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Glycosphingolipid deficiency affects functional microdomain formation in Lewis lung carcinoma cells†

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In view of the increasing evidence that gangliosides in membrane microdomains or rafts are closely associated with various signal transducing molecules including Src family kinases, we compared rafts in two subclones of 3LL mouse lung carcinoma cell line, J18 and J5, characterized by high and very low GM3 ganglioside contents, respectively. Rafts were isolated from cell lysates as low density detergent-insoluble microdomains (DIM) by sucrose density gradient centrifugation. J5 and J18 cells expressed comparable amounts of Src family kinases and the majority of Src kinases in both clones were concentrated in their DIMs, suggesting that GM3 is not necessary for DIM localization of Src kinases and there is no direct interaction between Src and GM3. However, the Src kinases were eliminated from DIMs after depletion of the major neutral GSLs of J5 cells, glucosylceramide and lactosylceramide, by an inhibitor of glucosylceramide synthase (D-PDMP), indicating that GSLs in general are required for Src kinase association to DIM. J5 and the D-PDMP-treated J5 cells had very similar DIM protein profiles and moreover cholesterol and sphingomyelin in the GSL-depleted cells were enriched in DIM similar to the untreated control cells. Interestingly, the levels of tyrosine-phosphorylated DIM proteins and cell proliferation of J5 cells were much lower than those of J18 cells, suggesting that GM3 might be involved in tyrosine phosphorylation of DIM proteins required for cell growth. Thus, our data suggest that GSLs are essential for functional raft formation.

Keywords: **glycosphingolipids, GM3 ganglioside, GlcCer synthase inhibitor (D-PDMP), Src kinases, raft, detergent-insoluble microdomain (DIM), GlcCer synthase inhibitor**

Abbreviations: **GSL, glycosphingolipid; GlcCer, glucosylceramide; GPI, glycosylphosphatidylinositol; LacCer, lactosylceramide; Gb3, Galα-4Galβ-4GlcCer; GM3, NeuAcα3Galβ-4GlcCer; SM, sphingomyelin; DIM, detergent insoluble microdomain; D-PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris buffered saline; TNE, 10 mM Tris-HCl (pH 7.5) 150 mM NaCl, 5 mM EDTA**

Introduction

In recent years, our knowledge of the characteristics and cellular function of low density, detergent-insoluble microdomains (DIMs) rich in glycosphingolipids (GSLs), cholesterol, sphingomyelin (SM), glycosylphosphatidylinositol (GPI)-anchored proteins, and lipid-modified protein, namely rafts, has considerably increased [1]. The general function of DIMs in signal transduction may be to concentrate receptors for interaction with ligands and effectors on both side of membranes, thus speeding up binding during signaling and preventing inappropriate crosstalk between signaling pathways. A close association of non-receptor type Src family tyrosine kinases with GSLs was indicated by several studies. For example, Lyn is co-immunoprecipitated with α-Gal-GD1b in rat basophilic leukemia cells [2] and with anti-GD3 in rat brain [3]. In B16 melanoma cells, c-Src, Ras, Rho and focal adhesion kinase are enriched in DIM and these signal transduction molecules are co-immunoprecipitated with the antibody against GM3 which is the major ganglioside of this cell line. Moreover, these signaling molecules are activated through GSL-GSL interaction [4,5]. It has been also reported that a neutral GSL, Galα-4Galβ-4GlcCer(Gb3), is expressed in human renal cell line associated with an Src family kinase Yes

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in DIMs, and Yes was activated by Shiga toxin which specifically binds to Gb3 [6]. These observations suggest that GSLs are required to form microdomains on the membranes and various signal transduction cascades elicited in DIMs might be the result of complex formation of Src family kinases with GSLs. However, a recent report using GM-95 cells, a GSL-deficient mutant clone of B16 melanoma cells treated with N-methyl-N'-nitrosoguanidine [7], suggested that GSLs are not essential for the organization of DIM components since GPI-anchored protein and Yes are retained in DIMs similar to the parent B16 cells [8].

We found previously that the wild type of Lewis lung carcinoma (3LL) cells consisted of several subpopulations differing in the degrees of GM3 expression [9]. Parental 3LL cells, like the wild type of B16 melanoma cells, express GlcCer, LacCer and GM3 as the major GSLs. Using a non-selective procedure (without mutagens), we isolated the J18 and J5 subclones which were characterized by high and very low GM3 contents, respectively [9]. We then conducted a series of experiments to address whether or not GSLs are essential for DIM organization and function. Our results indicate that 1) GM3 is not essential for the association of Src kinases with DIMs but might be involved in tyrosine phosphorylation of DIM proteins. 2) After depletion of GlcCer and LacCer in addition to GM3 from J5 cells by an inhibitor of glucosylceramide synthase, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), most of the Src proteins in DIMs were eliminated and shifted to the high density fractions, indicating that some type of GSL(s) or GSLs in general are essential for association of Src kinases with DIMs.

Experimental Procedures

GSLs, sphingolipids, antibodies, and inhibitor

GM3 prepared from bovine milk was purchased from Snow Brand Milk Products (Tokyo, Japan). GlcCer, LacCer, and SM were from Matreya (PA, USA). Neutral sphingolipid mix TLC standards containing GalCer, LacCer, and Gb3 were from Calbiochem. Anti-Src rabbit IgG (sc-18), which recognizes Src family kinases including Src, Yes, Fyn and Fgr, and anti-phosphotyrosine mouse monoclonal IgG_2b antibody (PY99) were purchased from Santa Cruz Biotechnology. D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) was synthesized as described previously [10].

Cell culture

The subclones of wild type 3LL Lewis lung carcinoma cells, J18 and J5 [9], were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% CO_2/95% air.

Preparation of low density detergent-insoluble microdomain particles

Cells were washed twice with PBS. For lysis of cells, the cells suspended in 1% Triton X-100, 10 mM Tris-HCl (pH 7.5) 150 mM NaCl, 5 mM EDTA (TNE), 2 mM $NaVO_4$, 75 U aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM PMSF, incubated for 40 min, scraped off the dish, and homogenized with Dounce apparatus (10 strokes). All lyzates were centrifuged for 5 min, at 1,300 X g to remove nuclei and large cellular debris. Lysates (2 ml) were diluted with 2 ml of 85% wt/vol sucrose in TNE. The diluted lysate was overlaid with 4 ml of 30% wt/vol and 4 ml of 5% wt/vol sucrose TNE solution in a Beckman SW41 centrifuge tube. The samples were centrifuged for 18 h at 200,000 X g at 4°C. A white, light-scattering band located between 5 and 30% sucrose was collected, and termed 'DIM.' All steps were done at 0–4°C.

Lipid analysis

Cells ($1\text{-}5 \times 10^7$) were collected, washed twice with PBS, and the lipids were extracted from the cell pellets by method of Macher and Klock [10] and fractionated by modified methods of Ledeen et al. [11]. Briefly, the total lipid extract was dissolved in chloroform/methanol/water (30:60:8, v/v/v), passed through a DEAE-Sephadex A-25 column (0.8×4.5 cm), and eluted with 5 volumes each of the same solvent (neutral lipid fraction), and chloroform/methanol/ 1 M aqueous Na acetate (30:60:8) (acidic lipid fraction). The fractions were evaporated to dryness, and the contained esters were cleaved with methanolic 0.5 M NaOH for 1 h at 40°C. The solution was neutralized with 1 M acetic acid in methanol and diluted with 6 ml of 50 mM NaCl solution, then desalted with Sep-Pak C18 reverse-phase cartridge (Waters). Sphingomyelin (SM) was isolated from the neutral fraction by HPTLC using chloroform/methanol/acetic acid/formic acid/water (42:18:7.2:2.4:1.2) and detected with 3% cupric acetate-8% phosphoric acid reagent. Neutral GSLs were separated by HPTLC using chloroform/methanol/acetic acid/formic acid /water (42:18:7.2:2.4:1.2) and detected with orcinol-sulfuric acid reagent. Gangliosides were separated by HPTLC of the acidic fraction using chloroform/methanol/0.5% $CaCl_2$ (60:40:9) and detected with resorcinol-HCl reagent. DIM and other fractions obtained by sucrose gradient centrifugation as described above were analyzed to determine the lipids. The chloroform-rich phase from Folch partition was subjected to alkaline methanolysis and the neutral GSLs and SM were analyzed by HPTLC as above. Gangliosides of each fraction without alkaline methanolysis were diluted with 10 ml of 50 mM NaCl aqueous and desalted with a Sep-Pak C18 cartridge and analyzed by HPTLC as above. The quantity of each sphingolipid was measured with a dual-wavelength flying spot scanner (CS9000, Shimadzu, Kyoto, Japan) in the reflectance mode at 500 nm with integrated areas. Cholesterol were determined cholesterol oxidase/p-chlorophenol system using Cholesterol CII assay kit (Wako, Tokyo, Japan).

Polyacrylamide gel electrophoresis and immunoblotting (Western blotting)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [13] after dissolving samples in 5 × loading buffer containing 10% SDS and 25% 2-mercaptoethanol. DIM and other protein fractions were separated on 10% PAGE gels and detected by silver staining using SIL-BEST Stain reagent (Nakalai tesque, Kyoto, Japan) or transferred electrophoretically to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) using a Trans-Blot apparatus (Bio-Rad). The membranes, blocked in Tris buffered saline (TBS) containing 0.05% Tween 20 and 5% nonfat dried milk for 1 h, were incubated overnight at 4°C with anti-Src rabbit IgG or anti-phosphotyrosine mouse IgG_{2b}, and then with appropriate horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using enhanced chemiluminescence (ECL, Amersham), then scanned using a flying spot scanner as above.

Protein assay

Proteins were determined by bicinchoninic acid protein assay kits (Micro BCA, Pierce) using bovine serum albumin as the standard.

Results

Sphingolipids, growth and morphology of J18 and J5 clones

Previously, we isolated the J18 and J5 subclones from 3LL Lewis lung carcinoma cells by *in vitro* non-selective procedure (without mutagens), which were characterized by high and very low GM3 contents, respectively [9]. Here, we first analyzed the GSLs and sphingolipids in total lipid extracts from J5 and J18 cells (Figure 1). As shown in Figure 1, the most distinctive difference in GSL composition in both cells was the GM3 contents (J18; 1.74 μg/mg protein vs. J5; 0.14 μg/mg protein), which are consistent with our previous study [9]. The lactosylceramide (LacCer) content of J5 cells was about 3 times higher than that of J18 cells. The accumulation of LacCer in J5 cells is probably due to the result of substrate accumulation because of lack of GM3 synthase. Not much difference in the expression of SM and GlcCer in both clones, was observed. The GlcCer, Gb3 and SM contents of J5 cells was slightly higher than those of J18 cells. These analytical data showed that the total sphingolipid contents of J5 cells, despite the very low level of GM3, was similar to that of J18 cells. Next we compared growth behavior and morphological differences. J18 cells were spread out on the culture dish, exhibiting the spindle-like morphology (Figure 2A), but on the other hand, J5 cells were all spherical (Figure 2B). When the growth rates of both cells were compared in the presence of 10% fetal calf serum, the cell growth of J5 cells was significantly lower than that of J18 cells at 4 days of culture (Figure 2C). J5 cells grew noticeably more slowly in a low density culture condition and attached weakly to the culture dish. These differences might be attributed to the very low expression of GM3 in J5 cells, since gangliosides

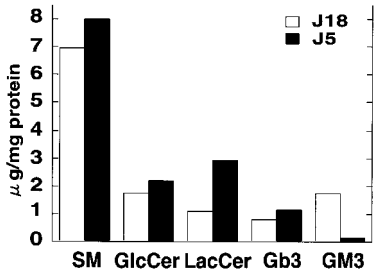

Figure 1. Sphingolipids analyses of J18 and J5 cells. J18 and J5 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum. Lipids were purified, separated by HPTLC and identified as described in Experimental Procedure.

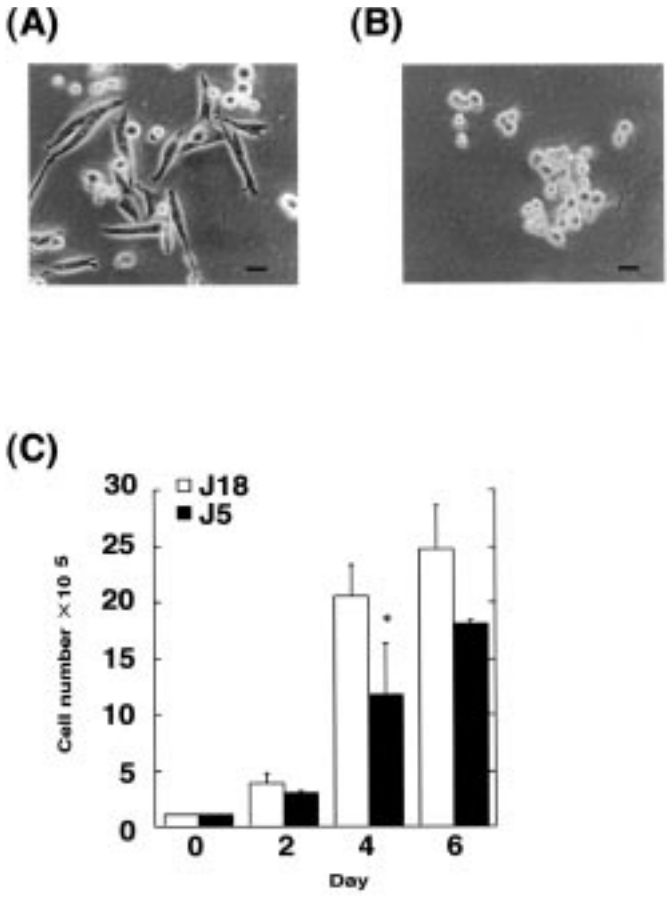

Figure 2. Cell morphology and growth of J18 and J5 cells. J18 (A) and J5 (B) cells were seeded at 1×10^5 cells per well, and on the next day were photographed. Sale bars represent 50 μm. Similarly, cells were seeded at 1×10^5 cells per well (triplicate) and incubated for 2, 4 and 6 days. The cells were then washed with PBS, trypsinized and counted under microscope (C). *, $P < 0.05$ compared to the growth rate of J18 cells.

including GM3 have been suggested to be involved in cell attachment [14] and in signaling of growth factor receptors [15] and tyrosine phosphorylation of Src kinases [4,5].

Distribution of sphingolipids and proteins of J5 and J18 in DIMs

Homogenization of J5 and J18 cells in TNE containing 1% Triton X-100, followed by sucrose density gradient centrifugation as described in Experimental Procedure, gave a white light-scattering band in the low density region which corresponds to the fractions 4 and 5. To compare recovery of DIMs from J18 and J5 cells, we measured cholesterol and protein contents (Figure 3). The recovery of cholesterol from J5 cells was about 10% higher than that of J18 cells (Figure 3A). Slightly less DIM protein was recovered from J5 cells than J18 cells. As shown in Figure 3B, we recovered $0.14 \pm 0.01\%$ of total cellular protein in DIMs from J5 cells and $0.19 \pm 0.05\%$ from J18 cells. Some proteins may have a slightly higher affinity for the DIMs of J18 cells, although we did not see significant differences in overall DIM protein composition (Figure 7B). Next, we analyzed sphingolipids in the sucrose gradient fractions and found that the most of them were concentrated in DIMs (Figure 4). Thus, we confirmed that DIMs prepared from J18 and J5 cells were very similar based on the recovery of DIM proteins and DIM lipids except for the loss of GM3 in J5 cells. This similarity supports the idea that maintaining the correct plasma membrane phase behavior is an important function of sphingolipids and cholesterol.

Distribution pattern of Src family kinases in sucrose gradient fractions

Since GM3 and c-Src kinase have been found to be closely associated in DIMs by immunoprecipitation [4,5], we analyzed

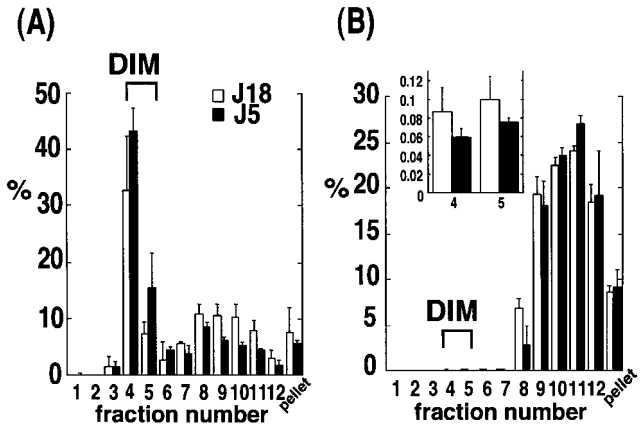

Figure 3. Cholesterol and protein distribution in sucrose gradient fraction from J18 and J5 cells. Relative quantities of cholesterol (A) and protein (B) are shown. Cholesterol were determined by cholesterol oxidase/p-chlorophenol system.

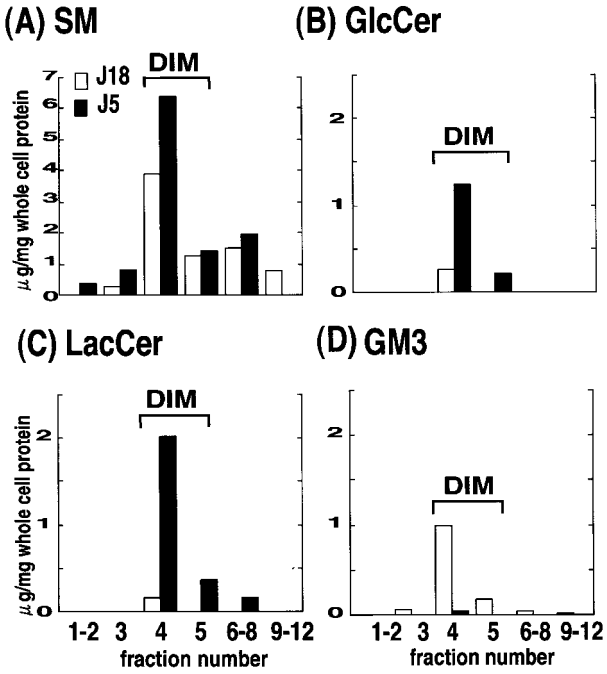

Figure 4. Sphingolipids distribution in sucrose gradient fractions from J18 and J5 cells. Fraction 1–12 and pellet were separated by sucrose gradient centrifugation in 1% Triton X-100 containing lysis buffer. Lipids were separated by HPTLC, visualized with 3% cupric acetate-8% phosphoric acid (A), orcinol-sulfuric acid (B and C), resorcinol-HCl (D), followed by densitometry.

the GM3-rich J18 and the GM3-deficient J5 DIMs for their distribution of Src family kinases by Western blotting using a polyclonal antibody (sc-18) recognizing with various Src family proteins. As shown in Figure 5, J5 and J18 cells expressed comparable amounts of Src family kinases and the majority of Src kinases in both clones were concentrated in their DIMs, suggesting that GM3 is not necessary for DIM localization of Src kinases. Since Do et al. reported the absence of c-Src mRNA in 3LL cells [16] and we could not detect c-Src in both cells by immunoblotting using the c-Src specific antibody, both clones may express Yes, Fyn and/or Fgr which are recognized by the sc-18 antibody.

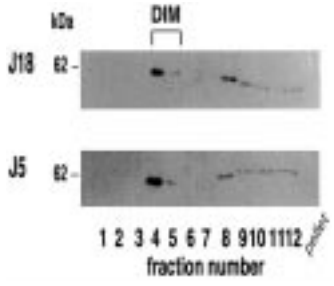

Figure 5. Distribution pattern of Src family kinase proteins in sucrose gradient fraction. Fraction 1–12 and pellet were separated by sucrose gradient centrifugation in 1% Triton X-100 containing lysis buffer. Src family kinase proteins in each fraction were detected by Western blotting.

The effects of GlcCer synthase unhibitor, D-PDMP

If GM3 is not essential for the association of Src kinases with DIMs as suggested in Figure 5, what is the role of the other GSLs in J5 cells? In order to answer this question, we employed the inhibitor of GlcCer synthase, D-PDMP [10]. When J5 cells were treated with 20 μM D-PDMP for 6 days, more than 90% of GlcCer and 80% of LacCer in whole cells as well as in DIMs were depleted and GM3 was not detectable in the D-PDMP-treated J5 cells (Table I). On the other hand, the whole cell contents of cholesterol and SM in the D-PDMP-treated cells were increased 120% and 30%, respectively, compared to the respective control. The increase in SM level by D-PDMP might be the result of enhanced conversion of ceramide to SM due to the substrate (ceramide) accumulation. The more than double accumulation of cellular cholesterol by D-PDMP suggests the importance of GSLs in cellular metabolism and transport of cholesterol (See Discussion). The levels of cholesterol and SM in DIM fractions in J5 and the D-PDMP-treated cells remained unchanged. Next, we examined the distribution of Src proteins in sucrose gradient fractions from the J5 cells treated with D-PDMP as in Table 1. Most of Src kinases in DIMs were eliminated and shifted to the high density fractions and pellet (Figure 6A). Although the recovery of DIM proteins from the D-PDMP-treated J5 cells was slightly lower than that of untreated J5 cells, we did not see any significant differences in overall DIM protein profile (Figure 6B).

Comparison of tyrosine-phophorylated proteins between J18 and J5 cells

As described above, the recovery of DIM proteins (Figure 3B and 7B) and DIM lipids except for the deficiency of GM3 in J5 cells, (Figure 1 and Table 1), was similar in both J5 and J18 cells, indicating that rafts are organized similarly in both cell types. We examined tyrosine-phosphorylated proteins in DIMs by Western blotting using anti-phosphotyrosine antibody. The levels of tyrosine-phosphorylated DIM proteins of J5 cells were much lower than those of J18 cells (Figure 7A). Marked differences were seen in especially 45–80 kDa proteins which are not yet identified. These results suggest that GM3 might be involved in the activation of tyrosine kinases in DIMs, possibly including that of Src family kinases.

Table 1. Lipid composition of J5 and D-PDMP-treated J5 cells

	whole cells		*DIM*	
Lipid	*control*	*D*-PDMP	*control*	*D*-PDMP
	μg/mg protein		μg/mg whole cell protein	
Cholesterol	20.03	44.86	12.48	10.85
SM	9.48	12.5	4.90	5.16
GlcCer	2.56	0.15	1.50	0.07
LacCer	2.95	0.50	2.17	0.42
Gb3	1.15	0.67	0.94	0.30
GM3	0.16	ND	0.03	ND

ND, not detected.
J5 cells were grown in RPMI 1640 medium containing 10% fetal calf serum alone (control), 20 μM D-PDMP for 6 days prior to quantitation of the major lipid species in whole cells and detergent-insoluble microdomain (DIM) as described under Experimental Procedure. Cholesterol was determined by cholesterol oxidase/p-chlorophenol system.

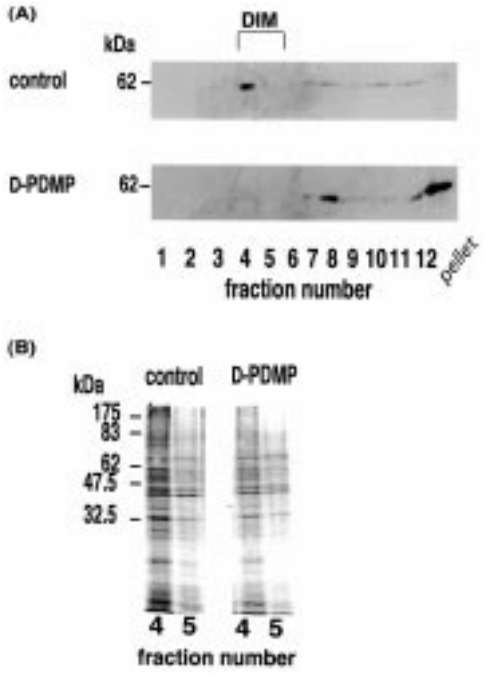

Figure 6. Distribution pattern of Src family kinase proteins in sucrose gradient fraction from J5 and D-PDMP-treated J5 cells. J5 were grown in RPMI 1640 medium containing 10% fetal calf serum alone (control), 20 μM D-PDMP for 6 days. Fraction 1–12 and pellet were separated by sucrose gradient centrifugation in 1% Triton X-100 containing lysis buffer. Src family kinase proteins in each fraction were detected by Western blotting (A). Fractions 4–5 were subjected to SDS-PAGE followed by silver staining (B).

Discussion

GSLs play important roles in cell recognition, signaling, and other processes [17] and are essential for mammalian development [18]. To understand the molecular mechanism of GSL function, it is essential to clarify the organizational status of GSLs at the cell surface membrane. Clustering of GSLs may occur in all cell membranes, since earlier studies showed that clustered GSLs to form microdomains in plasma membranes or in liposome membranes have been observed by ferritin-labeled anti-GSL antibodies [19,20]. Numerous subsequent studies have indicated that clustered components can be isolated as low density detergent insoluble particles by sucrose gradient ultracentrifugation. DIM components include cholesterol [21], sphingolipids, glycosylphosphatidylinositol-anchored proteins and signal transducer molecules such as Src

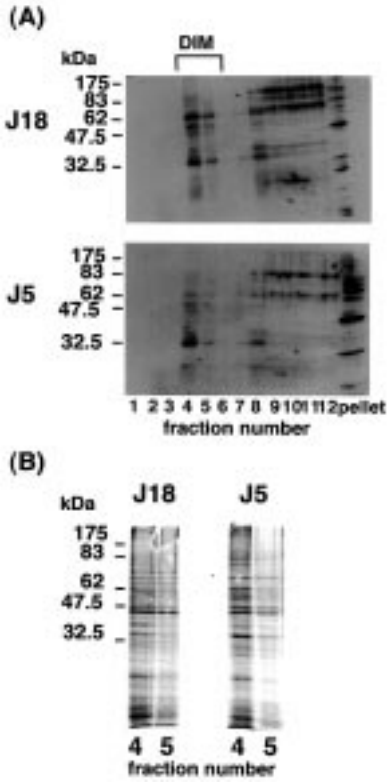

Figure 7. Distribution pattern of tyrosine-phosphorylated proteins in sucrose gradient fraction from J18 and J5 cells. Fractions 1–12 and pellet were separated by sucrose gradient centrifugation in 1% Triton X-100 containing lysis buffer. Tyrosine-phosphorylated proteins in each fraction were detected by Western blotting (A). Fractions 4–5 were subjected to SDS-PAGE followed by silver staining (B).

family tyrosine kinases [22–25]. A potential link between Src family kinases and GSLs in DIMs has been indicated by several studies. Src family kinases are anchored to the plasma membrane at cytoplasmic leaflet via myristoylation and/or palmitoylation at the N-terminal region, and clusters of basic residues near the anchoring site promote membrane binding [26]. In rat basophilic leukemia cells, Lyn and IgE receptor were co-immunoprecipitated by antibody AA4 directed to α-Gal-GD1b, suggesting a close association between these three components in membranes [2]. In rat brain, Lyn was co-immunoprecipitated by antibody R24 directed to GD3, suggesting an association of Lyn with GD3 in neuronal membranes. R24 induced the activation of Lyn and rapid tyrosine phosphorylation of several substrates in primary cerebellar cultures [3]. In B16 melanoma cells, c-Src enriched in DIM was co-immunoprecipitated with antibody DH2 against GM3 which is the major ganglioside of this cell line. Moreover, the Src in DIM was activated through GSL-GSL interaction or by the addition of DH2 [4,5]. In human renal cell line, Yes in DIM was activated by Shiga toxin which binds to Gb3 specifically [6]. These observations strongly suggest that GSLs, especially gangliosides, are required for functional raft formation by interacting with Src family kinases. In the present study, we isolated DIMs from the GM3-rich (J18) and the GM3-deficient (J5) subclones of 3LL mouse lung carcinoma cells [9] and compared them with their lipids and proteins. DIM lipid composition was very similar in the two cell lines, except for the loss of GM3 and the corresponding increases of its biosynthetic precursor, LacCer. Some proteins may have a slightly higher affinity for the DIMs of J18 cells that contain GM3, although we did not see significant differences in overall DIM protein composition including Src kinases. Thus, our results suggest that GM3 is not necessary for DIM localization of Src kinases and there is no direct interaction between Src and GM3. However, the levels of tyrosine-phosphorylated DIM proteins of J5 cells were much lower than those of J18 cells, suggesting that GM3 molecule are required for the activation of tyrosine kinases in DIM. The low levels of tyrosine-phosphorylated proteins in J5 DIMs might be due to the low Src kinase activities in DIMs, which correlate with the low cell proliferation rate and round cell morphology.

We are currently performing reconstitution experiments by transfecting GM3 synthase gene [27] into J5 cells to evaluate the role of GM3 in functional raft formation with special attention to Src and related proteins. We hope to obtain the important clues for still unanswered questions: 1) How does the expression of endogenous GM3 in J5 cells affect the functional raft formation (i.e., Src kinase activities)? 2) Are there any membrane-spanning molecules (phosphorylated or not ?) involved in the transmission of the GM3-dependent signal to Src which is located in the cytoplasmic site of the membrane? 3) In a physiological sense, what molecules can associate or interact with GM3 specifically in the cell surface?

The development of highly specific inhibitors of GlcCer synthase has permitted investigators to deplete cells of GSLs biosynthesized through GlcCer [10,28–30]. In the current study the GlcCer synthase inhibitor, D-PDMP, significantly depleted the total GSLs of J5 cells and the GSL-depleted J5 cells could not retain Src kinases in the DIM. The amounts of cholesterol and sphingomyelin and protein profiles in DIMs of both J5 and the D-PDMP cells were very similar. Thus, we showed here that GSLs are essential for localization of Src kinases in DIMs. However, a recent study, using a GSL-deficient mutant clone of B16 melanoma cells which lacks GlcCer synthase [7], reported that Src kinase Yes is concentrated in the DIMs of the GSL-deficient clone similar to the parent B16 cell [8]. In order to solve these disagreements, a more definitive experiment such as conditional knockout of GlcCer synthase will be required.

The finding of dramatic accumulation of cellular cholesterol in the GSL-deficient J5 cells treated with D-PDMP leads us to speculate that cholesterol may be trapped within GSLs-laden compartments as the result of the well known physical association of cholesterol with GSLs [31,32].

Considering the possible roles of rafts in various cellular functions [1], it is becoming increasingly important to

understand in molecular detail how particular proteins and particular lipids are organized into these domains. We demonstrate here that GSLs are essential for functional raft formation.

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