Separation of “Glycosphingolipid Signaling Domain” from Caveolin-containing Membrane Fraction in Mouse Melanoma B16 Cells and Its Role in Cell Adhesion Coupled with Signaling*

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Two membrane subfractions, one enriched in GM3 ganglioside and the other containing caveolin, were separated from low density detergent-insoluble membrane fraction prepared by sucrose density gradient centrifugation of postnuclear fraction of mouse melanoma B16 cells. The GM3-enriched subfraction, separated by anti-GM3 monoclonal antibody DH2, contained sphingomyelin, cholesterol, c-Src, and Rho A but not caveolin. In contrast, the caveolin-containing subfraction, separated by anti-caveolin antibody, contained neither GM3, c-Src, nor Rho A but did contain glucosylceramide, Ras, a very small quantity of sphingomyelin, and a very large quantity of cholesterol. The GM3/c-Src-enriched membrane subfraction was characterized by (i) maintenance of GM3-dependent adhesion and (ii) susceptibility to being activated for signal transduction through GM3. ³²P-Phosphorylation of c-Src (M, 60,000) together with two other components (M, 45,000 and 29,000) was enhanced in the fraction bound to dishes coated with asialo-GM2 (Gg3) or with anti-GM3 monoclonal antibody DH2, detected by incubation with [γ-³²P]ATP at 37 °C for 5 min. GM3-dependent adhesion of B16 cells to Gg3-coated dishes and associated signaling were not reduced or abolished in the presence of either filipin or nystatin, which are cholesterol-binding reagents known to abolish caveolae structure and function. B16 melanoma cells incubated with filipin (0.16–0.3 μg/ml) or with nystatin (25 μg/ml) for 30 min showed depletion of cholesterol in detergent-insoluble membrane fraction but were still capable of binding to Gg3-coated plate and capable of the associated signaling. Thus, the GM3-enriched subfraction, involved in cell adhesion and capable of sending signals through GM3, represents a membrane domain distinguishable from caveolin-containing subfraction or caveolae. This microdomain is hereby termed the “glycosphingolipid signaling domain” or “glycosignaling domain”.

Glycosphingolipids (GSLs)³ are known as cell surface anti-

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1 The abbreviations used are: GSL, glycosphingolipid; Cer, ceramide; DIM, detergent-insoluble membrane fraction; Gg3, asialo-GM2 (GalNAcβ3Galβ4Galβ3Glcb1Cer); GlcCer, Glcβ1Cer; GM3, NeuAcα3Galβ3-4Glcβ1Cer; Leα, Fucα2Galβ4Fucα3GlcNAcβ3; IP, immunoprecipitation; PBS, phosphate-buffered saline; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophore-

sis; SM, sphingomyelin; RIPA, radioimmuneprecipitation assay; FAK, focal adhesion kinase; PVDF, polyvinylidene difluoride; mAb, monoclonal antibody; GM1, Galβ3GalNAcβ3[NeuAcα3Galβ4Glcβ1Cer. 

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hereby termed the "glycosphingolipid signaling domain" or "glycosignaling domain."

EXPERIMENTAL PROCEDURES

GSls, Antibodies, and Other Reagents—GM3 (N-acetyl) was prepared from dog erythrocytes (28). Gg3 was prepared from guinea pig blood (29). GlcCer was purchased from Matreya, Inc. (Pleasant Gap, PA). Anti-GM3 mAb DH2 (mouse IgG3) (30), anti-Gg3 mAb 2D4 (mouse IgM) (31), and anti-Lcα2 mAb AH6 (mouse IgM) (32) were described as previously. Sources and properties of antibodies directed to caveolin, c-Src, and other molecules involved in signal transduction are described under the procedures using these antibodies. Phosphatidylethanolamine, phosphatidyserine, phosphatidylcholine, phosphatidinositol, SM, cholesterol, Cer, PMSF, filipin, and nystatin were purchased from Sigma.

Preparation of Low Density Detergent-insoluble Membrane Fraction (DIM)—Cells were harvested in 0.02% EDTA-PBS (PBS: 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, pH 7.4), lysed, homogenized, and subjected to sucrose density gradient centrifugation to separate low density light-scattering membrane fraction by a modification of the method described previously (14, 27). Briefly, 1–5 × 107 cells were suspended in 1 ml of lysis buffer (1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 75 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, pH 7.4), and suspended with 100 μg/ml of anti-caveolin rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and dried under gentle shaking on a rocker (20 movements/min) at room temperature. The coated dishes were subsequently placed again in a rotary mixer for 2 h. The beads were washed three times with IP buffer by centrifugation at 270 × g for 2 min, followed by washing with IP buffer by centrifugation at 270 × g for 2 min. The beads were washed three times with IP buffer by centrifugation at 270 × g for 2 min, followed by washing with IP buffer by centrifugation at 270 × g for 2 min.

The immunoprecipitates on beads were treated differently depending on subsequent analysis. (i) For Western blot analysis, the washed beads were suspended with 100 μl of sample buffer with 2-ME, heated to 95 °C for 3 min, and centrifuged at 1000 × g for 2 min, and the supernatant was subjected to SDS-PAGE. (ii) For dot-blot analysis, bound materials on beads were eluted by 1 ml of 0.2 M glycine-HCl (pH 2.3) containing 1% Triton X-100 with sonication in ice-water bath for 15 s. After centrifugation at 270 × g for 2 min, resultant supernatants were subjected to the assay. (iii) For the second immunoprecipitation analysis, immunoprecipitates on beads were eluted by mixing with 0.2 M glycine-HCl (pH 2.3) containing 1% Triton X-100 as above and centrifugation at 270 × g for 2 min. The supernatants were collected and immediately neutralized with 1 ml Tris-HCl (pH 10) to pH 7.4 and then diluted 10× with IP buffer and incubated with 1 μg/ml anti-caveolin IgG, anti-GM3 mAb DH2 or normal rabbit IgG in a rotary mixer at 4 °C overnight. The mixtures were added with 50 μl of protein G-Sepharose beads and placed again in a rotary mixer for 2 h. Beads were washed three times with IP buffer, by centrifugation at 270 × g for 2 min, and then suspended with 100 μl of sample buffer with 2-ME, heated to 95 °C for 3 min, and centrifuged at 1000 × g for 2 min, and the supernatant was subjected to SDS-PAGE.

Western blot analysis was performed as described previously (26, 27). Briefly, proteins separated on 5–15% linear gradient SDS-PAGE under reducing conditions were electrophoretically transferred to PVDF membranes. The membranes were incubated with anti-c-Src rabbit or goat IgG (Santa Cruz Biotechnology), anti-Rho A mouse mAb (Santa Cruz Biotechnology), anti-Ras rat mAb (Santa Cruz Biotechnology), or anti-FAK rabbit IgG (Santa Cruz Biotechnology) for 2 h at room temperature and then incubated with horseradish peroxidase-labeled proper secondary antibody. The reacted proteins were detected by SuperSignal chemiluminescent substrate. Dot blot analysis was performed as described above.

3. Src Activation Assay—Dim was 10× diluted with kinase buffer (30 mM HEPES (pH 7.5), 10 mM MgCl2, 2 mM MnCl2, 1 mM CaCl2) with a final protein concentration of 2 μg/ml. Aliquots (5 μl) of the diluted fraction were added to dishes coated with Gg3, GM3, or GlcCer, followed by centrifugation at 2000 × g at 4 °C for 30 min. The dishes were left for 15 h on ice to ensure membrane adhesion and then further incubated at 25 °C for 2 h with gentle shaking on roller (10 movements/min), and washed three times with Tris-NaCl-cation buffer after washing out of nonadhered material, adherent material on dishes was dosed by the addition of 5 ml of lysis buffer with sonication for 5 min in a water bath sonicator (Branson, Danbury, CT). The desorbed material was subjected to immunoblot assay to detect GM3 or caveolin using specific antibodies by the method of Towbin et al. (33) with modification. A PVDF membrane (Immobilon-P, Millipore Corp., Bedford, MA) was soaked in fixation buffer (62.5 mM Tris-HCl (pH 8.9), containing 5% methanol) for at least 2 h before assembly onto Bio-Dot apparatus (Bio-Rad, Hemel Hempstead, UK). The adsorbed fraction was loaded on PVDF membrane by aspiration. Next, the blotted membranes were washed in fixation buffer and dried. Dim diluted to various concentrations was also blotted and used as standards. After brief soaking in methanol, the membranes were blocked in 5% skim milk in TBS-T (10 mM Tris-HCl (pH 8.0), 150 mM NaCl containing 0.05% Tween 20) at room temperature for 3 h, and then incubated with 1 μg/ml anti-GM3 mAb DH2 or rabbit anti-caveolin IgG in TBS-T containing 1% normal goat serum at room temperature for 1 h. After extensive washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) or horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) (1:1000) for 1 h. The membranes were washed twice with TBS-T and developed using chemiluminescence method with Super-Signal Chemiluminescent Peroxidase (Pierce).
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1.0 ml of IP buffer, 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 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, those beads were washed five times with IP buffer containing 0.5 mM NaCl, and boiled with SDS-sample buffer containing 5% 2-ME. In some experiments, RIPA buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM NaVO₄, 50 mM NaF, 1 mM PMSF, 10 µg/ml peptatin A, 10 µg/ml leupeptin, 75 units/ml aprotinin) was used for solubilization after acetone precipitation and washing of immunoprecipitates. Instead of IP buffer (34). The samples were run on 5–15% SDS-PAGE, transferred, and blotted on PVDF membranes. Autoradiography was carried out by exposing the electroblotted membranes to Kodak XAR x-ray film at −80 °C with intensifying screens (DuPont Lightning Plus). After autoradiography, the blotted membranes were probed with anti-c-Src rabbit IgG to determine the amount of protein in each lane. The bound antibodies on PVDF membrane were stripped using 100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl (pH 6.7), and reprobed with anti-erb-1 rabbit IgG (Santa Cruz Biotechnology) and anti-mycosin light chain mAb (Sigma) to analyze proteins co-immunoprecipitated with c-Src.

Lipid Component Analysis of DIM and Subfractions—The membrane subfractions immunoseparated with antibodies were mixed with 1 ml of methanol, sonicated for 15 min, added with 2 ml of chloroform, and further sonicated for 5 min. To extract lipids from liquid samples, volumes of methanol was added, and the mixture was sonicated for 15 min in a water bath sonicator and then added with 20 volumes of chloroform, sonicated for 5 min, and centrifuged at 1500 × g at 4 °C for 15 min. The supernatants were collected, dried under an N₂ stream, sonicated in methanol, added with water, and sonicated again (final solution contained methanol/water, 3:7, v/v). The solutions were applied to Bond Elut packed columns (1 ml, C18, Analytichem International, Harbor, CA) washed with chloroform/methanol (2:1, v/v) and preequilibrated with methanol/water (3:7). The columns were extensively washed with distilled water, and bound materials were eluted with 2 ml of chloroform/methanol (2:1) followed by 1 ml of chloroform. The eluates were dried under N₂ and dissolved in 20 µl of chloroform/methanol (2:1).

Two-dimensional thin layer chromatography was carried out according to the method of Yokoyama et al. (35). In brief, samples were spotted at the lower left-hand corner of a 10 × 10-cm high performance thin layer chromatography plate (Merck). The first chromatographic run was performed with chloroform/methanol/formic acid/water (65:25:8.8:1, v/v) at 21 °C. The second run was performed with chloroform/methanol/4.4 N ammonia 50:40:10 (v/v/v) at a rotation of 90° from the first direction. The third run was performed with diethylthreitol in a direction opposite to that of second run. The plate was sprayed by 0.03% prilinum (Aldrich) in acetone/water (50:20, v/v) and photographed under UV light. For autoradiography, TLC plates were exposed to Kodak BioMax MS film at −80 °C with Kodak TranScreen-LE intensifying screen. Effects of Filipin and Nystatin on 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 Dulbecco’s modified Eagle’s medium at 1.25 × 10⁶ cells/ml. Aliquots of cell suspension were mixed with Me₂SO alone (final concentration 0.5%, as control) or Me₂SO containing various quantities of filipin (final concentration 0.16–2.5 µg/ml) or 25 µg/ml nystatin in polypropylene tubes and incubated for 30 min at 37 °C. After incubation, the reaction mixtures were centrifuged at 270 × g for 10 min, cell pellets were suspended in 1 ml of lysis buffer, and DIM was prepared as described above. For each DIM sample, cholesterol was isolated as described under “Lipid Component Analysis of DIM and Subfractions” and measured by the method of Gamble et al. (36).

RESULTS

Separation of Low Density Membrane Fraction into Two Distinct Subfractions, One Containing GM3 Exclusively and the Other Containing Caveolin Exclusively—The presence of two clearly distinguishable membrane subfractions in low density DIM prepared from B16 melanoma cells was indicated by two different separation methods, i.e. (i) separation based on antibody binding to the antigen expressed in membrane components (immunoseparation) and (ii) separation based on specific interaction between GM3 (on membrane) and GlcCer (coated on dish) (27), followed by immunoblot analysis of membrane subfractions using anti-GM3 and anti-caveolin antibodies. The DIM subfraction adhered to the GlcCer-coated dish, but not the subfraction nonspecifically adhered to the GM3- or GlcCer-coated dish, showed positive blotting with anti-GM3 mAb DH2. Pretreatment of the GlcC3-coated dish with anti-GM3 mAb 2D4, but not with isotype-matched control mAb AH6 (anti-Le¹), inhibited binding of the GM3-containing subfraction (Fig. 1A). The subfraction immunoprecipitated with anti-c-Src showed...
binding (positive blotting) with anti-GM3, whereas that immunoprecipitated with anti-caveolin IgG did not (negative blotting) (Fig. 1B). The subtraction bound to anti-caveolin and that unbound to anti-GM3-coated dish were positively immunoblotted with anti-caveolin (Fig. 1C). In Western blotting analysis with anti-caveolin antibody, the IP with anti-caveolin showed a band corresponding to caveolin (M_r ~ 21,000) (Fig. 1D, lane 2), but the IP with anti-GM3 or control normal rabbit IgG did not show such a band (lanes 1 and 5). A second IP with anti-GM3, using eluate from IP with anti-caveolin, did not show a caveolin band (lane 3), although a second IP with anti-caveolin showed a clear caveolin band (lane 4).

c-Src Is Associated with GM3 but Not with Caveolin, and Ras H Is Associated with Caveolin—In Western blotting analysis of DIM subfractions, a major band corresponding to c-Src was present in subfractions immunoprecipitated with anti-GM3 (Fig. 2A, lane 2) but not with anti-caveolin (lane 3). A band corresponding to caveolin was observed in a fraction immunoprecipitated with anti-caveolin (Fig. 2B, lane 3) but not with anti-c-Src (lane 1) or anti-GM3 (lane 2). The presence of Rho and FAK, probed by their respective antibodies in Western blot analysis, was found consistently in co-immunoprecipitated fraction from DIM of B16 melanoma cells (data not shown).

We demonstrated previously that anti-GM3 mAb DH2 co-immunoprecipitates c-Src, Rho A, and FAK (27). In the present study, caveolin was co-immunoprecipitated with Ras H but not with Rho A or FAK (Fig. 3). Under our experimental conditions, DH2 did not co-immunoprecipitate Ras H (data not shown), although enhanced Ras H activity in terms of GTP binding was induced by Glc3 (27).

32P Phosphorylation of c-Src and M_r 45,000 and 29,000 Bands in DIM Is Induced by Binding of GM3 to Its Ligands (Gg3 and Anti-GM3 mAb)—c-Src was rapidly 32P-phosphorylated with two other components with M_r 45,000 and 29,000, which co-immunoprecipitated with c-Src, when DIM was adhered to a Glc3-coated dish and incubated with [γ-32P]ATP at 37 °C for 5 min (Fig. 4A, lane 3). The co-immunoprecipitation of these two components with c-Src is not due to trichloroacetic acid precipitation, since the same co-immunoprecipitation pattern was observed without the trichloroacetic acid precipitation procedure (data not shown). Such induction of phosphorylation was not observed when [γ-32P]ATP was added to GlcCer-coated (lane 2) or nonstimulated DIM (lane 1). The quantities of c-Src loaded in each lane were nearly identical (Fig. 4B). Phosphorylation of c-Src and the M_r 45,000 and 29,000 components was greatly reduced when DIM was placed on a dish coated with Glc3, pretreated with anti-Gg3 mAb 2D4 (Fig. 4C, lane 3). Phosphorylation was not reduced, or was even enhanced, when the Glc3-coated dish was pretreated with isotype-matched irrelevant anti-Le^a mAb AH6 (lane 2). No band was phosphorylated when DIM was kept in polypropylene tubes without adhesion (lane 4). A 32P-phosphorylated c-Src band with minimal contamination was observed when the DIM adhered to the Glc3-coated dish was solubilized in RIPA buffer and immunoprecipitated with anti-c-Src (Fig. 4D, lane 3). Phosphorylation of this band was completely abolished in the presence of 100 μM Lavendustin C (lane 4).

A similar induction of phosphorylation of c-Src together with M_r 45,000 and 29,000 components was observed when DIM was bound to anti-GM3-coated dish and incubated at 37 °C for 5 min in the presence of [γ-32P]ATP (Fig. 5A, lane 2). Phosphorylation of bands with higher M_r was also observed under this condition (lane 2). No phosphorylated band was observed in
fractions added on dishes coated with normal mouse IgG (lane 3) or kept in polypropylene tubes (lane 1). The quantities of c-Src loaded in each lane were nearly identical (Fig. 5B). The $^{32}$P-phosphorylated c-Src band became more prominent, without appreciable associated bands, when DIM adhered to the anti-GM3-coated dish was solubilized in RIPA buffer and immunoprecipitated with anti-c-Src (Fig. 5C, lane 2). Phosphorylation of this band was abolished in the presence of 100 μM Lavendustin C (lane 3).

The $M_r$ 45,000 and 29,000 bands co-immunoprecipitated with anti-c-Src antibody seem to be Erk-1 and myosin light chain, respectively, based on immunoblotting analysis with anti-Erk-1 and anti-myosin light chain antibodies (data not shown). This tentative finding needs to be confirmed by further immunoprecipitation analysis using these antibodies.

Lipid Components Present in GM3-enriched Subfraction and in Caveolin-associated Subfraction (Caveolae)—Total lipid components, separated by two-dimensional high performance thin layer chromatography, present in whole cell membrane, DIM (fraction 5), and high density fraction (fraction 12), are shown in Fig. 6, A, B, and C, respectively. In terms of lipid composition, the GM3-enriched/DH2-bound subfraction contains GM3 and SM as major components and an uncharacterized spot 1 (corresponding to GlcCer) as a minor component (Fig. 6D). In contrast, the caveolin-enriched/anti-caveolin antibody-bound subfraction is characterized by a high quantity of cholesterol, absence of GM3, and, surprisingly, very small quantities of SM. This subfraction also contained uncharacterized spot 1 and spot 2 as minor components (Fig. 6E). $[^{3}H]$Ser labeling (for sphingolipids) confirmed the major presence of GM3, SM, and a small quantity of GlcCer in the GM3-enriched subfraction (Fig. 6F) and the presence of a trace quantity of SM in the caveolin-containing subfraction (Fig. 6G).

Effects of Filipin and Nystatin on GM3-dependent Adhesion and Signaling of B16 Melanoma Cells—The invaginated structure of caveolae and their function are disrupted by the cholesterol-binding reagents filipin and nystatin. Incubation of cells (e.g. endothelial cells) in medium containing subtoxic doses for 10 min disrupted the structure and endocytotic function of caveolae. However, caveolae function disrupted by these conditions could be restored by washing and incubation of cells in regular medium free of these reagents (23). Therefore, the same conditions were applied to observe the effects of these reagents on GM3-dependent adhesion of B16 cells to Gg3-coated dishes. Such adhesion was unchanged when cells were treated for 10 min with various doses (0.02–10 μg/ml) of filipin or when filipin-treated cells were reincubated with regular medium (Fig. 7A). Cell viability in terms of trypan blue exclusion was also unchanged under these conditions (Fig. 7B).

Effects of filipin and nystatin on caveolae structure and function can be probed by cholesterol content of DIM prepared after treatment of intact cells with appropriate concentrations of these reagents, as demonstrated recently (37). Treatment of B16 cells with 0.16–0.3 μg/ml filipin or with 25 μg/ml nystatin caused strong or nearly complete depletion of cholesterol from DIM but had no effect on GM3-dependent cell adhesion to the
Gg3-coated dish (Fig. 7C). However, cell adhesion was abolished, in parallel with loss of cell viability, at filipin concentration >0.5 μg/ml (Fig. 7, C and D).

The effect of filipin on signal transduction induced by GM3-dependent adhesion was further analyzed by monitoring FAK activity, since our previous study indicated that GM3-dependent adhesion of B16 cells to Gg3 stimulates FAK activity (27). Preincubation of B16 cells in the presence of 0.5 μg/ml filipin for 30 min at 37 °C did not cause any reduction of FAK activity (Fig. 8). No FAK activation was observed when cells were added to the GlcCer-coated dish, in agreement with our previous results (27).

**DISCUSSION**

A remarkable self-assembling ability of GSLs to form microdomains in plasma membrane (6) is characteristic of GSLs, since their clustering occurs in phosphatidylcholine liposome membrane without cholesterol, probed by ferritin-labeled monovalent anti-GSL antibodies under scanning EM (7, 8). Clustered GSLs have also been observed in brain neuronal tissues (38), T lymphocytes (39), and B16 melanoma cells2 by transmission EM using colloidal gold coated with anti-GSL antibodies or protein A. Clustering of GSLs may occur in all cell membranes. Functions of GSLs as antigens (1), receptors (2), and adhesion sites for bacteria (3) may depend on such clustered GSL microdomains.

In B16 cells, GM3 ganglioside in clustered form (but not nonclustered) is recognized as a melanoma-associated antigen (40, 41) and is capable of adhering to endothelial cells through GM3-Gg3 or GM3-LacCer interaction (42, 43), a process considered to initiate metastasis (44). In our previous study, a low density membrane fraction (fraction 5) containing clustered GM3 was isolated from B16 cells according to the procedure established for isolation of GEM or caveolae in the presence or absence of detergent or hypertonic salt solution. Fraction 5 contained caveolin, in addition to GM3 and four transducer molecules (c-Src, Rho, Ras, FAK) and was capable of sending signals through GM3 (26, 27). Caveolin, a scaffold membrane protein with a hydrophobic domain at the center, interacts with various molecules (e.g. small G-proteins, Src family kinases, and endothelial nitric oxide synthase) involved in signal transduction (for a review, see Ref. 18). Caveolae, with characteristic caveolin function, plays an essential role in endocytosis and signal transduction.

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3 On the other hand, immunoseparation of caveolin-associated components in endothelial cells indicates that various molecules involved in signal transduction (e.g. annexin II, protein

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2 K. Iwabuchi, K. Handa, and S. Hakomori, unpublished observation.
kinase C, heterotrimeric G-protein, small G-protein, c-Src, and endothelial nitric oxide synthase) are separated from caveolin-containing fraction (45). A recent study indicates that less than 1% of total cellular phosphatidylinositol 4-phosphate and its synthetase (4-kinase) in A431 cells is associated with caveolae; the majority of these components is present in noncaveolar low density membrane fraction (46). In these studies, however, no particular attention was paid to the presence of GSLs and sphingolipids. A close association of GM1 and caveolin in A431 and Madin-Darby canine kidney cells was indicated by use of a GM1-bearing photoactive cross-linker (47). It is therefore of interest to determine whether GM3, c-Src, and Rho found in fraction 5 of B16 melanoma cells (26, 27) are included in caveolae or exist as independent membrane components.

We conducted critical studies on (i) separation of GM3-enriched and caveolin-containing membrane subfractions of low density fraction 5 of B16 melanoma cells; (ii) distribution of major transducer molecules c-Src and Rho A in GM3- and caveolin-containing subfractions; (iii) effect of stimulation of GM3 in membrane fraction on phosphorylation of c-Src and other membrane components; and (iv) effect of filipin and nystatin on GM3-dependent adhesion and signaling. Results of the first two studies indicate that the caveolin-containing subfraction is distinct from the GM3-enriched membrane subfraction and that c-Src and Rho are associated with the latter but not the former subfraction. In study three, phosphorylation of c-Src and other components was observed when GM3-enriched subfraction was stimulated by Gg3 or anti-GM3, indicating that this subfraction represents a structural unit displaying GM3-dependent cell adhesion coupled with signaling, as observed in intact cells. Together, these findings suggest that the GM3-enriched subfraction is independent from caveolae and functionally involved in GM3-dependent adhesion and signaling through activation of c-Src. This idea is supported by the results of study four; i.e. GM3-dependent adhesion of B16 cells is not affected by filipin or nystatin at concentrations effective for cholesterol depletion in these cells.

Since 1993, more than 20 papers have appeared describing association of Src family kinases and other transducer molecules with low density membrane fractions similar to caveolae, enriched in sphingolipids, cholesterol, caveolin, and glycosphosphatidylinositol-anchored proteins (e.g. Refs. 12, 14, and 48). However, little attention was paid in these papers to the functional notion of GSLs involved in signal transduction, and no attention was paid to their role in cell adhesion. So far, only three studies have addressed the functional role of GSLs in microdomains as related to signal transduction. (i) In rat basophilic leukemia cells, Src family kinase p53/56



associated antigen GM3 plays a role in defining melanoma cell adhesion to endothelial cells and consequent metastatic processes (42–44). Subsequently, we found that GM3-dependent adhesion of melanoma is due to an adhesive property of GM3 present as clusters in a microdomain organized with several transducer molecules (c-Src, Rho, Ras, FAK) (26). Adhesion of melanoma to Gg3-coated dishes, which mimics adhesion to endothelial cells, activates FAK tyrosine phosphorylation and enhances GTP binding to Rho and Ras (27). These consequences of adhesion are regarded as a basis for enhanced motility of melanoma cells.

Results of the present study indicate a clear difference in lipid composition between the GM3-enriched subfraction and the caveolin-containing subfraction. The general functional differences between these two fractions are as follows. (i) the former is clearly involved in GSL-dependent cell adhesion coupled with signal transduction, whereas caveolae are not involved in cell adhesion. Examples are adhesion based on GM3 in B16 melanoma (27) and that based on Gb4 in human embryonal carcinoma 2102 (52). (ii) Invaginated structure and endocytotic function of caveolae are lost in the presence of filipin (23), nystatin, and methyl-β-cyclodextrin (24), which are capable of binding cholesterol and thereby destroying caveolae structure and function. The caveolin-enriched membrane fraction contains a large quantity of cholesterol but, surprisingly, only a small quantity of SM in B16 cells. It is therefore reasonable that caveolae function is sensitive to cholesterol binding reagents. Interestingly, the present study indicates that these reagents do not affect GM3-dependent cell adhesion and signaling. This supports the idea that self-aggregation of GSLs (in this case GM3) does not depend highly on cholesterol, as suggested initially by the morphological observations of Rock et al. (7, 8). Thus, GSL-enriched microdomain must be structurally and functionally independent from caveolae and capable of causing cell adhesion as well as signal transduction. We hereby propose the term “glycosignaling domain” for this structural unit of plasma membrane.

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