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Functional role of glycosphingolipids and gangliosides in control of cell adhesion, motility, and growth, through glycosynaptic microdomains

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Abstract

At cell surface microdomains, glycosyl epitopes, carried either by glycosphingolipids, *N*- or *O*-linked oligosaccharides, are recognized by carbohydrate-binding proteins or complementary carbohydrates. In both cases, the carbohydrate epitopes may be clustered with specific signal transducers, tetraspanins, adhesion receptors or growth factor receptors. Through this framework, carbohydrates can mediate cell signaling leading to changes in cellular phenotype. Microdomains involved in carbohydrate-dependent cell adhesion inducing cell activation, motility, and growth are termed “glycosynapse”. In this review a historical synopsis of glycosphingolipids-enriched microdomains study leading to the concept of glycosynapse is presented. Examples of glycosynapse as signaling unit controlling the tumor cell phenotype are discussed in three contexts:

(i) Cell-to-cell adhesion mediated by glycosphingolipids-to-glycosphingolipids interaction between interfacing glycosynaptic domains, through head-to-head (*trans*) carbohydrate-to-carbohydrate interaction.

(ii) Functional role of GM3 complexed with tetraspanin CD9, and interaction of such complex with integrins, or with fibroblast growth factor receptor, to control tumor cell phenotype and its reversion to normal cell phenotype.

(iii) Inhibition of integrin-dependent Met kinase activity by GM2/tetraspanin CD82 complex in glycosynaptic microdomain.

Data present here suggest that the organizational status of glycosynapse strongly affects cellular phenotype influencing tumor cell malignancy.

Keywords

Glycosynapse; glycosphingolipids microdomain; glycosilation; tetraspanin; ganglioside; integrin

Introduction

Glycosphingolipids (GSLs), including gangliosides, have been known for many years to function in animal cells as antigens, and receptors for microbial toxins, as well as mediators

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of cell adhesion and modulators of signal transduction [1–3] (see Table 1). Many functions of GSLs are assumed to be based on their unique property to form clusters, presumably due to their ability to act as hydrogen bond donor as well as acceptor, and thereby undergo side-by-side (*cis*) interaction based on the presence of hydroxyl and acetamide group [4]. Clustered GSLs may provide detergent-resistant properties [5,6], and interact with various functional components on cell membrane.

GSL clusters at the cell surface membrane interact with functional membrane proteins such as integrins, growth factor receptors, tetraspanins (TSPs), and non-receptor cytoplasmic protein kinases (*e.g.*, Src family kinases, small G-proteins) to form glycosynaptic domains controlling GSL-dependent or -modulated cell adhesion, growth, and motility, for review see [7–9]. In this review, we describe (i) a historical overview of GSL microdomains, leading to the concept of glycosynaptic domain which is functionally distinct from "raft", *i.e.*, signaling platform with high motility which is not directly involved in control of cell adhesion, growth, and motility; (ii) cell-to-cell adhesion mediated by GSL-to-GSL interaction between interfacing glycosynaptic domains, through head-to-head (*trans*) carbohydrate-to-carbohydrate interaction (CCI); (iii) GM3/ TSP CD9 complex inhibitory effect of cell motility and proliferation through modulation of integrin and FGFR activation; (iv) GM2 complexed with TSP CD82, and inhibitory effect of GM2/CD82 complex on hepatocyte growth factor (HGF)-induced activation of Met and its cross-talk with integrin $\alpha 3\beta 1$.

GSL-enriched microdomains: From caveolae, to raft, to glycosynapse

Soon after the lipid bilayer hypothesis, in which lipids function as solvents for membrane proteins randomly distributed on cell surface was proposed by Singer and Nicolson [10], a possible heterogeneity of bilayer due to differences in fatty acid composition (degree of unsaturation) was suggested [11]. Further, studies on epithelial cells showed that GSL [12–14] and glycosylphosphatidylinositol (GPI)-anchored proteins [15,16] were preferentially target to the apical membrane. Moreover, biologically active membrane proteins were found to be associated with surrounding GSLs [17]. Clustered GSLs were originally demonstrated on erythrocytes by scanning electron microscopy applied to "freeze-fractured" surface [18], and are widely observed in all types of cell membranes, including liposome membrane prepared from phospholipid without cholesterol [19–22]. The concept of "GSL-enriched microdomain" (GEM) evolved, based on detergent-resistant properties [5,6], association of GSLs at morphologically unique caveolar structures, which are also enriched in characteristic hydrophobic membrane protein caveolin [23,24] (for review see [25]), and transmission electron microscopy with anti-GSL antibodies [21,26]. Since similar composition, detergent-resistance, and cholesterol-dependent properties (*i.e.*, structure and function are disrupted by cholesterol-binding reagents β -cyclodextrin, filipin, and nystatin) were found in not only caveolae but also non-caveolar region, the term "raft" was proposed, representing "floating signaling platform" [27]. The hypothesis of signaling transduction through membrane microdomains arose when growth factor receptor tyrosine kinase (GFR) was found to be inhibited by surrounding gangliosides [28,29] and when Src kinase activity was demonstrated to be associated with GPI-anchored proteins [30].

Many adhesion molecules involved in monocyte adhesion, principally adhesion between T- or B-lymphocytes and antigen-presenting cells, are organized in microdomains on membrane, particularly TCR for T-cells, and CD15 for B-cells. Organizational framework of such membrane proteins has been thought to be similar to that of caveolae or raft. However, there are many differences in size, dynamic status, and detergent-resistance properties. Thus, the term "immunological synapse" was proposed for such microdomain, which is claimed to be different from caveolae or raft [31].

A membrane with composition similar to GEM but highly enriched in TSP or hydrophobic proteolipid protein, soluble in chloroform/ methanol, was found to be cholesterol-independent. Such membrane domain is involved in glycosylation-dependent cell adhesion with concurrent signaling, is therefore basically different from caveolae or raft, and is termed "glycosynapse" [32]. A historical overview of development of GSL microdomains and other membrane domains is shown in Fig. 1.

Three different glycosynapses have been so far described (Fig. 2), for review see [8]:

- i) Type-1 glycosynapse containing GSLs and associated signal transducers. GSL in glycosynapse 1 is involved in adhesion between interfacing cells through carbohydrate-carbohydrate interaction (CCI) (Fig. 2Bi), or through interaction of GSL with its binding protein (Fig. 2Bii). For example, Lewis^x (Le^x) mediates morula-stage embryonal stem cells to induce "compaction" [33,34], or adhesion of embryonal carcinoma F9 cells to induce autoaggregation. The process is essentially cooperation of Le^x-to-Le^x self-adhesion [34,35] and classically-known uvomorulin- [36] or E-cadherin (Ecad) [37]-mediated adhesion. GSL-dependent adhesion through carbohydrate-binding proteins can be exemplified by GM1/galectin 1 mediated cell interaction [38,39].
- ii) Type-2 having *O*-linked mucin type glycoproteins associated with Src family kinases involved in carbohydrate-dependent cell adhesion and signaling (Fig. 2C).
- iii) Type-3 enclosing a complex of integrin receptor, tetraspanin and gangliosides controlling carbohydrate-dependent cell adhesion to extra cellular matrix (ECM) (Fig. 2D). Inhibition of cell motility and proliferation through modulation of integrin and FGFR function by GM3/GM2 ganglioside complex is an example of type-3 glycosynapse.

CCI as a basis for glycosynapse modulated cell adhesion and signal transduction

In the glycosynaptic microdomain glycosyl epitopes, carried either by GSL, *N*- or *O*-linked oligosaccharides, are recognized by carbohydrate-binding proteins or complementary carbohydrates. In both cases, carbohydrate epitopes are clustered with specific signal transducers, adhesion receptors or growth factor receptors. Through this framework, carbohydrates can mediate cell signaling leading to changes in cellular phenotype.

Glycoconjugates on cell membranes may interact side-by-side (*cis*-interaction) forming clusters stabilizing the glycosynaptic microdomain, or interact through their carbohydrate heads between two interfacing membranes (*trans*-interaction).

CCI, either *trans* or *cis*, displays proper specificity and affinity required for cell adhesion and consequent signal transduction. Affinity of CCI varies from low to high ($K_a = 10^{-5} - 10^{-8}$) depending on degree of clustering. Actually, CCI low affinity is compensated by multivalent presentation of the ligands [34,40–43]. Both strength and specificity of the CCI are defined by degree of clustering, multivalency, epitope structures, and their orientation, for review see [44]. Binding of carbohydrate epitope to the ceramide moiety, liposomes, plastic surfaces, or nanoparticle, promotes the proper orientation for clustering [45]. Oligosaccharides without carrier do not show CCI, rather, in some cases, they may inhibit CCI [34]. It should be noted that Le^x epitope bound to ceramide displays a high degree of self-recognition, whereas that bound to glyceride does not. This implies that orientation of Le^x epitope bound to ceramide is different from the same Le^x epitope bound to glyceride [46].

A proposed model for CCI between two adjacent cells is that of a zipper [47] or a gear (a wheel with teeth around its circumference) in which the purpose of the teeth being to mesh with

similar teeth on another gear wheel. In this model a perfect fit between interacting carbohydrates (gear's or zipper's teeth) is assured when distance between epitopes on the GSL of one cell are identical to the distances between adhesion epitopes on the adjacent cell.

Molecular forces operating in the CCI are the same to those acting between other biological molecules. Van der Waals forces are the major forces acting in this system. Dipole-dipole and London forces as well as hydrogen bonds including participation of water were observed [48,49].

Other characteristic feature of CCI is dependency of bivalent cations, particularly Ca^{2+} . Although not all CCI require Ca^{2+} , it seems to be essential for CCI in most biological systems [34,40–43]. Ca^{2+} ions may lock the glycoside residues in an optimal configuration to provide an adequate conformation for interaction, or they may act as a bridge between hydroxyl groups enhancing the adhesion force of adjacent molecules (Fig. 3A illustrate one example of CCI mediated by Ca^{2+} , see below) [40].

New findings on carbohydrate-to-carbohydrate interaction as a basis of cell signaling through glycosynapse are discussed in this review.

Cell-to-cell adhesion mediated by GSL-to-GSL interaction between interfacing glycosynaptic domains through *trans*-CCI

Adhesion between the same type of cells (homotypic adhesion) mediated by CCI was initially found in two different cell systems: (i) adhesion of early mouse embryo (embryonal stem cells) to induce compaction, or of embryonal carcinoma F9 cells to induce auto-aggregation, mediated by Le^x -to- Le^x interaction [33–35,50]; (ii) species-specific autoaggregation of sponge cells, mediated by specific oligosaccharides (*e.g.*, 3- sulfo-GlcNAc β 3Fuc α or 4,6-isopropylidene-Gal β 3GlcNAc β 3Fuc α in *Microciona prolifera*) [51–54], for review see [55]. Both adhesion systems (i) and (ii) are Ca^{2+} -dependent.

The system (i) is also mediated by Ecad, which was extensively studied during three decades, for review see [56,57].

The molecular basis of Le^x -mediated adhesion has been demonstrated by several studies during past years [34,35,42,46,58,59]. The adhesion force between a single pair of Le^x molecules was determined by atomic force microscopy [58]. Thermodynamic and quantitative kinetic data for the Ca^{2+} mediated self-aggregation of the Le^x molecules were obtained by isothermal titration calorimetry [59] and by surface plasmon resonance [42] respectively. Adhesion energy of two interfacing membrane vesicles containing Le^x GSL or various other GSLs was determined calculating the contact angle of two vesicles when equilibrium was reached [46].

The atoms involved in Le^x - Le^x interaction were determined by nuclear magnetic resonance (NMR) spectroscopy using a dimer containing a flexible spacer between two Le^x trisaccharides epitopes (bis- Le^x) allowing several orientations for the Le^x moieties [40]. In absence of calcium ions no interaction between the trisaccharide moieties was observed. However, in presence of calcium the Le^x moieties assume a single conformation with hydrophobic contacts between the H2 of GlcNAc and the H2 of Gal', between the H1 of GlcNAc' and the CH₃ of Fuc, and between the CH₃ of Fuc CH₃ and the COCH₃ of GlcNAc'. These contacts result from a structure where the GlcNAc (**a** and **a'**) rings are coplanar and the Fuc ring (**b**) is located approximately 90° from Fuc ring (**b'**) (Fig. 3A). The conformation proposed for the two epitopes would allow the *trans*-contact between Le^x glycoconjugates from adjacent cell membranes (*trans*-CCI, Fig 3B left) or a *cis*-interaction between Le^x GSL within the same microdomain (*cis*-CCI, Fig 3B right).

Despite the fact that the Le^x- Le^x interaction has been clearly established on a biochemical and biophysical basis [34,35,42,46,58,59], the basis of Le^x-mediated adhesion in biological system was still not irrefutable mainly due to the complexity of cell membrane and the difficulty of rule out the possibility of co-occurrence of Ecad and/or other protein mediated interaction. A very recent study demonstrated unambiguously that Le^x-mediated adhesion and autoaggregation take place in F9 or embryonal stem cells (D3M) in which E-cadherin is completely knocked out (F9 Ecad (-/-) or D3M Ecad (-/-) cells). These cells strongly express Le^x as in wild-type F9 or D3M cells (Fig. 4) [43].

There have been several other studies of CCI systems in which the same or different types of carbohydrate epitopes are involved, for review see [44].

Glycosynapse modulated cell signaling through cis-CCI

CCI may not only mediate cell-to-cell adhesion based on interfacing glycosyl groups carried by clustered GSLs (*trans*-CCI), but also provides the basis for interaction of GSLs with other glycosyl residues present in the same membrane microdomain, *i.e.*, *cis*-CCI. A model *cis*-CCI in glycosynapse is illustrated in Fig. 5. For example, GM3 is known to interact with the extracellular domain of the epidermal growth factor receptor (EGFR) to inhibit receptor tyrosine kinase [29], but the mechanism and site of EGFR for such interaction has been unclear. GM3 was recently shown to interact with a specific *N*-linked glycan having multivalent GlcNAc termini [60]. The increased expression of *N*-glycans with GlcNAc termini in cells treated with swainsonine, which inhibits α -mannosidase-II, causing accumulation of hybrid-type structures having GlcNAc termini, was closely associated with the inhibitory effect of GM3 on EGFR tyrosine kinase (Fig. 6) [61]. These findings suggest that GM3 inhibits EGFR tyrosine kinase by binding to GlcNAc residues present on EGFR *N*-linked oligosaccharides in a *cis*-CCI process (as illustrated in Fig 5a). In agreement with this hypothesis is the finding that *N*-glycosylation is required for EGFR function [62].

Within the concept of *cis*-CCI, a GD1a/ GD1b complex, cause an unusual immunogenicity associated with Guillain-Barré Syndrome. The antibody present in sera of patients with this syndrome does not react with GD1a or GD1b, but reacts with the complex GD1a/ GD1b. The presence of such antibody was significantly associated with the neuropathy severity [63,64]. Similarly, a novel GM2/ GM3 complex assumed to be present in normal bladder epithelial cells might play an important role in TSP CD82-dependent inhibition of cell motility and growth (see "GM2 complexed with TSP CD82", below).

GM3/TSP CD9 complex inhibits cell motility and proliferation through modulation of integrin and FGFR activation

Both gangliosides and TSP are reported to locate at GSL microdomains in association with integrins [65–67]. Integrins have been implicated in regulating cellular processes such as adhesion, mobility, signaling, for review see [68]. Integrin function, including α/β -subunit interaction, is affected by *N*-glycosylation status (for review see [69]) and by interaction with TSP and/or gangliosides [7,66]. TSP are palmitoylated and *N*-glycosylated and associate with integrin receptors, gangliosides and signaling molecules forming a membrane multi molecular complex referred as tetraspanin web [66]; for review see [70].

Since TSP CD9 inhibits cell motility and its expression is down-regulated in various human cancers [71,72], a possibility was opened that CD9 function was affected by glycosylation. Id1D mutant of Chinese Hamster Ovary cells, defective in UDP-Glc: 4-epimerase, has been utilized for study of glycosylation of functional proteins [73,74]. Id1D cells with high CD9 expression were cloned after CD9 gene transfection. Motility of these Id1D/ CD9 cells was

greatly inhibited when cells were grown in serum-free medium (ITS: insulin/ transferrin/ selenium) containing galactose [65], allowing glycoproteins to be fully glycosylated and GM3 to be synthesized. A close association of GM3 with CD9 function was found in the following series of further studies, which will be discussed in more detail below:

(i) CD9 and integrin $\alpha 3$ were co-immunoprecipitated in ldlD/ CD9 cells when GM3 was synthesized (+Gal condition), but not when GM3 synthesis did not occur (–Gal condition). Interaction of GM3 with CD9, and CD9 with $\alpha 3$, were demonstrated by confocal microscopy. GM3/ CD9/ $\alpha 3$ are associated in the same microdomain, which is resistant to 1% Brij 98 but soluble in Triton X-100 [67]. Since CD9 is chloroform/ methanol soluble, its complex with GM3 or other gangliosides was expected, similarly to proteolipid protein [75,76].

(ii) Various colorectal tumor cell lines whose motility was clearly inhibited by exogenous GM3 addition were all characterized by high CD9 expression. Motility of a CD9-non-expressing tumor cell line was unaffected by GM3 addition, but became inhibitable by GM3 when CD9 was expressed by its gene transfection [66].

(iii) Addition of ^3H -labeled photoactivatable GM3 having ω -phenylazido acyl group to HRT18 cells, followed by UV irradiation, caused specific ^3H -labeling of CD9 but not other glycosynaptic proteins ($\alpha 3$, $\alpha 5$, or $\beta 1$ integrin). However, other proteins were labeled by the probe [66].

(iv) Down regulation of GM3 synthesis is associated with oncogenesis in v-Jun transformation. Transfection of GM3 synthase gene resulted in reversion of oncogenic to normal phenotype in v-Jun-transformed chicken and mouse fibroblasts and inhibition of motility and invasiveness through formation of GM3/ CD9/ $\alpha 5\beta 1$ complex [77].

(v) Human diploid embryonal lung WI38 fibroblasts are highly contact-inhibitable cells. They are biochemically unusual in having high level of CD9 and CD81, which are complexed with FGFR. GM3, the major ganglioside in these cells, interacts specifically with FGFR, whereas other gangliosides and GSLs do not. Since FGFR is closely associated with cSrc and GM3, cell contact induced by interaction of GM3 with FGFR may inhibit tyrosine kinase associated with FGFR as well as c-Src [78]. The exact mechanism for GM3 interaction with FGFR remains to be elucidated.

(vi) In a typical case with bladder cancer cells, decrease or depletion of GM3 by P4 (D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol) suppresses interaction of CD9 with integrin $\alpha 3\beta 1$, leading to enhanced motility and invasiveness [79]. Such conversion of less malignant to highly malignant cell phenotype was also caused by decrease of CD9 by RNAi. Besides, exogenous addition of GM3 resulted in inhibition of motility in YTS1 cells (Fig. 7A). These results suggest that integrin/ CD9/ GM3 organized in membrane, "glycosynapse 3" (for review see [32]), may define tumor cell invasiveness. This is also consistent with previous observations that highly invasive YTS1 is reverted to less-invasive phenotype by enhanced GM3 expression induced by brefeldin A [80]. Moreover, Mitsuzuka and coworkers [79] demonstrated that GM3 levels, in bladder cancer cells, define glycosynapse function by controlling the interaction of CD9 with integrin $\alpha 3$ (Fig. 7B); and by modulating c-Src activity. Enhanced levels of GM3 induce csk translocation into glycosynapse resulting in phosphorylation on Tyr 527 of c-Src with consequent inhibition of c-Src activity and cell motility (Fig. 7C).

GM2/ GM3/ CD82 complex inhibits hepatocyte growth factor (HGF)-induced activation of Met, and its cross-talk with integrin $\alpha 3\beta 1$

TSP CD82 was originally found as product of metastasis-suppressing gene Kal-1, highly expressed in normal epithelial cells such as prostate, bladder, or colorectal epithelia and down-regulated or depleted in their metastatic deposits [81–83]. CD82 is known to suppress cell invasiveness by inhibiting functional interaction of integrin with tyrosine kinase receptor for HGF, Met [84]. Met has been implicated in promotion of cancer cell motility and invasiveness; for review see [85]. In analogy with CD9, we expected to observe an effect of glycosylation on CD82-dependent motility inhibition [65]. (i) We initially observed that GM2, but not GM3 or Gb4, specifically interacted with CD82 in normal bladder epithelial cell line HCV29 (Fig. 8A), while GM3 showed specificity for CD9 (see preceding section). (ii) GM2/ CD82 complex physically interacted with Met (Fig. 8B) inhibiting functional interaction of integrin $\alpha 3$ or $\beta 1$ with Met, whereby HGF-induced Met tyrosine phosphorylation was strongly suppressed. (iii) Treating normal cells with P4, which depleted GM2, or abrogating CD82 expression by RNAi method, greatly enhanced HGF-induced Met phosphorylation and cell motility. In contrast, highly invasive bladder cancer cells, YTS1 (lacking CD82), were characterized by HGF-independent Met activation and cell motility. Met activation and cell motility were inhibited by co-expression and mutual interaction of GM2 with CD82 (see Fig. 9 and text below for details), as observed in YTS1 cells transfected with CD82 gene; or by the exogenous addition of GM2 (91).

(iv) YTS-1 cells, when adhered on LN5-coated plate, showed strong activation of Met phosphorylation without stimulation by HGF, and this process was promoted when gangliosides were depleted by P4 treatment of YTS-1 cells (Fig. 9). These results indicated that highly malignant cells are characterized by enhanced cross-talk between integrin and Met kinase. Such cross-talk in normal cells is minimal, but was greatly enhanced when GM2 was depleted by P4; *i.e.*, CD82/ GM2 complex plays a major role in inhibiting not only HGF-induced Met kinase activity (Fig. 10a) but also LN5-induced cross-talk between integrin and Met (Fig. 10b) [86].

The molecular mechanism of GM2 inhibition of the HGF-Met signaling pathway leading to cell motility may be controlling the distribution of CD82 in- and outside of the glycosynapse; and interacting with CD82 in the glycosynapse forming the GM2/CD82 complex which acts as a functional constituent of the microdomain. Fig. 9 shows a hypothetical scheme for this mechanism. Besides, inhibition of GM2/CD82 complex on Met activation, or on $\alpha 3$ -to-Met interaction, may involve *cis*-CCI between GM2 and *N*-linked glycan of CD82 (Fig. 5b), since partial deletion of three *N*-linked glycans (at Asn129, 157, and 198) from mutant CD82 caused remarkable change in interaction with $\alpha 3$ and $\alpha 5$ integrins [87].

Further studies on effects of various gangliosides, and their combinations, on HCV29 cell motility, clearly indicate that GM2 together with GM3 (but not other gangliosides or GSLs, or their combinations) show stronger binding to CD82, compared to GM2 or GM3 alone, based on the following observations: (i) GM2 binding to CD82 was greatly enhanced by addition of GM3, although GM3 *per se* did not bind to CD82 [88]. (ii) Cells expressing CD82, when cultured with silica nanospheres co-coated with GM2 and GM3, displayed much stronger inhibition of cell motility than those cultured with silica nanospheres coated with GM2 alone. (iii) GM2/ GM3 combination in the above process strongly inhibited phosphorylation of Src and MAPK. (iv) Id1D mutant cells transfected with GM2 synthase gene showed greatly reduced motility when endogenous synthesis of both GM2 and GM3 occurred, as compared with cells grown under conditions in which only one of these gangliosides was synthesized. In addition to functional changes (i) to (iv) as above, a physical and chemical basis for interaction of GM2 and GM3 was provided by (a) electrospray ionization mass spectrometry [88], and (b) *in situ*

cross-linking of cell surface GM2 and GM3 by periodate oxidation followed by succinyl dihydrazide (unpubl. results).

Taken together, these results suggest the existence of heterotypic *cis* carbohydrate-to-carbohydrate interaction of GM2 and GM3, providing a basis for control of cell motility through inhibition of signal transduction.

Conclusions

Several studies have demonstrated that glycosylation plays a central role in defining tumor progression; for review see [89]. Results discussed in this review demonstrate that glycosylation dictates the organizational status of glycosynapse, which in turn strongly affects cellular phenotype influencing tumor cell malignancy. These observations open a new possibility that malignancy is not defined by tumor-specific molecules or their genes but rather is caused by disorganization of cell membrane components. Supporting this hypothesis, a classic work demonstrate that highly malignant teratocarcinoma kills the host when intraperitoneally inoculated, but it undergo to normal development when inoculated in blastocyst [90,91]. More recently Bissell group has demonstrated that misregulation of signaling pathways involved in epithelial cell communication with neighboring cells and ECM results in loss of tissue organization contributing to tumor formation and progression [92,93]. These works furnish an unequivocal example of malignance arising from disorganization, rather than from changes in gene structure.

Therefore, modulation of glycosynapse functions can lead to new strategies in cancer therapy. Noteworthy is the inhibition of GM3-dependent adhesion [94] and α EGFR signaling [95] by lyso-GM3. Elucidation of the molecular mechanism of interaction among components in glycosynaptic domain will require extensive physical studies and might bring insights into approaches to disrupt or promote such interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GFR, growth factor receptor tyrosine kinase
 GPI, glycosylphosphatidylinositol
 GSL, glycosphingolipids

CCI, carbohydrate-to-carbohydrate interaction
Ecad, Ecadherin
EGFR, epidermal growth factor receptor
ECM, extra cellular matrix
FGFR, fibroblast growth factor receptor
GEM, GSL-enriched microdomain
HGF, hepatocyte growth factor
LN, lamin
Le, Lewis
Neu3, ganglioside-specific sialidase
NMR, nuclear magnetic resonance
nOe, nuclear overhauser effect
TD, signal transducers
TSPs, tetraspanins

Fig. 1.

Overview of key steps during the development of glycosynapse concept. **1.** First observations of polarized sorting of GSL [12–14] and glycoproteins [15,16] in epithelial cells. **2.** Observation of GSL organization associated to biological function in cell surface membranes [17]. **3.** Demonstration of GSL clustering in cell surface membranes by freeze-fracture [18], and at the surface of GSL-phosphatidylcholine liposome [19,20]. **4.** Concomitantly, it was found that clustered GSL were insoluble in 1 % Triton X-100 due to their association with cytoskeleton components and extra cellular matrix [5,97]. **5.** Demonstration that tyrosine phosphorylation of growth factor receptor is affected by neighboring GSLs in microdomains [28,29]. **6.** Stefanova and coworkers [30] found that proteins anchored to the membrane by glycosphosphatidylinositol (GPI) anchor associate with protein tyrosine kinase related to Src family. **7.** Presentation of rafts as new structure of cell membranes [23,27]. **8.** Current work.

Fig. 2.

Illustration of three types of glycosynapses proposed by Hakomori [32]. **A.** Glycosyl epitopes involved in cell-to-cell adhesion are found in glycosynapse 1 (**B**) and 2 (**C**), while carbohydrates implicated in cell-to-ECM adhesion compose glycosynapse 3 (**D**). **B.** Glycosynapse 1 is based on GSL-to-GSL interaction (**Bi**) or GLS-to-binding protein interaction (**Bii**), in which GSL are clustered with signal transducers (TD). **C.** Glycosynapse 2 is based on interaction between O-linked oligosaccharides of mucin like glycoproteins and carbohydrate binding proteins associated with TD. **D.** Glycosynapse 3 is established by carbohydrate dependent association of N-glycosylated adhesion receptor (e.g. integrin receptor), TSP, GLS

and TD mediating cell adhesion to ECM. Cell adhesion and motility are greatly inhibited when glycosynapse 3 complex is stabilized by glycosylation of integrin receptor or TSP.

Fig. 3.

A. Solution conformation of bis-Le^x calcium complex. GlcNAc (**a**, **black**), Fuc (**b**, **blue**) Gal (**c**, **red**) and the methylene spacer, linking the GlcNAc, is shown in green. The same conformational minimum is shown from two different points of view. Observe that rings **a** and **a'** are coplanar (left), and ring **b** is located approximately 90° from ring **b'** assuming a cross like orientation which is more visible from a different direction (right). **B.** Proposed Le^x-Le^x interaction between glycolipids anchored to the same microdomain (*cis*-CCI) or anchored to adjacent microdomains (*trans*-CCI). Two Le^x pentasaccharides, a Le^x trisaccharide (filled pyranose rings) plus a lactose moiety (open pyranose rings), were sketched with the relative orientation of the Le^x epitopes as demonstrated in **A**, above, were the **GlcNAc** (**a** and **a'**) rings are coplanar and the **Fuc** ring (**b**) is located approximately 90° from **Fuc** ring (**b'**). The

orientation proposed in **A** allows for *cis*- and *trans*-CCI between Le^x glycoconjugates. Data from Geyer et al. [40].

Fig. 4.

Le^x mediates homotypic adhesion of embryonal cells independently from E-cad. Adhesion of: (1) F9 Ecad (-/-), (2) D3M Ecad (-/-) and control (3) PYS-2 cells to plates coated with Le^x GSL (Le^x GSL (Gal β 4(Fuc α 3)GlcNac β 3Gal β 4Glc β Cer)). Both cells F9 Ecad (-/-), (2) D3M Ecad, in the absence of Ecad expression, adhered in a dose-dependent manner to plates coated with 0.25, 0.5, 1.0, or 2.0 $\mu\text{g/ well}$ of Le^x GSL. PYS-2 cells, a cell line derived from mouse 129 embryonal carcinoma OTT6050 (from which F9 cells were derived) but do not express Le^x glycan, did not show adhesion to Le^x GSL-coated plates. Data from Handa et al. [43].

Fig. 5. Illustration of *cis*-CCl₄ controlling glycosylation-dependent cell motility and/or growth. Integrin subunits α and β , TSP and GFR interaction mediated by *N*-linked glycans and surrounding GSL. GSL associate with Src family kinases and other signal transducers. **a.** GFR and its interaction with surrounding gangliosides. **b.** TSP interaction with gangliosides.

Fig. 6. Interaction of EGRF with GM3 but not GM1 or Gb4 and inhibition of EGRF/GM3 interaction by Fr. B but not by cellobiose. EGRF, from A431 cell lysate, shows strong binding to GM3 coated polystyrene beads (*lane d*), weak or no binding to beads coated with GM1 (*lane b*), Gb4 (*lane c*) or control beads. Binding of GM3 to EGRF was abrogated by co-incubation with a N-linked glycan with five to six GlcNAc termini, Fr.B (*lane f*) but was not inhibited by the Glc disaccharide, cellobiose (*lane e*) [61].

Fig. 7.

Effects of GM3 levels, in bladder cancer cells, glycosynapse.

A. Exogenous addition of GM3 (50 μ M) resulted in significant inhibition of phagokinetic cell motility in YTS1 cells as compared with control and GM1 (50 μ M) treated cells.

B. GM3 mediates interaction of $\alpha 3$ integrin with CD9. Exogenous addition of GM3 to YTS1 cells causes enhanced co-immunoprecipitation of $\alpha 3$ integrin and CD9. No effect was observed when GM1 was added to the cells. **Upper panel:** Cell lysates were immunoprecipitated with anti- $\alpha 3$ or anti-CD9, as control, and analyzed by Western blot with anti-CD9. **Bottom panel:** Densitometry of $\alpha 3$ band co-immunoprecipitated with CD9 in control and GM3- or GM1-treated cells.

C. Increased GM3 levels on c-Src phosphorylation and csk localization in fractions obtained by sucrose density centrifugation. Enhanced GM3 levels did not cause significant change in Src phosphorylation or csk level in the total extract (PNF). However, an increased

phosphorylation at Tyr-527, with clear translocation of csk to GEM fractions 4–6 (maximum at 3 h) associated with a simultaneous decrease in phosphorylation of Tyr-416 was observed [79].

Fig. 8.

GM2 complexes with CD82 and promotes CD82 interaction with Met

A. CD82, specifically interacts with GM2-coated polystyrene beads (d). No interaction was observed when YTS1/CD82⁺ cell lysate was incubated with noncoated (a) Gb4-coated (b) or GM3-coated (c) beads. (e) protein load (30 μg).

B. Effect of GM2 on CD82 and Met interaction on CD82 expressing cells HCV29 and YTS1/CD82 cells. Exogenous addition of GM2 to HCV29 and YTS1/CD82 cells enhances interaction between CD82 and Met (b and e) while ganglioside depletion by P4 significantly decreases this interaction (c and f) when compared with medium treated cells (a and d). Data from Todeschini et al. [86].

Fig. 9.

Hypothetical associations among components of glycosynapse from bladder epithelial cells. Bladder epithelial cells express two major receptors as follows: (i) HGF receptor Met and its kinase (shown at left), which is inhibited by GM2-CD82 complex ("a"); (ii) integrin receptor $\alpha 3 \beta 1$, which binds to extra cellular matrix component LN5/10–11 upon cell adhesion (shown at right). $\alpha 3 \beta 1$ activation is blocked by GM3-CD9 complex in bladder epithelial cells ("b") [79]. The functional interaction between systems i and ii is blocked by GM2-CD82 complex ("c"). Signaling shown for both systems is arbitrary, based on a few previous reviews or studies by others and by our group [79,85]. Grb2 and Gab1 are initial signaling molecules that may lead to activation of extra cellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), phosphatidylinositol 3-kinase (PI3K), or focal adhesion kinase (FAK) [85], controlling cell growth and motility. $\alpha 3 \beta 1$ may act through Src family kinases (which are inhibitable by Csk) [78,79], and lead to Rac/phosphatidylinositol 3-kinase/Akt signaling [99], controlling cell adhesion and motility. From Todeschini et al. [86].

Fig. 10.

Effect of integrin mediated adhesion of HCV29, YTS1, or YTS1/CD82 cells to Ln5 on Met kinase activation. The degree of Met activation (rate of Met phosphorylation relative to total Met level) of HCV29 (A), YTS1 (B), and YTS1/CD82 cells (C) was determined for cells in suspension (*lanes 1 and 2*) versus adhesion to LN5-coated plates (*lanes 3 and 4*). Met activation was also determined after GLS depletion by P4 (black versus gray columns). First row at bottom: intensity of Met tyrosine phosphorylation probed by Py20 in Western blot. Second row: Met level by Western blot. Third row: suspension without (-) or with (+) LN5 adhesion. Fourth row: pre-incubation without (-) or with (+) P4. Data were expressed as mean $\pm$ SD. Significance of differences indicated by bracket: *** $p < 0.001$. Data from Todeschini et al. [86]. Observe that adhesion to LN5 of normal HCV29 cells did not affect Met tyrosine kinase phosphorylation, apparently due to ganglioside inhibition since, ganglioside depletion increases Met phosphorylation upon adhesion to LN5 (*lane 4 versus 1-3*). Adhesion of YTS1 and YTS1/CD82 cells to LN5-coated plate greatly increased Met tyrosine phosphorylation, as compared to suspension culture (*lane 3 vs. 1 B and C*). The degree of enhancement of Met kinase activity by LN5-dependent adhesion was increased when gangliosides were depleted by P4 treatment (*lane 4 vs. 3 in B and C*).

Table 1

General functions of GSLs [100]

1.	GSL antigens	<ul style="list-style-type: none"> • blood group ABH; Lewis (Le^a, Le^b, Le^c, Le^d, etc); I/i; P, P^k and p antigens; • heterogenic Forssman; Hanganutziu-Deicher (NeuGc epitope); Galα1$\rightarrow$3Galβ1$\rightarrow$3GlcNAc (di-Gal); • cell type specific antigens (CD15, CD76, CD75, CD71, CD57, CD65; • developmentally regulated [stage-specific embryonic antigen-1 (SSEA-1), Le^x, SSEA-3, SSEA-4, etc]; • tumor-associated antigens [Gb3, Gal-Gb4, Globo-H, GD3, GD2, fucosyl GM1, sialosyl-Le^c, sialosyl-Le^a, etc).
2.	GSL receptors for microbial infection and toxins, e.g.	<ul style="list-style-type: none"> • cholera toxin (GM1); • Shigella or verotoxin (Gb3); • Botulinum neurotoxin (GT1b, GD1a as co-factors of protein receptor); • <i>E. coli</i> infection (globo-series GSL); • <i>Staphylococcus</i> infection (Gg4; asialo-GM1).
3.	GSLs in cell adhesion/ recognition	<ul style="list-style-type: none"> i. <u>Mediated by GSL-binding protein, e.g.</u> <ul style="list-style-type: none"> • neuronal cell adhesion to myelin sheath membrane mediated by gangliosides and myelin-associated glycoproteins; • myelogenous GSLs to selectin; • GM1 mediating adhesion through galectin-1. ii. <u>Mediated by GSL-GSL interaction, e.g.</u> <ul style="list-style-type: none"> • GM3-LacCer/ Gg3 interaction mediating melanoma adhesion to endothelial cells (initiation of metastasis); • Gb4-GalGb4/ nLc₄ interaction mediating human teratocarcinoma cell interaction (model of human embryo compaction); • Le^x-Le^x interaction mediating mouse embryogenesis. • Glycan-to-glycan interaction mediating species-specific sponge cell aggregation in proteoglycan [54,101]; • GalCer/ GalCer-sulfatide interaction in myelin membrane mediating signal transduction in oligodendrocytes [102,103]; • sperm (KDN)GM3 interaction with Gg3-like epitope, expressed on micropyle of vitelline envelope in egg, mediating sperm-to-egg interaction in rainbow trout fertilization [104].
4.	GSLs controlling cell motility and signal transduction	<ul style="list-style-type: none"> i. <u>GSL to TSP interaction, e.g.</u> <ul style="list-style-type: none"> • GM3/TSP CD9 complex [73–79] • GM2/GM3/CD82 complex [86]. ii. <u>GSL to GSL interaction through cis-CCI</u>