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Cell adhesion/recognition and signal transduction through glycosphingolipid microdomain

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Glycosphingolipids (GSLs) and sphingomyelin in animal cells are clustered and organized as membrane microdomains closely associated with various signal transducer molecules such as cSrc, Src family kinases, small G-proteins (e.g., RhoA, Ras), and focal adhesion kinase. GSL clustering in such microdomains causes adhesion to complementary GSLs on the surface of counterpart cells or presented on plastic surfaces, through carbohydrate-to-carbohydrate interaction. GSL-dependent cell adhesion in microdomain causes activation of the signal transducers, leading to cell phenotypic changes. A retrospective of the development of this concept, and current status of our studies, are presented.

Keywords: **glycosphingolipid signaling domain (GSD), cell adhesion, detergent-insoluble material (DIM), signal transducer molecules, transcription factors, GM3, Gal-globoside**

Abbreviations: **DIM, detergent-insoluble material; GEM, GSL-enriched microdomain; GPI, glycosylphosphatidylinositol; GSD, glycolipid signaling domain; GSL, glycosphingolipid; PC, phosphatidylcholine; SM, sphingomyelin.**

Glycosphingolipids (GSLs) are multifunctional molecules associated with membranes, particularly at the cell surface. They confer antigenicity and adhesive properties of cells, bind to microorganisms and their toxins, and initiate or modulate signal transduction (Figure 1). Some of these functions are better understood based on clustered structural units of GSLs, termed 'GSL-enriched microdomain (GEM)' or 'GSL signaling domain (GSD).'

In this brief review, I will describe first the background of how this concept developed, then our current studies in this area, with a few comments on future trends.

Retrospective

Clustering of GSLs

Presence of GSLs in a clustered state at the erythrocyte surface was first observed by Young, Tillack, et al. in 1983 through scanning electron microscopy (EM) with freeze-fracture technique, using ferritin-labeled or gold-sol coated antibodies directed to globoside or Forssman antigen. GSL clusters were separable from glycoprotein clusters labeled with Con A [1]. Subsequent studies indicated that GSL clusters are observable even for liposomes made from GSLs and phosphatidylcholine (PC) in the absence of cholesterol [2,3]. GSL clustering in membrane was confirmed by transmission EM [4,5].

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DIM or DRM: Behavior of clustered GSL in detergent or high-ionic salt solution

Purified GSLs having > 4 sugar residues, or having sialic acid (gangliosides), are soluble in pure water or in aqueous media containing suitable detergent, but are insoluble in salt solution with high ionic strength. When GSLs are organized in membrane in the presence of phospholipid and cholesterol, they become insoluble in aqueous detergent. This solubility change may be based on GSL clustering in PC/cholesterol membrane. In our original studies in the early 1980s, we observed that pericellular matrix proteins, nucleus, and cytoskeletal components, as well as GSLs of human and hamster fibroblasts, were essentially insoluble in 2% zwitterionic detergent Empigin BB. GSLs, particularly GM3 ganglioside, were enriched at focal adhesion sites where actin filaments converged (Figure 2). Cell adhesion sites left in the glass dishes after cells were detached by EDTA treatment were found to be enriched in GSLs (GM3 in the case of BHK cells) and other membrane components termed 'detergent-insoluble substrate adhesion matrix (DISAM).' When detached cells were treated with detergent, GSLs and other compounds were found and termed 'detergent-insoluble material (DIM)' (Figure 3).

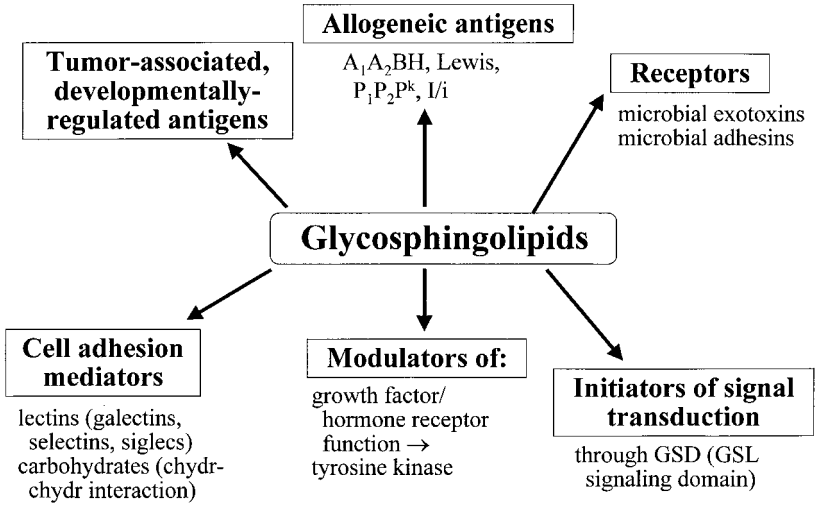

Figure 1. GSL functions. Six functions are shown schematically. Each has been reviewed:
•Allogeneic antigens: A_1A_2BH [46], $P_1P_2P^k$ [47], Lewis [48]. **Note**: A_1A_2, $P_1P_2P^k$, and Lewis antigens expressed in erythrocytes are exclusively GSL, not glycoprotein.
•Receptors [49–51].
•Tumor-associated, developmentally-regulated antigens: [52,53].
•Cell adhesion mediators: (a) lectins [54–56]; (b) carbohydrate-carbohydrate interaction [57,58]
•Modulators of growth factor receptor function [34,35]
•Initiators of signal transduction (major topic of this article) [59].

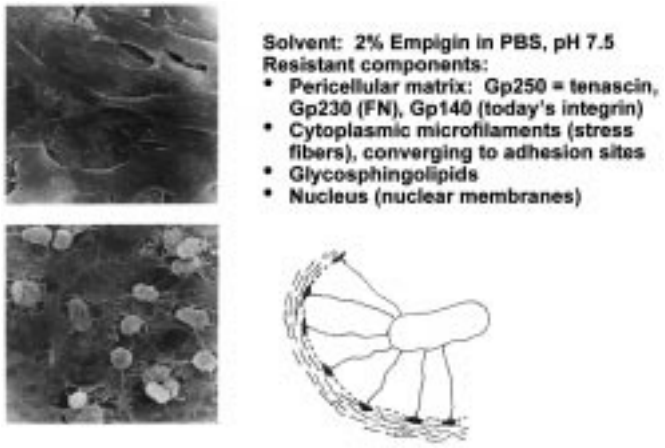

Figure 2. Detergent-resistant components in human and hamster fibroblasts. Microphotograph at upper left shows monolayer of intact WI38 cells; lower left shows same monolayer treated with Empigin BB in PBS, pH 7.5. The insoluble residues include the components listed. Lower right: schematic illustration of possible organization of insoluble residues. Numerous microfilaments connect nuclear membrane to a part of cell surface insoluble residues which presumably correspond to focal adhesion sites where adhesion receptors (integrins) and their associated cytoplasmic components are organized. GSLs may be closely associated with focal adhesion sites (see Fig. 3). Figure constructed from data in Carter WG & Hakomori S, *JBC* 256: 6953–60, 1981; Okada Y, et al., *Exp Cell Res* 155: 448–56, 1984).

Difference in lipid composition of plasma membrane, termed 'polarity,' was initially indicated by difference of GSL, PC, and SM composition in microvillus membrane, brush border membrane, and whole mucosa of rat intestinal epithelia [6]. GSLs, particularly fucose-containing GSLs, showed very high levels in microvillus membrane separated from brush border and whole mucosa [7]. This discovery was made soon after existence of fucose-GSLs was established [8,9]. Polarity of lipid distribution in 'apical' vs. 'basolateral' surface membrane was claimed, but based on indirect evidence, i.e. envelope virus, grown in continuous epithelial cell lines such as MDCK, which supposedly buds from apical surface, is relatively enriched in SM, GSL, and cholesterol, as compared to whole cell (see for review [10]. A number of subsequent studies based on extraction in detergent-containing medium indicate that components supposedly present at apical surface, i.e. SM, GSLs, cholesterol, and GPI-anchored proteins, are detergent resistant or insoluble, particularly in 1% Triton X-100 [11–14].

Detergent resistance was also observed for invaginated membrane domain ('caveolae') characterized by presence of the scaffold protein caveolin. Caveolar membrane showed lipid composition similar to that of 'detergent-insoluble membrane (DIM)' [15], 'detergent-resistant membrane (DRM)' [16], or 'rafts' [17], i.e. high levels of sphingolipids, cholesterol, and GPI-anchored proteins. [Note: The term 'raft' should be limited to membranes not involved in cell adhesion, even though GSL domains are always involved in cell adhesion (see 'Discussion and future trends').] A number of studies indicate that caveolae play an essential role in

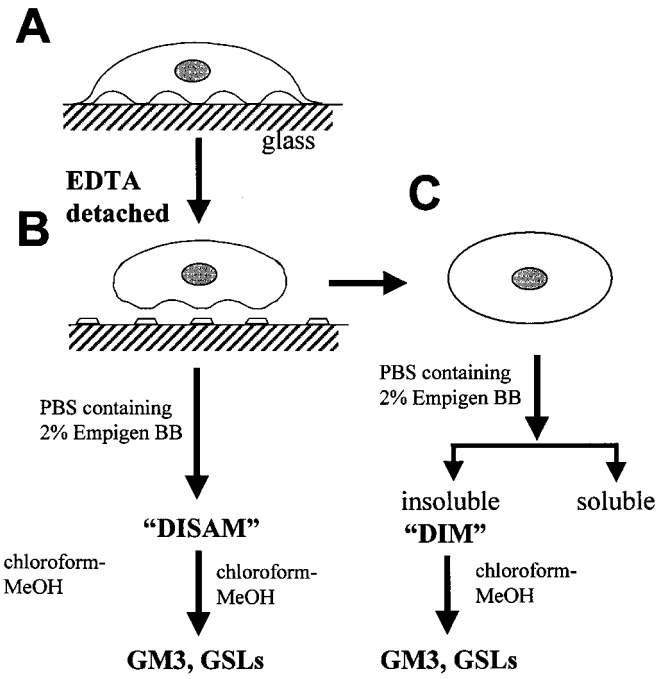

Figure 3. Association of GSLs, particularly GM3 ganglioside, in detergent-insoluble material (DIM) and detergent-insoluble substrate adhesion matrix (DISAM). BHK fibroblasts were grown on glass Petri dishes (**A**) and detached with EDTA, whereby cells were released leaving attachment sites on the glass surface (**B**). The attachment sites were treated with PBS containing 2% Empigen BB. The insoluble residue was termed 'DISAM', and was enriched in GSLs, particularly GM3 ganglioside. Cells detached from the glass surface contained detergent (Empigen BB)-insoluble material (DIM). GSLs, particularly GM3, were also detected in DIM by chloroform-methanol extraction. The data indicates that GSLs are enriched in not only DIM but also adhesion sites corresponding to focal adhesion sites. Figure constructed from data in Okada Y, et al. *Exp Cell Res* 155: 448–56, 1984.

endocytosis and signal transduction, and their function is blocked by cholesterol-binding reagents filipin, nystatin, and β-cyclodextrin (for review see [18]. It was unclear until recently whether GSLs are always associated with caveolae, and whether caveolae, DIM, and DRM (each characterized by the presence of GSL, GPI-anchor, and caveolin) are identical or distinct.

In fact, all these components are co-localized in detergent-insoluble, low-density membrane fraction after cells are Dounce homogenized in Tris buffer containing 1% Triton X-100 or high salt concentration, and 'post-nuclear' fraction is subjected to sucrose or Ficoll density gradient centrifugation. We found recently that this fraction can be separated into two, one containing GSL, the other highly enriched in cholesterol and containing caveolin (see 'Our current studies . . .').

Association of cytoplasmic transducer molecules with GPI-anchor or anchored proteins

The possible association of Src family kinases with specific membrane components was originally suggested by studies of how GPI-anchored proteins induce signal transduction when ligand binds to GPI-anchored receptors such as CD59, CD55, CD48, and Thy-1. Stefanova et al. found that $p56^{lck}$ is associated with GPI-anchored receptors as above in mouse T cells [19], while Thomas et al. showed that GPI-anchored Thy-1 in T cell hybridomas 2D4 and BW5147 is associated with $p60^{fyn}$ [20]. Stimulation of GPI-anchored proteins by their ligand induces activation of signal transducers. Soon afterward, both GPI-anchored proteins and Src family kinases were found to be components of DIM and/or caveolae (for review see [18].

Association of cytoplasmic transducer molecules with GSLs in DIM or caveolae

Although the presence of GSLs or gangliosides together with GPI-anchored receptors in DIM or caveolae has been documented, little attention has been paid to possible association of GSLs with transducer molecules, or functional significance of such association. Four independent studies have shed some light on this subject:

(i) Rat basophilic leukemia cells are characterized by presence of α-Gal GD1b, GD3, and other GSLs, although only α-Gal GD1b can be coimmunoprecipitated with the Src family kinase $p53/56^{lyn}$ [21]. (ii) Similarly, GD3 in rat brain is associated with $p53/56^{lyn}$, and both are present in DIM and coimmunoprecipitated with anti-GD3 mAb R24 [22]. (iii) GM3 in human peripheral lymphocytes and T cell lines is clustered at the cell surface, as observed by transmission EM, is found in DIM, and is coimmunoprecipitated with CD4 and $p56^{lck}$ [5]. (iv) GM3 in B16 melanoma cells, previously identified as melanoma-associated antigen [23], mediates adhesion of melanoma cells to endothelial cells to initiate metastasis [24]. > 90% of GM3 in B16 cells is found in DIM, and is coimmunoprecipitated with cSrc, Rho A, and focal adhesion kinase (FAK) (25). These data indicate that GSL clusters are organized with cytoplasmic signal transducer molecules, as illustrated schematically in Figure 4.

Our current studies: GSL function through 'GSD' in defining cell adhesion, signaling, and differentiation

Separation of GSD, and its involvement in cell adhesion coupled with signal transduction

A characteristic function of GSLs is to mediate GSL-dependent cell adhesion through various lectins or through complementary carbohydrates on one hand, and on the other to initiate or modulate signal transduction (see Figure 1). Our initial studies were focused on: (i) association of GM3 with cSrc, Rho A, Ras, and FAK in low-density, buoyant membrane fraction ('Fraction 5') prepared from B16 melanoma cells using media containing detergent (Triton X-100), or containing salt solution with high ionic strength at high pH without detergent; (ii) effect of GM3-dependent adhesion on activity of associated signal transducers cSrc, Rho A, etc. Essentially all

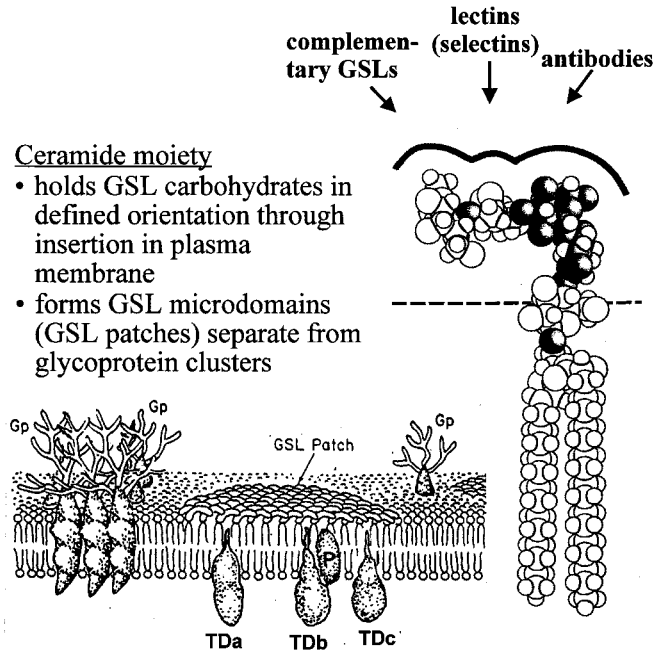

Figure 4. Minimum energy conformational model of GSLs and their organization in membrane. Upper right panel: Minimum energy conformational structure of typical GSL (globoside). Axis of oligosaccharide is perpendicular to axis of ceramide (N-fatty acyl sphingosine). Antibodies, lectins, and complementary GSLs interact with the outer surface profile of oligosaccharide. Lower left panel: Proposed self-aggregation of GSLs in lipid bilayer of plasma membrane ('GSL patch'). GSL patches are separated from clusters of glycoprotein (Gp). A few signal transducer molecules (TDa, TDb, TDc) may be associated with GSL clusters. Yet-unidentified hydrophobic protein (P) may also be associated with GSL clusters, and have some role in connecting transducer molecules to GSLs.

cellular GM3 was found in Fr. 5 from both preparation methods as above. Distribution patterns of various signal transducers and certain other membrane proteins (e.g. caveolin, proteolipid protein [PLP]) were similar regardless of preparation method (Table 1). GM3-dependent adhesion causes enhanced tyrosine phosphorylation of cSrc and FAK, and enhanced GTP binding of Rho A and Ras [26]. Enhanced motility of melanoma cells caused by GM3-dependent adhesion to endothelial cells was regarded as the initial step of melanoma cell metastasis [24]. Enhanced activity of cSrc, Rho A, etc. following adhesion of B16 cells through GM3-Gg3 interaction may provide an important pathophysiological rationale for this initial step of the metastatic process.

Through application of immunoseparation technique using anti-GM3 antibody DH2 or anti-caveolin antibodies, Fr. 5 was divided into two subfractions: (i) the fraction bound to anti-GM3 and not bound to anti-caveolin antibody contained GM3, sphingomyelin (SM), PC, cholesterol, cSrc, and Rho A, whereas (ii) the fraction not bound to anti-GM3 but bound to anti-caveolin contains no GM3, cSrc, nor Rho A, but does contain a small quantity of SM and PC, and large quantities of cholesterol, caveolin, and Ras (Table 2). A question subsequently arose whether membrane fraction or vesicles enriched in GM3, cSrc, etc. maintain GM3-dependent adhesion and associated activation of cSrc and other signal transducers, e.g. Fak. Lipid membrane or vesicles immunoseparated by anti-GM3 showed GM3-dependent adhesion to Gg3-coated plates (but not GlcCer-coated plates), leading to enhanced activation of cSrc and Fak [26].

Another example of GSL-dependent adhesion coupled with signal transduction is that of human teratocarcinoma 2102 cells, or Tera2 cells, which show clear autoaggregation

Table 1. Distribution patterns of cellular protein, GM3, and transducers in low-density microvesicular fraction (Fr. 5; GEM) and high-density fractions (Fr. 10–12).

	in medium cont'g 1% Triton X-100		*in hypertonic sodium carbonate*	
	GEM (Fr. 5)	*Fr. 10–12*	*GEM (Fr. 5)*	*Fr. 10–12*
	% composition relative to total extract			
Protein	*< 0.5*	*> 90*	*< 2*	*> 90*
GM3	*> 90*	*0*	*> 95*	0
	*distribution index**			
cSrc	~90	5	~90	5
Rho A	~50	~50	~40	~60
Ras H	~95	< 5	ND	ND
FAK	~10	~90	25	75
caveolin	85	< 10	95	< 5
PLP	100	0	ND	ND

*Relative intensity of band in Western blotting, given by equivalent protein content in each fraction (~2.5 μg)

Table 2. Differences between GSL microdomain (glycosignaling domain) and caveolae in B16 melanoma cells, in terms of chemical composition and function.

	glycosignaling domain	*caveolae*
GM3	+++	−
GlcCer	+	+
SM	++	±
cholesterol	+	+++
caveolin	−	++
cSrc, Rho, Ras	++	−
Ras H	−	+
PLP (proteolipid protein*)	+	?
GSL-dependent cell adhesion	++	−
c-Met receptor**	−	−
integrin receptor	−	−
effect of cholesterol binders filipin, nystatin	no effect on adhesion and signaling	destroy structure, block signaling

*Chloroform-methanol soluble protein with low molecular mass (~15 kDa) showing binding affinity to GM3.
**Major tyrosine kinase receptor expressed in B16 melanoma cells. Synonyms: hepatocyte growth factor receptor; scatter factor receptor. Present in high-density protein fraction (Fr. 12).

mimicking the compaction process of early embryogenesis, in which globoside (Gb4) and Gal-globoside (SSEA-3) play a major role in cell adhesion. The process occurs through Gb4-to-GalGb4 or Gb4-to-nLc_4 interaction, whereby signal transduction is initiated to activate transcription factor CREB or AP1 [27]. DIM fractions of both 2102 and Tera2 cells contain signal transducers such as cSrc, $p53/56^{lyn}$, Csk, and Rho A, together with GSLs such as Gb4, GalGb4, etc. [28].

GSD as target for induction of differentiation by exogenous GSL: Role of Csk in regulatory effect of cSrc in Neuro2a GSD

Neuronal cell differentiation leading to neuritogenesis is one of the central themes of neurobiology. Of particular interest is ganglioside-induced neuritogenesis as observed in neuroblastoma Neuro2a cells, which are nonsusceptible to Nerve Growth Factor (NGF) because they lack Trk A and $p75^{ntr}$, but are highly susceptible to exogenous ganglioside addition leading to neuritogenesis. GSD of Neuro2a cells contains GM1, GM3, LacCer, and Gb4 as GSL components, and cSrc, Csk, and $p53/56^{lyn}$ as signal transducers, but no caveolin. The presence in Neuro2a GSD of Csk, a physiological inhibitor of cSrc, is in striking contrast to the absence of Csk in B16 cell GSD. Csk and cSrc are mutually coimmunoprecipitated, and cSrc was coimmunoprecipitated with anti-GM3 mAb, from Neuro2a GSD. GM1 and GM3 exogenously added to culture medium of Neuro2a cells are preferentially incorporated in GSD, causing decrease of Csk and consequent relative increase of cSrc, and subsequent activation of mitogen-activated protein kinase (MAPK) leading to neuritogenesis. Tyrosine kinase inhibitor Lavendustin C inhibited neuritogenesis induced by GM3 or GM1 [29].

Inhibitory effect of lyso-GM3 and sialyl 2→1 Sph on GSD function: GSL-dependent cell adhesion coupled with signal transduction

Structure and function of caveolae are known to be disrupted by the cholesterol-binding reagents filipin [30], nystatin, and β-cyclodextrin [31]. GM3-dependent adhesion of B16 melanoma cells to Gg3-coated plates was not inhibited by subtoxic doses of filipin (0.1–0.3 μg/ml) or nystatin (15–30 μg/ml), although these doses greatly decreased cholesterol content of the cells (decrease of 90–95% by filipin, and > 95% by nystatin). Enhancement of FAK activity of B16 cells associated with GM3-dependent adhesion was essentially unchanged in the presence of 0.5 μg/ml filipin. These observations indicate that GSD structure and function of B16 cells, in contrast to caveolae, are relatively insensitive to filipin and nystatin [26].

We therefore looked for reagents capable of disrupting GSD structure and function. GSLs having no fatty acid (i.e. glycosylsphingosine, or lyso-GSL), and phospholipids having a single fatty acid (lyso-phospholipid), are known to be cytolytic, although the exact mechanism of lysis is not known. We compared the effect of various lyso-GSLs and lyso-phospholipids on GM3-dependent adhesion of B16 cells, and associated enhancement of FAK and cSrc activity. Lyso-GM3 and sialyl 2→1 Sph were found to inhibit both these processes, whereas other lyso-compounds such as lactosyl-Sph, galactosyl-Sph (psychosine), and lyso-PC had no effect at subtoxic doses. Compounds with substituted amino group (i.e. N-monochloroacetyl or N,N-dimethyl Sph linked to sialic acid), also had virtually no effect on these processes. N-dichloroacetyl substitution of amino group of sialic acid in lyso-GM3 (i.e. NeuNdcAc-lyso-GM3) showed essentially no cytolytic or cytotoxic effect, even at 100 μM, in striking

contrast to lyso-GM3 which showed cytolytic effect at 15–20 μM. Interestingly, NeuNdcAc-lyso-GM3 had a clear inhibitory effect on GM3-dependent adhesion and associated FAK and cSrc enhancement at 20–50 μM (Figure 5). It is therefore likely that lyso-GM3 and sialyl $2 \rightarrow 1$ Sph have specific affinity to GSD of B16 cells, where GM3 is the major component, and that they disrupt GSD structure and function [32,33].

Reconstitution of membrane simulating GSD function: GM3-dependent adhesion coupled with cSrc activation

In order to assess the essential components which display GSD function, membranes with properties similar to those of GSD were reconstituted using GM3, SM, and cSrc, with or without other lipid components. Lipid mixtures with various compositions were dissolved in Tris buffer containing 50 mM octylglucoside, mixed with an appropriate quantity of cSrc (molar ratio of GM3 to cSrc 1:1000–1:2000), and subjected to extensive dialysis to eliminate octylglucoside. During dialysis, lipid vesicles complexed with cSrc are formed. Sufficient quantity of cSrc was produced from insect cell line SF9. The heterogeneous vesicles thus formed were subjected to sucrose density gradient centrifugation to separate the fraction containing low-density, buoyant membrane vesicles. Reconstituted membrane prepared in this way had lipid composition similar to that of natural GSD prepared from DIM fraction of B16 melanoma cells, i.e. high level of SM and GM3, low level of PC and cholesterol (Figure 6A), and bound cSrc was immunoprecipitated with anti-GM3 mAb (Figure 6B). These membranes also displayed GM3-dependent adhesion to plates coated with Gg3 or anti-GM3 mAb, resulting in enhanced cSrc phosphorylation (cSrc phosphorylation response). This response in reconstituted membrane depends on GM3 concentration and was not observed when GM3 was absent or replaced with LacCer (Figure 7), or with other gangliosides GM1 or GD1a [33]. The GM3-dependent cSrc phosphorylation response was enhanced when cholesterol and PC were added, suggesting that some quantity of cholesterol and PC may act as an auxiliary factor to stabilize membrane, while GM3, SM, and cSrc are essential for maintaining structure and function of reconstituted membrane simulating GSD function. GSD function in terms of GM3-dependent adhesion and signaling was blocked in the presence of lyso-GM3, sialyl $2 \rightarrow 1$ Sph, or their analogues, but not psychosine, lactosyl-Sph, or lyso-PC [32,33]. Such suscept-

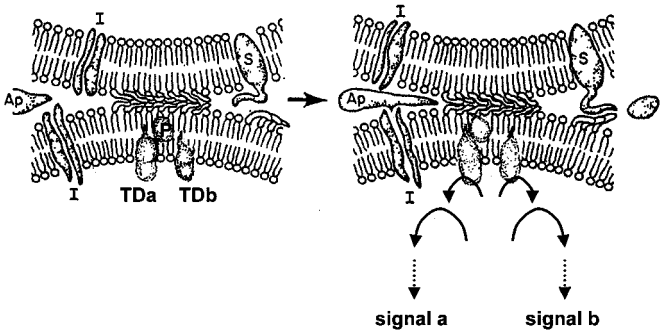

Figure 5. GSL-dependent adhesion between GSLs in GSD may activate signal transducers (TDa, TDb), creating a 'cascade' of signals.

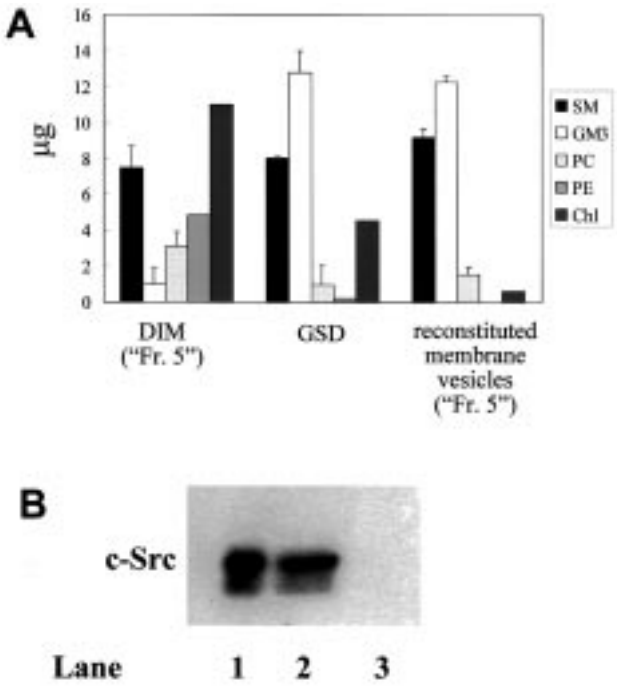

Figure 6. Composition of reconstituted membranes. **Panel A**: Lipid composition of reconstituted membrane as compared to low-density, detergent-insoluble membrane ('DIM') and 'glycosignaling domain' (GSD) immunoseparated from DIM. **Left group**: lipid composition of DIM isolated from 2×10^7 B16 cells as 'Fr. 5.' **Middle group**: composition of GM3- and c-Src-containing fraction (corresponding to GSD) immunoseparated from DIM (from 2×10^8 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 μg respectively), and separated as 'Fr. 5' by sucrose density gradient centrifugation. Note that ratio of SM:GM3:PC in resulting reconstituted membrane is similar to that in GSD separated from total DIM, although the lipid composition of total DIM is very different. Black bar, SM. White bar, GM3. Light gray bar, PC. Medium gray bar, phosphatidyl-ethanolamine. Dark gray bar, cholesterol (Chl). Values shown are means of two independent experiments, with standard variation indicated. **Panel B**: c-Src present in reconstituted membrane. Aliquots of reconstituted membrane purified by sucrose density gradient centrifugation were (i) 10x diluted with RIPA buffer, immunoprecipitated with goat anti-c-Src antibodies after preclearance, and Western blotted (**lane 1**); (ii) 10x diluted with IP buffer containing 0.1% Triton X-100, immunoprecipitated with anti-GM3 mAb DH2, washed with IP buffer containing 0.5 M 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. Result shown is from one typical experiment.

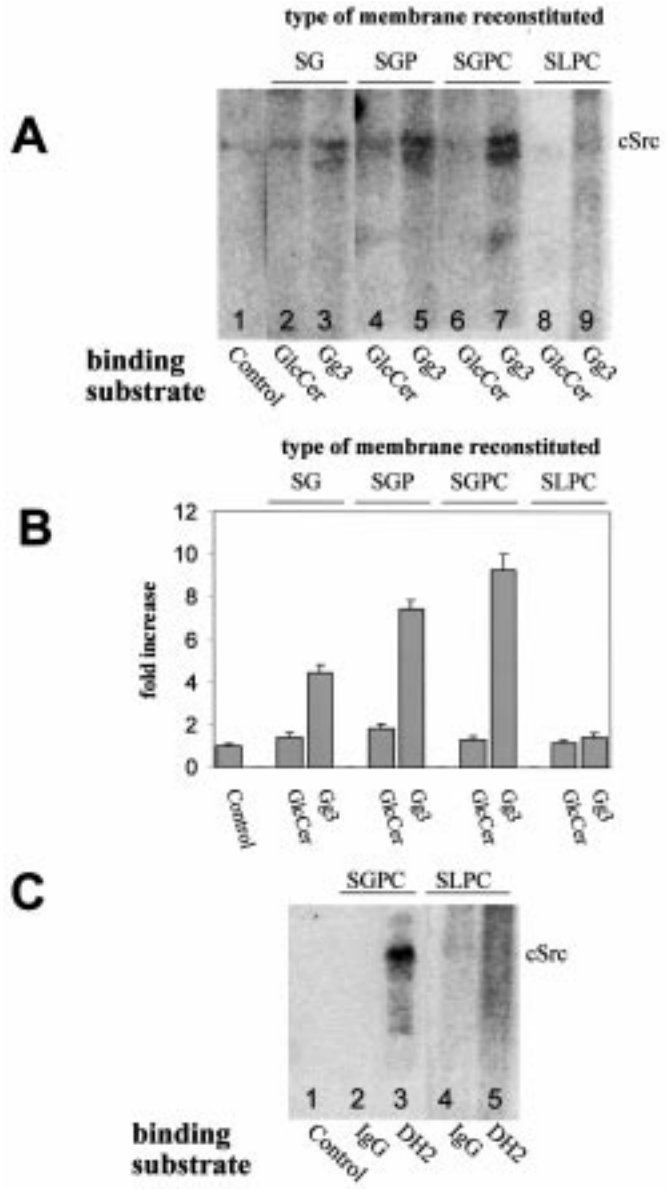

Figure 7. c-Src phosphorylation response of reconstituted membranes with four different lipid compositions when placed on dishes coated with GlcCer, Gg3, or anti-GM3 mAb. Membranes with different lipid components and proportions were reconstituted with c-Src as described in the text and purified by sucrose density gradient centrifugation. **Panel A**: c-Src phosphorylation response to adhesion of these reconstituted membranes to Gg3-coated dish, as compared to placement on GlcCer-coated dish. Reconstituted membranes with four different lipid compositions (as described in Materials & Methods) are abbreviated as follows. SG: SM + GM3, SGP: SM + GM3 + PC, SGPC: SM + GM3 + PC + cholesterol, SLPC: SM + LacCer + PC + cholesterol. Control value is 'SGPC' membrane placed in nonadherent polypropylene tube. Results shown are from one of two experiments. **Panel B**: c-Src phosphorylation response quantitated as fold increase of phosphorylation relative to control (defined as 1); mean ± SD from two independent experiments. **Panel C**: c-Src phosphorylation response to adhesion of reconstituted 'SGPC' membrane to dishes coated with anti-GM3 mAb DH2 (lane 3) or normal mouse IgG (lane 2). The response of 'SLPC' membrane is used for comparison (lanes 4 and 5). Control lane as in Panel A.

ibility of reconstituted GSD to lyso-GM3 and other lyso-compounds is the same as GSD of original B16 cells (Figure 8). Thus, functional organization of the reconstituted membrane closely simulates that of GSD in B16 cells, which is based on clustered GM3 organized with cSrc as essential components [33].

Discussion and future trends

Cell social function in multicellular systems, in terms of cell recognition and signal transduction, is one of the major themes of study in current cell biology. Previous studies by us and others suggest that GSLs and gangliosides play a major role in these processes [34,35]. The mechanism for GSL involvement in cell adhesion/recognition coupled with initiation of signal transduction has been partially clarified by studies of GSL-enriched microdomains.

Such microdomains show common properties such as detergent resistance, low density (buoyancy), enrichment in GPI anchor, GSLs, SM, and cholesterol, and presence of specific types of signal transducers (Src family kinases, small G-proteins). Association of Src family kinase ($p56^{lck}$) with GPI anchor, observed originally in T lymphocytes by Horejsi and colleagues [19], was extended to various signal transducers associated with GSLs in microdomains [5,21–23], although the functional significance of such association in terms of GSL-dependent adhesion was not clarified until recently [26,36]. Such microdomains are obviously not a single component, but consist of different entities. Among them, caveolae are the most extensively studied, as an invaginated morphological feature or a structural unit for endocytosis and signal transduction [18]. Since caveolar membranes show properties similar to those of the biochemically ill-defined mixture of membranes, termed 'rafts' by Kai Simons [17] or 'detergent-resistant membrane (DRM)' by Deborah Brown [16], these terms (particularly raft) have been often applied to mixtures of membrane domains regardless of chemical, biochemical, or cell biological identity. However, in the case of the microdomain involved in cell adhesion and signaling, the term 'raft' is inappropriate since this microdomain is immobile and does not resemble a raft, i.e. floating platform. We have used the term 'GSL-enriched microdomain (GEM),' to emphasize that enrichment of GSL is of central importance for the domain. The term 'GSL signaling domain (GSD)' is used if GSL-dependent signaling is clearly defined. GSD is separate from caveolar membrane, which is enriched in cholesterol but not GSLs, and is not involved in cell adhesion or signaling, as is obvious from its morphology. The major adhesive receptors, integrins, are usually excluded from caveolae [18], although a portion of integrins (20–40% of total) is detectable in DIM fraction depending on detergent concentration and type of cell. Tetraspan membrane proteins (TMP) such as CD9 and CD82, the facilitators of many membrane receptors [37], are also found in DIM fraction prepared under specific conditions, and are closely associated

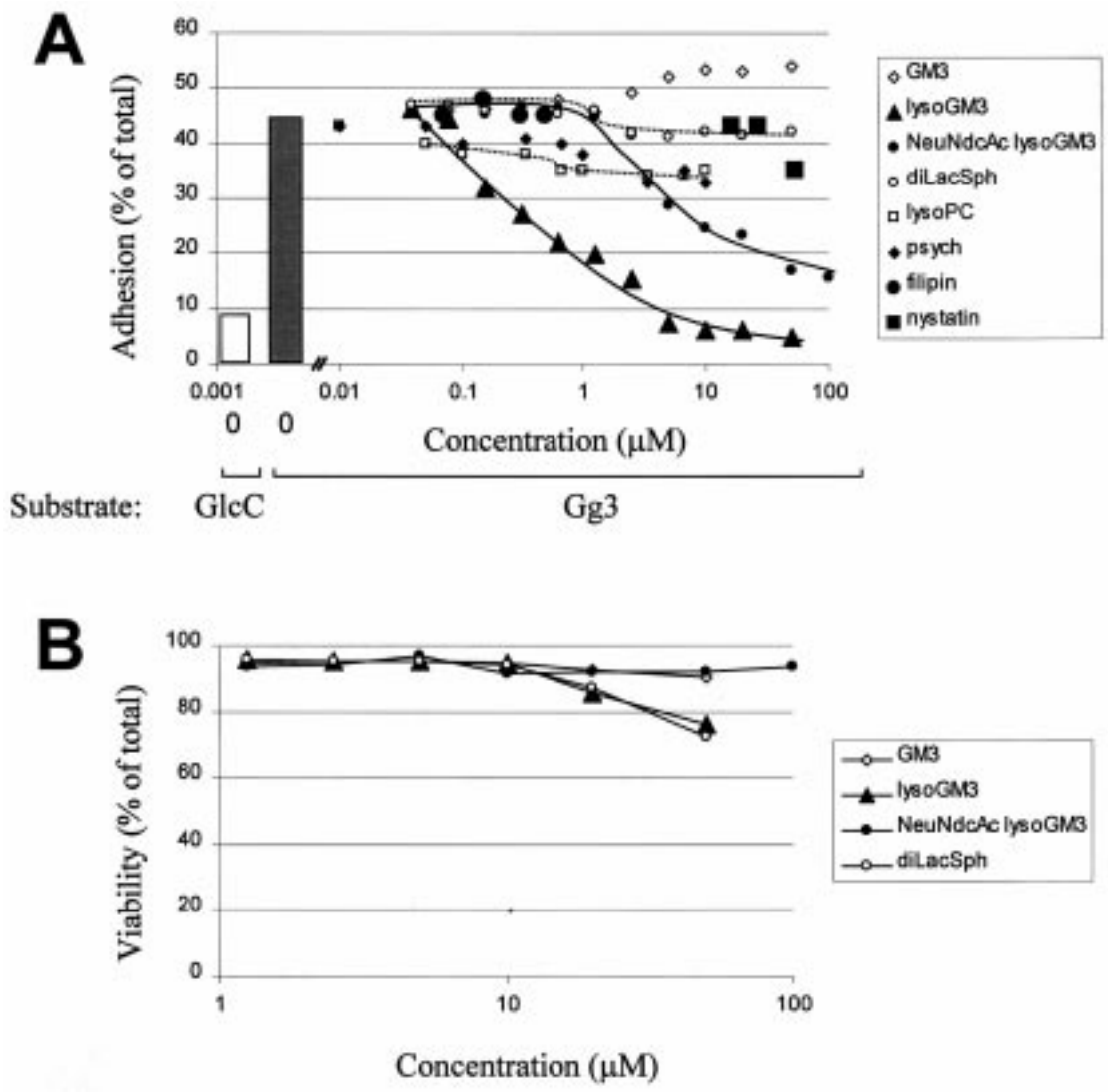

Figure 8. Effect of lyso-GM3, other lyso-GSLs, and lyso-PC on GM3-dependent adhesion and viability of melanoma B16 cells. **Panel A**: Effect on B16 cell adhesion to Gg3-coated dishes. B16/F10 cells detached by EDTA/trypsin were preincubated for 30 min with various concentrations (0.05–50 μM) of GM3 (◇), lyso-GM3 (▲), NeuNdcAc lyso-GM3 (small ●), dilactosyl-Sph (○), lyso-PC (□), psychosine (◆), filipin (large ●), or nystatin (■) in DMEM using DMSO as vehicle (final concentration of DMSO in DMEM 0.5%), followed by washing with DMEM, and plated on Gg3- or GlcCer-coated 24-well dishes for adhesion assay. Solid bar at left, Gg3-coated dish as positive control. Open bar, GlcCer-coated dish as negative control. Adhesion was expressed as percent of total cells added on dish. **Panel B**: Effect on viability of cells determined by Trypan Blue exclusion test. Cells were treated with various lyso-compounds as in Panel A and subjected to viability test. These cells were the same samples treated with lyso-compounds and used for adhesion assay. Symbols for reagents as in Panel A. Assays for cell adhesion and for viability are described in ref. [26].
For both Panels 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 $< 10\%$.

with GM3 ganglioside and integrins (Ono M, et al., unpubl. data). These observations indicate that there is another microdomain besides GSD and caveolae, which contains integrin and TMP and may be involved in control of cell adhesion and motility.

GSLs, particularly gangliosides, are well documented to inhibit or promote tyrosine kinases associated with growth factor or hormone receptors [38–42]. EGF and PDGF receptors are found in caveolar membrane, prepared under detergent-free conditions, and are claimed to be quickly translocated out of caveolae after their activation by growth factor [43–45]. How growth factor (or hormone) receptors and their associated tyrosine kinases are influenced by gangliosides through their microdomain remains to be clarified. Perhaps an entirely new experimental design is necessary to answer this question unambiguously.

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