Glycosphingolipid-enriched Signaling Domain in Mouse Neuroblastoma Neuro2a Cells

MECHANISM OF GANGLIOSIDE-DEPENDENT NEURITOGENESIS*

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Dedicated to Pierre Sinay (Ecole Normale superieure, Paris) in honor of his festive occasion.

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Glycosphingolipids (GSLs),1 particularly gangliosides, have been implicated as mediators of cell adhesion and modulators of signal transduction (1). There has been considerable interest in the functional significance of GSLs in neuronal cells and tissues. Ganglioside patterns in the nervous system display dramatic changes during development, neurite outgrowth, synaptogenesis (2, 3), and malignant transformation. Sphingolipid biosynthesis is necessary for neuritogenesis in primary cultures of hippocampal neurons (4), and induced expression of GD32 synthetase in Neuro2a neuroblastoma cells is followed by neurite outgrowth (5). The discovery that the exogenous addition of gangliosides prevents neurodegeneration in vivo and induces neuritogenesis and maintains neurotrophic effects in several cell systems of neural origin (6), including neuroblastoma (7, 8), led to the hypothesis that GSLs and gangliosides play essential roles in the maintenance of the structure and function of neuronal cells. Numerous studies along this line followed (for review, see Refs. 9 and 10). A Neuro2a cell model, in contrast to other neuronal cell lines, is unusual in that neuritogenic differentiation is induced readily by various gangliosides (7), although cells are not susceptible to stimulation by nerve growth factor (NGF) (11) and do not contain NGF receptor. The exact mechanism by which gangliosides trigger the molecular events leading to neuronal differentiation remains unexplored.

Specific association of c-Src with synaptic vesicles in PC12 cells (12) and early activation of c-Src or Src family kinases in neuroblastoma cells in response to differentiation induction by phorbol esters (13) or by anti-GM3 antibody (14) indicate an important role of c-Src or Src family kinases in neuronal cell differentiation and signal transduction.

Recent studies have revealed a novel organization of GSLs and gangliosides in cell membrane, i.e. the majority of them are clustered and associated closely with single or multiple signal transducer molecules. Examples are GM3 organized with c-Src, Rho, FAK, and Ras in B16 melanoma cells (15, 16) and GD3 associated with Lyn in rat brain (17). Such structural units consisting of GM3, c-Src, and Rho can be separated from caveo-

1 The abbreviations used are: GSL(s), glycosphingolipid(s); NGF, nerve growth factor; GSD, glycosphingolipid signaling domain (this indicates a functional entity); MAPK, mitogen-activated protein kinase; LacCer, lactosylceramide; PC, phosphatidylcholine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GM, glycosphingolipid-enriched microdomain (this indicates a physical or chemical entity); MES, 4-morpholinooethanesulfonic acid; SM, sphingomyelin; HPTLC, high performance thin layer chromatography; PAGE, polyacrylamide gel electrophoresis.

2 Glycosphingolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature ((1977) Lipids 12, 455–463); however, the suffix -OseCer is omitted. Gangliosides are abbreviated according to Svennerholm (1964) J. Lipid Res. 5, 145–155.)
### Glycosphingolipid Microdomain in Neuro2a Cells

**Experimental Procedures**

#### Reagents

GM3 was prepared from dog erythrocytes (20). Gg3 was prepared from guinea pig erythrocytes (21). GM1 from bovine brain was from Fidia Research Laboratories (Italy). GB4 and lactosylceramide (LacCer) were from human erythrocytes (22). GM1 and GB4 were radiolabeled at the terminal sugar residue using the galactose oxidase-[3H]NaB procedure (23). GM3 radiolabeled at C-3 of the long chain base was kindly provided by Prof. S. Sonnino (University of Milan, Italy) (24). STEAROYL-2-ARACHIDONYL \[\text{PC}\] (specific activity 175 Ci/mmol) was from NEN Life Science Products. L-1-\(^{32}\)P\[\text{ATP}\] (3,000 Ci/mmol) and \[^{3}H\]NaB (204.1 mCi/mmol) were from NEN Life Science Products.

#### Cell Culture

Mouse Neuro2a neuroblastoma cells (CCL-131, American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% FBS (HyClone, Logan, UT), 4 mM L-glutamine, 1 mM pyruvic acid, 4.5 mg/ml d-glucose, 100 units/ml potassium penicillin G, and 100 µg/ml streptomycin sulfate in a 5% CO\(_2\), 95% air humidified atmosphere.

#### GEM Preparation

Membrane fraction presumably corresponding to the GSL-enriched microdomain (GEM) was prepared from Neuro2a cells by ultracentrifugation on a discontinuous sucrose gradient after lysis and homogenization in the presence of 1% Triton X-100 (27) or in hypertonic sodium carbonate medium (28) by modification of original procedure as described below. After ultracentrifugation, 1-ml fractions were collected starting from the top of the tube. GEM was also prepared after stimulation of Neuro2a cells with different GSLs as described under *Effect of Gangliosides on e-Src Activation in Intact Cells.*

**Detergent Method**—Cells were harvested in phosphate-buffered saline containing 0.4 mM Na\(_2\)VO\(_4\), lysed, homogenized, and subjected to sucrose density gradient centrifugation to separate the low density light-scattering membranous fraction (16, 27). Briefly, 1–5 \(\times\) 10\(^7\) cells were suspended in 1 ml of 10 mM Tris buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na\(_2\)VO\(_4\), containing 1% Triton X-100, Dounce homogenized, and the “postnuclear fraction” was subjected to gradient ultracentrifugation (16), i.e. the fraction was mixed with an equal volume of 85% sucrose (w/v) in the same buffer. The resulting diluent was placed at the bottom of a discontinuous sucrose concentration gradient (30–5%) in the same buffer. Samples were centrifuged for 17 h at 200,000 \(\times\) g at 4 °C. A white light-scattering band under light illumination located between 5 and 30% sucrose interface was collected and used as the GEM fraction. The entire procedure was performed at 0–4 °C (in ice immersion). The protein content of each fraction was determined using MicroBCA kit (see *Experimental Procedures*).

#### Lipid Analysis

Lipids were separated by HPTLC, visualized by orcinol-sulfuric acid (for GSLs), by mercury/ammonium molybdate (for phospholipids), or by sulfuric acid (for cholesterol), followed by densitometry (see *Experimental Procedures*). Relative quantities of protein (A), gangliosides (B), neutral GSLs (C), SM (D), glycerophospholipids (E), and cholesterol (F) are shown. Ordinate, quantity of given component as a percent of total components (defined as 100%). Right panel, lipid components, shown by TLC, present in each sucrose gradient fraction (lanes 1–12 correspond to fractions 1–12) obtained by detergent method. The positions of gangliosides (b), neutral GSLs (c), SM (d), glycerophospholipids (e), and cholesterol (f) are indicated in the left margin.
a MicroBCA kit (Pierce Chemical Co.).

Hypertonic Sodium Carbonate Method—Cells were harvested in 500 mM sodium carbonate, pH 11.0 (2–4 × 10^7 cells/2 ml) and homogenized using a loose fitting Dounce homogenizer (20 strokes), a Polytron tissue grinder (three 10-s bursts), and a bath sonicator (three 20-s bursts). 1.5 ml of the cell homogenate thus obtained was mixed with an equal volume of 90% sucrose in 25 mM MES, pH 6.5, 150 mM NaCl and overlaid with a discontinuous sucrose gradient (30–5% in the same buffer containing 250 mM sodium carbonate). Samples were submitted to ultracentrifugation, and the light-scattering band just above the 5–30% sucrose interface was collected and designated as the GEM fraction as above. The protein content of each fraction was determined as above.

Determination of Distribution Patterns of Glycosphingolipids, Sphingomyelin, Glycerophospholipids, and Cholesterol in Fractions Obtained from Sucrose Gradient Centrifugation

GEM and other fractions obtained by sucrose gradient centrifugation as described above were analyzed to determine the lipid content. Each fraction was dialyzed against water to eliminate sucrose and then lyophilized. Residues were extracted with chloroform/methanol (2:1), and the lipid extracts were subjected to repeated Folch-Pi partition (29). The resulting aqueous phases were purified further using C18 Bond elut packed columns (1 ml, Analyticchem International, Harbor, CA) (30) and subjected to HPTLC. Gangliosides were visualized using orcinol-sulfuric acid staining. GM3 was detected by immunostaining using anti-GM3 monoclonal antibody DH2 and a Vectastain ABC kit (Vector, Burlingame, CA) using biotinylated goat anti-mouse IgG as secondary antibody and diaminobenzidine substrate for the final staining (31). The organic phases from the Folch-Pi partition were subjected to alkaline methanolysis (32) to remove interfering glycerophospholipid, and the content of neutral GSL and SM was analyzed by HPTLC. Glycerophospholipids and cholesterol were separated directly from the lower phase of the Folch-Pi partition without alkaline methanolysis and were subjected to HPTLC. Neutral GSLs and gangliosides were separated by TLC with solvent chloroform/methanol/water 5:4:1 and visualized by spraying with 0.5% orcinol in 10% sulfuric acid. SM and glycosphingolipids were separated by TLC in solvent chloroform/methanol/acetone/acidic acid 90:20:2:1 and revealed with phosphomolybdate spray (33). Cholesterol was separated by TLC in solvent hexane/diethyl ether/acetic acid 80:20:1 and visualized by spraying with 15% solution of concentrated sulfuric acid in 1-butanol. In all cases, the quantity of lipids and their ratio were determined by densitometry in comparison with a known quantity of standard lipid using the Scion Image program (Scion Corporation, Frederick, MD). For determination of 3H-labeled GSLs, TLC autoradiography was performed by exposure to Kodak BioMax MS film at ~80 °C with Kodak TranScreen-LE intensifying screen.

Distribution of Signal Transducer Molecules in Fractions Obtained from Sucrose Gradient Centrifugation

For analysis of distribution of transducer molecules, GEM and other fractions were subjected to SDS-PAGE followed by Western immuno blotting (34) using commercially available specific antibodies as described previously (16). In some experiments, aliquots of GEM (containing ~30 μg of protein) were diluted 10-fold in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 75 milliunits/ml aprotinin, 1% Triton X-100) and immunoprecipitated by adding 1 μg/ml rabbit anti-Csk polyclonal IgG, 1 μg/ml rabbit anti-c-Src polyclonal IgG, or 1 μg/ml normal rabbit IgG (as negative control). Immunoprecipitates were recovered by adding protein G-Sepharose beads, washed with IP buffer, suspended with 100 μl of SDS-sample buffer, heated to 95 °C for 5 min, subjected to SDS-PAGE, and analyzed by Western blotting.

Coimmunoprecipitation of GM3 and c-Src

Neuro2a cells were harvested in phosphate-buffered saline and lysed in lysis buffer (500 μg of protein/ml of buffer) containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, and 75 milliunits/ml aprotinin and allowed to stand for 20

![Fig. 2. Distribution patterns of signal transducer molecules in GEM (fractions 5 and 6) and high density fraction (fraction 12). Fractions 5, 6, and 12 were separated by sucrose gradient centrifugation in 1% Triton X-100-containing lysis buffer (panel A) or in 500 mM sodium carbonate (panel B). Signal transducer molecules present in each fraction were detected by Western blotting using respective antibodies, indicated at the top of each panel, as described under “Experimental Procedures.”](image)

![Fig. 3. Association of GM3 with c-Src. Aliquots of Neuro2a cell lysate were immunoprecipitated with anti-GM3 DH2 (ascites form was used in this case) or control mouse IgG. Immunocomplexes were analyzed by two-dimensional electrophoresis followed by Western blotting (see “Experimental Procedures”). Left panel, Western blot pattern with anti-c-Src antibody. Right panel, Western blot pattern with mouse myeloma SP2 ascites (used as nonimmune mouse IgG control).](image)
min. The cell suspension was Dounce homogenized, lysate was centrifuged for 5 min at 1,300 × g, and 1 ml of supernatant was mixed with protein G-Sepharose beads (50 μl packed) and stirred by rotary shaker for 2 h at 4 °C to precipitate nonspecific binding. After centrifugation (500 × g for 1 min), the supernatant was added to 20 μl of DH2 ascites or 20 μl of mouse myeloma SP2 ascites as negative control. The mixtures were placed overnight in a rotary stirrer at 4 °C, added to protein G-Sepharose beads (50 μl packed), and placed again in a rotary mixer for 2 h. Beads were washed three times with IP buffer, recovered by brief weak centrifugation (270 × g, 2 min), suspended in 100 μl of SDS-sample buffer, heated to 95 °C for 3 min, and centrifuged (1,000 × g, 2 min).

Immunoprecipitated proteins were analyzed by two-dimensional SDS-PAGE, with the first run performed through 5–15% gradient gel under nonreducing conditions. The second run was performed through 8% gel under reducing conditions. Subsequently, proteins were transferred electrophoretically to polyvinylidene difluoride membranes and immunodetected as described previously (34). The purpose of the two-dimensional SDS-PAGE procedure was to improve detection of c-Src avoiding interference from the presence of mouse IgG (from DH2 antibody).

Incorporation of Exogenous Gangliosides, GSLs, and PC in GEM and Other Membrane and Soluble Fractions from Neuro2a Cells

Preconfluent Neuro2a cells cultured in 150-mm dishes were washed three times with serum-free DMEM and incubated in the same medium for 10 min or 1 h in the presence of 10 μM [3H]-Sphingolipid (‘H-GM3, ‘H-Gal)GM1, ‘H-Gal)GM3, or triacetylated [3H]IPC (10 μM, 0.25 μCi/ml, specific activity 0.025 Ci/μmol). For specific activity of [3H]-labeled GSLs and PC applied for incorporation into cells, we followed the protocol described previously (35). Briefly, [3H]-GM3 solution in ethanol (76 μl, containing 73,000 dpm/μl; specific activity 2 Ci/μmol; equivalent to 2.5 μCi and 1.25 nmol) was mixed with 9.57 μl of 10 mM solution of cold GM3 solution in ethanol. Lipid solution was concentrated under a nitrogen stream to near dryness (−10 μl) and 10 μl of DMEM was added, sonicated, and allowed to stand at 37 °C for 2 h. DMEM solution of [3H]-labeled GM1 or GB4 was prepared in the same way, with approximately the same specific activity. For preparation of DMEM solution of [3H]-labeled PC, 25 μl of [3H]IPC solution (0.1 Ci/ml; specific activity 175 Ci/μmol) was mixed with 200 μl of 0.5 mM ethanol solution of cold PC prepared from bovine brain and concentrated under a nitrogen stream to near dryness, and 10 μl of DMEM was added, sonicated, and allowed to stand at 37 °C as above. Thus, the DMEM solution of [3H]-labeled lipids (10 μM) with a specific activity 0.025 Ci/μmol, corresponding to radioactivity of 0.25 μCi/μl, was obtained. After incubation, cells were washed three times with 10% FBS and DMEM and incubated for 30 min in the same medium to remove the loosely bound portion of lipid (35). Cells were then rinsed twice with ice-cold phosphate-buffered saline and harvested in the same buffer. Two dishes were pooled for each experimental point and subjected to GEM preparation by the detergent method described above. Radioactivity associated with postnuclear supernatant and sucrose gradient fractions was determined by liquid scintillation with a Beckman LS6000IC counter.

Effect of GM3 on c-Src Activation and Src/Csk Interaction in Isolated GEM

GEM was diluted 10× with kinase buffer (30 mM HEPEs, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM CaCl₂) (protein content 7–10 μg/ml), and 5-ml aliquots of diluted GEM suspension were used for stimulation by GM3, by the following procedure. The stock solution of GM3 was prepared as a 10 mM solution in absolute ethanol. 5 μl of the stock solution (0.1 μCi/ml; specific activity 6 Ci/ml, 100 μCi) were added to 5 ml of GEM suspension to obtain, respectively, 50, 100, or 200 μg of GM3. Aliquots of GEM suspension were also evaluated by Western blotting. In some experiments, incubation of GEM with GM3 was carried out in the absence of radioactive ATP for different times. After adding stop buffer, samples were immunoprecipitated with anti-c-Src or anti-Csk antibodies as described above, and immunoprecipitates were analyzed by SDS-PAGE followed by Western blotting.

Effect of Gangliosides on c-Src Activation in Intact Cells

Preconfluent Neuro2a cells cultured in 150-mm dishes were washed extensively with serum-free DMEM and incubated in the presence of 10 μM GM3, GM1, or LacCer (from 10 mM stock solution in ethanol) in serum-free DMEM for 5, 15, or 30 min. Cells were harvested, and GEM was prepared from stimulated cells using the detergent method described above. Aliquots of GEM from different samples containing approximately the same amount of protein (typically 30 μg) were diluted to 500 μl with water and added to the same volume of 2 × IP buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na₂VO₄, 2 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100). Mixtures were precleared with protein G-Sepharose. Supernatants were recovered by centrifugation, added to 1 μg/ml goat anti-c-Src IgG, and incubated at 4 °C overnight with rotation. Immunoprecipitates were recovered by centrifugation after adding protein G-Sepharose beads, and the immunocomplex kinase assay was performed as described above.

Effect of Lavendustin C on Ganglioside-dependent Neuritogenesis in Neuro2a Cells

Neuro2a cells (5,000 cells/cm²) were incubated in 2% FBS and DMEM in the absence or presence of 100 μM lavendustin C. After 1 h, cells were incubated further in the presence of 10 μM GM3 or GM1 in the same medium with or without lavendustin C, and the degree of morphological differentiation was assessed by phase-contrast microscopy. Cell viability was assessed by the trypan blue exclusion test.

FIG. 4. Association of c-Src with Csk in Neuro2a GEM fraction, indicated by reciprocal coimmunoprecipitation. Aliquots of GEM obtained by the detergent method from Neuro2a cells were first immunoprecipitated by polyclonal rabbit antibodies to c-Src (middle lane in both panels), antibodies to Csk (right lane in both panels), or normal rabbit IgG (left lane in both panels, as control). Each immunoprecipitated fraction was subjected to SDS-PAGE followed by Western blotting using anti-Csk antibodies (left panel), or the same immunoprecipitated fraction was subjected to SDS-PAGE followed by Western blotting using anti-c-Src antibodies (right panel). WB, Western blotting; IP, immunoprecipitation; α, anti-.

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**Measurement of MAPK Activation in Neuro2a Cells**

Neuro2a cells were plated in 60-mm dishes (15,000 cells/cm²) and cultured 24 h in 10% FBS and DMEM. Cells were washed three times with serum-free DMEM and incubated in the presence of 10 μM GM3, GM1, or LacCer in serum-free DMEM for various times (0–60 min). Cells were rinsed twice with phosphate-buffered saline containing 0.4 mM Na₂VO₄, scraped in 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 25 mM β-glycerophosphate, 2 mM EGTA, 1 mM Na₃VO₄, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20 mg/ml leupeptin, 75 units/ml aprotinin), and sonicated for 10 s 10 times. Lysates were centrifuged at 15,000 rpm for 10 min at 4 °C and precleared for nonspecific binding with protein G-Sepharose. Supernatants were recovered by centrifugation, added to 1 mg/ml goat anti-ERK1 IgG, and incubated at 4 °C overnight with rotation. Immunoprecipitates were added to protein G-Sepharose columns, recovered by centrifugation, washed twice with lysis buffer, and resuspended in 40 μl of kinase buffer (50 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM EGTA, 10% glycerol, 20 mM ATP), containing 1 μCi of γ⁻³²P[ATP] and 0.5 mg/ml myelin basic protein. Samples were incubated for 10 min at 25 °C, reactions were stopped by adding 2 × SDS-sample buffer, and mixtures were analyzed by SDS-PAGE on 12.5% gel. Gel was dried and subjected to autoradiography to visualize phosphorylated myelin basic protein.

To assess the effect of lavendustin C on ganglioside-induced MAPK activation, in some experiments Neuro2a cells were incubated in the presence of 100 μM lavendustin C for 2 min at 37 °C before treatment with gangliosides and MAPK activation assay.

**RESULTS**

**Sphingolipids Are Present Predominantly in Low Density Membrane Fraction in Neuro2a Cells**—Neuro2a neuroblastoma cell homogenate prepared in lysis buffer containing 1% Triton X-100 or in 500 mM sodium carbonate and subjected to centrifugation on discontinuous sucrose gradient gave a sharp light-scattering band near the 5 and 30% sucrose interface. The appearance and position of the band were nearly identical under both methods. TLC immunostaining of GM3 in fractions obtained from both methods showed nearly identical patterns, as observed previously for mouse melanoma B16 cells (16); therefore, these data are not shown. Neuro2a cells contained 4.12 ± 0.86 nmol of gangliosides/mg of protein. The GM3 content was 1.00 ± 0.09 nmol/mg of protein (24.3%), GM2 was 2.10 ± 0.57 nmol/mg of protein (51.0%), and GM1 was 1.02 ± 0.18 nmol/mg of protein (24.7%). Total amounts of neutral GSL and SM were, respectively, -1.2 and 3.65 nmol/mg of protein.

Fraction 5 and adjacent fraction 6 contained >80% of GM3 present in the cell homogenate prepared from both detergent-containing and 500 mM sodium carbonate-containing medium, although the protein content of fractions 5 and 6 represented only a small portion of total protein amount loaded on gradient (0.5–2%). We therefore analyzed in greater detail sphingolipid distribution patterns of GEM and other fractions (fractions 1–12) prepared by the detergent method. All sphingolipids present in Neuro2a cells were highly enriched in GEM, i.e. fractions 5 and 6 (Fig. 1). More than 60% of gangliosides (mainly GM3, GM2, and GM1 in these cells) (Fig. 1B), 70% of neutral GSLs (Gg3, Gg4, and smaller amounts of GlcCer and LacCer) (Fig. 1C), and 45% of SM (Fig. 1D) loaded on gradient were recovered in fraction 5. Fraction 6 also contained lower but significant amounts of gangliosides (18%) and neutral GSLs (21%), but the content of SM in fraction 6 was greater (55%) than that in fraction 5 (Fig. 1D). In contrast, about 30% of cholesterol (Fig. 1F), >50% of glycerophospholipids (mainly PC and phosphatidylethanolamine) (Fig. 1E), and >95% of proteins (Fig. 1A) were found in high density fractions 10–12. TLC patterns of various fractions with regard to cholesterol and...
glycerophospholipid are shown in Fig. 1, e and f. Presence of Signal Transducer Molecules in GEM from Neuro2a Cells—SDS-PAGE followed by immunoblotting analysis revealed that Neuro2a GEM, regardless of preparation method in the presence or absence of detergent, is enriched in various signal transducer molecules, including the Src family tyrosine kinases c-Src and Lyn, Csk tyrosine kinase, and the GDP/GTP-binding proteins Rho A and Ha-Ras (Fig. 2). Ha-Ras was detectable by the detergent method but undetectable by the sodium carbonate method, as we observed previously in B16 melanoma cells (16). The majority of c-Src and Lyn in Neuro2a cells were found to be present in both fractions 5 and 6, in similar quantity, but only trace quantities were found in high density fractions 10–12 (Fig. 2A).

Considering that GEM contains only a very small portion of total protein, enrichment of these signal transducer molecules in GEM was remarkably high (300-fold in the case of c-Src). Remarkably, similar enrichment of c-Src in GEM prepared under detergent-containing or detergent-free conditions was also detectable in other cell lines of neural origin, including GOTO human neuroblastoma cells and PC12 pheochromocytoma cells (data not shown). Other signal transducer molecules such as protein kinase C\(\alpha\), phospholipase C-\(\beta\)2, focal adhesion kinase, and cell adhesion kinase were present only in minimal amounts in Neuro2a GEM and were almost quantitatively recovered in the high density fraction of the gradient. Caveolin was not detectable by immunoblotting in Neuro2a total lysates or in sucrose gradient fractions (data not shown).

Association of c-Src and GM3 in Neuro2a Cells—Aliquots of cell lysate were immunoprecipitated by adding anti-c-Src monoclonal antibody DH2 and protein G-Sepharose beads. Immunocomplexes were eluted from the beads and analyzed by two-dimensional electrophoresis as described under “Experimental Procedures.” Subsequent Western blotting using anti-c-Src antibody revealed the presence of c-Src in DH2 immunoprecipitates, whereas c-Src was not detectable in control experiments with the addition of mouse myeloma ascites or nonspecific mouse IgG (Fig. 3).
Association of c-Src and Csk in GEM from Neuro2a Cells—Aliquots of GEM fraction prepared from Neuro2a cells by gradient ultracentrifugation in the presence of Triton X-100 were immunoprecipitated with anti-c-Src or anti-Csk antibodies followed by SDS-PAGE with Western blotting by one of the antibodies used for immunoprecipitation, as described in the Fig. 4 legend. The immunoprecipitate with anti-c-Src gave a band corresponding to Csk when subjected to Western blotting with anti-Csk (Fig. 4, left panel, middle lane). Reciprocally, the immunoprecipitate with anti-Csk gave a band corresponding to c-Src when subjected to Western blotting with anti-c-Src (right panel, right lane). Control rabbit IgG did not give any band (left lane in both panels). Because Csk kinase has high sequence homology with and an inhibitory effect on c-Src, the close association of these two signal transducers in Neuro2a GEM is biologically significant (see “Discussion”).

Exogenous GSLs Become Associated with GEM, whereas Exogenous PC Does Not, When Added in Culture Medium of Neuro2a Cells—To assess the possibility that exogenous gangliosides exert their effects on Neuro2a cells through interaction with GEM, cells were incubated in the presence of 10 μM [3H-Sph]GM3, [3H-Gal]GM1, [3H-GalNAc]Gb4, or [arachidonoyl-3H]PC for 10 or 60 min. After incubation and washing of pericellularly bound GSL or PC, cell lysates obtained in the presence of Triton X-100 were subjected to sucrose gradient centrifugation and radioactivity associated with each fraction was measured. At both 10 and 60 min the majority of radioactivity, incorporated from [3H]-labeled GSLs added to culture medium, associated with the postnuclear supernatant, was detected in GEM (in the case of GM3, 63 and 66% at 10 and 60 min, respectively) (Fig. 5, A–C). A smaller amount of radiolabeled lipid was found in fractions 6 and 7, whereas other fractions, including fraction 12, contained negligible radioactivity. In striking contrast, [3H]PC added to culture medium and incubated under the same conditions as for [3H]GSLs was not incorporated in GEM fraction; rather, essentially all radioactivity was found in high density fractions 10–12 (Fig. 5D).

Addition of GM3 to GEM Isolated from Neuro2a Cells Leads to c-Src Activation—To evaluate the possible effect of GM3 ganglioside on c-Src kinase activity, GEM prepared from Neuro2a cells was incubated with GM3, and c-Src autophosphorylation was measured after immunoprecipitation of c-Src using anti-c-Src antibody. c-Src autophosphorylation in isolated GEM from Neuro2a cells was enhanced strongly after brief (5 min) incubation with 10 μM GM3 (Fig. 6A), whereas the quantity of c-Src protein detectable in immunoprecipitates by Western blotting was essentially unchanged (Fig. 6B). Under the same experimental conditions, LacCer (which has no effect on neurite outgrowth in these cells) had no effect on c-Src autophosphorylation (Fig. 6A). Treatment with lavendustin C (50–150 μM), a potent inhibitor of tyrosine kinases (particularly for c-Src), completely blocked c-Src kinase activation induced by GM3 under these conditions (Fig. 6C).

GM3-induced Reduction of Csk in Neuro2a GEM—We investigated the effect of GM3 treatment on Csk level (quantity) in GEM. Levels of Csk measured by Western blotting after immunoprecipitation with anti-Csk antibody decreased significantly in Neuro2a GEM incubated in the presence of 10 μM GM3 for up to 30 min (Fig. 7A), although the level (quantity) of c-Src under these experimental conditions was constant (Fig. 7B). Thus, the Csk/c-Src ratio in c-Src immunoprecipitates obtained from Neuro2a GEM was reduced significantly during treatment with GM3 (Fig. 7C), indicating that GM3 can induce decrease of Csk in GEM, presumably through dissociation of the Csk-c-Src complex (for notion and possible mechanism, see “Discussion”).

c-Src Activation Occurs in GEM during Ganglioside Stimulation of Neuro2a Cells—To assess the possible role of c-Src during early stages of ganglioside-induced neuronal differentiation of Neuro2a cells, cells were treated with a neuritogenic dose (10 μM) of GM1 or GM3 for 5–15 min. Immediately, GEM was prepared from stimulated cells at 0–4 °C under detergent-containing conditions. c-Src kinase activity was measured in GEM from resting and ganglioside-stimulated cells by an in vitro autophosphorylation assay in immunoprecipitates with anti-c-Src antibody. c-Src kinase activity was almost undetectable in nonstimulated cells. Autophosphorylation of c-Src was enhanced significantly for both GM1- and GM3-stimulated cells (Fig. 8A). The maximal effect of these gangliosides was observed after 5 min of stimulation, and c-Src kinase activity returned almost to basal level after 15 min of incubation. Treatment of Neuro2a cells with LacCer under these conditions did not induce c-Src autophosphorylation. The total amount of c-Src in GEM was essentially unchanged during GSL treatment (Fig. 8B).

Ganglioside-dependent Neuritogenesis in Neuro2a Cells and

Glycosphingolipid Microdomain in Neuro2a Cells
Fig. 10. Time course of MAPK activation by GM3 and GM1 in Neuro2a cells. Cells were treated for the indicated times with 10 μM LacCer, GM3, or GM1. MAPK activity was measured as phosphorylation of myelin basic protein after immunoprecipitation with anti-ERK1 antibody as described under “Experimental Procedures.” Right panel, intensity data from autoradiograms expressed graphically.

Fig. 11. Effect of lavendustin C on GM3-induced MAPK activation in Neuro2a cells. Cells were incubated in the presence of 100 μM lavendustin C (LVC) before treatment with vehicle (Control) or 10 μM GM3 for the indicated times. MAPK activity was measured as described for Fig. 10. Right panel, intensity data from autoradiograms expressed graphically.

Its Inhibition by Lavendustin C—Incubation of Neuro2a cells for 6 h in 2% FBS and DMEM in the presence of 10 μM GM3 (Fig. 9C) or GM1 (Fig. 9E) induced neurite outgrowth in the majority of cells. Pretreatment with 100 μM lavendustin C for 1 h blocked neuritogenesis of both GM3 (Fig. 9D) and GM1 (Fig. 9F) but had no effect on the viability of the cells.

Gangliosides Induce Rapid MAPK Activation in Neuro2a Cells—To investigate the possible involvement of the MAPK pathway in ganglioside-induced signaling in Neuro2a cells, MAPK activity was measured in cell lysates from LacCer-, GM3- and GM1-treated cells after immunoprecipitation with anti-ERK1 antibody. Treatment of Neuro2a cells with GM3 resulted in prompt and prolonged activation of MAPK. A significant increase of MAPK activity was observed after a 5-min incubation. The maximal value of MAPK activity was reached within 10 min after the addition of GM3 and was maintained for up to 60 min (Fig. 10). A similar MAPK activation curve was observed after incubation with GM1. LacCer treatment did not cause any change in MAPK activity in these cells.

Lavendustin C Prevents GM3-induced MAPK Activation in Neuro2a Cells—To evaluate the possible dependence of ganglioside-induced MAPK activation on c-Src activation, Neuro2a cells were incubated in the presence of 100 μM lavendustin C before stimulation with GM3. Under this condition, GM3-induced MAPK activation was almost completely blocked (Fig. 11).

DISCUSSION

A peculiar feature of the mouse neuroblastoma Neuro2a cell line is its high susceptibility to induction of differentiation by the exogenous addition of gangliosides and its lack of susceptibility to differentiation by NGF. Differentiation is typically observed as neuritogenesis, as originally described by Roisen et al. in 1981 (37), followed by many subsequent studies along the same line, including primary neuronal cell culture (3, 7, 38, 39; for review see 9). Interestingly, Neuro2a cells do not express Trk A and p75NGFR (9, 11) for NGF and do not require NGF to maintain cell growth or neuritic differentiation.3 In striking contrast, the majority of neuronal cells depend on NGF and function of its receptor. Neuro2a cells are unique among neuronal cells in that they are capable of induction of differentiation and neuritogenesis by the addition of GM3 or GM1 to the culture medium. Thus, Neuro2a cells provide a model for study of the neurobiological effects of gangliosides, independent of the NGF effect or its receptor function.

The studies described in this paper are focused on the mechanism for the above effect of GM3 and GM1, operating through specific organization of gangliosides with defined signal transducer molecules within GEM. The majority of GSLs and gangliosides present in the plasma membrane are clustered and can be recovered as low density, light-scattering membrane fractions when cells are homogenized in 1% Triton X-100 or hypertonic salt solution (500 mM Na2CO3) followed by sucrose density gradient centrifugation. In Neuro2a cells, five signal transducer molecules (c-Src, Lyn, Csk, Rho A, and Ha-Ras) were found to be organized in this low density GEM fraction. This observation is similar to that we made previously for B16 melanoma cells, in which we found GM3, c-Src, Rho, and FAK to be concentrated in a low density GEM fraction (15, 16). Stimulation of GSLs by binding of their ligands causes activation of various signal transducers; therefore, GEM can be termed “glycosphingolipid signaling domain” (19), particularly in view of the fact that GEM is separable from caveolae, the other membrane domain active in signal transduction and endocytosis (18).

How does exogenous GM3 or GM1 added in culture medium induce differentiation leading to neuritogenesis? Initial experiments indicate that exogenously added 3H-labeled GM3 or GM1 is concentrated and recovered in fractions 5 and 6 (GEM), whereas 3H-labeled PC, in striking contrast, is incorporated in high density fractions 10–12 and is essentially absent in GEM (Fig. 5). Thus, the target of the stimulatory effect of GM3 or GM1 is presumably the GEM component, particularly c-Src, because a close association of GM3 and c-Src was demonstrated in this and previous studies (18). Therefore, a crucial experiment on the effect of GM3 on c-Src in GEM was undertaken,

3 The absence of Trk A and p75NGFR in Neuro2a cells and the nonsusceptibility of these cells to NGF were confirmed. However, the cells appear to contain Trk B (receptor for brain-derived nerve factor) even though the cells do not respond to any type of neurotrophic factor except gangliosides (A. Prinetti, unpublished observation).
employing the isolated GEM membrane fraction, i.e. the membrane fraction was stimulated by exogenous addition of GM3 or GM1 followed by determination of c-Src phosphorylation. The c-Src phosphorylation was clearly stimulated by the addition of GM3 or GM1. Similarly, a previous study showed that c-Src activation (tyrosine 527 phosphorylation) in human neuroblastoma SH-SY5Y cells is induced by phorbol ester (13).

Is the effect of GM3 or GM1 on activation of c-Src observable when intact Neuro2a cells are stimulated by exogenous ganglioside? Such an experiment appears to be very difficult because a brief stimulation by exogenous ganglioside followed by separation of GEM (which takes overnight even at 0–4 °C) may not maintain the change of c-Src activity in GEM. Surprisingly, however, the enhanced c-Src activity is still observed when Neuro2a cells are briefly (~5 min) stimulated by GM3 followed by separation of GEM (Fig. 6, A and B), i.e. the impact of brief GM3 treatment causing c-Src activation lasts many hours at low temperature after GM3 stimulation. This result was unexpected; therefore, four independent experiments were performed, and essentially the same result was observed. Interestingly, if GM3 treatment is prolonged (15–60 min), c-Src activation is no longer observable. This response is similar to that in isolated Neuro2a GEM, in which c-Src activation is only observable within 5 min of GM3 stimulation but no longer observable after 15–60 min. Thus, GM3 stimulation has only transient impact on c-Src response. This event is followed by a series of signal transduction events leading to activation of MAPK, which triggers neuritogenesis. In our previous study of B16 melanoma cells, c-Src activation was also observed within 5 min after GM3 stimulation, before activation of other protein kinases, i.e. FAK (18). c-Src activation may therefore be the earliest event. Consequent changes in downstream signal transduction, represented by enhancement of MAPK, are initiated and affected by c-Src activation, as clearly demonstrated by the inhibitory effect of lavendustin C. The fact that GM3, c-Src, and Csk are closely associated in GEM and that stimulation of GM3 causes decrease of Csk, the inhibitory regulator of c-Src (40, 41), suggest that c-Src activation by GM3 stimulation is due to decrease of Csk.

A major question that remains is how exogenous GM3 or GM1 stimulation stimulates c-Src. Does any gangliophilic receptor exist in GEM or GSD? We have no clear answer at this time. However, the close association among GM3, c-Src, and Csk in Neuro2a GEM and that stimulation of GM3 causes decrease of Csk, the inhibitory regulator of c-Src (40, 41), suggest that c-Src activation by GM3 stimulation is due to decrease of Csk. GM3 causes a decrease of Csk is unknown, but this phenomenon could result from enhanced degradation of Csk or more likely from translocation of Csk from GEM. However, translocation of Csk in fraction 12 (high density fraction) was not clearly observed. The fact that translocation of Csk suppresses or activates c-Src is well documented (42). Whatever the mechanism, c-Src activation induced by GM3 or GM1 takes place at GSD and initiates a series of signal transduction events leading to MAPK activation.

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