BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 218–222 (1997)
ARTICLE NO. RC976933

A Close Association of GM3 with c-Src and Rho in GM3-Enriched Microdomains at the B16 Melanoma Cell Surface Membrane: A Preliminary Note

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Received June 3, 1997

B16 melanoma is characterized by high content of GM3 ganglioside, which has been recognized as a melanoma-associated antigen defined by specific monoclonal antibodies. We report now that GM3 is present predominantly (>90%) in the 1% Triton X-100-insoluble, low-density microvesicular fraction ("detergent-insoluble glycosphingolipid-enriched microdomain"; DIGEM) separated on sucrose density-gradient centrifugation. Associated with DIGEM, many signal transducer molecules such as c-Src, FAK, and the low-molecular-weight G-proteins Rho A and H-Ras were also found. Rho A and FAK were found in part, and PLC-β2 and Gαs were found exclusively, in the high-density fraction. Immunoprecipitation of GM3 present in DIGEM by anti-GM3 antibody DH2, followed by Western blotting, revealed co-precipitation of Rho A and c-Src with GM3. These findings suggest (i) a specific organization of GM3 in close association with Rho A and c-Src within DIGEM at the melanoma cell surface; and (ii) such organizational units may be directly involved in signal transduction, in which glycosphingolipids receive signals which are subsequently transduced by associated transducer molecules.

Glycosphingolipids (GSLs) have been implicated as modulators of signal transduction and mediators of cell adhesion (1). The detailed molecular basis of these two different but interrelated functions remains unclear. Previously, during studies on functional role of GSLs in cell adhesion, we found that GM3 is particularly enriched in detergent-insoluble cell adhesion matrix, and that GSLs in general are enriched in the insoluble fraction remaining after treatment with the zwitterionic or neutral detergent; this fraction was termed "DIM" (detergent-insoluble material) or "DISAM" (detergent-insoluble substrate attachment matrix) (2). GSLs at the cell surface form "GSL microclusters" as observed by electron microscopy with freeze-fracture technique (3,4). Many recent studies indicate that the low-density, detergent-insoluble fraction of plasma membrane contains membrane vesicles enriched in GSLs, sphingolipids, GPI anchors, and other components, suggesting that "detergent-insoluble vesicles" and "GSL microclusters" may be identical or partly overlapping; see for review (5), and see "Discussion". In rat brain, GD3 ganglioside was co-immunoprecipitated with a 53/56 kDa protein identified as the Src-family tyrosine kinase Lyn. Co-transfection of genes for GD3 synthesis and for Lyn in cerebellar neurons resulted in co-immunoprecipitation of GD3 and Lyn. These results suggest a close association of GD3 and Lyn (6). Nevertheless, the functional organization of GSLs in plasma membrane, in general, remains unclear.

GM3 plays essential roles in two major processes: (i) modulation of signal transduction through FGF receptor (7), EGF receptor (8), and mouse insulin receptor (9); (ii) cell adhesion, through direct interaction with LacCer and Gg3Cer (10,11). Its organization in plasma membrane is therefore of pivotal importance. The present study reveals a close association of c-Src with GM3 or Rho A with GM3 in "detergent-insoluble glycosphingolipid-enriched microdomain" (DIGEM), as evidenced by co-existence and co-immunoprecipitation of these components in DIGEM from mouse B16 melanoma cells. DIGEM in which GSLs and transducer molecules are co-organized may act as structural/functional units capable of receiving stimuli with simultaneous signal transduction.

Abbreviations: DIGEM, detergent-insoluble glycosphingolipid-enriched microdomain; Fr., fraction(s); GPI, glycosylphosphatidylinositide; GSL, glycosphingolipid; mAb, monoclonal antibody. Glycosphingolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Lipids* **12,** 455–463, 1977). Gangliosides are abbreviated according to Svennerholm (*Eur. J. Biochem.* **79,** 11–21, 1977).

0006-291X/97 $25.00

MATERIALS AND METHODS

GSLs, transducer molecules, and antibodies directed to them. GM3 was prepared from dog erythrocytes. Its specific mAb DH2 (IgG_3) was established as described previously (12). Specific polyclonal or monoclonal antibodies suitable for "Western" immunoblotting and directed to various transducer molecules such as c-Src, H-Ras, Rho A, Fak, Gαs, and PLC-β2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies directed to c-Src, FAK, and PLC-β2 were rabbit polyclonal, antibody directed to Rho A was mouse monoclonal, and antibody directed to H-Ras was rat monoclonal.

Preparation of detergent-insoluble fraction containing GM3-enriched microdomain from B16 melanoma. Cells were harvested, lysed, homogenized (Dounce homogenizer, 10 strokes), and subjected to sucrose density-gradient centrifugation as described previously (13). Briefly, 1-5$\times 10^7$ cells were suspended in 1 mL of lysis buffer containing 1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 75 U aprotinin, and allowed to stand for 20 min. When phosphorylation assay was included, 1 mM $NaVO_4$ was added to inhibit phosphatase. The cell suspension was Dounce homogenized as above, and lysate was centrifuged for 5 min at 1300×g to remove nuclei and large cellular debris. The entire procedure was performed at 0-4 °C (in ice immersion). The lysate after centrifugation was mixed with an equal volume of 85% sucrose (wt/vol) containing 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA, with or without 1 mM $NaVO_4$ ("Tris-NaCl-EDTA buffer"). The resulting diluent was placed at the bottom of a sucrose concentration gradient with Tris-NaCl-EDTA buffer. Samples were centrifuged for 18 h at 200,000×g at 4 °C. White bands under light illumination (presumably due to "Tyndall phenomenon" of microvesicles) located at ~5-7% sucrose were separated from other fractions at different sucrose concentrations (see Fig. 1). Protein content of each fraction was determined using a MicroBCA kit (Pierce Chemical Co., Rockford, IL).

Determination of distribution of GM3 and transducer molecules in fractions obtained from sucrose density-gradient centrifugation. For GM3, each fraction was dialyzed to eliminate sucrose, and lyophilized. The residue was extracted with chloroform-methanol (2:1), placed on HPTLC plate, developed, and immunostained using anti-GM3 mAb DH2 and Vectastain ABC kit (Vector, Burlingame, CA) as described previously (14), *i.e.* using biotinylated goat anti-mouse Ig as secondary antibody. Final TLC staining was made with metal enhanced DAB substrate (Pierce). GM3 band on TLC was also detected by orcinol-sulfuric acid staining. For detection of transducer molecules, DIGEM and other protein fractions were subjected to SDS-PAGE with "Western" immunoblotting; *i.e.* protein bands separated on SDS-PAGE were transferred electrophoretically to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, and 15% methanol at 200 mA for 2 h. The membranes were incubated overnight at 4 °C in PBS containing 5% defatted milk, washed in PBS containing 0.05% Tween-20, and incubated 2 h with various antibodies directed to specific transducer molecules. After incubation with primary antibody with appropriate dilution (see Fig. 2 legend), the membrane was washed twice in PBS containing 0.05% Tween-20, and incubated with goat anti-rabbit, anti-rat, or anti-mouse Ig conjugated with horseradish peroxidase for 45 min for detection of rabbit, rat, or mouse primary antibodies. The membrane was washed in PBS 0.05% Tween-20 five times, and developed using chemiluminescence method with a substrate kit (Super-Signal™-CL-HRP; Pierce).

Co-immunoprecipitation of GM3 and transducer molecules in DIGEM fraction. Approximately 500 μL of DIGEM (containing 25-30 μg protein) was mixed with protein A-Sepharose beads (50 μL packed) and stirred by rotary shaker for 2 h at 4 °C to pre-clear nonspecific binding. After centrifugation (500×g for 1 min), the supernatant was added with 10-20 μL anti-GM3 mAb DH2 ascites or with 10-20 μL mouse myeloma SP-2 or NS/1 ascites as negative control. The mixtures were placed overnight in a rotary mixer at 4 °C, added with protein A-Sepharose beads (50 μL packed), and placed again in a rotary mixer for 2 h. Beads were washed 3 times with PBS containing 0.01% Tween-20, by brief weak centrifugation (500×g for 1 min), then suspended with 100 μL sample buffer with mercaptoethanol, heated to 95 °C for 3 min, and centrifuged (1000×g for 2 min). The supernatants were subjected to SDS-PAGE, transferred electrophoretically to PVDF membranes, and incubated with various antibodies directed to specific transducer molecules, under the same conditions as described in the preceding section. Transducer molecules were detected with secondary antibody by chemiluminescence method as described in the preceding section.

RESULTS

GM3 is predominantly present in low-density microvesicular fraction. B16 melanoma cell homogenate in Tris buffer containing 1% Triton X-100 subjected to sucrose density-gradient centrifugation gave a distinctive white under light illumination in the low-density (~ 5-10% sucrose) region, *i.e.* fraction 5 (Fig. 1, left panel). Over 90% of GM3 in the homogenate was found in this vesicular band (Fig. 1, right panel), although the band contained only 0.5% of total cellular protein. GM3 was detected only minimally in various other fractions including Fr. 11 and 12, the major high-density, protein-containing fractions (~ 20-40% sucrose region), which contained ⩾90% of total protein (Fig. 1, right panel).

Distribution pattern of transducer molecules and other proteins in fractions separated by sucrose density-gradient centrifugation. Quantity of protein present in Fr. 5 was only 50-60 μg (~0.5% of total protein). Essentially all c-Src and Ras present in the cells were found in Fr. 5, whereas Rho and FAK were found in both Fr. 5 and 12. PLC-β2 and Gαs were found exclusively in Fr. 12. Some of these results are summarized in Fig. 2. Caveolin from the cells was also found predominantly in Fr. 5, and was virtually absent in Fr. 12 (data not shown).

The major protein-containing fractions (11 and 12) were found at the high-density region. 65% of total protein was found in Fr. 12 and 25% in Fr. 11. Fr. 6, 7, 8, 9, and 10 contained 2.0, 2.3, 1.3, 1.4, and 1.1% of total protein respectively. Fr. 5, in contrast to other fractions, has the peculiar property of containing most of the GM3, c-Src, and Ras present in the cells, and being greatly enriched in Rho and FAK, in spite of its very low protein quantity.

Association of GM3 and transducer molecules in DIGEM. Aliquots of DIGEM were immune separated with anti-GM3 mAb DH2 and protein A-Sepharose beads. GM3 and its possible complex with transducer molecules present in DIGEM were eluted from the beads, and subjected to Western blotting with various antibodies directed to transducer molecules, as described in Materials and Methods. With this approach, c-Src and Rho A were detected on Western

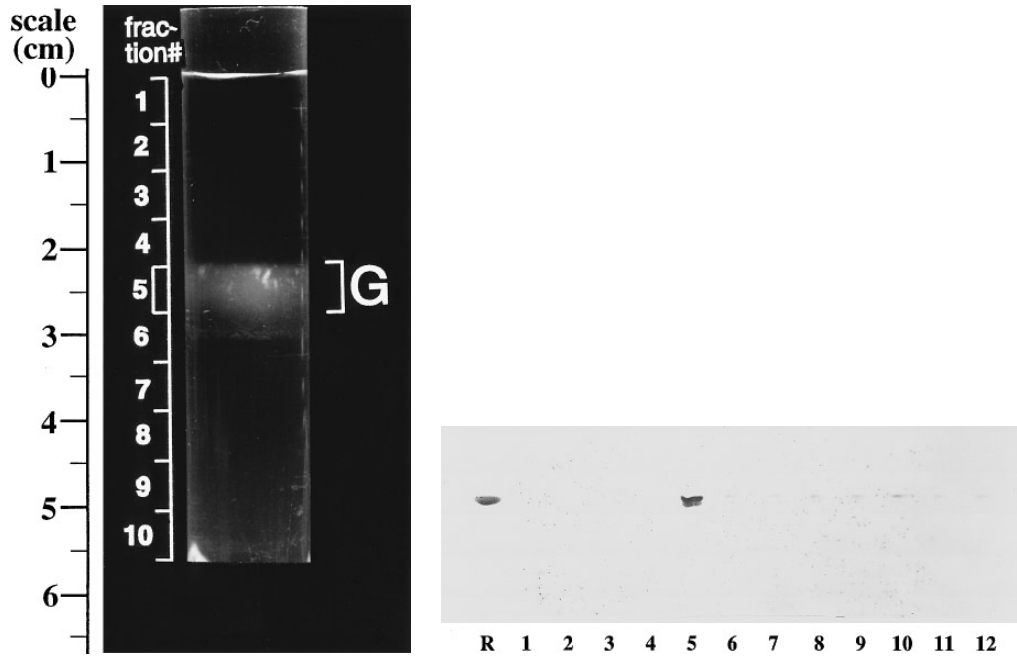

FIG. 1. (Left panel) Distinct white band revealed by light illumination (Tyndall phenomenon; marked as G), separated as detergent (1% Triton X-100)-insoluble, low-density vesicles on sucrose density gradient centrifugation. G is in Fr. 5, when a series of 1 mL aliquots (fractions) is taken starting from the top. The major cellular protein is located in Fr. 12 at the bottom of the tube (not seen). (Right panel) TLC immunostaining of GM3 in each fraction separated as above. Each fraction was dialyzed to eliminate sucrose, lyophilized, extracted with chloroform:methanol (2:1), and immunostained with anti-GM3 mAb DH2. Lane R, reference GM3. Lanes 1 through 12, Fr. 1 through 12. Note that only Fr. 5 showed a clear band of GM3.

blotting (Fig. 3). In control experiments, addition of mouse myeloma ascites or non-specific mouse IgG to DIGEM did not result in detectable levels of c-Src or Rho A (Fig. 3).

DISCUSSION

Glycosphingolipids (particularly gangliosides) have been implicated as modulators of signal transduction through their effect on tyrosine kinase activity associated with growth factor receptors (7-9). A remarkable inherent property of GSLs (and perhaps sphingolipids in general) is their ability to interact with each other to form microclusters at cell surface membranes or liposomal membranes, as initially suggested by electron microscopic studies with freeze-fracture technique (3,4). These microclusters appear to be detergent-insol-

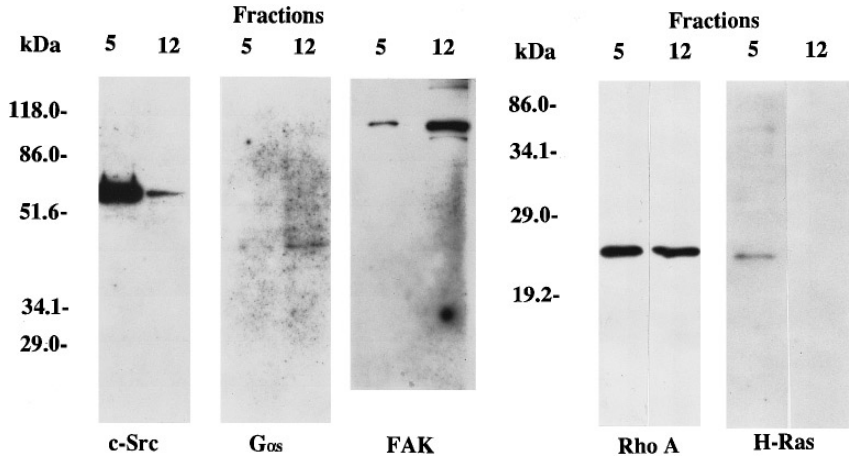

FIG. 2. Detection of transducer molecules in DIGEM fraction (Fr. 5 in Fig. 1) and Fr. 12. Among various fractions separated on sucrose density gradient centrifugation, over 90% of total protein was present in Fr. 12, and ~0.5% in Fr. 5. Therefore, only Fr. 5 and 12 were analyzed by SDS-PAGE and Western blotting, using specific antibodies directed to various transducer molecules. (Left panel) Blotting patterns with antibodies to c-Src (left), Gαs (middle), and FAK (right). (Right panel) Blotting patterns with antibodies to Rho A (left) and H-Ras (right).

uble. Our previous studies showed that GSLs are present in BHK cells in detergent-insoluble form, and that GM3 is enriched in detergent-insoluble matrix (2). We termed the material "DIM" (detergent-insoluble material) or "DISAM" (detergent-insoluble substrate attachment matrix). The possibility that microclusters of GSLs or sphingolipids observed at the plasma membrane and in detergent-insoluble vesicular fraction are identical or partially overlapping has been investigated in recent years. GSL clusters were assumed to function in cell-to-cell adhesion based on GSL-GSL interaction, since multivalency is essential to effect GSL-dependent cell adhesion (15).

Signal transduction, indicated by enhanced expression of the transcription factors Ap1 and CREB, is induced by GSL-dependent adhesion of human teratocarcinoma 2102 cells to Gb4-coated plates (16). GSL-enriched microdomains play essential roles in initiation of this signal transduction. Gb4 and globo-series GSLs were found in low-density, detergent-insoluble vesicles isolated from 2102 cells. This vesicle fraction was also enriched in various transducer molecules such as c-Src and the small G-proteins Rho A and H-Ras. Thus, GSL-enriched microdomains at the cell surface may be the sites where GSLs interact with GSLs on counterpart cells, and stimuli may be transformed through transducer molecules organized in these microdomains. This concept is compatible with the observation that GD3 is associated and co-immunoprecipitated with the Src family transducer molecule Lyn in rat brain (6).

Certain cell types are characterized by presence of caveolae, invaginations of the plasma membrane which play an essential role in endocytosis and signal transduction. Many studies since 1992 have indicated that caveolae are enriched in GPI-anchored protein, the characteristic protein caveolin, and Src family and GTP-binding proteins, in addition to GSLs and sphingomyelin/cholesterol (5,17,18). However, composition of microdomains is inconsistent. Some microdomains from endothelial cells are enriched in GPI-anchored protein but devoid of caveolin; others are enriched in GM1 and GPI-anchored protein (19). The presence of GM1, in this and many other studies, was inferred based simply on cholera toxin binding, but was not conclusively demonstrated by solid chemical or physical criteria. Demonstration of GSLs in "glycolipid-enriched domains" has been obscure. Often, "glycolipid" microdomain implies GPI anchor microdomain, since GPI is regarded as a "glycolipid." Some "glycolipid" mi-

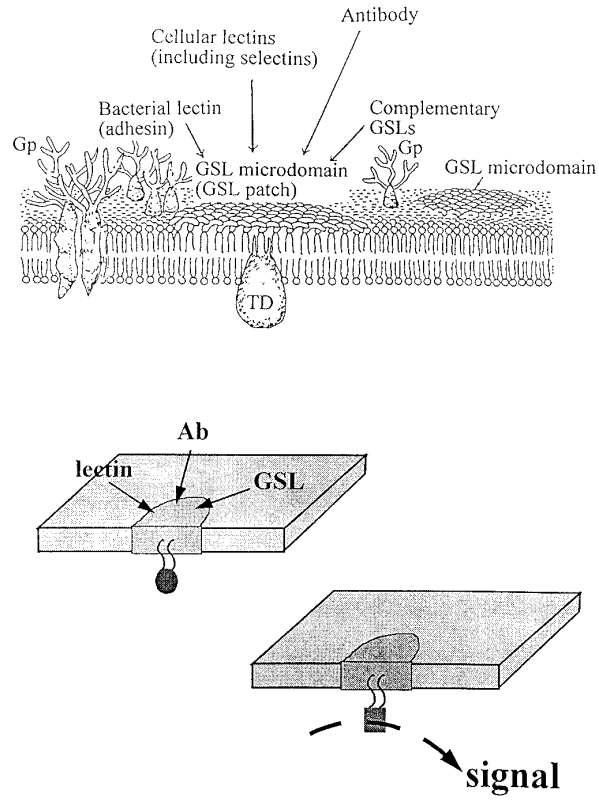

FIG. 4. DIGEM as a structural and functional unit at the plasma membrane. (Upper panel) Clusters of GSLs (GSL patches) present at outer leaflet of plasma membrane, forming DIGEM, where transducer molecules (TD) are also located at cytoplasmic sites. The interaction of GSLs in DIGEM with TD may take place through a hydrophobic domain (*e.g.* fatty acyl residue) of TD. Clusters of glycoproteins (Gp) are located separately from DIGEM. (Lower panel) Conceptual scheme of signal transduction through TD associated with DIGEM. Various stimuli (lectins, antibodies, complementary carbohydrates) to GSLs in DIGEM are converted by TD to create intracellular signals, typically associated with conformational change of TD (symbolized by change from circle to square).

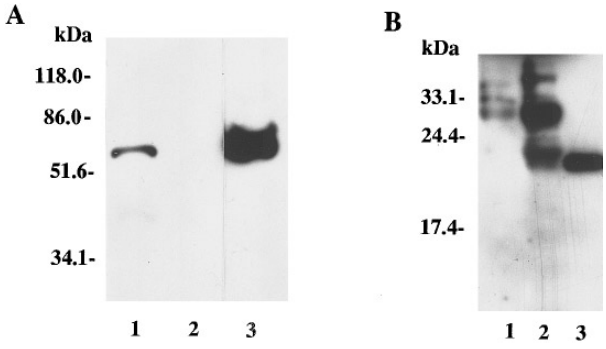

FIG. 3. Co-immunoprecipitation of transducer molecules present in DIGEM fraction with anti-GM3 mAb DH2. Freshly-prepared DIGEM fraction was incubated with Sepharose-protein A conjugate in order to pre-clear nonspecific components, then incubated with DH2 (or control mouse ascites), then added and incubated with Sepharose-protein A conjugate. The bound components, eluted and subjected to SDS-PAGE, were "Western blotted" as described in Materials & Methods. (Panel A) Lane 1, immunoprecipitated with DH2 and probed by anti-c-Src. Lane 2, treated with control mouse ascites and Sepharose-protein A conjugate and probed by anti-c-Src. Lane 3, control c-Src from DIGEM fraction. (Panel B) Lane 1, treated with control mouse ascites and Sepharose-protein A conjugate and probed by anti-Rho. Lane 2, immunoprecipitated with DH2 and probed by anti-Rho. Lane 3, control Rho A from DIGEM fraction.

crodomains may be independent of microdomains associated with caveolae, since they are present in cells not containing caveolae (20).

Results of the present study indicate clearly that (i) GM3, the major ganglioside of B16 melanoma, is predominantly present in the microvesicular fraction isolated after Triton X-100 treatment, hereby termed "detergent-insoluble glycosphingolipid-enriched microdomain" (DIGEM); (ii) two major transducer molecules, c-Src and Ras, are found exclusively in DIGEM; and (iii) there is a close association of GM3 with c-Src or with Rho A in DIGEM, as suggested by the co-immunoprecipitation of these transducer molecules with GM3. Since caveolin is present in DIGEM from B16 cells, DIGEM may partially overlap with caveolae.

Since GM3 is greatly enriched in DIGEM and associated with various transducer molecules, its direct involvement in signal transduction through DIGEM appears plausible. Studies along this line are in progress. DIGEM is considered to be a functional as well as structural unit, where GSLs function as receptors and affect transducer molecules to initiate signal transduction. This hypothesis is shown schematically in Fig. 4.

ACKNOWLEDGMENTS

We thank Dr. Stephen Anderson for scientific editing and preparation of the manuscript. This study was supported by National Cancer Institute Outstanding Investigator Grant CA42505 (to S.H.).

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