Reconstitution of Membranes Simulating “Glycosignaling Domain” and Their Susceptibility to Lyso-GM3*

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GM3 ganglioside at the surface of mouse melanoma B16 cells is clustered and organized with signal transducer molecules c-Src, Rho A, and focal adhesion kinase (FAK) to form a membrane unit separable from caveolae, which are enriched in cholesterol and caveolin but do not contain GM3 or the above three signal transducers. The GM3-enriched membrane units are involved in GM3-dependent cell adhesion coupled with activation of c-Src, Rho A, and FAK and are termed the “glycosphingolipid signaling domain” or the “glycosignaling domain” (GSD). In order to assess the essential components that display GSD function, membranes with properties similar to those of GSD were reconstituted using GM3, sphingomyelin, and c-Src, with or without other lipid components. The reconstituted membrane thus prepared displayed GM3-dependent adhesion to plates coated with Gg3 or anti-GM3 antibody, resulting in enhanced c-Src phosphorylation (c-Src phosphorylation response). This response in reconstituted membrane depends on GM3 concentration and was not observed when GM3 was absent or replaced with other gangliosides GM1 or GD1a, or with LacCer. The GM3-dependent c-Src phosphorylation response was enhanced when cholesterol and phosphatidylcholine were added. Although GM3, sphingomyelin, and c-Src are essential for GSD function, a small quantity of cholesterol and phosphatidylcholine may act as an auxiliary factor to stabilize membrane. GSD function in terms of GM3-dependent adhesion and signaling was blocked in the presence of lyso-GM3 or its analogue but not psychosine, lactosyl-sphingosine, or lyso-phosphatidylcholine. Such susceptibility of reconstituted GSD to lyso-GM3 and other lyso compounds is the same as GSD of original B16 cells. Thus, functional organization of the reconstituted membrane closely simulates that of GSD in B16 cells, which is based on clustered GM3 organized with c-Src as the essential components.

GM3 ganglioside in mouse melanoma B16 cells is clustered and organized with c-Src, Rho A, and focal adhesion kinase (FAK)1 and causes GM3-dependent cell adhesion coupled with activation of these signal transducers (1–3), leading to enhanced cell motility and invasiveness (4, 5). The term “glycosphingolipid signaling domain” or “glycosignaling domain” (GSD) has been assigned to such structural and functional membrane unit (3, 6). GSD appears to be a basic membrane unit found in various types of cells closely associated with GSL function in terms of antigenicity and cell adhesion/recognition coupled with signal transduction (7–9). However, such units may have been overlooked among (or mixed up with) other membrane units having similar properties such as cholesterol-rich, caveolin-containing units (caveolae) involved in endocytosis and signal transduction (10), sphingomyelin (SM)/cholesterol-rich microdomains termed “rafts” (11), or those containing glycosphingosylphatidylinositol (GPI) anchors bearing a number of functionally well-defined receptors (12; for review see Ref. 13).

GSD of B16 cells is enriched in GM3 and SM but has a surprisingly low quantity of cholesterol and phospholipid, whereas caveolae have a large quantity of cholesterol and surprisingly a very small quantity of SM. c-Src, Rho A, and FAK but not caveolin are associated with GSD, whereas Ras and caveolin but not other signal transducers are associated with caveolae (3). To assess the minimal essential components showing GSD function, membranes simulating the properties of GSD were successfully reconstituted by a specific procedure as described in this paper, based on the concept of reconstitution of membrane-simulating organelle function (14). In addition, effects of various synthetic glycosylsphingosines derivatives on GSD function in B16 cells and in reconstituted membranes are compared. Results indicate that sialyl-glycosylsphingosines but not lactosyl-Sph, galactosyl-Sph, or lyso-PC, are capable of disrupting GSD structure and function in B16 cells and reconstituted membranes.

1 The abbreviations used are: FAK, focal adhesion kinase; DIM, detergent-insoluble membranes separated as low density fraction by density gradient centrifugation; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco’s modified Eagle’s medium; GSD, glycosphingolipid signaling domain (“glycosignaling domain”) immunoseparated from DIM by anti-GSL antibodies; Lac-Sph, lacto(sphingosine) (Galβ1–4Glcβ1–1sphingosine); diLac-Sph, dilacto(sphingosine) (Lacβ1–1[Lacβ1–2])sphingosine); lyso-GM3, NeuAcα2–3Galβ1–4Glcβ1–1sphingosine; lyso-PC, lyso-phosphatidylycholine (1-acylglycerophosphorylcholine); NeuNdcAc lyso-GM3, N-dichloroacetyleuroneumylinolβ2–3Galβ1–4Glcβ1–1sphingosine; lyso-PC, lyso-phosphatidylcholine; psychosine, Galβ1–1sphingosine; PAGE, polyacrylamide gel electrophoresis; SM, sphingomyelin; Sph, sphingosine; mAβ, monoclonal antibody; RIPA, radioimmunoprecipitation assay; PBS, phosphate-buffered saline.
Reconstitution of Membranes Simulating GSD

MATERIALS AND METHODS

GSLs, Lyso-GSLs, Lyso-phospholipids, Antibodies, and Other Reagents

An outline of the synthetic scheme for N-acetyleneuraminyl a2→3Galβ1→4Glcβ1→1Sph (lyso-GM3) and N-dichloroacetamino a2→3Galβ1→4Glcβ1→1Sph (lyso-GM3) is shown in Fig. 1. Details of their synthesis will be described elsewhere.2 Sialylla 2→1Sph (SA-Sph), lactosylβ1→1Sph (Lac-Sph) and lactosylβ1→4Glcβ1→1Sph (diLac-Sph) were chemically synthesized as described elsewhere.2

GM3 ganglioside from dog erythrocytes (15), Gg3 (asialo-GM2) from guinea pig erythrocytes (16), LacCer and GlnCer from bovine erythrocytes, and anti-GM3 mAb DH2 (17) were prepared in this laboratory. Pseudochinase was prepared from GelCer by alkaline degradation in butanol (18).

Preparation of c-Src

Mouse c-Src cDNA in pUSE amp was purchased from Upstate Biotechnology (Lake Placid, NY), excised by NotI + HindIII, cloned into a pFastBac HTa NotI-HindIII site, and propagated in insect SF9 cells (ATCC, Rockville, MD) using a baculovirus system (Bac-to-Bac; Life Technologies, Inc.). Infected cells were grown for 4 days, harvested, and washed with phosphate-buffered saline (PBS). Cells were resuspended (107 cells/ml) in PBS containing 1 mM diisopropyl fluorophosphate, 10 μg/ml leupeptin, and 100 μg/ml apotinin. Sonication was performed on ice in a sonifier (Sonifier 450; Branson Equip-

2 Zhang, Y., Hakomori, S., and Sinay, P., unpublished data.
2 Zhang, Y., Toyokuni, T., and Hakomori, S., unpublished data.

Reconstitution of the Membrane with Composition and Properties Similar to Those of GSD

For membrane reconstitution, types of lipids, their proportions, and c-Src quantity were based on those observed originally in GSD fraction (3). Reconstitution of membranes with standard lipid composition, and varying quantities of GM3, other gangliosides, and c-Src, was performed according to the method used for reconstituting the PC-cholesterol membrane with transmembrane receptor (19, 20), with some modification as described below.

Standard Composition of Lipids and c-Src in Reconstituted Membranes—Four different combinations of lipids in chloroform/methanol (2:1, v/v) solution: (i) GM3 (55 μg) and SM (55 μg) (referred to as “SG”); (ii) GM3 (55 μg), SM (55 μg), and PC (55 μg) (referred to as “SGP”); (iii) GM3 (55 μg), SM (22 μg), PC (10 μg), and cholesterol (6.4 μg) (referred to as “SGPC”); (iv) LacCer (50 μg), SM (22 μg), PC (10 μg), and cholesterol (6.4 μg) (referred to as “SLPC”) were mixed separately and dried under a stream of N2. Each of the four dried residues was dissolved in 968 μl of TBS (50 mM Tris-HCl [pH 7.4], 0.14 mM NaCl, 1 mM EDTA, and 50 mM (−1.4%) octyl glucoside. This buffer composition was used for reconstitution of membrane vesicles with properties similar to those of GSD.
Determination of Membrane Reconstituted Components

Lipid Composition of Reconstituted Membranes—Lipids were extracted from reconstituted membrane suspension (1 ml) separated on density gradient centrifugation with 10 ml of methanol and 20 ml of chloroform, sonicated for 15 min, and then centrifuged. The supernatant lipid extract was removed, and the precipitate was extracted again with 10 ml of chloroform/methanol (2:1). The first and second extracts were combined and evaporated, and the residue was dissolved in methanol/water (3:7, v/v), applied to a Bond Elut-packed C18 column (Analyt映chem International, Harbor City, CA), and washed with the same solvent. Finally, lipids were eluted with chloroform/methanol (2:1). Aliquots of total lipid from reconstituted membrane (10–20% of total) were separated by thin layer chromatography (either one- or two-dimensional). The quantity of lipids and their ratio were determined by densitometry and compared with a known quantity of stanched lipid using the Scion Image program (Scion Corporation, Frederick, MD) as described previously (3, 9). Lipid components of reconstituted membrane were compared with those from total detergent-insoluble membranes (DIM) and from the GSD fraction separated by anti-GM3 mAb DH2 (3).

c-Src Associated with Reconstituted Membrane—This was determined by (i) immunoprecipitation with anti-c-Src antibody and (ii) co-immunoprecipitation of c-Src with anti-GM3 mAb DH2. In the procedure for (i), low density reconstituted membrane fraction was 10× diluted with radioimmunoprecipitation assay (RIPA) buffer (30 mM HEPES (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 2 mM sodium 2-mercaptoethanol, 0.5% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin), sonicated 10 min at 4 °C, precleared with Protein A-Sepharose beads, and immunoprecipitated with goat anti-c-Src antibody. RIPA buffer was used to destroy lipid vesicles. In the procedure for (ii), the same membrane fraction was 10× diluted with IP buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 1 mM PMSP, 75 units/ml aprotinin, 0.1% Triton X-100), precleared as above, co-immunoprecipitated with DH2, and washed with IP buffer containing 0.5 mM NaCl. This procedure was necessary to maintain lipid vesicle structure, which is resistant in this medium. The immunoprecipitated c-Src was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting.

Determination of c-Src Phosphorylation Response to Adhesion of Reconstituted Membrane and Effects of Lyso-sphingolipid Derivatives

Basic Procedure for Determination of c-Src Phosphorylation Response—The reconstituted membrane fraction, purified by sucrose density gradient centrifugation, corresponding to Fr. 5 was 10× diluted with kinase buffer (30 mM HEPES (pH 7.5), 10 mM MgCl2, 2 mM MnCl2, 1 mM CaCl2). The c-Src phosphorylation response of membranes, adhered to 10-cm dishes coated with Gg3 or anti-GM3 antibody, was determined by ECL (2). Briefly, 5-ml aliquots of 1% digitonin in Me2SO in order to achieve final 0.001% digitonin concentration of 0.001%. This digitonin concentration makes the membrane vesicles permeable but does not destroy membrane structure. The mixture was then incubated at 37 °C for 5 min, and the reaction was stopped by placing on ice and by addition of 5 ml of stop buffer (15 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1% Triton X-100, 1 mM PMSP). The soluble fraction was transferred to a centrifuge tube (part 1), and the adhered membrane was desorbed and solubilized in RIPA buffer (part 2). Parts 1 and 2 were combined in the test tube (these were termed “solubilized reconstituted membrane” fractions). The c-Src fraction was separated preliminarily by 10% trichloroacetic acid and further immunoprecipitated in RIPA buffer, followed by SDS-PAGE with autoradiography as described previously (3).

Alternative Method for Comparative c-Src Response in Reconstituted Membrane with Varying Content of GM3, Other Gangliosides, and c-Src—The c-Src phosphorylation response of reconstituted membranes having various quantities of GM3 and c-Src was determined by membrane adhesion to Gg3-coated dish. Each response was simultaneously quantified by 32P radioactivity present in immunoprecipitate with anti-c-Src antibody, without separation of c-Src by SDS-PAGE.

The solubilized reconstituted membrane fractions prepared from 32P-labeled c-Src in the phosphorylation reaction mixture in each dish were precipitated with trichloroacetic acid at a final concentration of 10%. The precipitates were centrifuged, washed twice with acetone to eliminate trichloroacetic acid, dissolved in 1 ml of RIPA buffer, pH checked and adjusted to 7.4, mixed with 20 μl of protein G-Sepharose, and placed in a rotary mixer at 4 °C for 2 h. After centrifugation at 270×g for 5 min, the supernatants were collected and added with anti-c-Src goat IgG at a final IgG concentration of 1 μg/ml, incubated at 4 °C overnight, added with 20 μl of protein G-Sepharose, and incubated at 4 °C for 2 h. Next, Sepharose beads were washed five times with RIPA buffer, and the remaining radioactive counts were measured using a scintillation counter (Beckman LS6800 Scintillation System, Fullerton, CA).

Imaging of GM3 Expression in Mouse Melanoma B16/F10 Cells

B16/F10 cell lines were obtained from Dr. I. J. Fidler, M.D. Anderson Cancer Center, University of Texas, Houston, TX and cultured in DMEM supplemented with 10% fetal calf serum. GM3 clusters and intensity expressed at the melanoma B16 cell surface were observed by immunofluorescence with anti-GM3 mAb DH2 as described previously (17), 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 the Fred Hutchinson Cancer Research Center. The degree of immunofluorescence intensity was also determined by flow cytometry using mAb DH2.

Effects of lyso-GSL compounds on GM3 imaging were determined as follows. Detached cells were washed 2× with DMEM, and 2.5× 106 cells per ml of DMEM were mixed with DMEM solution containing lyso-GM3, Lac-Sph, diLac-Sph, SA-Sph, or lyso-PC (each at 5 μM concentration), or 50 μM NeuNacAc lyso-GM3 (MeSO as vehicle; final concentration 0.5%). After 30-min incubation, cells were washed 1× with DMEM and subjected to immunofluorescence followed by microscopy or flow cytometry as described above.

Change of FAK in B16 Cells and of c-Src in Reconstituted Membranes Associated with GM3-dependent Adhesion

The GM3-dependent adhesion of B16 cells to Gg3-coated dishes and the associated change of FAK were determined as described previously (2). The enhanced c-Src activity of the DIM membrane fraction in response to GM3-dependent adhesion of membrane to the Gg3-coated dish was determined as described previously (3). The effects of preincubation with Lac-Sph and lyso-GM3 on FAK response of the B16 cells associated with GM3-dependent cell adhesion were determined (see the legend of Fig. 7A) as well as the c-Src phosphorylation response associated with GM3-dependent adhesion of DIM membrane (see the legends of Figs. 7 and 8).

RESULTS

Reconstituted Membranes Simulating the Glycosignaling Domain (GSD)—Although the lipid composition and associated signal transducer molecules in GSD of mouse melanoma B16 cells are well documented and distinct from those of caveolin-containing fraction (caveolae) (3), the components essential for GSD function are not clearly identified. To better identify these components, a mixture of membrane lipids and c-Src was processed to obtain reconstituted membranes showing GM3-dependent cell adhesion and associated activation of c-Src, by the procedure described under “Materials and Methods.”

Lipid composition of reconstituted membranes, separated as low-density fraction, is shown in Fig. 2A, in comparison with the composition of the DIM fraction and the GSD membrane.
were (i) 10

stituted membranes purified by sucrose density gradient centrifugation
cated. In means of two independent experiments, with standard variation indi-

3

lated as Fr. 5). All lane 3

precipitated with anti-GM3 mAb DH2, washed with IP buffer contain-

ing 0.5M NaCl, and Western blotted (

\( \text{phosphatidylethanolamine; Chl} \)

DIM, although the lipid composition of total DIM is very different.
reconstitured membrane is similar to that in GSD separated from total

composition was determined using aliquots of 10–20% total lipids as

tion. Total lipids of reconstitured membrane were extracted, and

osition of reconstitured membranes as compared with the low density,

tured to dishes coated with GlcCer, Gg3, or anti-GM3 mAb. Membranes

e with four different lipid compositions when placed on
dishes coated with GlcCer, Gg3, or anti-GM3 mAb. Membranes

e different lipid components and proportions were reconstituted

c-Src as described in the text and purified by sucrose density

GM3, but distinctively different from those in total DIM (Fr. 5)
moves similar to those of the GSD fraction separated by anti-

GM3, and c-Src-containing fraction (corresponding to GSD) immunoseparated

from DIM (from \( 2 \times 10^{6} \) B16 cells) by anti-GM3 mAb DH2. Right group,

composition of reconstituted membrane vesicles prepared from a mixture

of SM/GM3/PC/cholesterol/c-Src (22, 55, 10, 6.4, and 3 \( \mu \)g, re-

spectively) and separated as Fr. 5 by sucrose density gradient centrifuga-

tion. Note that the ratio of SM:GM3:PC in the resulting

reconstitured membrane is similar to that in GSD separated from total

DIM, although the lipid composition of total DIM is very different. PE,

phosphatidylethanolamine; Chl, cholesterol. Values shown are the

means of two independent experiments, with standard variation indi-

cated. B, c-Src present in reconstitured membranes. Aliquots of recon-

stituted membranes purified by sucrose density gradient centrifugation

were (i) 10\times diluted with RIPA buffer, immunoprecipitated with goat

anti-c-Src antibodies after preclearance, and Western blotted (lane 1);

(ii) 10\times diluted with IP buffer containing 0.1% Triton X-100, immuno-

precipitated with anti-GM3 mAb DH2, washed with IP buffer contain-

0.5M NaCl, and Western blotted (lane 2); (iii) same as ii, but using

normal mouse IgG instead of DH2, and Western blotted (lane 3).

All three lanes were Western blotted with rabbit anti-c-Src antibodies. For

the rationale for differential use of RIPA and IP buffer, see “Materials

and Methods,” c-Src Associated with Reconstitured Membrane.” Results

shown are from one typical experiment.

separated by the anti-GM3 antibody (DH2) from \( 2 \times 10^{8} \) mel-

anoma B16 cells. The lipid composition of reconstituted mem-

branes shown in Fig. 2A was based on 55 \( \mu \)g GM3, 22 \( \mu \)g SM,

10 \( \mu \)g PC, 6.4 \( \mu \)g cholesterol, and 3 \( \mu \)g c-Src. The quantity

of GM3 and SM recovered in reconstitured membrane, separated

by sucrose density gradient centrifugation, was \( \sim 22\% \) and

\( \sim 43\% \), respectively, of the original GM3 and SM used for

brane reconstitution. It is noteworthy that the proportions of

GM3, SM, and PC in the reconstitured membrane fraction were

very similar to those of the GSD fraction separated by anti-

GM3, but distinctively different from those in total DIM (Fr. 5)

prepared from B16 cells. Phosphatidyserine and phosphati-

dyethanolamine were present in the DIM fraction but absent

in the reconstitured membrane because we did not include

these phospholipids. c-Src was clearly present in reconstitured

membrane and was co-immunoprecipitated with GM3 (Fig.

2B). This finding indicates a close association of these compo-

nents in the reconstitured membrane, similar to GSD.
The degree of c-Src phosphorylation induced by adhesion of reconstituted membrane to Gg3-coated versus GlcCer-coated dishes was quantified by densitometric scanning of autoradiography bands (Fig. 3B). The c-Src phosphorylation response occurred only in membrane containing GM3/SM/c-Src and was higher following addition of PC or PC/cholesterol. Optimal response was observed for the composition GM3 (55 μg), SM (22 μg), PC (10 μg), and cholesterol (6.4 μg) (referred to as “SGPC”) and hereby termed “standard composition”). There was no response for the membrane with LacCer in place of GM3 (SLPC).

Strong c-Src phosphorylation was also observed when GM3/SM/PC/cholesterol/c-Src-reconstituted membrane was adhered to the DH2-coated dish (Fig. 3C, lane 3). No response was observed for membrane without GM3, i.e. LacCer/SM/PC/cholesterol/c-Src (lane 5), or placed in a nonadherent polypropylene tube (lane 1), or on a dish coated with normal mouse IgG (lanes 2 and 4).

Effect of Varying Quantities of GM3 and c-Src, and Substitution of GM3 by Other Gangliosides, on c-Src Phosphorylation Response of Reconstituted Membrane—The c-Src phosphorylation response of the reconstituted membrane was optimal with the standard composition and c-Src quantity of 3 μg (50 pmol) as described above. The response decreased significantly when GM3 quantity was decreased, and decreased slightly when GM3 quantity was doubled (from 55 to 110 μg). When the c-Src quantity was decreased to 1.5 μg (25 pmol) or 0.75 μg (12.5 pmol), response also decreased significantly (Fig. 4A).

When GM3 in reconstituted membrane (with a fixed quantity of other lipids and c-Src) was replaced with GM1 or GD1a and the membrane was placed on the Gg3-coated dish, c-Src phosphorylation response was very low (Fig. 4B). This is consistent with the previous observation that only GM3, not GM1, interacts with Gg3 (4).

Effects of Lyso-GM3 and Its Analogues on GM3 Expression Pattern and on GM3-dependent B16 Cell Adhesion—In a search for specific compounds that disrupt GSL clusters, which play a central role in maintenance of structure and function of GSD, lyso-GSLs and their derivatives were considered to interrupt cis-interaction between GSLs (see “Discussion”). The effects of various lyso-compounds (lyso-GM3, NeuNdcAc lyso-GM3, SA-Sph, Lac-Sph, diLac-Sph, psychosine, lyso-PC) on GSL function, in terms of GM3-dependent B16 cell adhesion associated with FAK enhancement and c-Src phosphorylation response, were studied initially.

Preincubation of B16 cells with 0.5–10 μM synthetic lyso-GM3 followed by washing with DMEM strongly inhibited the subsequent adhesion of cells to the Gg3-coated dish. Similarly, preincubation of cells with NeuNdcAc lyso-GM3 inhibited this adhesion at much higher concentrations (5–50 μM). In contrast, psychosine, lyso-PC, Lac-Sph, and diLac-Sph had no inhibitory effect even at 10 μM (Fig. 5A). Filipin at 0.07–0.47 μM, and nystatin at 16–54 μM, which at these concentrations destroy caveola function, did not inhibit this GM3-dependent cell adhesion (Fig. 5A). Based on the reduced viability of cells determined by Trypan Blue exclusion test, lyso-GM3 had no cytotoxic effect up to 10 μM but became cytotoxic at >20 μM. NeuNdcAc lyso-GM3 had no cytotoxic effect even at 100 μM (Fig. 5B).

The inhibitory effect of 1–5 μM lyso-GM3 or 50 μM NeuNdcAc lyso-GM3 on GM3-dependent B16 cell adhesion is ascribable to reduced anti-GM3 mAb binding, as was observed clearly by confocal microscopy (Fig. 6A) and by fluorescent microscopy with digital imaging (data not shown). The expression pattern was not changed when cells were pretreated with 1–20 μM lactosyl-Sph or 1–10 μM lyso-PC. Pretreatment of cells with 50 μM NeuNdcAc lyso-GM3 clearly reduced GM3 expression observed by flow cytometry (Fig. 6B), and anti-GM3 mAb DH2 did not cross-react with this compound (Fig. 6C). The great reduction of GM3 expression by pretreatment of cells with even higher concentrations of lyso-GM3 (5–10 μM) was not due to release of GM3 from the cell surface, because this treatment did not change the cell GM3 content (Fig. 6D).

Effects of Lyso-GM3 and Its Analogues on Cellular FAK Activity and on Membrane c-Src Activity Associated with GM3-dependent Adhesion—An increase of FAK activity associated with the GM3-dependent adhesion of B16 cells to the Gg3-coated dish was observed as described previously (2). This
effect was blocked by preincubation of B16 cells with 1–5 μM lyso-GM3 or 25–50 μM NeuNdcAc lyso-GM3 followed by washing and adhesion but not by preincubation with 10 μM lactosyl-Sph (Fig. 7A).

The great enhancement of c-Src activity in low density membrane fraction (DIM fraction 5) upon adhesion of the membrane to the Gg3-coated dish was inhibited by 1–10 μM lyso-GM3 or 50 μM NeuNdcAc lyso-GM3 but was unaffected by 10 μM lactosyl-Sph (Fig. 7B).

Susceptibility of Reconstituted Membrane to Lyso-GM3 Is Similar to That of GSD of B16 Cells—Analogous to GSD of native B16 cells (see above), c-Src phosphorylation response in the reconstituted membrane was strongly blocked in the presence of 1 or 5 μM lyso-GM3 (Fig. 8, lanes 6 and 7), 50 μM NeuNdcAc lyso-GM3 (lane 5), or 1 or 5 μM SA-Sph but was maintained when the membrane was incubated with GM3 or Lac-Sph (lanes 3 and 4) or psychosine (lane 8).

These results indicate that the reconstituted membrane containing GM3, SM, and c-Src as basic components was capable of c-Src activation response to GM3-dependent adhesion and the response was disrupted by lyso-GM3 and its analogue, similarly to naturally occurring GSD in melanoma B16 cells.

**DISCUSSION**

Cellular polarity based on uneven distribution of membrane sphingolipids, e.g., in apical and basolateral epithelial membranes (21), gave rise to the concept that membranes consist of different subdomains. Topological characteristics of glycosphingolipids (GSLs) are indicated by large-scale clustering of

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**FIG. 5.** Effect of lyso-GM3, other lyso-GSLs, and lyso-PC on GM3-dependent adhesion and viability of melanoma B16 cells. A, effect on B16 cell adhesion to Gg3-coated dishes. B16/F10 cells detached by EDTA/trypsin were preincubated for 30 min with various concentrations (0.05–50 μM) of GM3 (○), lyso-GM3 (▲), NeuNdcAc lyso-GM3 (small ●), dilactosyl-Sph (□), lyso-PC (■), psychosine (●), filipin (large ●), or nystatin (●) in DMEM using Me2SO as vehicle (final concentration of Me2SO in DMEM, 0.5%), followed by washing with Me2SO and plating on Gg3- or GlcCer-coated 24-well dishes for adhesion assay. Open bars at left, GlcCer-coated dishes as positive control. Open bar, GlcCer-coated dishes as negative control. Adhesion was expressed as percentage of total cells added on dish. B, effect on viability of cells determined using the Trypan Blue exclusion test. Cells were treated with various lyso compounds as in A and subjected to a viability test. These cells were the same samples treated with lyso compounds and used for adhesion assay. Symbols for reagents are the same as those in A. Assays for cell adhesion and for viability are described in Ref. 3. For both A and B, values for lyso-GM3 and NeuNdcAc lyso-GM3 are means from three independent experiments, values for other compounds are means from two independent experiments, and standard variation is <10%.

**FIG. 6.** Imaging of GM3 distribution in melanoma B16 cells treated with various lyso-GSLs. A, fluorescent images of GM3 distribution pattern by confocal microscopy. Control: cells in DMEM containing 0.5% Me2SO. Others: cells treated with DMEM containing various reagents, with Me2SO as vehicle (final concentration, 0.5%). Lac-Sph, 5 μM; Lyso-PC, 5 μM; Lyso-GM3, 5 μM; SA-Sph, 5 μM; NeuNdcAc lyso-GM3, 50 μM. Cells were incubated for 30 min, washed, then immunostained with anti-GM3 mAb DH2. After incubation with a second antibody, cells were washed 3× with PBS, fixed with 1% paraformaldehyde (4 °C, 30 min), washed 1× with PBS, placed on a chamber slide (Nunc Inc., Naperville, IL), affixed by centrifugation (900 rpm, 3 min), mounted with Fluorogard reagent (Bio-Rad), and subjected to confocal microscopy. B, flow cytometry of B16 cells treated with 50 μM NeuNdcAc lyso-GM3 (peak 2) as compared with untreated cells (peak 1) and control cells without antibody (peak 1). Cells treated in the same way as for the adhesion assay were washed 3× with PBS, incubated with PBS containing mAb DH2 (1 μg/ml) at 4 °C for 1 h, washed 2× with PBS, incubated with PBS containing fluorescein isothiocyanate goat anti-mouse IgG at 4 °C for 1 h, and subjected to flow cytometry. C, reactivity of anti-GM3 mAb DH2 with GM3 (spot 1), lyso-GM3 (spot 2), NeuNdcAc lyso-GM3 (spot 3), Lac-Sph (spot 4), and diLac-Sph (spot 5). Dot blot analysis was performed using a “Bio-dot” apparatus (Bio-Rad) as described previously (5). D, B16 cells (2.5 × 10⁶) were incubated in 1 ml of DMEM containing 0.5% Me2SO without (as control) or with 10 μM lyso-GM3 (final concentration) for 30 min at 37 °C. Cells were centrifuged, and the cell pellet (of the same protein quantity) was extracted using chloroform/methanol (2:1). The total GSL fraction was developed on thin layer chromatography with orcinol-sulfuric acid spray. Note that both control and lyso-GM3-treated cells show the same GM3 duplet. Other bands are not identified.
Reconstitution of Membranes Simulating GSD

GSLS at the cell surface (22) and the insolubility of GSLs in buffer solution containing neutral or zwitterionic detergent (12, 23). A number of subsequent studies indicate that sphingolipids and cholesterol are associated with GPI anchors and Src family kinases to form structural and functional domains (for reviews see Refs. 11 and 13), similar to membranes derived from caveolae, which are involved in endocytosis and signal transduction (for review see Ref. 10). However, little attention has been paid to the functional notion of GSLs in either sphingolipid/cholesterol domains or caveolae.

Our previous studies provide evidence that clustered GM3 or other GSLs, organized with c-Src, Rho A, and FAK at the surface of mouse melanoma B16 cells, form structural and functional units involved in GM3-dependent cell adhesion as well as signal transduction (1, 2) to promote cell motility (5). Such structural units coexist in detergent-insoluble, low density membrane fraction (DIM) with, but can also be immunoprecipitated by blotting of the same sample with anti-FAK antibody (lower gel, anti-FAK), as described previously (2). Cells were preincubated in DMEM (with MeSO4 as vehicle; final concentration, 0.5%) containing lyso-GM3 (1 or 5 μM; lanes 3 and 4), NeuNdAc lyso-GM3 (1, 10, 25, 50 μM; lanes 5–8), or Lac-Sph (10 μM; lane 10) for 30 min, washed with DMEM, added to GlcCer-coated dishes, and adhered by centrifugation. Cells placed in polypropylene tubes (no adhesion; resting cells, lane 1); cells added to GlcCer-coated dishes (negative control, lane 2); or cells added to GlcCer-coated dish without preincubation in any reagent (positive control, lane 9). B, effect of various lyso compounds on enhanced c-Src activity in membrane, associated with adhesion of membrane to the GlcCer-coated dish. DIM containing GSLs, preincubated in DMEM without reagent but containing 0.5% MeSO4, were added on the GlcCer-coated dish (negative control, lane 7), or cells added to GlcCer-coated dish without preincubation in any reagent (positive control, lane 9). B, effect of various lyso compounds on enhanced c-Src activity in membrane, associated with adhesion of membrane to the GlcCer-coated dish. DIM containing GSLs, preincubated in DMEM without reagent but containing 0.5% MeSO4, were added on the GlcCer-coated dish (negative control, lane 7), or cells added to GlcCer-coated dish without preincubation in any reagent (positive control, lane 9).

Reconstituted membranes are characterized by the following properties: (i) lipid composition (major lipids GM3 and SM; much smaller quantities of PC and cholesterol) is very similar to that of naturally occurring GSD membranes immunoseparated from caveolae fraction present in DIM fraction; (ii) c-Src in the membrane is closely associated with GM3 as shown by co-immunoprecipitation; (iii) the membranes bind to the solid phase coated with GlcCer or anti-GM3 mAb, with consequent activation of c-Src phosphorylation, but do not bind to the GlcCer-coated solid phase, and hence no c-Src phosphorylation occurs; (iv) optimal adhesion and c-Src phosphorylation response are observed with a certain composition of GM3, c-Src, and other lipid components, but the response is lower when the quantity of GM3 or c-Src is reduced; (v) the reconstituted membrane in which GM3 is replaced by GlcCer, GM1, or LacCer does not show binding to GlcCer or anti-GM3 mAb, or consequent c-Src phosphorylation response; (vi) addition of cholesterol and PC significantly increases the c-Src phosphorylation response; and (vii) binding to solid-phase GlcCer and c-Src phosphorylation response is blocked by lyso-GM3 or its derivative, but the binding is not affected by lyso-PC, psychosine, or Lac-Sph. All these properties of reconstituted membranes are very similar to those of GSD in B16 cells (2, 3), indicating that GM3, SM, and c-Src are essential components of GSD, whereas PC and cholesterol are auxiliary components that enhance or stabilize GSD function.
A major question remaining to be elucidated is the mechanism by which c-Src incorporated in the lipid bilayer is activated when external GM3 is stimulated by its ligand. In view of recent findings on crystal structure, c-Src activation is thought to be based on disordering of autoinhibitory interaction between intramolecular subunits, leading to Tyr416 autophosphorylation. This is associated with distortion of an ordered helical loop located between the “N-lobe” and the “C-lobe” of the kinase domain (25). Activation does not necessarily always depend on dephosphorylation of Tyr527 at the C terminus, which was previously considered to lock the molecule in an inhibited conformation (26). Tyr527-independent activation of Src kinase through sulfhydryl group modification was proposed recently (27).

c-Src produced in SF9 cells using the baculovirus system is an inactive form that lacks Tyr416 phosphorylation. Such inactive c-Src is activated by external ligand bound to SH3 and SH2 domains (25, 28). It is crucial to elucidate the mechanism causing activation of c-Src, bound to the lipid bilayer, by enhanced clustering of GM3. The mechanism may be analogous to activation of cytoplasmic tyrosine kinase induced by growth factor-dependent clustering of its receptor (29).

Glycosyl-Sph, having sialic acid and N-unsubstituted Sph, was found to disrupt GSD function, as indicated by inhibition of GM3 immunostaining by anti-GM3 DH2, and inhibition of GM3-dependent adhesion and associated FAK activity and c-Src phosphorylation response. The simplest compound of this type is SA-Sph, as previously found (30). In the present study, two other compounds, lyso-GM3 and NeuNdeClyso-GM3, were found to also disrupt GSD function. Other lyso-GSLs and lyso-PC had no effect at similar concentration. The effect of glycosyl-Sph on GSD is in striking contrast to the effect of cholesterol-binding reagents that disrupt caveolae structure and function. Significantly, the disruptive effect of lyso-GM3 and its analogue on GSD in reconstituted membrane is similar to that in native B16 cells.

The effect of lyso-GM3 on GSD function may well be due to disruption of cis interaction of GM3 and consequent dispersion of GM3 clustering, although further extensive study is needed to clarify the exact mechanism. Preincubation of cells with 1–5 μM lyso-GM3, followed by washing, greatly reduced staining with anti-GM3 mAb DH2 and GM3 clustering, without changing the cellular level of GM3. The reduction of DH2 staining was not due to competitive inhibition of DH2 binding to the cell surface by lyso-GM3, because cells were washed before the DH2 was added, lyso-GM3 cross-reacts with DH2 (Fig. 5C), and a large quantity of lyso-GM3 was inserted in the membrane even though the GM3 content was unchanged (Fig. 5D). Lyso-GM3, therefore, may cause a change in GM3 organization (e.g. dispersion of GM3 clustering as above). The blocking effect of lyso-GM3 and its analogue on FAK activity and c-Src phosphorylation response must be considered a consequence of GM3 organizational change in GSD, in both B16 cells and in reconstituted membrane.

Activated c-Src often appears as a doublet band on SDS gel, analogous to membrane-bound c-Src (31) (see legend of Fig. 3A). An effect on GSD similar to that observed for lyso-GM3 was found previously for sialylα1–2Sph (30). Neither filipin nor nystatin had an effect on GM3-dependent adhesion and associated FAK activation (3) at doses that disrupt caveolae structure and function (32, 33). Interestingly, the reconstituted membrane showed the same susceptibility to lyso-ganglioside analogues as the original B16 cells or isolated GSD membrane fraction.

In summary, reconstituted membranes show all the characteristic properties of GSD from melanoma B16 cells. GSD from other cell types is characterized by different major GSLs in combination with different signal transducers (7–9, 24). A close association and interaction between clustered GSLs and signal transducers, particularly Src family kinases, small G-proteins, and FAK, play a central role in cellular interactions coupled with signal transmission in general.

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