Effect of Synthetic Sialyl 2→1 Sphingosine and Other Glycosylsphingosines on the Structure and Function of the "Glycosphingolipid Signaling Domain (GSD)" in Mouse Melanoma B16 Cells†

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ABSTRACT: Mouse melanoma B16 cells are characterized by a high concentration of GM3 ganglioside, which has been identified as a melanoma-associated antigen and is present as a clustered microdomain organized with major signal transducers, c-Src, small G-protein (Rho A), and focal adhesion kinase (FAK), to form a "glycosphingolipid signaling domain" or "glycosignaling domain" (GSD) separable from cholesterol- and caveolin-enriched microdomain, "caveolae." Cholesterol-binding reagents, filipin and nystatin, disrupt the structure and function of caveolae, but have no effect on GSD function [Iwabuchi, K., et al. (1998) *J. Biol. Chem. 273*, 33766−33773]. In this study, we searched for compounds which disrupt the structure and function of GSD in B16 cells. Such compounds should have structural features analogous to those of GM3, destroy or reduce clustering of GM3 in GSD, and inhibit GM3-dependent adhesion and signaling. The simplest compound so far found with these properties is sialyl α2→1 sphingosine (Sph). We describe the synthesis of this compound and its analogues, and their effects on GM3 expression pattern and GSD function, in comparison with effects of lyso-GM3 and other lyso compounds, in B16 cells. Incubation of B16 cells with 0.5−10 μM sialyl α2→1 Sph or 1−5 μM lyso-GM3 reduced GM3 clustering and GM3-dependent adhesion, and inhibited adhesion-dependent cellular FAK activity. The c-Src activation response of GSD isolated from B16 cells was inhibited strongly by sialyl α2→1 Sph. Substitution of the Sph amino group with a chloroacetyl or *N*,*N*-dimethyl group strongly reduced the inhibitory effect of sialyl α2→1 Sph on GM3-dependent adhesion, FAK, and c-Src response. Other lyso compounds such as lyso-phosphatidylcholine, galactosyl-Sph (psychosine), and lactosyl-Sph at 0.5−10 μM did not show the same effect as sialyl α2→1 Sph. Thus, adhesion coupled with signal transduction, initiated by clusters of GM3 in GSD, is blocked by sialyl α2→1 Sph or lyso-GM3. Analogues with N-substitution of Sph in sialyl α2→1 Sph, other lyso-phospholipids, and galactosyl- or lactosyl-Sph did not block such adhesion, coupled with activation of c-Src and FAK.

Glycosphingolipids (GSLs) have been implicated as mediators of cell adhesion and as modulators of signal transduction (*1*). GSLs are known to form clusters at the cell surface (*2*) and even at the surface of liposomes made from GSLs and phosphatidylcholine without cholesterol (*3*, *4*). Such clustering is due to the intrinsic physical properties of GSLs, rather than preparation artifact (see Discussion). Sphingolipids are distributed in a unique and characteristic fashion giving rise to "polarity" of surface structure (e.g., apical vs basolateral surface of epithelial cells) (*5*, *6*), and are insoluble in aqueous buffer containing neutral or zwitterionic detergent (*7*, *8*), similarly to extracellular matrix and cytoskeletal components (*9*). A number of studies during the past decade indicate that sphingolipids and cholesterol are organized with GPI-anchored proteins and c-Src family kinases to form microdomains involved in signal transduction (e.g., *8*; for reviews, see *10*, *11*). There has been ambiguity regarding the possible identity of such sphingolipid microdomains with another membrane domain, caveolae, derived from morphologically distinct invagination of plasma membrane, enriched in caveolin and involved in endocytosis and signal transduction (for review, see *12*). In all these studies,

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little attention has been paid to the functional significance of GSLs in these microdomains.

Our recent studies indicate that clustered GSLs associated with c-Src, Rho A, and FAK are involved in GSL-dependent cell adhesion coupled with activation of these signal transducers (*13*, *14*). A typical example is GM3 ganglioside clustering at the cell surface of mouse melanoma B16, forming a "glycosignaling domain" (GSD) separable from cholesterol- and caveolin-enriched membrane fractions derived from caveolae (*15*). GM3 in B16 cells is also recognized as a melanoma-associated antigen (*16*, *17*), and may have a role in initiating adhesion of melanoma to endothelial cells, the first step in metastasis (*18*) (see Discussion). Antigenicity, mediation of adhesion, and initiation of signaling through GM3 ganglioside at the B16 cell surface are thought to be maintained by GM3 clustering in GSD. If GM3 clustering in GSD is inhibited, antigenicity, adhesion, and signaling through GM3 could be blocked. We undertook a novel approach to observe disruptive effects of chemically synthesized GSL analogues on GSD structure and function. As the initial step in this approach, we synthesized the simplest ganglioside analogue, sialyl α2→1 sphingosine, and its derivatives. We hereby report the effects of these compounds, in comparison with other lyso-GSLs and lysophospholipids, on GM3 clustering, GM3-dependent adhesion, and signaling.

MATERIALS AND METHODS

Outline of Synthesis of Sialyl α2→1 Sph and Its Analogues. Sialyl α2→1 Sph ("SA-Sph";[1] structure **8**) was synthesized by sialylation of O-protected azidosphingosine as outlined in Figure 1, Schemes 1 and 2. *O-tert*-Butyldiphenylsilyl azidosphingosine was synthesized as described previously (*19*) in the laboratory of Prof. Tomoya Ogawa (Department of Agricultural Chemistry, University of Tokyo, Japan). Sialyl α2→1 (*N*-chloroacetyl) Sph ("SA-Nc-Sph"; structure **9**) and sialyl α2→1 (*N*,*N*-dimethyl) Sph ("SA-NMe-Sph"; structure **10**) were synthesized as in Figure 1, Scheme 2. Details of the synthesis procedure and properties of products are described in the following six subsections. Synthetic lyso-GM3 was provided by Prof. Pierre Sinaÿ (Department de Chimie, Ecole Normale Superieure, Paris, France). Method of synthesis and chemical properties of intermediates and final products will be published elsewhere.[2]

Lactosyl Sph ("Lac-Sph"; Galβ1→4Glcβ1→1 Sph) was synthesized by a modification[3] of the procedure described previously (*20*). Dilactosyl Sph ("diLac-Sph"; Galβ1→4Glcβ1→1[Galβ1→4Glcβ1→2] Sph) was synthesized as described elsewhere.[3]

General methods used for identification of synthetic products were as follows. Optical rotations were measured at 589 nm (Na line) at 20 ± 2 °C with a Perkin-Elmer model 241MC digital polarimeter, using a 10 cm, 1 mL cell. Mass spectra were obtained with a JMS-700 spectrometer for chemical ionization (CI) and fast atom bombardment (FAB), or a VG-Platform mass spectrometer for electrospray ionization (ESI). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker WM-500 spectrometer or a Bruker DRX 400 spectrometer for solutions in $CDCl_3$, or CD_3OD at ambient temperature. Chemical shifts (δ) are given in ppm, and coupling constants in hertz. Assignments were aided by COSY experiments. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60 F_{254} and detection by charring with orcinol in sulfuric acid solution. Flash column chromatography was performed on silica gel 60 (230−400 mesh).

*(A) [Methyl (5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-*D*-glycero-β-*D*-galacto-2-nonulopyranosyl)onate]-(2→1)-(2S,-3R,4E)-2-azido-3-O-tert-butyldiphenylsilyl-4-octadecene-1,3-diol (**3**) and [Methyl (5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-*D*-glycero-α-*D*-galacto-2-nonulopyranosyl)onate]-(2→1)-(2S,3R,4E)-2-azido-3-O-tert-butyldiphenylsilyl-4-octadecene-1,3-diol (**4**).* A mixture of donor **1** (165 mg, 0.276 mmol), acceptor **2** (113 mg, 0.2 mmol), 4 Å powdered molecular sieves (1 g), and dry $CH_3CN-CH_2Cl_2$ (11.5 mL, 2:1) was stirred at room temperature under argon for 50 min. AgOTf (77 mg, 0.3 mmol) and di-*tert*-butylpyridine (57 mg, 0.3 mmol) were added. The reaction mixture was cooled to −70 °C. PhSCl (35 μL, 0.3 mmol) in CH_2Cl_2 (0.2 mL) was added very slowly. After stirring at −70 °C for 1 h and then at −60 °C for 2 h (monitoring by TLC), the reaction mixture was diluted with a suspension of silica gel (2 g) in EtOAc (20 mL), filtered through Celite, washed with saturated $NaHCO_3$ solution, water, and saturated NaCl solution, dried over $MgSO_4$, and concentrated. The residue obtained was flash-chromatographed, eluting with 1:2.5 hexane/ethyl acetate to afford **3** (21 mg, 10%) as a syrup: $[\alpha]_D$ −36° (*c* 1.7, $CHCl_3$); TLC (1:2.5 hexane/ethyl acetate): R_f 0.44; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69−7.63 (m, 4 H, aromatic), 7.48−7.35 (m, 6 H, aromatic), 5.39 (dd, 1 H, H-7, $J_{7,6}$ = 2.2, $J_{7,8}$ = 3.6), 5.35 (dd, 1 H, H-d, $J_{d,c}$ = 8.2, $J_{d,e}$ = 15.4), 5.25 (d, 1 H, NH, $J_{NH,5}$ = 10.2), 5.22 (ddd, 1 H, H-8, $J_{8,9}$ = 2.6, $J_{8,9'}$ = 7.8), 5.19 (ddd, 1 H, H-4, $J_{4,3ax}$ = 11.7, $J_{4,3eq}$ = 4.9, $J_{4,5}$ = 10.4), 5.07 (dt, 1 H, H-e, $J_{e,f}$ = 6.7), 4.71 (dd, 1 H, H-9, $J_{9,9'}$ = 12.3), 4.14 (ddd, 1 H, H-5, $J_{5,6}$ = 10.5), 4.12 (dd, 1 H, H-9′), 4.08 (dd, 1 H, H-c, $J_{c,b}$ = 4.9), 4.03 (dd, 1 H, H-6), 3.78 (ddd, 1 H, H-b, $J_{b,a}$ = 3.2, $J_{b,a'}$ = 9.0), 3.72 (s, 3 H, OMe), 3.44 (dd, 1 H, H-a, $J_{a,a'}$ = 10.0), 3.29 (dd, 1 H, H-a′), 2.44 (dd, 1 H, H-3eq, $J_{3eq,3ax}$ = 12.9), 2.14, 2.05, 2.02, 1.98 (4 s, 12 H, 4 OAc), 1.90 (s, 3 H, NAc), 1.87 (dd, 1 H, H-3ax), 1.75 (m, 2 H, H-f, H-f′), 1.29 (s, 9 H, CMe_3), 1.10−1.08 (m, 22 H, 11 CH_2), 0.88 (t, 3 H, CH_3, J = 6.8); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 170.82, 170.52, 170.44, 170.28, 170.12, 167.00 (6 C═O), 136.14, 135.99, 135.93, 133.49, 133.29, 129.74, 129.57, 127.63, 127.35, 127.03 (C-d, C-e, and aromatic C), 98.26 (C-2), 75.34, 71.85, 71.66, 68.79, 68.41, 66.60 (C-4, C-6, C-7, C-8, C-b, C-c), 64.09, 62.37 (C-9, C-a), 52.72 (OMe), 48.97 (C-5), 37.25 (C-3), 32.04, 31.89, 29.67−29.63, 29.55, 29.45, 29.34, 29.19, 28.55 (11 CH_2), 26.87 (CMe_3), 23.18 (NAc), 22.66 (CH_2), 21.04, 20.87, 20.77, 20.76 (4 OAc), 19.20 ($SiCMe_3$), 14.11 (CH_3); MS (CI) *m*/*z*: Calcd for $C_{54}H_{80}N_4O_{14}Si$, 1036; Found, 1054 $(M+NH_4)^+$. Solvent used: dichloromethane/ammonia.

Second eluted was compound **4** (125 mg, 60%), also as a syrup: $[\alpha]_D$ −19.7° (*c* 1.5, $CHCl_3$); TLC (1:2.5 hexane/ethyl

[1] Abbreviations: diLac-Sph, dilactosyl sphingosine (Galβ1→4Glcβ1→1[Galβ1→4Glcβ1→2] Sph); Lac-Sph, lactosyl sphingosine (Galβ1→4Glcβ1→1 Sph); lyso-PC, lyso-phosphatidylcholine; SA-Sph, sialyl α2→1 sphingosine; SA-Nc-Sph, sialyl α2→1 (*N*-chloroacetyl) sphingosine; SA-NMe-Sph, sialyl α2→1 (*N*,*N*-dimethyl) sphingosine.

[2] Zhang, Y., and Sinaÿ, P., unpublished data; manuscript in preparation.

[3] Zhang, Y., Toyokuni, T., and Hakomori, S., unpublished data; manuscript in preparation.

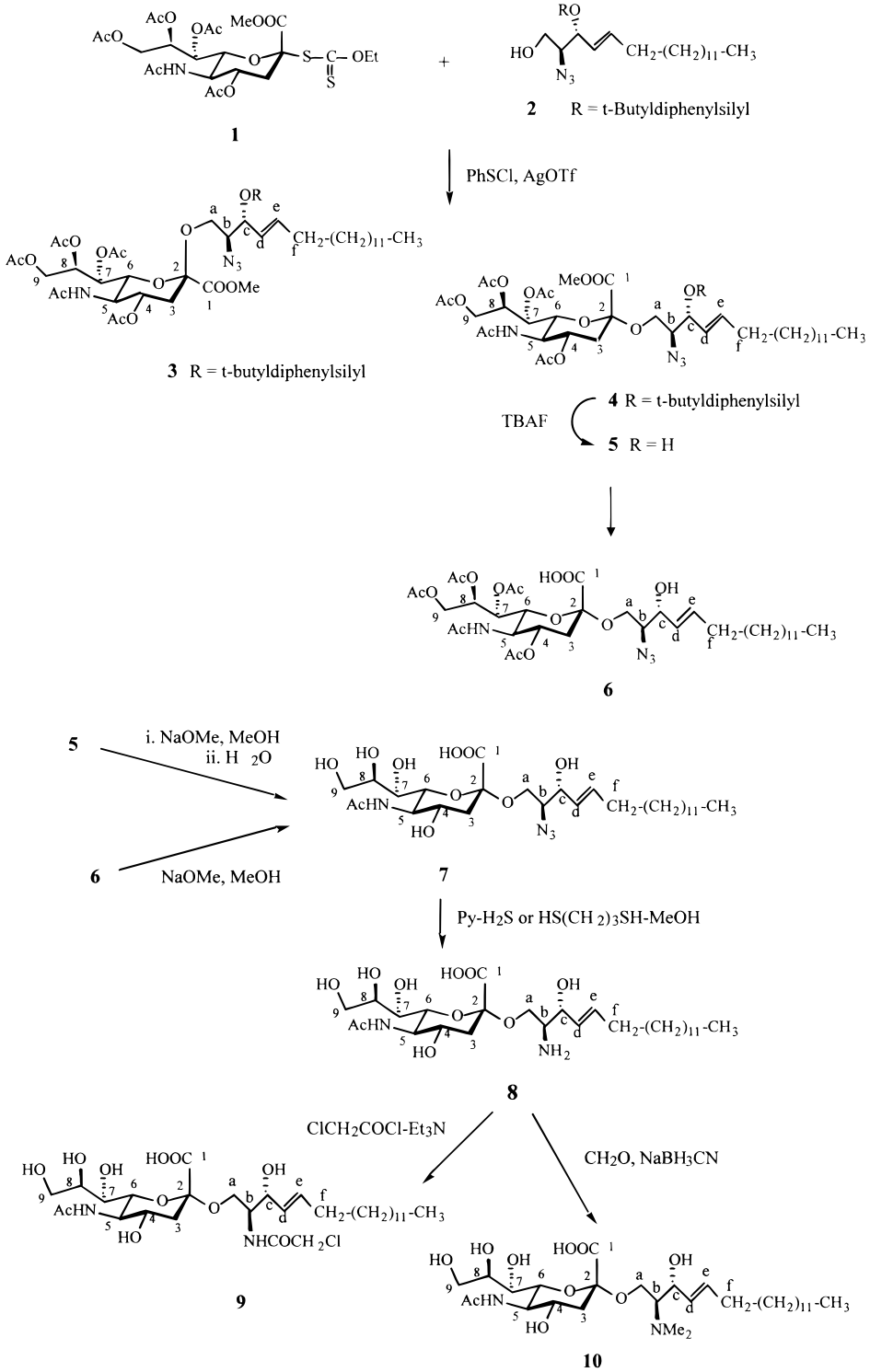

FIGURE 1: Steps for synthesis of sialyl α2→1 Sph and its analogues.

acetate): R_f 0.37; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68−7.63 (m, 4 H, aromatic), 7.46−7.34 (m, 6 H, aromatic), 5.38 (dd, 1 H, H-d, $J_{d,c}$ = 7.9, $J_{d,e}$ = 15.4), 5.32 (m, 2 H, H-7, H-8), 5.18 (dt, 1 H, H-e, $J_{e,f}$ = 6.7), 5.13 (d, 1 H, NH, $J_{NH,5}$ = 9.4), 4.86 (m, 1 H, H-4), 4.25−4.20 (m, 2 H, H-9, H-c), 4.10−4.01 (m, 3 H, H-6, H-9′, H-5), 3.83 (dd, 1 H, H-a, $J_{a,a'}$ = 10.2, $J_{a,b}$ = 3.3), 3.77 (s, 3 H, OMe), 3.54 (ddd, 1 H, H-b, $J_{b,a'}$ = 8.1, $J_{b,c}$ = 4.3), 3.30 (dd, 1 H, H-a′), 2.61 (dd, 1 H, H-3eq, $J_{3eq,3ax}$ = 12.8, $J_{3eq,4}$ = 4.5), 2.12 (s, 6 H, 2 OAc), 2.04, 2.02 (2 s, 6 H, 2 OAc), 1.90 (dd, 1 H, H-3ax, $J_{3ax,4}$ = 12.6), 1.89 (s, 3 H, NAc), 1.78 (m, 2 H, H-f, H-f ′), 1.27 (s, 9 H, CMe_3), 1.14−1.03 (m, 22 H, 11 CH_2), 0.88 (t, 3 H, CH_3, J = 6.7); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 170.92, 170.52, 170.23, 169.96, 169.57, 167.74 (6 C═O), 135.93, 135.84, 135.40, 133.52, 133.21, 129.57, 129.36, 127.41, 127.21, 127.15 (C-d, C-e, and aromatic C), 98.59 (C-2), 74.63, 72.27, 68.92, 68.06, 66.87, 65.89 (C-4, C-6, C-7, C-8, C-b, C-c), 64.26, 62.04 (C-9, C-a), 52.65 (OMe), 48.98 (C-5), 37.71 (C-3), 31.93, 31.82, 29.60−29.59, 29.46, 29.39, 29.28, 29.04, 28.48 (11 CH_2), 26.76 (CMe_3), 23.06 (NAc), 22.58 (CH_2), 20.96, 20.77, 20.71, 20.67 (4 OAc), 19.12 (SiCMe$_3$), 14.05 (CH_3); MS (CI) *m/z*: Calcd for $C_{54}H_{80}N_4O_{14}$-

Si, 1036; Found, 1054 $(M+NH_4)^+$. Solvent used: dichloromethane/ammonia.

*(B) [Methyl (5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)onate]-(2→1)-(2S,3R,4E)-2-azido-4-octadecene-1,3-diol (**5**) and (5-Acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic Acid)-(2→1)-(2S,3R,4E)-2-azido-4-octadecene-1,3-diol (**6**).* To a solution of **4** (68 mg, 0.065 mmol) in THF (1 mL) was added, at 0 °C, tetra-*n*-butylammonium fluoride (TBAF, 0.15 mmol, 1 M solution in THF), and the mixture was stirred at room temperature for 2 days. After concentration, the residue was flash-chromatographed, eluting with 1:4 hexane/ethyl acetate to afford **5** (15 mg, 28%) as a syrup: $[\alpha]_D$ −23° (*c* 1, $CHCl_3$); TLC (ethyl acetate): R_f 0.45; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.78 (dt, 1 H, H-e, $J_{e,d} = 15.3$, $J_{e,f} = 6.7$), 5.52 (dd, 1 H, H-d, $J_{d,c} = 7.2$), 5.42−5.28 (m, 2 H, H-7, H-8), 5.11 (d, 1 H, NH, $J_{NH,5} = 9.7$), 4.88 (m, 1 H, H-4), 3.82 (s, 3 H, OMe), 2.63 (dd, 1 H, H-3eq, $J_{3eq,3ax} = 12.7$, $J_{3eq,4} = 4.4$), 2.15, 2.14, 2.03, 2.02 (4 s, 12 H, 4 OAc), 1.88 (s, 3 H, NAc), 1.40−1.30 (m, 22 H, 11 CH_2), 0.88 (t, 3 H, CH_3, $J = 6.7$). Second eluted with 3:1 ethyl acetate/methanol was compound **6** (36 mg, 70%), also as a syrup: $[\alpha]_D$ −9.3° (*c* 1, MeOH); TLC (3:1 ethyl acetate/MeOH): R_f 0.45; ^{1}H NMR (500 MHz, CD_3OD) δ 5.79 (dt, 1 H, H-e, $J_{e,d} = 15.4$, $J_{e,f} = 7.1$), 5.55 (dd, 1 H, H-d, $J_{d,c} = 7.6$), 5.03 (m, 1 H, H-4), 2.69 (dd, 1 H, H-3eq, $J_{3eq,3ax} = 12.2$, $J_{3eq,4} = 4.8$), 2.12 (s, 6 H, 2 OAc), 2.03, 1.99 (2 s, 6 H, 2 OAc), 1.87 (s, 3 H, NAc), 1.75 (t, 1 H, H-3ax), 1.49−1.33 (m, 22 H, 11 CH_2), 0.92 (t, 3 H, CH_3, $J = 6.9$).

*(C) (5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic Acid)-(2→1)-(2S,3R,4E)-2-azido-4-octadecene-1,3-diol (**7**).* To a solution of **5** (15 mg, 0.019 mmol) in MeOH (3 mL) was added NaOMe (8 mg), and the mixture was stirred at room temperature for 15 h. H_2O was added at 0 °C. After stirring at room temperature for 1 h, the mixture was neutralized with Amberlite IR 120 resin, filtered, and concentrated. Compound **7** was obtained (11.5 mg, quantitative) as a white amorphous solid: TLC (4:2:1 ethyl acetate/2-propanol/H_2O): R_f 0.37; ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD 1:2) δ 5.76 (dt, 1 H, H-e, $J_{e,d} = 15.4$, $J_{e,f} = 6.7$), 5.50 (dd, 1 H, H-d, $J_{d,c} = 7.3$), 4.11 (dd, 1 H, H-c, $J_{c,b} = 6.2$), 4.01 (dd, 1 H, H-a, $J_{a,a'} = 10.4$, $J_{a,b} = 3.4$), 3.62 (dd, 1 H, H-a′, $J_{a',b} = 6.7$), 3.46 (ddd, 1 H, H-b), 2.85 (dd, 1 H, H-3eq, $J_{3eq,3ax} = 12.6$, $J_{3eq,4} = 4.0$), 2.06 (m, 2 H, H-f, H-f ′), 2.04 (s, 3 H, NAc), 1.65 (dd, 1 H, H-3ax, $J_{3ax,4} = 10.5$), 1.43−1.27 (m, 22 H, 11 CH_2), 0.89 (t, 3 H, CH_3, $J = 6.7$); MS (ESI) *m*/*z*: Calcd for $C_{29}H_{52}N_4O_{10}$, 616.7; Found, 615.7 $(M-H)^-$. Solvent used: dichloromethane/methanol. Treatment of **6** with NaOMe in MeOH as described above also gave quantitatively compound **7**.

*(D) (5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic Acid)-(2→1)-(2S,3R,4E)-2-amido-4-octadecene-1,3-diol (**8**).* To a solution of **7** (25 mg, 0.042 mmol) in dry MeOH (1 mL) were added under argon propane-1,3-dithiol (0.1 mL) and triethylamine (0.1 mL), and the mixture was stirred at room temperature for 4 days. A white precipitate was formed. After filtration, and washing with MeOH, the filtrate was concentrated. The residue obtained was flash-chromatographed, eluting with 2.5:1:0.15 CH_2Cl_2/MeOH/NH_4OH to afford **8** (22.2 mg, 90%) as an amorphous solid: $[\alpha]_D$ +0.5° (*c* 1, MeOH); TLC (2.5:1:0.1 CH_2Cl_2/MeOH/NH_4OH): R_f 0.2; ^{1}H NMR (400 MHz, CD_3OD) δ 5.92 (dt, 1 H, H-e, $J_{e,d} = 15.3$, $J_{e,f} = 7.0$), 5.67 (dd, 1 H, H-d, $J_{d,c} = 7.2$), 4.18 (dd, 1 H, H-c, $J_{c,b} = 6.5$), 4.13 (dd, 1 H, H-a, $J_{a,a'} = 10.0$, $J_{a,b} = 3.7$), 3.89 (m, 1 H, H-4), 3.67 (dd, 1 H, H-a′, $J_{a',b} = 9.5$), 3.03 (ddd, 1 H, H-b), 3.02 (dd, 1 H, H-3eq, $J_{3eq,3ax} = 12.3$, $J_{3eq,4} = 4.0$), 2.26 (m, 2 H, H-f, H-f ′), 2.20 (s, 3 H, NAc), 1.81 (dd, 1 H, H-3ax, $J_{3ax,4} = 11.3$), 1.62−1.44 (m, 22 H, 11 CH_2), 1.10 (t, 3 H, CH_3, $J = 7.0$); ^{13}C NMR (100.6 MHz, CD_3OD) δ 175.95, 174.43 (2 C═O), 136.87 (C-e), 128.65 (C-d), 102.53 (C-2), 74.87, 73.06, 71.43, 70.36, 69.55 (C-4, C-6, C-7, C-8, C-c), 64.76, 62.72 (C-9, C-a), 57.36, 54.39 (C-5, C-b), 42.10 (C-3), 33.73, 33.39, 31.12−31.07, 30.95, 30.79, 30.76, 30.51, 24.05 (12 CH_2), 22.88 (NAc), 14.75 (CH_3); MS (ESI) *m*/*z*: Calcd for $C_{29}H_{54}N_2O_{10}$, 590.4; Found, 589.4 $(M-H)^-$. Solvent used: methanol.

*(E) (5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic Acid)-(2→1)-(2S,3R,4E)-2-chloroacetamido-4-octadecene-1,3-diol (**9**).* To a solution of **8** (5 mg, 8.5 μmol) in dry MeOH (1 mL) under argon were added at 0 °C triethylamine (7 μL, 50 μmol) and chloroacetyl chloride (1.6 μL, 20 μmol), and the mixture was stirred at room temperature for 30 h. After concentration, the residue was flash-chromatographed, eluting with 7:2:1 EtOAc/*i*-PrOH/H_2O to afford **9** (4.7 mg, 83%) as an amorphous solid: $[\alpha]_D$ +1.5° (*c* 0.47, 1:1 $CHCl_3$/MeOH); TLC (6:2:1 EtOAc/*i*-PrOH/H_2O): R_f 0.48; ^{1}H NMR (400 MHz, 1:5 $CDCl_3$/CD_3OD) δ 5.91 (dt, 1 H, H-e, $J_{e,d} = 15.3$, $J_{e,f} = 6.8$), 5.65 (dd, 1 H, H-d, $J_{d,c} = 7.4$), 4.25−4.18 (m, 2 H, H-c, H-a), 4.03 (s, 2 H, CH_2Cl), 3.66 (m, 1 H, H-b), 2.94 (dd, 1 H, H-3eq, $J_{3eq,3ax} = 12.6$, $J_{3eq,4} = 4.7$), 2.24 (m, 2 H, H-f, H-f ′), 2.19 (s, 3 H, NAc), 1.97 (dd, 1 H, H-3ax, $J_{3ax,4} = 12.1$), 1.65−1.45 (m, 22 H, 11 CH_2), 1.08 (t, 3 H, CH_3, $J = 6.80$); MS (ESI) *m*/*z*: Calcd for $C_{31}H_{55}ClN_2O_{11}$, 666 and 668; Found, 665.7 and 667.7 (3:1, $M-H)^+$. Solvent used: dichloromethane/methanol.

*(F) (5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic Acid)-(2→1)-(2S,3R,4E)-2-N,N-dimethylamido-4-octadecene-1,3-diol (**10**).* To a solution of **8** (5.9 mg, 10 μmol) in CH_3CN/MeOH (0.4 mL, 1:1) were added aqueous formaldehyde (0.1 mL) and $NaBH_3CN$ (6.2 mg, 0.1 mmol), and the mixture was stirred at room temperature for 1 h. AcOH was added (20 μL) to make the pH near neutrality. After stirring at room temperature for 14 h, the mixture was concentrated, and the residue was flash-chromatographed, eluting with 3:2:1 EtOAc/*i*-PrOH/H_2O to afford **10** (6.1 mg, 98%) as an amorphous solid: $[\alpha]_D$ −5° (*c* 0.5, MeOH); TLC (3:2:1 EtOAc/*i*-PrOH/H_2O): R_f 0.31; ^{1}H NMR (400 MHz, CD_3OD) δ 6.06 (ddt, 1 H, H-e, $J_{e,c} = 0.8$, $J_{e,d} = 15.3$, $J_{e,f} = 6.8$), 5.71 (dd, 1 H, H-d, $J_{d,c} = 5.8$), 4.78 (m, 1 H, H-c), 4.35 (dd, 1 H, H-a, $J_{a,a'} = 12.2$, $J_{a,b} = 3.2$), 4.10 (dd, 1 H, H-a′, $J_{a',b} = 8.7$), 3.59 (ddd, 1 H, H-b, $J_{b,c} = 2.7$), 3.17 (s, 6 H, NMe_2), 3.00 (dd, 1 H, H-3eq, $J_{3eq,3ax} = 12.2$, $J_{3eq,4} = 4.3$), 2.29 (m, 2 H, H-f, H-f ′), 2.21 (s, 3 H, NAc), 1.84 (dd, 1 H, H-3ax, $J_{3ax,4} = 11.2$), 1.65−1.45 (m, 22 H, 11 CH_2), 1.20 (t, 3 H, CH_3, $J = 6.9$); ^{13}C NMR (100.6 MHz, CD_3OD) δ 175.91, 174.20 (2 C═O), 135.74 (C-e), 129.81 (C-d), 102.29 (C-2), 74.98, 73.15, 70.58, 70.37, 70.15, 69.58 (C-4, C-6, C-7, C-8, C-b, C-c), 64.79, 59.81 (C-9, C-a), 54.35 (C-5), 42.79 (NMe_2), 42.34 (C-3), 33.65, 33.39, 31.12−31.09, 30.95, 30.79, 30.76, 30.57, 24.05 (12 CH_2), 22.86 (NAc), 14.75 (CH_3); MS (FAB) *m*/*z*:

Calcd for $C_{31}H_{58}N_2O_{10}$, 618.5; Found, 619.5 $(M+H)^+$, 641.5 $(M+Na)^+$. Solvent used: methanol.

GSLs, Antibodies, and Other Reagents. GM3 (*N*-acetyl) was prepared from dog erythrocytes (*14*). Gg3 was prepared from guinea pig blood (*21*). GlcCer was purchased from Matreya, Inc. (Pleasant Gap, PA). Psychosine was prepared from brain cerebroside by alkaline degradation in butanol (*22*). Anti-GM3 mAb DH2 (mouse IgG3) (*23*) and anti-LacCer mAb T5A7 (*24*) were established as described previously. Antibodies directed to c-Src, FAK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oleoyl- and palmitoyl-lyso-PC, PMSF, filipin, and nystatin were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells, Images of GM3 Distribution Patterns at the Cell Surface, and Their Intensity. Mouse melanoma B16/F10 cells were cultured as described previously (*15*). GM3 clusters and intensity expressed at the melanoma B16 cell surface were observed by immunofluorescence with anti-GM3 mAb DH2, and the pattern was digitally imaged using a DeltaVision microscope (Applied Precision, Inc.) or Leica TCS-SP confocal laser scanning microscope in the Image Analysis Lab at Fred Hutchinson Cancer Research Center. The common procedure for microscopy and flow cytometry was as follows. Detached cells were washed 3× with PBS, incubated with DH2 (1 μg/mL PBS containing 1% normal goat serum) in ice or at 4 °C for 1 h (*23*), washed 2× with precooled PBS, incubated with 2 μg/mL PBS containing FITC goat anti-mouse IgG for 30 min, and washed 2× with PBS. Washed cells were subjected directly to flow cytometry, or subjected to microscopy after being fixed with 1% paraformaldehyde for 30 min, washed 2× with PBS, suspended in 100 μL of PBS, placed on a coverglass affixed to a "coverglass chamber" (Nunc, Inc., Naperville, IL), and mounted on objective glass. Prefixation of cells with paraformaldehyde did not change the pattern, but reduced the intensity of immunostaining.

Effects of SA-Sph, lyso-GM3, Lac-Sph, lyso-PC, and psychosine on GM3 imaging were determined as follows. Detached cells were washed 2× with DMEM, and 2.5 × 10^6 cells per milliliter of DMEM were mixed with the above compounds (1, 5, or 10 μM concentration; DMSO as vehicle; final concentration 0.5%). After 30 min incubation, cells were washed 1× with PBS and subjected to immunofluorescence followed by microscopy or flow cytometry as described above.

GM3-Dependent B16 Cell Adhesion and Associated Changes of FAK and c-Src. GM3-dependent adhesion of B16 cells to dishes coated with Gg3 or GlcCer and the associated change of focal adhesion kinase (FAK) and c-Src phosphorylation response were determined as described previously (*14, 15*). Briefly, 10 cm dishes (Corning, Cambridge, MA) were coated with Gg3 or GlcCer by placing 2 mL of GSL solution in ethanol (100 μg/mL) and drying under gentle shaking on a rocker (20 movements per minute) at room temperature. The coated dishes were subsequently treated with 5 mL of 0.1% BSA in PBS at room temperature for 1 h with gentle shaking. The dishes were washed 5× with ice-cold PBS, and used immediately for adhesion experiments.

Cells added on dishes and adhered by centrifugation were harvested in ice-cold PBS containing 1 mM $NaVO_4$ and 0.1 mM diisopropyl fluorophosphate. The harvested cells (containing 20−30 μg of protein) were lysed by 1 mL of RIPA (radioimmunoprecipitation assay) buffer (for composition, see ref *15*) with sonication for 10 s, and centrifuged for 5 min at 1300*g* (2500 rpm). The resulting supernatant was precleared by mixing with protein G−Sepharose beads and immunoprecipitated with anti-FAK antibody, followed by Western blotting using anti-tyrosine phosphate mAb PY99 (Santa Cruz) as described previously (*14*).

Effects of SA-Sph, Its Analogues, and Lac-Sph on GM3-Dependent Adhesion and Signaling of B16 Melanoma Cells. B16 cells were detached with 0.05% trypsin/0.5 mM EDTA/PBS and suspended in serum-free DMEM at 2 × 10^6 cells per milliliter. Aliquots of cell suspension were mixed with DMSO alone (final concentration 0.5%, as control) or with DMSO containing various quantities of GM3, lyso-GM3, SA-Sph, SA-Nc-Sph, SA-NMe-Sph, psychosine, or Lac-Sph, and incubated for 30 min at 37 °C. A 500 μL aliquot of the preincubated cell suspension was added to each well of 24-well plates (Corning) which were precoated with Gg3 or GlcCer and blocked with 0.1% BSA to observe cell adhesion, followed by centrifugation at 100*g* for 10 s. The same aliquots were added to polypropylene tubes (to avoid cell adhesion) as control. After incubation of cells at 37 °C for 30 min in a CO_2 incubator, each well was washed 3 times with warmed PBS to remove nonadherent cells. The quantity of adherent cells was estimated by protein determination using a Micro-BCA kit.

Determination of Levels of FAK and Its Tyrosine Phosphate in Cells. To determine the level of tyrosine phosphate associated with FAK, cell lysate was immunoprecipitated by anti-FAK antibodies. Cells stimulated through adhesion to Gg3-coated plates or control cells added on GlcCer-coated plates were harvested using ice-cold PBS containing 1 mM $NaVO_4$ and 0.1 mM DFP. The harvested cells (containing 20−30 μg of protein) were lysed by 1 mL of RIPA buffer with sonication for 10 s, and centrifuged for 5 min at 1300*g* (2500 rpm). The resultant supernatant was mixed with protein G−Sepharose beads (20 μL packed; Pharmacia Biotech) and rotated for 2 h at 4 °C. After centrifugation (1300*g* for 5 min), the supernatant was added with 2 μL of 100 μg/mL rabbit anti-FAK IgG (Santa Cruz), rotated overnight at 4 °C, and then incubated with protein G−Sepharose beads (20 μL packed) for 2 h at 4 °C. The mixture was centrifuged at 1300*g* for 5 min, and pelleted beads were washed 3 times with RIPA buffer and boiled 5 min in SDS−PAGE sample buffer containing 5% 2-mercaptoethanol. The FAK content of each SDS−PAGE sample was measured by Western blotting using an Ultroscan XL densitometer (Pharmacia LKB Biotechnology). Samples containing equal amounts of FAK were run on 7.5% SDS−PAGE and transferred to PVDF membranes. The membranes were probed by anti-tyrosine phosphate mAb PY99 (Santa Cruz) and developed using a chemiluminescence method, followed by densitometric analysis.

Determination of c-Src Phosphorylation Response in Detergent-Insoluble, Low-Density Membrane Fraction (DIM). Cells were lysed, the postnuclear supernatant fraction was subjected to sucrose density gradient centrifugation, and the detergent-insoluble, low-density light-scattering membrane fraction (DIM) was collected as described previously (*14*). DIM was 10× diluted with kinase buffer (for composition, see ref *15*), with final protein concentrations of 2−3 μg/mL. Five milliliter aliquots of this solution were mixed with

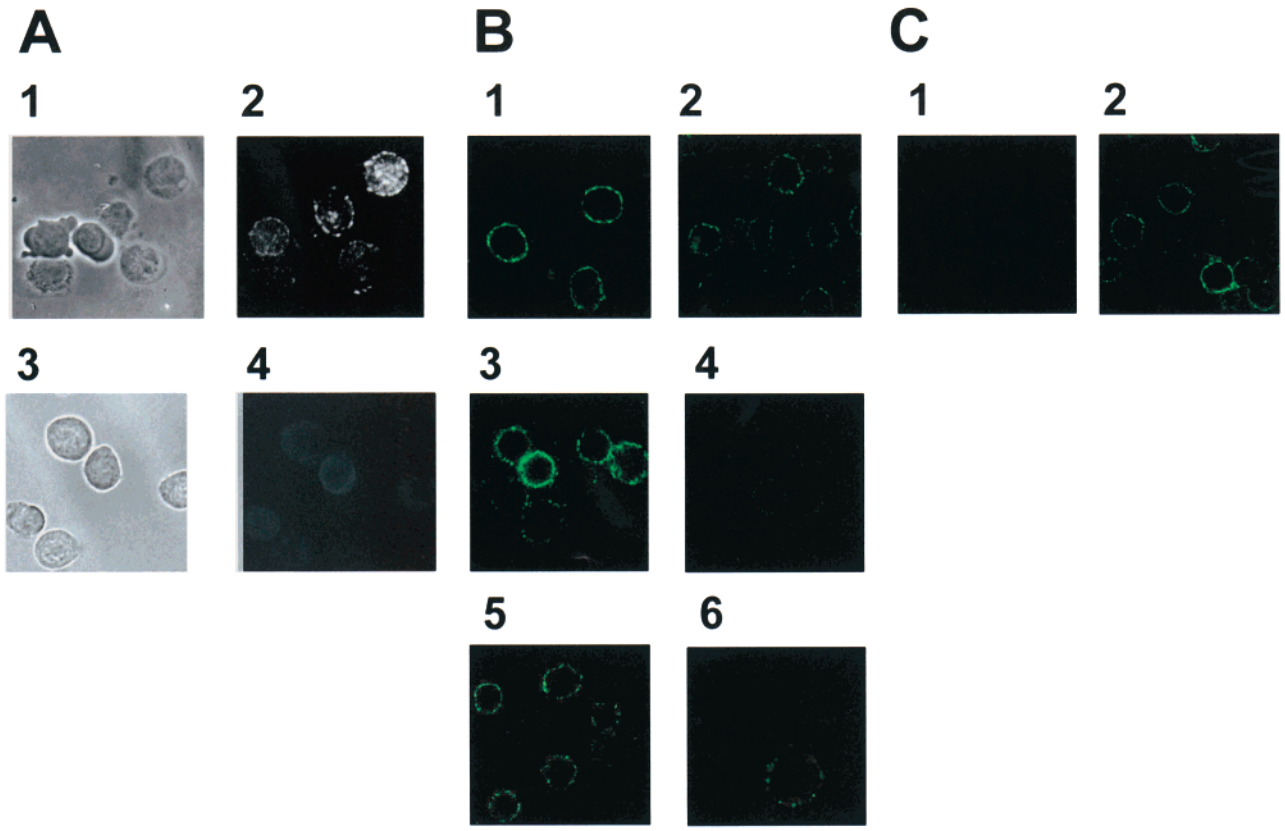

FIGURE 2: GM3 distribution pattern in mouse melanoma B16 cells revealed by immunofluorescence, and the effect of sialyl α2→1 Sph and other compounds. Detached cells were processed for immunofluorescence as described under Materials and Methods, using anti-GM3 IgG3 mAb DH2 with or without preincubation in various concentrations of SA-Sph or other compounds. Panel A: 1 and 3: Pattern from light microscopy. 2 and 4: Pattern from immunofluorescence microscopy. 1 and 2: Control cells. 3 and 4: Cells treated with 5 μM SA-Sph. Panel B: Pattern from confocal microscopy of immunofluorescence with mAb DH2. 1: Control cells. 2: Cells treated with 5 μM Lac-Sph. 3: 5 μM oleoyl lyso-PC. 4: 5 μM SA-Sph. 5: 1 μM lyso-GM3. 6: 5 μM lyso-GM3. Panel C: Pattern from microscopy of immunofluorescence with mAb T5A7. 1: Control cells. 2: Cells treated with sialidase.

or without GM3, SA-Sph or its derivatives, psychosine, or Lac-Sph (for concentration, see Figure 7 legend), and then added to GSL-coated 10 cm dishes, followed by centrifugation at 2000*g* (3000 rpm) at 4 °C for 30 min. The dishes were kept on ice for 15 h with gentle rocking (10 strokes/min). To determine phosphorylation of control DIM, the same amount of DIM in a polypropylene tube was kept on ice for 15 h. The c-Src activity of the membrane fraction in each dish and the control tube was determined after addition of 50 μCi of [γ-^{32}P]ATP (corresponding to 370 GBq/mmol, NEN), incubation at 37 °C for 5 min, and stopping the reaction by stop buffer (for composition, see ref *15*). Desorption and solubilization of membranes adhered to dishes, collection of c-Src proteins in both the membrane and the soluble portion by trichloroacetic acid precipitation, and determination of ^{32}P activity associated with the c-Src fraction separated by immunoprecipitation in RIPA buffer were performed as described previously (*15*).

RESULTS

Synthesis and Properties of Sialyl α2→1 Sph and Its Analogues. SA-Sph (compound **8** in Figure 1, Scheme 2) was synthesized as described under Materials and Methods. At the initial sialylation of O-protected azido-Sph with *N*-acetylneuraminic acid xanthate as sialyl donor (*25*), promoted by phenylsulfenyl trifluorate (*26*), approximately 10% and 60% of β- and α-isomers, respectively, were yielded. Determination of their anomeric configuration was based on the empirical ^{1}H NMR rules using differences between the anomeric isomers (*27, 28*): δ α H-3eq (2.61 ppm) > δ β H-3eq (2.44 ppm), and δ α H-4 (4.86 ppm) < δ β H-4 (5.19 ppm).

Deprotection of the *tert*-butyldiphenylsilyl group in α-isomer **4** (Figure 1, Scheme 1) was performed by treatment with tetra-*n*-butylammonium fluoride (TBAF) in tetrahydrofuran (*29, 30*) to give alcohol **5** (28%), and its acid derivative **6** (70%), derived from hydrolysis of the methyl ester of compound **5**.

The removal of silyl and acetyl groups and saponification of α-isomer **4** led quantitatively to compound **7** (Figure 1, Scheme 2). For selective reduction of azide in **7**, we first used hydrogen sulfide (*31*) in pyridine/water (7:3), which gave after 2 days less than 40% yield of amine **8**. However reduction with propanedithiol/triethylamine (*32*) has proved to be more efficient; a 90% yield of **8** was obtained. N-Chloroacetylation of **8** was performed by using chloroacetyl chloride, in the presence of triethylamine, to afford amide **9** in 83% yield. Introduction of dimethyl groups into amine group in **8** was performed by reductive methylation (*33*) with formaldehyde and sodium cyanoborohydride to provide **10** in 98% yield (Figure 1, Scheme 2).

Effect of Sialyl α2→1 Sph and Its Analogues on GM3 Immunofluorescence. GM3 clusters in melanoma B16 cells can be observed as intense fluorescent loci distributed over

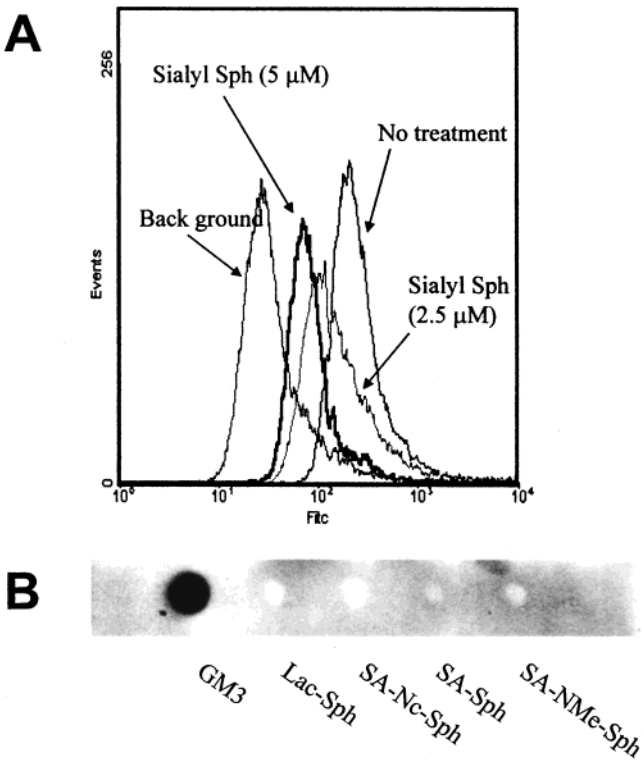

FIGURE 3: Effects of sialyl α2→1 Sph on GM3 expression in melanoma B16 cells, and absence of DH2 cross-reactivity with lyso-compounds. Panel A: Effect of 2.5 and 5.0 μM SA-Sph on GM3 expression in B16 cells, shown by flow cytometry. Panel B: Specific reactivity of anti-GM3 mAb DH2 to GM3, and absence of reactivity to Lac-Sph, SA-Nc-Sph, SA-Sph, and SA-NMe-Sph, as indicated by dot blotting with PVDF membrane assembled in a Bio-Dot apparatus (Bio-Rad Laboratory, Cambridge, MA) as described previously (*15*, *41*).

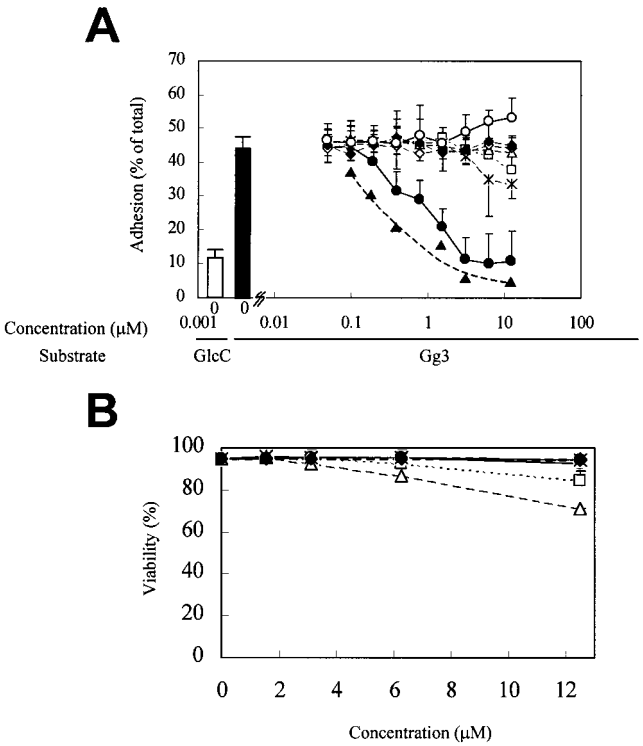

FIGURE 4: Effects of sialyl α2→1 Sph and other lyso-sphingolipids and lyso-phospholipids on GM3-dependent adhesion of melanoma B16 cells. Panel A: Open bar at left, cell adhesion upon addition of B16 cells to GlcCer-coated dish. Solid bar, GM3-dependent adhesion of B16 cells to Gg3-coated dish. Adhesion of B16 cells pretreated with various subtoxic concentrations (0.075−15 μM) of the following compounds at 37 °C for 1 h (see Materials and Methods and other parts of text) is indicated by line graphs at right: (●) SA-Sph; (▲) lyso-GM3; (□) SA-Nc-Sph; (×) SA-NMe-Sph; (△) Lac-Sph; (○) GM3; (◇) oleoyl lyso-PC; (◆) palmitoyl lyso-PC. Galactosyl β1→1 Sph (psychosine) had no effect on GM3-dependent adhesion [curve overlaps those of (□) and (◆); data not shown]. Panel B: Effect of various lyso-compounds at concentrations up to 15 μM (shown on abscissa) on viability (shown on ordinate) of B16 cells as tested by Trypan blue exclusion. Symbols as in panel A. Only Lac-Sph (△) and SA-Nc-Sph (□) showed a weak cytotoxicity, at concentrations of 6−15 μM. All compounds tested showed cytotoxicity at concentrations above 25−50 μM (data not shown). Galactosyl β1→1 Sph (psychosine) had no cytotoxicity at concentrations below 15 μM (data not shown).

the cell surface following treatment with anti-GM3 mAb DH2. Such loci are diminished or disappear following treatment with 1−5 μM SA-Sph or lyso-GM3,[4] as observed by both regular fluorescence microscopy (Figure 2A4) and confocal fluorescence microscopy (Figure 2B4). Lac-Sph and lyso-PC had little or no such effect (Figure 2B2,3). Similar to the effect of SA-Sph, lyso-GM3 reduced immunofluorescence with DH2 (Figure 2B5,6). As a control, anti-LacCer antibody showed no immunofluorescence (Figure 2C1), since LacCer is completely absent in B16 cells. Reactivity with anti-LacCer antibody was weakly positive following treatment of cells with sialidase **(**Figure 2C2).[5] The intensity of fluorescence also decreased as determined by flow cytometry (Figure 3A). mAb DH2 reacts strongly with GM3 but not with SA-Sph, its derivatives, or lactosyl Sph (Figure 3B).

Effect of Sialyl α2→1 Sph and Its Analogues on GM3-Dependent Adhesion of B16 Cells. SA-Sph, as well as lyso-GM3, inhibited GM3-dependent adhesion at 0.1−1.0 μM concentration, and inhibition was nearly complete at 5−10 μM. The degree of inhibition was much less when the amino group of Sph in SA-Sph was substituted with a chloroacetyl or *N*,*N*-dimethyl group. There was no inhibition by galactosyl or lactosyl Sph, or by oleoyl- or palmitoyl-lyso-PC, even at 10−15 μM. GM3 had an enhancing rather than inhibitory effect on the adhesion (Figure 4A).

The inhibitory effect of SA-Sph or lyso-GM3 on GM3-dependent adhesion was not due to cytotoxicity, since these compounds did not reduce viability as tested by Trypan Blue exclusion even at 10−15 μM. Lac-Sph, in contrast, showed a slight cytotoxic effect at 6 and 12 μM (Figure 4B), and a clear effect at >15 μM (data not shown), even though this compound had no inhibitory effect on GM3-dependent adhesion. Other compounds mentioned above had no cytotoxic effect even at 15 μM.

Effect of Sialyl α2→1 Sph and Its Analogues on Cellular FAK Activity Associated with GM3-Dependent Cell Adhesion. SA-Sph strongly inhibited the enhanced FAK phosphorylation associated with GM3-dependent adhesion of B16 cells to Gg3-coated dishes. In contrast, SA-Nc-Sph moder-

[4] Effects of lyso-GM3 and its derivatives N-substituted at the amino group of Sph and of various lyso-phospholipids and lyso-sphingolipids, on reconstituted membrane simulating GSD and on GM3-dependent adhesion and signaling, have been systematically studied and will be published elsewhere (Iwabuchi, K., Zhang, Y., Handa, K., Sinay, P., and Hakomori, S).

[5] Treatment of B16 cells with sialidase made them highly susceptible to lysis by SA-Sph or Lac-Sph even at 1−5 μM concentration. Therefore, we could not test the effects of these compounds on LacCer expression in sialidase-treated cells.

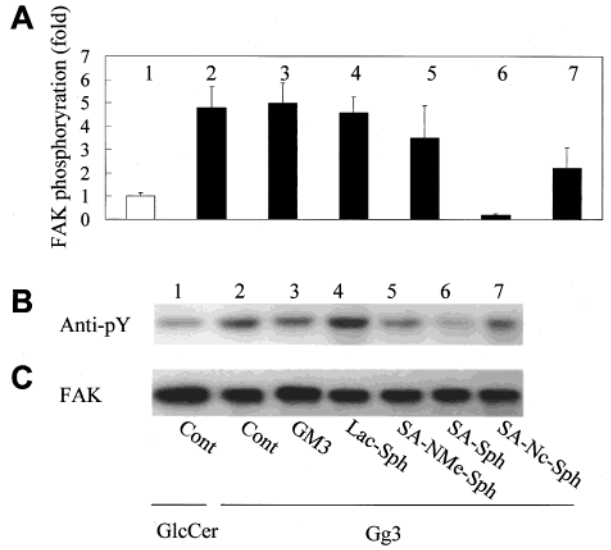

FIGURE 5: Effects of sialyl α2→1 Sph and its analogues on FAK kinase activity associated with GM3-dependent adhesion of B16 cells to Gg3-coated dish. Panel A: FAK kinase activity in terms of degree of tyrosine phosphorylation of B16 cells, preincubated with 10 μM GM3, LacCer, SA-NMe-Sph, SA-Sph, or SA-Nc-Sph, and adhered to Gg3-coated dish, is shown in columns 3 through 7 at top. Control cells without preincubation added to GlcCer-coated dish are shown as open column 1 (defined as 1). Degree of tyrosine phosphorylation of control cells added to Gg3-coated dish is shown as column 2. Panel B: Intensity of tyrosine phosphate associated with FAK band blotted by anti-pY antibody. Panel C: Intensity of FAK band blotted with anti-FAK antibody.

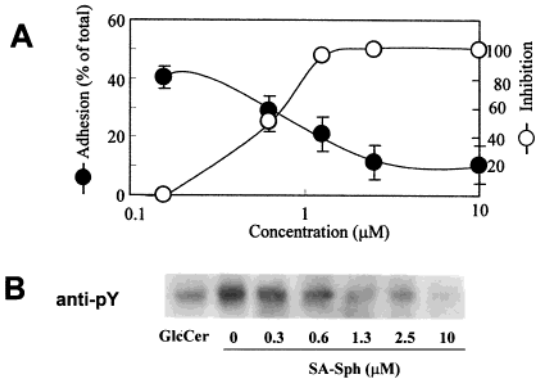

FIGURE 6: Dose-dependent effect of sialyl α2→1 Sph on GM3-dependent adhesion of B16 cells to Gg3-coated dish, and associated inhibition of FAK phosphorylation. Panel A: B16 cell adhesion to Gg3-coated dish, expressed as percent of adhered cells relative to total cell number added (left ordinate), and percent inhibition of FAK phosphorylation (right ordinate). Abscissa, concentration of SA-Sph (μM). Change of B16 cell adhesion is shown by curve with (●), and change of FAK kinase inhibition is shown by curve with (○). Panel B: Western blotting pattern of FAK band with anti-pY antibody in B16 cells added to GlcCer-coated dish, and in B16 cells preincubated with 0−10 μM SA-Sph.

ately inhibited, SA-NMe-Sph weakly inhibited, and Lac-Sph had no effect on this FAK phosphorylation, as determined by Western blotting with anti-tyrosine phosphate (anti-pY) mAb (Figure 5A,B). None of the above compounds affected the FAK level as indicated by blotting with anti-FAK antibody (Figure 5C).

The dose-dependent effect of SA-Sph on cell adhesion and FAK-associated tyrosine phosphorylation is shown in Figure 6A,B. FAK phosphorylation was inhibited strongly even at 1 μM concentration, and completely at 10 μM. The degree

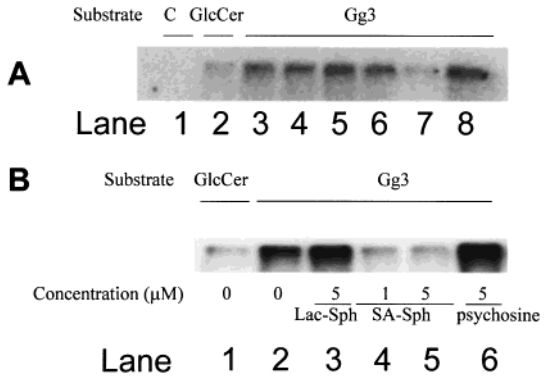

FIGURE 7: Effect of sialyl α2→1 Sph, lactosyl Sph, lyso-PC, and psychosine on c-Src phosphorylation response. Response was defined by enhancement of c-Src phosphorylation upon adhesion of detergent-insoluble, low-density membrane fraction (DIM) to Gg3-coated dish (lane 3 in panel A, lane 2 in panel B) as compared to GlcCer-coated dish (lane 2 in panel A, lane 1 in panel B) or to control membrane without adhesion (lane 1 in panel A). Panel A: Effect of 5 μM Lac-Sph (lane 4), oleoyl lyso-PC (lane 5), palmitoyl lyso-PC (lane 6), SA-Sph (lane 7), or galactosyl β1→1 Sph (psychosine) (lane 8). Complete inhibition was observed only for SA-Sph (lane 7). Panel B: Dose-dependent effect of SA-Sph (1 and 5 μM; lanes 4 and 5). Effect of 5 μM Lac-Sph (lane 3) and psychosine (lane 6).

of FAK inhibition was closely correlated with the degree of inhibition of cell adhesion (Figure 6A).

Effect of Sialyl α2→1 Sph and Its Analogues on c-Src Phosphorylation in Response to Membrane Adhesion. Enhanced c-Src activity in detergent-insoluble, low-density membrane fraction (DIM) upon adhesion to Gg3-coated dishes was strongly inhibited by 5 μM SA-Sph (Figure 7A, lane 7). Oleoyl- or palmitoyl-lyso-PC, or psychosine at 5 μM had no effect on c-Src phosphorylation. Membrane fraction added to a polypropylene tube (control) showed no c-Src phosphorylation, and that added to a GlcCer-coated dish had minimal phosphorylation. A similar inhibitory effect of SA-Sph on the c-Src phosphorylation response was observed with both 1 μM and 5 μM concentrations of SA-Sph (Figure 7B, lanes 4, 5); 0.1−0.3 μM concentrations of SA-Sph showed the same intensity of c-Src phosphorylation as control without SA-Sph (data not shown). No effect was observed for 5 μM Lac-Sph or psychosine (Figure 7B). Thus, only SA-Sph but no other lyso-compound affected the c-Src phosphorylation response of the membrane fraction representing GSD.

DISCUSSION

Clustering of glycosphingolipids (GSLs) together with signal transducer molecules to form a "GSL signaling domain" or "glycosignaling domain" (GSD) is of central importance for understanding the functional significance of GSLs in cell adhesion (*34*, *35*) and signal transduction (*13*−*15*, *36*). GM3, which is highly expressed in mouse melanoma B16 cells, was identified as a melanoma-associated antigen (*16*). The fact that mAb M2590 reacts with a type of cell having GM3 concentration above a threshold value, in "all-or-none" fashion, explains the observation that this antibody reacts preferentially with high GM3 expressors such as B16 melanoma cells (*17*). In contrast, anti-GM3 mAb DH2 used in this study shows reactivity proportional to the quantity of GM3 antigen presented in vitro or at the cell surface, and

does not recognize a clear "threshold value" of antigen concentration (*24*). High reactivity with DH2 indicates a high concentration of GM3 per unit area (density) at the cell surface, i.e., clustering. Low DH2 reactivity indicates a low level of clustering. GM3 clustering was observed under both regular and confocal fluorescence microscopy at low temperature (<4 °C). In some cases, glutaraldehyde-prefixed cells were examined; they also showed GM3 clustering, indicating that such clustering is naturally occurring rather than due to antibody-induced lateral movement. The natural occurrence of GSL clustering in the membrane has been supported by results from scanning electron microscopy with the freeze−fracture technique, even at the liposome surface, using ferritin-labeled Fab fragments of anti-GSL antibodies (*3*, *4*).

High density of clustered GM3 at the B16 cell surface is instrumental in adhesion of the cells to the mouse endothelial cell surface to initiate the metastatic process through a novel interaction of GM3 (on the B16 cell) with LacCer or Gg3 (on the endothelial cell) (*18*), and liposomes with GM3 or Gg3 block melanoma metastasis (*37*). GM3 clustering at the native cell surface is not due to the artifactual effect of preparation, but rather to intrinsic properties causing cis GM3-to-GM3 interaction (for review, see *36*).

In view of this background, and current development of concepts on GSL organization with signal transducer molecules in GSD, we investigated the structure and function of B16 cell GSD, which is characterized by a high content of GM3, sphingomyelin, c-Src, Rho A, and FAK, and is separable from another membrane domain, caveolae, which is enriched in cholesterol, the scaffold protein caveolin, and Ras H, but lacks GM3, c-Src, Rho A, and FAK (*15*). Both membrane domains, GSD and caveolae, are insoluble in neutral or zwitterionic detergent, and can be isolated as low-density, light-scattering membrane components by sucrose density gradient centrifugation. A number of recent studies do not distinguish these two domains, and regard cholesterol as an essential GSL domain component (for reviews, see *10*, *11*). Caveolae are involved in endocytosis and signal transduction, and their structure and function are disrupted by cholesterol-binding reagents such as filipin, nystatin, and β-cyclodextrin (*38*, *39*; for review, see *12*). The susceptibility of caveolae to these reagents is highly advantageous for their functional study. We therefore looked for reagents which disrupt the structure and function of GSD. One possibility is GSL analogues which preferentially disrupt GSL clustering in GSD and thereby reduce GSL-dependent adhesion and inhibit associated signal transduction.

Theoretically, GSL clustering is based on GSL-to-GSL cis interaction, which may be blocked by GSL analogues inserted in GSD in the membrane. A candidate for such a reagent is lyso-GSL having the same or similar structure as GSL in GSD. The simplest such compound, sialyl $\alpha 2 \rightarrow 1$ Sph (SA-Sph), and its two derivatives having *N*,*N*-dimethyl or *N*-chloroacetyl substitution at the amino group of Sph were synthesized and compared in their effect on (i) the GM3 distribution pattern at the cell surface; (ii) GM3-dependent cell adhesion; (iii) enhanced FAK activity; and (iv) c-Src phosphorylation response upon GM3-dependent adhesion of GSD membranes to Gg3-coated dishes.

A remarkable inhibitory effect on all four of these parameters was clearly observed for SA-Sph and lyso-GM3 at subtoxic (0.5−15 μM) doses. However, its *N*,*N*-dimethyl- or *N*-chloroacetyl-substituted derivatives had much less effect, even though their cytotoxic dose was similar to that of SA-Sph. The free amino group of Sph, and terminal sialic acid, may be important for insertion of SA-Sph and lyso-GM3 into GSD to disrupt GM3-to-GM3 cis interaction, although the exact mechanism remains to be elucidated. Interestingly, Lac-Sph, galactosyl Sph (psychosine), and lyso-PC (either oleoyl or palmitoyl form) had no effect on the four above parameters at concentrations up to 15−20 μM. This indicates that reagents capable of disrupting GSD structure and function in B16 cells must have sialic acid at the hydrophilic terminus, and Sph with a free amino group at the hydrophobic domain. Lyso-PC and psychosine are well-known to be cytolytic. This study indicates, surprisingly, that these compounds are not cytotoxic at concentrations below 15 μM and do not disrupt cell adhesion and signal transduction through GSD structure.

Reduction of clustered GM3 immunofluorescence by SA-Sph or lyso-GM3 is not due to release of GM3, since treated cells showed the same chemical level of GM3 as nontreated cells (unpublished data), and SA-Sph does not cross-react at all with anti-GM3 mAb DH2. Therefore, the effect of SA-Sph on GSD is presumably due to reduction of GM3 clustering, leading to reduced binding of anti-GM3 mAb at the cell surface. Further studies on the mechanism of this effect are in progress.

In view of the important role of GM3-dependent adhesion through GSD of B16 cells to endothelial cells (*18*) (see above), and associated enhanced motility of B16 cells (*40*), it seems likely that adhesion coupled with signal transduction triggers the process of metastasis (*18*). Therefore, B16 melanoma metastasis and progression may well be blocked by subtoxic doses of SA-Sph, a nonphysiological compound obtainable only by synthetic chemistry. In this aspect, application of synthetic chemistry is essential to obtain efficient reagents for disruption of GSD function, possibly providing a means to inhibit B16 melanoma metastasis.

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