (19). Because exogenously added gangliosides induced differentiation of neuronal cells in vitro (20), various gangliosides are thought to have neurotrophic effects, and have been administrated to experimental animals after generating artificial neurological damages or disorders by mechanical or chemical manipulation (21), by the injection of toxic reagents (22) or by ischemic treatment (23). Although various gangliosides have often had positive effects presumably due to their neurotrophic activity, it has been difficult to elucidate the molecular mechanisms for the effects of gangliosides endogenously generated in the cells or tissues.

A number of studies have been performed to investigate the effects of gangliosides on the function of nerve growth factor receptors (12, 13, 24–27). Physical and functional association of GM1 with NGF receptor TrkA was reported (24–26). Activation of TrkA with GM1 (24, 26), or enhancement of the TrkA dimerization due to NGF with GM1 (25) was also reported. Ferrari et al. (13) reported that GM1 alone could at least partly replace the activity of NGF. In particular, Mutoh et al. (12) reported that exogenously added GM1 tightly binds to TrkA and enhances the phosphorylation of TrkA when stimulated with NGF. Rabin et al. (27) showed that GM1 activated Trk receptors via the induction of neutrophin release. However, it is not clear whether exogenously added GM1 actually acts in the same way as endogenously generated GM1, because the density and molecular topology of the added GM1 on the cell membrane are hard to be determined. Its molecular form and effect in the liquid on the cultured cells are also difficult to be precisely determined.

Using a cloned GM1/GD1b/GA1 synthase gene (5), we have tried to modify the ganglioside composition of PC12 cells and succeeded in obtaining clones expressing higher levels of GM1. Although the gene product had activity to produce not only GM1 but GD1b (5), the resulting transfectant cells expressed increased levels of GM1 and fucosyl-GM1, and reduced levels of GT1b. Consequently, we could analyze the effects of GM1 derived from introduced cDNA on the response to NGF.

In the response to NGF, GM1+ cells showed much reduced reaction in both the neurite extension and the activation of TrkA/ERK1/2 signaling pathway. The vector control cells showed fairly good neurite formation and prompt phosphorylation of TrkA upon NGF treatment. ERK1/2 were also activated quickly with a peak at 5 min after NGF treatment. In
contrast, GM1+ cells showed no neurite extension and no or a
very faint response of TrkA phosphorylation. Activation of
ERK1/2 was also scarcely detectable. Thus, expression of GM1
showed suppressive effects on NGF-induced differentiation.
Recent progress in the analysis of membrane microdomains
indicates that even neuronal cells have caveolae-like domains
containing Shc, Ras, caveolin, and TrkA (28), and GEM/rafts
are structurally unique components of plasma membranes,
crucial for neural development and function (29). Differentia-
tion signals from NGF could be transduced only through the
NGF receptors localized in GEM/rafts showing a different be-
model presented by Miljan et al. (33), i.e. ganglioside modulation of
ligand binding, ganglioside regulation of receptor dimerization,
and ganglioside implication with receptor activation state and
subcellular localization, the latter two seemed likely in the
results of our study.
There have been a number of reports to indicate the changes
in the intracellular localization of receptors and signal mole-
cules following the modification of components in lipid rafts
(34). In Swissa3T3 cells, overexpression of GM1 synthase gene
induced clear reduction of cell growth. It also resulted in the
reduction of phosphorylation levels of PDGFR and ERK1/2
after PDGF treatment (8). The mechanisms behind these phe-
notypic changes should be the dramatic changes in the intra-
cellular localization of receptor molecules in the cells, and this
was also the case in PC12 as demonstrated in this study.
Originally, NGF induces neuronal differentiation through the
TrkA/Ras/MEK/ERK pathway in PC12 cells (35–40). Our
results indicated that overexpression of GM1 modulated the ini-
tial events in TrkA activation just after binding of NGF, i.e.
TrkA dimerization and phosphorylation. This means that TrkA
outside of GEM/rafts is hard to be activated in GM1+ cells,
suggesting that appropriate physicochemical circumstances of
GEM/rafts are needed for the early step of its activation. Not
only TrkA but also p75NTR and Ras underwent translocation
from GEM/rafts to non-raft compartment. Although the roles of
p75NTR are not clear, it might be important in the full reaction
of PC12 cells to NGF, and its cotransfer with TrkA may en-
trast with these systems, Trk receptors usually show constant localization and activation in
GEM/rafts during the signal transduction. We also confirmed
no apparent changes in the subcellular localization of NGF
receptors after NGF treatment (data not shown). Cell type-
specific factors might determine which pattern of regulation
individual receptor molecules undergo. Structures of receptors
are also very important in the regulation of the intracellular
localization. However, little is known about the universal prin-
ciple determining the behavior of receptors inside/outside of
GEM/rafts, and it remains to be analyzed.
Epidermal growth factor (EGF) induces cell proliferation
through the EGF receptor/Ras/MEK/MAPK pathway, too. The
reason why these two factors, NGF and EGF, can induce dif-
cerent cellular events via the same signaling pathway has been
explained based on the different kinetics of ERK1/2 activation
after their binding to the individual receptors (46). Therefore,
the response of the GM1+ cells to EGF might be very interest-
ing, and remains to be analyzed.
As for the reason for the difference in the effects of exogenous
GM1 and cDNA-derived GM1, four possibilities should be con-
sidered. First, exogenously added GM1 may exert effects on
the cell surface molecules (such as TrkA) in quite a different way
from that of cis-existing glycolipids, although some portion
of the added GM1 is certainly incorporated into the membrane
and expressed (data not shown). Second, the expression levels
of GM1 may vary resulting in the different effects on TrkA
molecular function as observed in the experiments of in vitro
kinase of the precipitated TrkA (Fig. 8). The artificial control of
the expression levels of the transfected gene is not so easy at
this moment, although it is needed to establish multiple trans-
fectants with various levels of GM1 expression to confirm the
cose-dependent effects in the future. Third, the altered NGF
signal may come not from increased GM1, but from other ch-
anges of glycolipid components in the transfected cells, i.e.
increased fucosyl-GM1 or decreased GT1b. These additional
changes should not be present in the cells treated with exoge-
 nous GM1. Fourth, differences in the duration of the exposure
cells to GM1 might be crucial to determine the fates of cells
as suggested by the experiments for alcohol toxicity (47).
Although an important role of glycosphingolipids in the for-
mation of the membrane microdomains has been discussed
(48–50), the quantitative effects of glycosphingolipids have not
been well understood. Little is also known about the effects
of ganglioside expression on the membrane fluidity. The effects
of exogenous gangliosides on the cell membrane fluidity have
been examined in a few system using the technique of FRAP.
HL-60 cells showed decreased fluidity after GM3 treatment
(51). In the analysis of the ethanol effects on the mobility and
viability of embryonal neural crest cells, added GM1 decreased
membrane fluidity, resulting in the protection of the cell death
(52). Furthermore, the membrane fluidity of the reconstituted
liposomes increased with dioleoylphosphatidylcholine (liquid
crystal phase) compared with distearoylphosphatidylcholine
(gel phase), resulting in the increased kinase activity of EGFR
(47). Taken together, it seems reasonable that overexpression
of GM1 results in the decreased membrane fluidity and in the
reduction of the differentiation signal with NGF/TrkA as dem-
onstrated in this study. However, how the membrane fluidity
associated with the nature of the GEM/raft is not well under-
stood and remains to be investigated.
In conclusion, our findings suggest that glycosphingolipids
produced and expressed in the cells play critical roles in
the modulation of the quality and quantity of signals for the cell
differentiation/proliferation and probably for death. Although
the mechanisms by which glycosphingolipids regulate the sig-
nals have been poorly understood, the results presented here
clearly indicate one example of the modes for such regulation.
Namely, the microdomain is important as a place for the inter-
action between glycolipids and signaling molecules, and carbo-
hydrate moieties in the glycosphingolipids contain much more influence than expected on the intracellular localization of various receptors and signal molecules. This means that we need to recognize glycolipids as critical factors determining the cell fates and to understand their roles in malignant tumors and neuronal degeneration when we construct strategies to apply glycosphingolipids and their synthetic machineries for the treatment of cancers and neurological degenerative diseases.

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