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The combination of carbohydrate and lipid generates unusual molecules in which the two distinctive halves of the glycoconjugate influence the function of each other. Membrane glycolipids can act as primary receptors for carbohydrate binding proteins to mediate transmembrane signaling despite restriction to the outer bilayer leaflet. The extensive heterogeneity of the lipid moiety plays a significant, but still largely unknown, role in glycosphingolipid function. Potential interplay between glycolipids and their fatty acid isoforms, together with their preferential interaction with cholesterol, generates a complex mechanism for the regulation of their function in cellular physiology.

The chemical identification of sphingosine/sphingomyelin by Thudichum (1884) marks the beginning of the enigma in terms of glycosphingolipid (GSLs) function. Their extensive compositional characterization, defines more than 300 species (Stults et al. 1989; Hakomori 2008). However, this large complement of chemically defined GSLs, containing on average 1–8 sugars, may significantly underrepresent the total GSL "glycome" because polyglycosyl ceramides, containing up to 60 sugar residues, have been described by Karlsson and colleagues (Miller-Podraza et al. 1993, 1997) but have not been followed up since their initial isolation.

Despite early compositional definition, functional studies on GSLs lag behind other macromolecular biomolecules, (e.g., proteins, or even glycoproteins). Indeed, the revolution in molecular biology and structural biology seem to have largely by-passed GSLs. GSL crystal structures are extremely rare (Pascher and Sundell 1977), much rarer than membrane proteins, for example (Loll 2003). Three dimensional GSL structures have been attained within protein complex crystals, rather than as separate entities (Zajonc et al. 2003; Malinina et al. 2006; Wu et al. 2006), and these resolve structures largely incompatible with lamellar membrane presented GSLs.

DIVERSITY AND SYNTHESIS OF GSLs

Ninety percent of mammalian GSLs are based on glucosyl ceramide. Galactosyl ceramide is the precursor for the remainder, essentially composed of galactosyl ceramide itself, its 3′ sulfate ester, sulfatide (sulfogalactosyl ceramide), and galabiosyl ceramide. The major GSL series are defined by their internal core carbohydrate

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sequence. These are the ganglio (galNAcβ1-4 gal), globo (galα1-4gal), lacto (galβ1-3glcNAc-β1-3gal), and neolacto (galβ1-4glcNAcβ1-3gal) series of GSLs, and gangliosides, which are the sialic acid α2-3Gal linked acidic GSLs, for the most part, based on the ganglio GSL series (Fig. 1). Lactosyl ceramide provides the branch point for the synthesis of all these GSL series. Thus, glucosyl ceramide synthase (GCS), which generates the ceramide monohexoside precursor of lactosyl ceramide, is a major control point for the regulation of GSL biosynthesis in toto. For each GSL, the ceramide fatty acid composition is heterogeneous because of fatty acid selective ceramide synthases (Teufel et al. 2009). The functional significance of this lipid heterogeneity has yet to be defined but plays a role in membrane organization (Panasiewicz 2003) and modulation of GSL receptor function (Lingwood 1996; Panasiewicz et al. 2003).

GLUCOSYL CERAMIDE SYNTHASE LOCATION PRESENTS A PROBLEM FOR GSL SYNTHESIS

Of GSL glycosyl transferases, only glucosyl ceramide synthase (GCS) is cytosolic (Futerman and Pagano 1991; Jeckel et al. 1992; Lannert et al. 1994). The remaining glycosyl transferases are at the site of carbohydrate extension, i.e., membrane proteins facing the Golgi lumen. An exception is galactosyl ceramide synthase within the ER lumen (Carruthers and Carey 1983; Sprong et al. 1998). Thus, glucosyl ceramide is synthesized using ceramide embedded within the cytosolic surface of the Golgi. The mechanism by which Golgi ceramide, rather than ceramide within the ER or elsewhere, is targeted by GCS is yet unknown. Knockdown studies show FAPP2, a small, PH domain containing protein with homology to glycolipid transfer protein, plays a key role

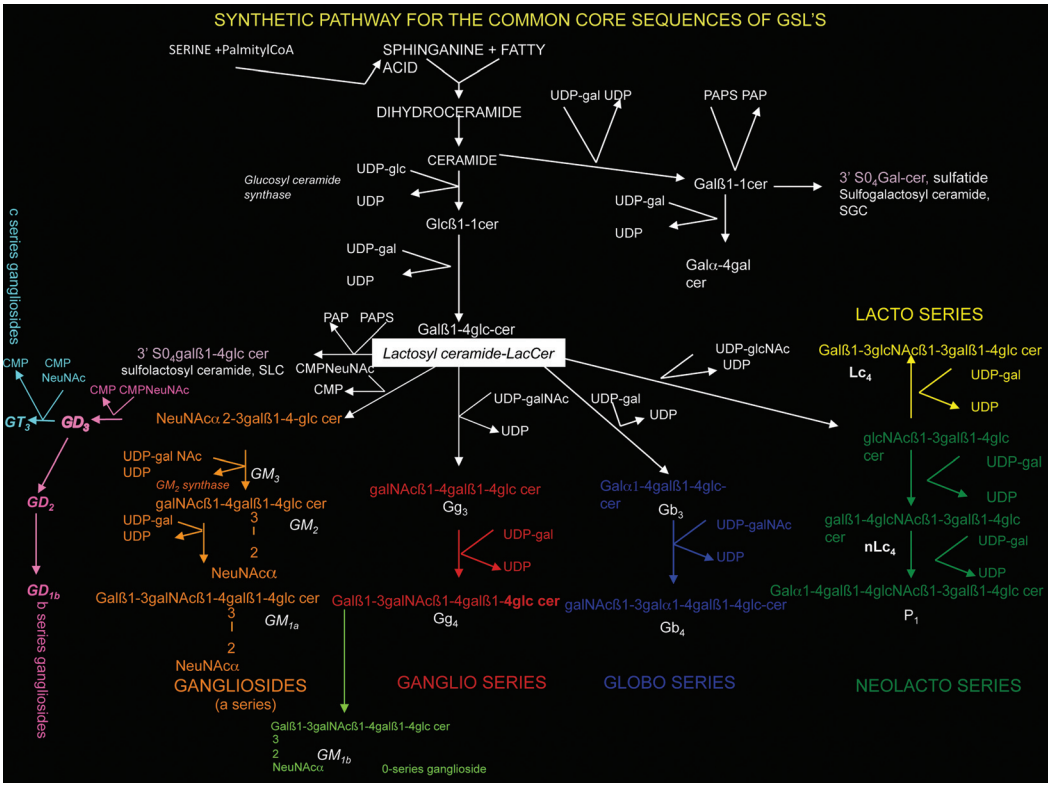

Figure 1. Synthetic pathways for the major GSL species. Glucosyl ceramide is the key precursor for most GSLs and lactosyl ceramide provides the branch point for the different GSL series.

(D'Angelo et al. 2007). It is proposed that cytosolic membrane GlcCer is trafficked from *cis*- to *trans*-Golgi stacks by FAPP2, but the mechanism by which glucosyl ceramide is subsequently flipped to the Golgi lumen remains undefined. Cytosolic Golgi GlcCer may also be retrogradely transported to the ER by FAPP2 (Halter et al. 2007), to spontaneously flip across the less ordered ER membrane and once lumenal, undergo vesicular transport to the Golgi for extension into more complex GSLs. However, the results of FAPP2-induced Golgi tubulation, independent of GSL binding (Cao et al. 2009; Lenoir et al. 2010), in these processes has yet to be assessed.

The MDR1 drug efflux pump (ABCB1) can flip GlcCer, but not lactosyl ceramide analogs across model membranes (Eckford and Sharom 2005). MDR1 is expressed in Golgi (Molinari et al. 1998; De Rosa et al. 2004) and inhibitors of MDR1 can reduce cellular GlcCer levels and neutral GSLs (Lala et al. 2000; De Rosa et al. 2004; Smith et al. 2006). The MDR1 inhibitor cyclosporin can reduce the levels of Gb_3 in tissues of the Fabrys mouse (Mattocks et al. 2006). It has been questioned whether MDR1 can flip native GlcCer (Halter et al. 2007), but other studies report both ceramide monohexoside and lactosyl ceramide as MDR1 substrates (Mizutani et al. 2008). Cyclosporin was effective to inhibit microsomal conversion of exogenous GlcCer to lactosyl ceramide (De Rosa et al. 2004). The specificity of cyclosporin however, still remains a question. Nevertheless, inhibition did not result in a reduction of ganglioside biosynthesis, despite a major reduction in GlcCer and lactosyl ceramide levels (De Rosa et al. 2004). Thus, gangliosides and neutral GSLs may be derived from different precursor pools.

THE FUNCTIONS OF GLYCOSPHINGOLIPIDS

GSL functions can be divided into (1) those in which overall inhibition of cell GSL synthesis has an effect, and (2) those in which a specified GSL plays a specific role.

Inhibition of GSL Synthesis

Several selective inhibitors of GCS have been developed which allow assessment of depletion of most GSLs (those based on GlcCer). These are either iminosugar derivatives (Andersson et al. 2000; Mellor et al. 2004) or inhibitory product mimics (Inokuchi et al. 1989; Lee et al. 1999; Abe et al. 2001).

GSLs and Intracellular Protein Trafficking

GSL biosynthesis was first shown to be important to the intracellular trafficking of proteolipids to the myelin membrane (Pasquini et al. 1989). Later studies showed a dramatic effect of GSL depletion on tyrosinase mislocalization in melanocytes (Sprong et al. 2001). Without GSLs, tyrosinase, necessary for melanin synthesis, did not reach melanosomes but accumulated in the Golgi. It was later proposed that GSL/cholesterol trafficking plays a more pervasive role in protein sorting during intracellular vesicular trafficking (Sillence et al. 2002). Inhibition of cellular GSL biosynthesis results in the loss of cell surface immunodetection of MDR1 (Wojtal et al. 2006; De Rosa et al. 2008) and its loss from lipid rafts (Kamau et al. 2005), suggesting an intimate relationship between these processes, potentially mediated by the lipid raft requirement for MDR1 function (Kamau et al. 2005). In MDR1-MDCK cells, cell surface MDR1 colocalized with globotriaosyl ceramide (Gb_3), and a soluble analog of Gb_3 proved an MDR1inhibitor (De Rosa et al. 2008). In plant cells, secretory products accumulate in swollen Golgi after GSL synthesis inhibition, and overexpression of such products results in a compensatory increase in GlcCer and sterol synthesis (Melser et al. 2010). This is consistent with evidence that protein cargo sorting during Golgi transit is based on lipid gradients, rather than time-dependent cysternal transit (Patterson et al. 2008). A two-phase membrane system was proposed (Lippincott-Schwartz and Phair 2010), whereby new protein processing occurs in a (GSL/cholesterol deficient) central Golgi compartment, whereas "mature" cargo exit occurs from GLS/cholesterol enriched distal Golgi domains (Orci et al. 1981). This model is

based on the different dimensions of cholesterol enriched versus depleted membranes, and the propensity of cholesterol to form more ordered (GSL-enriched), membrane domains. Golgi traffic would be effected via a biosynthetic compartment, restricted to less ordered, GSL/cholesterol-poor membranes, and an exit compartment within more ordered, thicker, GSL/cholesterol-enriched membranes (Lippincott-Schwartz and Phair 2010; Sharpe et al. 2010). The differential apical versus basolateral membrane GSL sorting (van Meer et al. 1987) that first gave rise the concept of lipid rafts, may be the simplest of a complex lipid-based system for differential membrane domain function during intracellular vesicular trafficking (Jackson 2009).

GSL Metabolism Affects Ceramide Levels

GCS glycosylates the primary hydroxyl of ceramide to form glucosyl ß1-1 ceramide. As such, GSL synthesis can deplete the ceramide pool and because ceramide and its metabolites are extensively involved in mechanisms of cellular growth control (Lin et al. 2006), GCS can indirectly affect these pathways. Indeed, cytotoxic drug-induced ceramide accumulation, to effect growth control in various tumor cells, can be subverted by increased GCS activity (Liu 2001), such that GCS can play a crucial role in the development of drug resistance in cancer chemotherapy (Gouaze et al. 2004; Liu et al. 2008c; Patwardhan et al. 2009). Tumor cells up-regulate GSC to deplete (drug-induced) ceramide pools, which would otherwise prove cytotoxic, via conversion to a more benign GSL format (Gouaze-Andersson and Cabot 2006).

GSL-Deficient Mice

GCS knockout mice die in utero (Yamashita et al. 1999), indicating a crucial role for GSLs during embryogenesis. Endoderm, mesoderm, and ectoderm were formed but tissue differentiation was blocked. GCS was detected in eight cell embryos and GCS k/o blocked embryogenesis during gastrulation by ectodermal apoptosis (Yamashita et al. 2002). Indeed, several globoseries GSLs expressed at this time, have been identified as stage-specific antigens in embryogenesis (Kannagi et al. 1982b; 1983a; Andrews 1987; Sekine et al. 1987) and several, particularly SSEA-4, are used as the major markers (Muramatsu and Muramatsu 2004) and means to isolate, human pleuripotent stem cells (Table 1) (Venable et al. 2005; Gang et al. 2007). Knockout of GM2 synthase to deplete all complex gangliosides, resulted in only a minor loss of neural nerve conductance (Takamiya et al. 1996). The compensatory increase in simple gangliosides (GM3, GD3) observed may ameliorate overt deficiency-induced differentiation blockages. Deletion of GM3 synthase (Yamashita et al. 2003) similarly results in mice with little overt pathology, but these mice still contained gangliosides GM1b, in which the terminal galactose of Gg_4 is sialated, and GD1α (GalNAc of GM1b is 2,6 sialated). In contrast, mice in which GM3 synthase and GM2 synthase are deleted and no gangliosides are made, show severe neurological pathology and die soon after birth (Yamashita et al. 2005). Nevertheless, these mice show a less severe phenotype than GCS deficient mice, suggesting the importance of neutral GSLs. Depletion of lactoseries GSLs is embryonic lethal via prevention of embryo implantation (Biellmann et al. 2008). Epithelial-to-mesenchymal cell transition is also GSL (Gg_4, GM2)-dependent (Guan et al. 2009). In contrast, depletion of globoseries GSLs is without overt pathological effect (Okuda et al. 2006), consistent with retention of stem cell pleuripotency after SSEA3/SSEA4 depletion (Brimble et al. 2007).

GalCer and SGC are the major GSLs of the myelin membrane and implicated in oligodendrocyte differentiation (Coetzee et al. 1998). Knockout the ceramide galactosyl transferase (Coetzee et al. 1996; Dupree et al. 1998), and then the galactosyl ceramide sulfotransferase (Honke et al. 2002) in mice showed GalCer and SGC are required for paranode formation in the axonal sheath, and SGC is a negative regulator of oligodendrocyte differentiation to form myelin (Hirahara et al. 2004). Compensatory increase in GlcCer, allowed normal differentiation but lack of the stabilizing GalCer and

Table 1. Stage-specific glycosphingolipid antigens

Stage-specific embryonic antigen (SSEA)	GSL structures	Occurrence	References
SSEA-1	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβl-4GlcNAcβl-3Galβ1-4 Glc ceramide, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβl-4 GlcNAcβ1-3Galβl-4GlcNAcβl-3Galβ1-4 Glc ceramide Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβl-4(Fucα1-3)GlcNAcβ1-3Galβl-4GlcNAcβl-3Galβ1-4 Glc ceramide Galβ1-4GlcNAcβ1-6GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-ceramide	4-8-cell embryo blastomeres erythrocytes, mouse kidney, astrocytes, oviduct, edometrium and epididymis human granulocytes, renal tumors, glioblastoma initiating cells	Solter and Knowles 1978; Fox et al. 1981; Kannagi et al. 1982; Knowles et al. 1982; Lagenaur et al. 1982; Liebert et al. 1987; Sekine et al. 1987; Son et al. 2009
SSEA-2		4–8-cell embryo blastomeres mouse spermatozoa	Shevinsky et al. 1981
SSEA-3	Galβ1-3GalNAcβ1-3Galα1-4 Galβ1-4 Glc ceramide	Ova, early cleavage stage embryonic cells, visceral endoderm, mouse kidney, human (not mouse) embryomal carcinoma cells, embryonic stem cells, testicular germ cells, non-metastatic seminoma, breast cancer stem cells, dorsal root ganglia	Damjanov et al. 1982; Jessell and Dodd 1985; Sekine et al. 1987; Ohyama et al. 1995; Thomson et al. 1998; Chang et al. 2008
SSEA-4	NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4 Galβ1-4 Glc ceramide	Pleuripotent embryonic stem cells, dorsal root ganglia, kidney, renal carcinoma, testicular germ cell tumors	Holford et al. 1994; Cooling et al. 1995; Thomson et al. 1998; Saito et al. 2003; Gang et al. 2007

SGC carbohydrate–carbohydrate interaction (Boggs et al. 2000), reduced conduction progressing to paralysis with age (Coetzee et al. 1996). Sulfoglycolipid synthesis is a marker of spermatogenesis (Lingwood 1985) and the male infertility of these knockouts confirmed their importance in this process (Fujimoto et al. 2000; Honke et al. 2002).

Insights from GSL Storage Diseases

While knowledge of the functional properties of specific GSLs is restricted, abnormalities in GSL physiology are apparent in several diseases that provide clues as to function. Most notable among these are the GSL lysosomal storage diseases (LSDs) (see also Schulze and Sandhoff 2011). These are diseases in which lysosomally located carbohydrate hydrolases, which mediate GSL catabolism, are defective and therefore substrate GSL accumulates. The diseases are categorized according to the accumulating GSL: GM2 gangliosidosis (Sandhoff disease, Tay-Sachs disease), globotriaosyl ceramide (Fabry disease), glucosyl ceramide (Gaucher disease), galactosyl ceramide (Krabbe disease), sulfogalactosyl ceramide (multiple leukodystrophy), and, although not a GSL storage disease, another important LSD is Niemann Pick type C disease. The gangliosidoses surprisingly, result not from

sialidase deficiency but rather β-hexosaminidase. Hexosaminidase comprises one α and one β subunit. Mutations in the β subunit give Tay-Sachs disease, whereas mutations in the α subunit result in Sandhoff disease. Defects in α-galactosidase mediate Fabrys disease and defects in β-glucocerebrosidase induce Gauchers disease. In these LSDs, subcellular GSL multivesicular inclusions can be observed. Thus, a primary feature of excess GSL is abnormal vesicular membrane structures. Intracellular GSL trafficking is aberrant, combined with altered cholesterol trafficking. In Niemann Pick type C, the primary defect is in cholesterol (Ribeiro et al. 2001). Cholesterol accumulates, and undergoes, together with GSLs, aberrant intracellular traffic (Puri et al. 1999). The retrograde transport of endogenous GSLs or exogenous fluorescent GSL analogs from the plasma membrane to the Golgi, seen in normal cells, is disrupted in LSD cells and GSLs are instead targeted to endosomes and lysosomes (Pagano 2003). Cholesterol accumulation and altered GSL trafficking was found for cells from all GSL LSDs (except Gaucher disease [Puri et al. 1999]) and intracellular trafficking of Bodipy-LacCer was proposed as a diagnostic (Chen et al. 1999). The increased lysosomal GSL causes lysosomal/endosomal cholesterol accumulation and ER cholesterol depletion (Puri et al. 2003) to alter lipid traffic. In Niemann-Pick type C, the increased lysosomal cholesterol (Ribeiro et al. 2001) causes GSL accumulation. Inhibition of GSL synthesis corrects cholesterol homeostasis (Lachmann et al. 2004). Similarly, depletion of excess cholesterol in LSD cells also corrects the GSL trafficking abnormalities (Puri et al. 1999). Similar cholesterol, and hence GSL, accumulation/trafficking is seen in cystic fibrosis (White et al. 2004; Gentzsch et al. 2007; Manson et al. 2008). These studies show the tight linkage between GSL and cholesterol metabolism and trafficking.

Specific Membrane GSL Functions

These can be essentially divided into those in which GSLs serve as a membrane receptor for an extracellular GSL binding ligand, and those in which membrane GSLs interact laterally with other components of the cell membrane, particularly growth factor receptors, to modify signal transduction. Examples of primary receptor function include the bacterial subunit toxins, Vero(Shiga) toxin, cholera toxin, and the heat labile *E.coli* toxins LT-1, LT-IIa, LT-IIb, CTx and LT-1 bind GM1, LTIIa GD1b> GD1a>GM1, LT-IIb, and GD1a only. Gangliosides also provide the primary receptor for the lectin, myelin associated glycoprotein, mediating the inhibitory effect of this glycoprotein on axonal regeneration. The second category primarily comprises the effect of gangliosides on NGF and EGF signal transduction, and more recently, on insulin signaling.

Membrane GSL Receptors for Exogenous Microbial Virulence Factors

Cholera Toxin. CTx is the cause of cholera. Cholera still represents a major health threat in the developing world. There is yet still no specific therapy but careful electrolyte management has greatly reduced mortality. CTx is an AB_5 subunit toxin (Gill 1976), the small B subunit pentamer mediating pentavalent binding to its receptor ganglioside GM1. CTx binding was the first described GSL receptor function (Heyningen 1974). The B subunit–GM1 oligosaccharide has been cocrystallized to resolve the binding site (Merritt et al. 1994), but within this site, only tyrosine 12 was found crucial for GM1 binding (Jobling and Holmes 2002). B subunit–GM1 binding mediates the internalization of the holotoxin and its subsequent retrograde transport through endosomes, *trans*-Golgi network, and Golgi to the endoplasmic reticulum, in which the A subunit separates and is transported through the Sec61 translocon into the cytosol (Schmitz et al. 2000). It has recently been found that CTx can bypass the Golgi to access the ER (Spooner et al. 2008). CTx is an ADP ribosyl transferase (Gill and Meren 1978), which ribosylates the stimulatory α subunit of the heterotrimeric G protein that activates adenylate cyclase to stimulate the CFTR chloride transporter, responsible for the massive water loss characteristic of cholera (Thiagarajah and

Verkman 2003). Retrograde ER transport is an intrinsic property of GM1 because mutational studies, which altered the binding specificity to GD1a, resulted in the loss of retrograde ER targeting and effective ADP ribosylation (Wolf et al. 1998). Although the mechanism of GM1 retrograde transport is largely unknown, acyl saturation and fatty acid chain length promote GM1 association with lipid rafts in cells (Panasiewicz et al. 2003). Coupling GM1 oligosaccharide to various lipid backbones showed variable efficacy in mediating cholera toxin cytopathology (Pacuszka et al. 1991), indicating that the retrograde transport pathways is a function of both the carbohydrate and lipid moieties, but an endogenous ligand for GM1 has yet to be defined. In contrast, GM1 sugar coupled to protein is ineffective to mediate internalization (Pacuszka and Fishman 1992). GM1 accumulates in microdomains, both in model (de Almeida et al. 2005), and plasma membrane bilayers (Chinnapen et al. 2007)

CTx B subunit provides mucosal adjuvant activity via GM1 binding-mediated signal transduction (Schnitzler et al. 2007), largely independent of A subunit action, and has been used in clinical trials (Sun et al. 2010). This adjuvant property is shared with the ganglioside binding *E.coli* heat labile toxins (Connell 2007). GM1 binding (crosslinking) is key to their immunomodulatory activity. Differential immune activation (CTxB,LT-I:Th2, LT-11a,b:Th1/Th2) is attributed to the different gangliosides bound (Arce et al. 2005; Connell 2007).

Verotoxins (VTs, Shiga Toxins). These are a family of *E. coli* elaborated AB_5 subunit toxins responsible for the pathology of hemolytic uremic syndrome (HUS) (Karmali et al. 2010). This life-threatening disease is primarily a renal glomerular nephropathy that results in anemia and thrombocytopenia (Ray and Liu 2001). The systemic toxins target the endothelial cells of the microvasculature, primarily in the pediatric renal glomerulus and cell death results in blood vessel occlusion and renal infarct. Despite extensive studies over the last 20 years, no specific treatment for this infection is yet available and HUS retains approximately 5% mortality. Outbreaks of gastrointestinal VTEC infection remain a serious threat, particular in the developed world (Rangel et al. 2005; Uhlich et al. 2008). The cow is the animal reservoir and infection results from ingestion of fecal contaminated foodstuffs (Erickson and Doyle 2007). *Shigella dysenteriae* infections involving the VT homolog, Shiga toxin 1, are largely confined to unsanitary conditions within the developing world, with Shiga toxin-induced HUS being only one component in the etiology of dysentery, with increasing importance in children (Nathoo et al. 1998; Bennish et al. 2006). The B subunit pentamer of VT binds to Gb_3. Gb_3 synthase knockout mice are completely protected (Okuda et al. 2006), indicating Gb_3 is the only function receptor for these toxins. Verotoxin 1 and VT2 (approximately 60% homologous to VT1) are primarily responsible for clinical disease but VT2 is associated with a more severe prognosis (Werber et al. 2003; Kawano et al. 2008a). This, despite the fact that VT2 is less cytotoxic than VT1 in vitro (Tam et al. 2008). VT2, more than VT1, is able to bind Gb_3 in neurological tissue (Fujii et al. 2001).

While both toxins bind Gb_3, the binding affinity of VT2 is somewhat lower than that of VT1 (Itoh et al. 2001; Nakajima et al. 2001). VT1 and VT2 recognize overlapping but also distinct epitopes within the Gb_3 carbohydrate (Chark et al. 2004). There are at least two, and perhaps three, Gb_3 binding sites within each subunit of the B-pentamer (Ling et al. 1998). These were shown in the VT1B-Gb_3 oligosaccharide cocrystal, but Gb_3 glycolipid binding is distinct from the lipid-free sugar (Soltyk et al. 2002). Verotoxin-Gb_3 binding is dependent on both Gb_3 carbohydrate and lipid moiety and its membrane environment. This effect has been termed aglycone modulation of GSL receptor function (Lingwood 1996). Since GSLs are heterogeneous in their fatty acid composition and membrane organization, a significant potential for an effect of membrane organization on the availability of the carbohydrate for ligand binding, such as VT1 and VT2, exists. Thus, not all Gb_3 containing cells are sensitive to VT. Toxin-Gb_3 binding results in

internalization by both clathrin dependent (Torgersen et al. 2005) and independent (Nichols et al. 2001) mechanisms. In model bilayers, VT1 can cluster bound Gb_3 to change the membrane curvature (Windschiegl et al. 2009), and induce plasma membrane invagination/tabulation (Romer et al. 2007), independent of other effector molecules. Tubule scission is cholesterol and Gb_3 (hydroxy) fatty acid dependent (Römer et al. 2010). Nevertheless, the prevailing view is that following internalization, VT engages in clathrin dependent (Saint-Pol et al. 2004) retrograde transport via endosomes, TGN, and Golgi to the ER (Falguieres et al. 2001). Here the proteolytically cleaved A1 subunit separates from the holotoxin and is translocated to the cytosol to inactivate protein synthesis by depurination of the 28S RNA of the 60S ribosomal subunit (Saxena et al. 1989). In addition, transmembrane src kinase activation by toxin binding and clustering of Gb_3 in lipid microdomains has been shown (Katagiri et al. 1999; Mori et al. 2000), suggesting signal transduction pathways distinct from the process of inhibition of protein synthesis, may be involved in apoptosis induced by this toxin (Fujii et al. 2003; Tetaud et al. 2003).

Cell Activation via Lactosyl Ceramide. Lactosyl ceramide (LacCer) is becoming increasingly recognized as a bioactive GSL in human disease and aspects of differentiated cell physiology. Two mechanisms predominate, one of activating src kinase family members and the other Erk1/2 activation to affect proliferation of various cell types (Won et al. 2007; Chatterjee and Pandey 2008).

(1) Neutrophil Lyn Kinase: The interdigitation of the acyl chains of long chain LacCer with the cytosolic bilayer leaflet to activate lyn-kinase has been strongly implicated in ligand activation of human neutrophil phogocytosis (Nakayama et al. 2008). CD11b/CD8 integrin neutrophil binding requires LacCer containing lipid rafts to induce zymosan phagocytosis. HL60 cells cannot undergo this activation process. Analysis of their LacCer content showed that HL60 cell LacCer primarily comprised C16 fatty acid isoform. Long chain C24 and C24:1 fatty acids LacCers were absent but present in primary neutrophils. Supplementation of HL60 cell membrane with exogenous C24:0 or C24:1 fatty acid containing LacCer resulted in the activation of Lyn kinase and induction of this phagocytosis pathway (Sonnino et al. 2008). It is proposed that the longer fatty acid isoform of LacCer are able to interdigitate with components of the cytosolic leaflet of the bilayer and thereby activate Lyn kinase (Sonnino et al. 2008). Some evidence of interdigitation of long fatty acid chain containing GSLs has been obtained in model phospholipid membranes (Florio et al. 1990; Boggs and Koshy 1994; Nabet et al. 1996) dependent on relative GSL/PL chain length (Boggs and Koshy 1994). Transmembrane signaling mediated by ligand binding to membrane LacCer implies a mechanism (partial reverse of aglycone modulation of GSL receptor function?), in which binding to the carbohydrate of LacCer must result in a change in the thermodynamic properties of the distal carbons of the long fatty acid acyl chain which is recognized in some way by the Lyn src kinase on the cytosolic membrane surface.

(2) Neuronal Inflammation: LacCer has been found to play a role in the induction of proinflammatory cytokines in both glial cells and neutrophils (Iwabuchi et al. 2008). Inhibition of LacCer synthase reduced glial cell proliferation and production of iNOS and this is selective reversed by exogenous LacCer (Won et al. 2007). Neuroinflammation is a consequence of neural injury. Inhibition of GSL synthesis, and LacCer synthase in particular, prevents TNFα induction of Ras/Erk 1/2 mediated astrocyte activation (Pannu et al. 2005), which otherwise restricts recovery from neural cell damage (Won et al. 2007). LacCer also activates this pathway in smooth muscle cells (Mu et al. 2009). VEGF activation of endothelial cells in angiogenesis provides another example of LacCer mediated signal transduction (Kolmakova et al. 2009). The increased precursor LacCer could increase Gb_3 to provide the explanation why verotoxin, which targets vascular endothelial cells, also targets neovascular endothelial cells (Heath-Engel and Lingwood 2003).

Ganglioside Regulation of Axonal Growth. Gangliosides are highly expressed in neuronal tissues and abnormal GSL biosynthesis, such as in the lysosomal storage diseases, induces severe neuropathy. Myelin associated glycoprotein (MAG) is an oligodendrocyte lectin (Siglec-4 [Varki and Angata 2006]), in the axon opposing layer of the insulating myelin sheath, which binds axonal gangliosides, GD1a and GT1b. This binding serves as a negative signal transduction mechanism preventing axonal growth after injury (Vyas et al. 2002). These gangliosides themselves also inhibit neurite outgrowth in vitro and this is dependent on complexing with the Nogo receptor, NgR1 (Williams et al. 2008), providing a mechanism for MAG–NgR1 interaction. MAG and NGR1 signal transduction is mediated via activation of the RhoA GTPase pathway (Niederost et al. 2002; Mimura et al. 2006). Oligosaccharide analogs of these sialated glycoconjugates promote axon outgrowth from cerebellar neurons in vitro (Vyas et al. 2005), and novel small molecule inhibitors based on these structures (Mesch et al. 2010), provide improved potential targets for future paraplegia treatment.

Ganglioside Modification of Cell Membrane Receptor Function. GSLs can serve a lateral, in addition to a transmembrane, receptor function. Specific receptor tyrosine kinase signaling can be modified by a lateral interaction of the receptor kinase with glycolipids within the membrane.

(a) NGF. The first case in which this potential was described was nerve growth factor signaling. Gangliosides, particularly GM1, promoted neurite outgrowth in neuronal cells (Ferguson and Williams 1988), which is blocked by CTx (Mutoh et al. 1993). Neurite outgrowth is initiated via a laminin–GM1 interaction (Ichikawa et al. 2009). GM1 has neurotrophic factor-like activity and increases the NGF dependent autophosphorylation of Trk, the receptor tyrosine kinase activated by NGF (Mutoh et al. 1995). GM1 and Trk were coprecipitated, suggesting binding. GM1 protects against neuronal cell injury and apoptosis (Ferrari and Greene 1996; Huang et al. 2009), and promotes dimerization of Trk after NGF binding. This requires glycosylated Trk (Mutoh et al. 2000), and may indicate a similar carbohydrate–carbohydrate interaction as for GM3 binding the EGF receptor (below). GM1 promotes neuronal cell regeneration in vivo and has therapeutic activity in Parkinson's disease (Schneider 1998). GM1 also promotes cell uptake of αsynuculein, mutated in Parkinson's (Park et al. 2009), and αsynuculein has been identified as a GSL binding protein (Di Pasquale et al. 2010).

(b) EGF. GM3 ganglioside has been long implicated in cell growth (Lingwood and Hakomori 1977). GM3 ganglioside can modify the signaling of the EGF receptor tyrosine kinase (Zhou et al. 1994). This transmembrane receptor kinase becomes autophosphorylated at three sites in the carboxy-terminal domain when ligated by extracellular epidermal growth factor (Honegger et al. 1988). In GM3 containing cell membranes, receptor phosphorylation is inhibited (Zurita et al. 2001). Significantly, this inhibitory activity of GM3 depends on the lipid moiety of the ganglioside, because lysoGM3, particularly lysoGM3 dimers and their mimics, are more effective (Haga et al. 2008). In contrast, de-*N*-acetylGM3 actually stimulated EGF receptor tyrosine kinase activity (Hanai et al. 1988). The lateral interaction between GM3 and EGF is thought to be mediated via carbohydrate–carbohydrate interaction between the sialated galactose of GM3 and the GlcNAc of N-linked oligosaccharides of the EGF receptor (Yoon et al. 2006; Kawashima et al. 2009). Modification of the lipid moiety of GM3 may alter the conformation of the GM3 carbohydrate modulating this interaction. Defective cellular GM3 synthase resulting in ganglioside depletion, leads to a reduction in EGF binding to normal levels of EGFR and a reduction in growth and cell migration response (Liu et al. 2008b), suggesting that this GSL interaction also influences ligand-EGFR binding.

(c) Insulin Signaling. The third example in which lateral GSL interaction modifies membrane signaling is that of GM3 in the development of insulin resistance.

The insulin receptor becomes autophosphorylated on insulin binding and this serves to recruit insulin receptor substrate (IRS1, IRS2), which becomes phosphorylated to activate PI3 kinase, which, in turn, activates PKC. The PIP3 generated activates PKB, which together with PKC, stimulates increased membrane GLUT4 insertion for rapid glucose uptake (Pessin and Saltiel 2000). Plasma membrane organization is important in this process (Muller et al. 2001; Ikonen and Vainio 2005; Inokuchi 2010). The recruitment of the insulin receptor into caveolae via interaction with caveolin-l, necessary for signal transduction (Karlsson et al. 2004), can be reduced by a competing interaction with GM3 ganglioside (Kabayama et al. 2007).

GM3 is found to be up-regulated in insulin resistant cells (Tagami et al. 2002), and insulin IRS-1phosphorylation is blocked (Langeveld and Aerts 2009). Exogenous GM3 also reduced insulin dependent IRS-1phosphorylation (Tagami et al. 2002). Inhibition of GSL synthesis in resistant cells reverses inhibition of insulin mediated signaling (Zhao et al. 2007; Langeveld and Aerts 2009). Mice genetically deficient in GM3 are hypersensitive to insulin (Yamashita et al. 2003), and inhibition of GSL synthesis improves insulin sensitivity in animal models of type II diabetes (Aerts et al. 2007; van Eijk et al. 2009). Increased resistance to insulin is also found in Gauchers disease (Langeveld et al. 2008), in which GlcCer and GM3 accumulate (Ghauharali-van der Vlugt et al. 2008).

GSLs and HIV Infection. The receptor role for GSLs in HIV infection is complex. The HIV adhesin gp120 forms a highly glycosylated trimeric complex on the viral membrane (Liu et al. 2008a). In addition to its CD4 receptor on T cells and chemokine coreceptor (CXCR4, for X4 HIV1 strains, and CCR5 for R5 HIV1 strains), gp120 binds to several GSLs. Galactosyl ceramide was the first identified, together with its 3′ sulfate ester, sulfatide (Bhat et al. 1993). Other GSLs found to bind gp120 include GM3, GD3, and Gb_3. Gp120s from dual tropic R5X4 HIV strains bind more selectively to GM3, whereas gp120s from X4 HIV strains bound preferentially to Gb_3 (Hammache et al. 1999). The GSL binding site was identified within the V3 loop of gp120. This is the same loop that binds the chemokine receptor. CD4 binding induces a conformational shift opening the V3 loop to allow chemokine coreceptor recognition (Wu et al. 1996; Zhang et al. 1999). The presence of soluble CD4 also increases gp120-GSL binding, consistent with improved access within the V3 loop (Hammache et al. 1998), but unlike chemokine receptor binding, gp120-GSL binding can be detected without prior CD4 binding.

Cholesterol/GSL lipid rafts are important for HIV infection (Liao et al. 2001), both in terms of initial interaction with the host cell receptors (Manes et al. 2000), and for budding of new virions from the infected cell surface (Nguyen and Hildreth 2000; Ono and Freed 2001). Since the nascent virus is encapsulated in host cell membrane, down-regulation of host cell receptors used to target the virus to infect cells is necessary for the generation of new infectious particles. The viral protein Nef is used to induce down-regulation of CD4 (Alexander et al. 2004). Nef connects CD4 to the intracellular protein sorting pathways (Mangasarian et al. 1997), and CD4 is depleted via induction of ER associated degradation (Binette et al. 2007). Host cell transfection with Nef has also been shown to modify clathrin mediated Gb_3 retrograde transport (Johannes et al. 2003), which may also favor the budding of more infectious virions.

Gp120 binding to GalCer has been strongly implicated as a means by which HIV can infect CD4 negative cells, such as neural (Bhat et al. 1991; Harouse et al. 1991) and epithelial cells within the GI tract (Yahi et al. 1992) or reproductive epithelium. It is questionable however, whether epithelial cells actually support a highly productive HIV infection. Rather, such cells may provide a latent pool or reservoir of viral DNA that likely plays a more long-term role. Analogs of GalCer have proven effective in preventing HIV infection of both CD4 +ve and −ve GalCer containing cells (Fantini et al. 1997). In these studies, modification of the lipid moiety was crucial in defining efficacy of

infection inhibition. The GSL binding specificity of gp120 is however, fairly promiscuous and an octamer of the conserved peptide GPGRAF, from the V3 loop (Fig. 2), has been shown to at least in part mimic the GSL binding specificity of gp120 in vitro (Delezay et al. 1996).

The GSL binding sequence within the V3 loop comprises an aromatic amino acid flanked by two α helixes. A sphingolipid binding motif was defined based on this sequence and used to search for GSL binding sequences in other proteins (Mahfoud et al. 2002a). Using this sphingolipid binding site model, two other GSL binding proteins were identified, that of β-amyloid, the precipitates of which are responsible for Alzheimer disease, and a similar sequence was found in the prion protein of bovine spongiform encephalitis. Both these proteins were later identified as bona fide GSL binding species (Levy et al. 2006). This motif has now been found in αsynuclein (Di Pasquale et al. 2010), mutated in Parkinson's Disease (PD). αSynuclein binds GM3 (Di Pasquale et al. 2010) (and other gangliosides [Schlossmacher et al. 2005]) and the binding affinity is increased for the familial mutant form. GM3 binding corrects the ion channel defect of this PD form. In an as yet, undefined manner, this may relate to the finding that mutations in glucocerebrosidase, responsible for Gaucher's disease (in which glucosyl ceramide and other GSLs, potentially GM3, accumulate) are also a risk factor for the development of PD (Velayati et al. 2010). Inhibition of glucocerebrosidase increases α-synuclein in cells and mice (Manning-Bog et al. 2009).

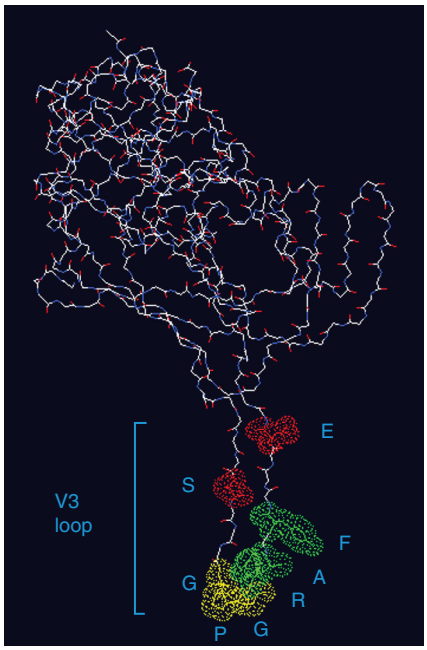

Figure 2. The glycolipid binding motif in the HIV adhesin gp120 is contained within the chemokine receptor binding sequence of the V3 loop. Red amino acids are required for chemokine receptor binding, green for GSL binding, and yellow are required for both. GSL sugar stacking against the ring of the aromatic amino acid is the key to binding.

The GSL binding site within the V3 loop of gp120 is in the center of the chemokine receptor binding site (Fig. 2) (Xiao et al. 1998), indicating a complex role for GSLs recognition. Many studies have shown that lipid raft assemblies within the host cell plasma membrane play a role in HIV susceptibility (Liao et al. 2001; Luo et al. 2008). CD4 and CCR5 are contained within detergent resistant membranes (DRMs) (Popik et al. 2002), whereas CXCR4 is found in the non-DRM fraction (Kozak et al. 2002; Popik et al. 2002). DRMs are formed by detergent extraction in the cold and are nonphysiological insoluble membrane residues that enrich for lipids (primarily GSLs) and proteins thought to be important for constructing dynamic raft-based membrane heterogeneity in the living cell (Lingwood and Simons 2007). Thus, DRM location indicates potential association with such organization. In studies examining the role of GSLs, it was proposed that GSL binding facilitated the colocalization of CD4 and chemokine receptor within the same DRM assembly to facilitate simultaneous gp120 binding to both receptors (Fantini et al. 2000). Studies showing GalCer can serve as alternative receptor in CD4 negative cells (Fantini et al. 1993), would suggest that GSL binding may serve as an alternative means to achieve viral internalization. This may explain why initial studies on Gb_3 gp120 binding showed augmentation of fusion using a

nonhuman system in which CD4 was not bound (Fantini et al. 1993). Since the GSL binding site is in the center of the chemokine receptor-binding site (Fig. 2), GSL binding should inhibit gp120-chemokine receptor binding. A soluble Gb_3 analog generated by exchanging the fatty acid of Gb_3 for an adamantane frame, proved a potent receptor for gp120 (Mahfoud et al. 2002b) and an inhibitor of HIV infection in vitro, both for primary lymphocyte and cultured cell HIV infection (Lund et al. 2006). AdamantylGb_3 was effective against both R5 and X4 HIV1 infection and against drug resistant HIV-1 strains. Similar effects are seen with other soluble Gb_3 analogs (Harrison et al. 2010). AdamantylGb_3 inhibited gp120 mediated HIV–host cell fusion. This inhibitory activity is consistent with blocking chemokine receptor binding.

Subsequent studies using HIV susceptible cultured cells, showed that increase in cellular levels of Gb_3 resulted in decreased HIV susceptibility (Lund et al. 2005) and depleting cellular Gb_3 resulted in a significant increase in HIV susceptibility. Increasing cellular levels of Gb_3 by inhibition of α-galactosidase resulted in decreased HIV susceptibility and depleting cellular Gb_3 via inhibition of GCS, resulted in a significant increase in HIV cell susceptibility (Ramkumar et al. 2009). This inverse relationship between cellular Gb_3 content and HIV susceptibility was verified using lymphocytes from individuals with different genotypes within the P blood group system (Spitalnik and Spitalnik 1995). Small *p* individuals lacked functional Gb_3 synthase and therefore express no P antigens. P1k individuals have a defective Gb_4 synthase and therefore Gb_3 (p^k antigen) accumulates on their cells. p lymphocytes were found to be significantly (up to 1000-fold) more susceptible to R5 and X4 HIV1 infection in vitro than normal controls whereas P1k lymphocytes were $<$50-fold more resistant to R5/X4 HIV-1 infection (Lund et al. 2009). Transfection of CD4-HeLa cells with Gb_3 synthase resulted in a selective Gb_3 increase and reduced HIV susceptibility, whereas siRNA against Gb_3 synthase reduced Gb_3 and increased HIV susceptibility (Lund et al. 2009). Thus, target cell Gb_3 may be a negative risk factor for HIV susceptibility within the general population.

Aglycone Regulation of GSL Presentation

GSL are clearly important players in cell physiology. It also seems equally clear that their bioactivity, i.e., glycone functionality, is in some way modulated by the underlying membrane matrix or aglycone. The molecular details of this regulation are becoming apparent.

GSL Conformational Change

From early reports of immuno- and enzymatic inaccessibility, it has been concluded that a large proportion of GSL is somehow masked in the plasma membrane (Hakomori 1981). These studies can involve the lack of antibody binding to membrane GSLs (Crook et al. 1986; Stewart and Boggs 1990) and has been ascribed to obstruction via membrane proteins, glycoproteins (Hakomori 1981), and sialyated glycoconjugates (Wiels et al. 1984). However, the basis of this masking of GSL carbohydrate structure from ligand binding has not been rigorously studied. Crypticity can be reproduced in model lipid membranes (Hamilton et al. 1994), indicating that it involves an inherent property of lipid-mediated GSL carbohydrate masking. Membrane GSL crypticity can correlate with the structure of the lipid moiety (Kannagi et al. 1982a,b; Kiarash et al. 1994). Several bacterial pathogens were shown to bind to lactosyl ceramide only if the ceramide contained hydroxy fatty acids (Stromberg and Karlsson 1990; Ångström et al. 1998). Non-hydroxy fatty acid containing lactosyl ceramide was cryptic. Molecular modeling studies suggested that hydroxy fatty acid containing ceramide GSLs could adopt a different carbohydrate conformation in the membrane (Calander et al. 1988) to explain this selective ligand binding.

More recently, a role for cholesterol as a modulator of GSL availability is becoming established. In the plasma membrane up to 40 mol% of surface membrane lipids are cholesterol molecules (Kalvodova et al. 2009), which shows preferential interaction with GSL

(Lingwood and Simons 2010). Here, their flat planar ring structure packs more effectively with the longer unsaturated acyl chains more common to GSL. Moreover, the GSL head group acts as an additional attractant by shielding cholesterol from unfavorable interaction with water. It is becoming increasingly apparent that this lipid–lipid interaction has consequences for GSL head group presentation. Atom scale molecular dynamics simulations have shown that the interaction of cholesterol with sphingomyelin (Niemela et al. 2004) alters the head group conformation. The cholesterol OH was more deeply buried in the sphingolipid compared to glycerolipid equivalent, such that the head group became preferentially orientated parallel, rather than perpendicular to the plasma membrane. Thus, cholesterol-bound GSL could show a remarkable difference in carbohydrate availability for ligand binding or lateral *cis*-interactions. Molecular simulation of the cholesterol/GalCer complex, showed the cholesterolOH formed an H-bond network to bend the sugar linkage to generate a membrane parallel carbohydrate conformer (Yahi et al. 2010). Vattulainen's group has found a similar effect for GalCer (Hall et al. 2010) and GM1-cholesterol (Lingwood et al. 2011). This conformer of GalCer preferentially interacted with the Alzheimer's β amyloid protein (Yahi et al. 2010) to promote the conformational change associated with amyloid formation. GSL/cholesterol enriched lipid microdomains are central to this process (Ehehalt et al. 2003; Han 2005). The amyloid β protein contains a sphingolipid binding site (Mahfoud et al. 2002a; Levy et al. 2006), and, lactosyl ceramide (Levy et al. 2006) and several gangliosides can bind (Yanagisawa and Ihara 1998; Mandal and Pettegrew 2004), to promote the conformational change, making this a therapeutic target (Fantini 2007).

However, membrane parallel GSL carbohydrate is unlikely to mediate GSL receptor function. Addition of cholesterol to GSL phospholipid liposomes results in a reduction in the binding capacity, but not affinity for both cholera toxin and verotoxin, consistent with a conformational change for the cholesterol complexed GSL carbohydrate (Lingwood et al. 2011). Membrane cholesterol can mask GSLs to prevent ligand binding in tissue immunohistology (Chark et al. 2004; Khan et al. 2009). Indeed, acetone (extracts cholesterol) commonly enhances GSL immunohistochemistry (Kolling et al. 2008; Sakumoto et al. 2009). VT1 and CTx binding to human renal tissue is markedly increased following cholesterol depletion of tissue sections (Lingwood et al. 2011). This may provide a risk factor for the induction of GSL-binding toxin-induced disease (Chark et al. 2004; Khan et al. 2009). Cholesterol masking of membrane GSL is an important feature reversed during sperm capacitation (Kawano et al. 2008b; Lingwood et al. 2011), a process of cholesterol depletion essential for fertility (Visconti et al. 1999).

Lateral Organization

The organization of GSLs, cholesterol and a subset of membrane proteins into lipid microdomains or rafts in the membrane of living cells has been the subject of intense study. Heterogeneous membrane organization allows for the specialization of functional membranes domains, for example for signal transduction pathways (Kasahara and Sanai 2000), host–microbial pathogen interactions (Heung et al. 2006), immune recognition and intracellular vesicle trafficking (Luo et al. 2008). Although membrane model studies and phase separation of liquid ordered and liquid disordered domains do not fully reflect the cell membrane at equibrium, they provide a valuable probe of the potential thermodynamic forces at play in membrane organization. The demonstration that such forces are resistant to detergent extraction in the cold has provided a valuable, but controversial, tool for the study of membrane organization in cell physiology. Although the DRM fraction represents an artificial pool of potentially separate cell surface domains, detergent resistance has proven a useful probe of aglycone parameters affecting GSL receptor presentation and potential functionality therein.

In this context, membrane-based regulation of GSL receptor function is supported by

differences in detergent resistance at the cell surface. Detergent resistance of Gb_3 is positively correlated its receptor activity for VT1 and subsequent cellular toxicity (VT retrograde transport to the Golgi and ER) (Falguieres et al. 2001). Cells in which Gb_3 is detergent soluble, recruit a different intracellular trafficking pathway that delivers the Gb_3-bound toxin to the lysosome, rather than endoplasmic reticulum, in which it is degraded to prevent cytotoxicity (Falguieres et al. 2001). Thus, a membrane basis for differential sorting of Gb_3 from the plasma membrane is crucial in defining cell susceptibility to VT cytotoxicity. Interestingly, this property appears to be a protective function in the bovine host, wherein Gb_3 of the GI tract is present in the nonDRM fraction and VT internalized into mucosal epithelial cells is degraded, essentially preventing systemic verotoxemia (Hoey et al. 2003). In humans, mucosal cells contain no Gb_3 and VT can be transcytosed to target Gb_3 expressing submucosal endothelial cells (Jacewicz et al. 1999) to induce hemorrhagic colitis, a prodrome of HUS (Andreoli et al. 2002), allowing verocytotoxemia which may progress to HUS. In human kidney sections, glomerular Gb_3 is, unlike tubular Gb_3, presented in a cholesterol-dependent, detergent-resistant format, predisposing glomeruli to VT cytopathology (Khan et al. 2009).

What is the underlying membrane organization that correlates GSL receptor function and detergent resistance? Surprisingly, a number of GSL receptors (Gb_3, GM1, GalCer, and SGC) can be separated into two vesicle populations within a single DRM preparation. For seven of their known ligands (including cholera toxin), a ligand-binding vesicle fraction, and a ligand-unreactive vesicle fraction can be separated from both model and cell membrane DRMs (Mahfoud et al. 2009, 2010). The unreactive fraction is particularly compelling, as it comprises most of the GSL, (only an IgM Mab antiGb_3 bound both vesicle populations). It is not yet clear if GSL is rendered "invisible" by changes in conformation or lateral receptor density (Shi et al. 2007), but this property is maintained irrespective of whether ligand is applied to the vesicles themselves or to the living cell prior to DRM formation, suggesting a physiological basis for this difference in receptor presentation. Indeed, depletion of cellular cholesterol or actin dissociation allows ligands to bind the major ("invisible") GSL vesicle fraction, suggesting that membrane raft heterogeneity serves to organize GSL geography, and in so doing, regulates their receptor action.

At the model membrane level, this potential for fine tuning GSL receptor function can be observed through the compositional modulation of receptor activity that underlies the ligand-binding and the ligand-unreactive vesicle fractions. For the reactive former, VT1 and gp120 binding depends on Gb_3 fatty acid chain length, in that C16, C22, and C24 but not (C17), C18, and C20 isoforms were recognized (Mahfoud et al. 2009). A similar transition for the interaction of glycerolipids with cholesterol, i.e., at C17, was ascribed to the minimum hydrophobic mismatch between cholesterol and the lipid acyl chains at this carbon number (McMullen et al. 2009). VT2 binding was independent of Gb_3 fatty acid chain length, consistent with a less selective aglycone requirement (Tam et al. 2008). Significantly, in Gb_3 fatty acid mixtures, the VT1, or gp120 nonbound Gb_3 fatty acid isoforms were dominant negative for ligand binding. Mixtures including C18 or C20 Gb_3, were not bound ("off" switch), but inclusion of C24:1 Gb_3 induced ligand binding to any mixture. Thus, C24:1 Gb_3 was a dominant positive ("on" switch for VT1/gp120 binding). This was most dramatically illustrated by gp120 binding to C18 and C24:1 Gb_3. For each isoform alone, no binding was observed, but strong binding to an equimolar mixture of these two isoforms was obtained (Fig. 3).

In the case of ligand-unreactive GSL vesicles, inclusion of GalCer or GlcCer (both found to bind Gb_3) together with Gb_3 unveiled "invisible" receptor, allowing VT binding to the ligand-unreactive vesicle fraction (Mahfoud et al. 2010). Thus, specific lateral GSL–GSL interactions may be tailored to selectively counter aglycone modulation of GSL receptor function. Interestingly, cellular GlcCer is required to

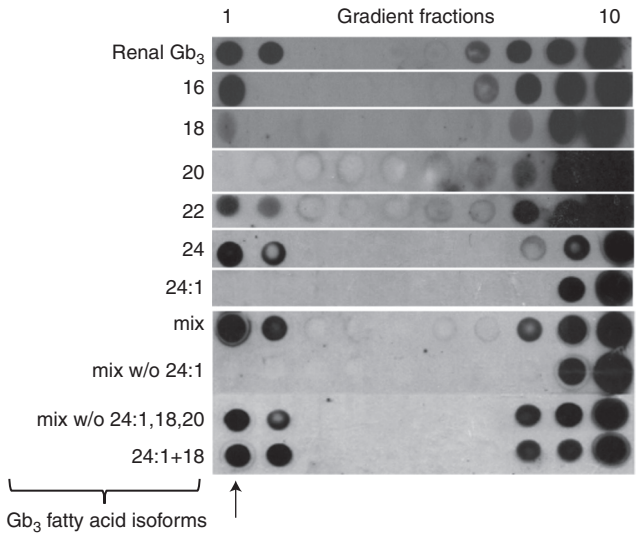

Figure 3. GSL fatty acid mixing can "switch on" ligand binding. Gp120 bound Gb_3/cholesterol vesicles separated to the top of a sucrose gradient (fraction 1) after equilibrium ultracentrifugation. Gp120 binding in this fraction varied according to the Gb_3 fatty acid content and the mixture of Gb_3 fatty acid isoforms present. Effective binding to the human renal Gb_3 mixture was seen. The C16 Gb_3 fatty acid isoform was also effectively bound but C18 and C20 alone were not recognized. Binding to C22 and C24 Gb_3 was effective but no binding to C24:1 Gb_3 was detected. A mixture of all these Gb_3 fatty acid isoforms (mix) was effectively bound but removal of the C24:1 Gb_3 (mix w/o 24:1) resulted in a loss of gp120 binding. Removing in addition, the C18 and C20 Gb_3 fatty acid isoforms (mix w/o24:1, 18, 20), each of which alone are unbound by gp120, induced gp120 binding. Thus, the presence of C18 and C20 together can "switch off" gp120-Gb_3 binding, whereas C24:1 Gb_3 can "switch on" gp120-Gb_3 binding. Remarkably, the combination of C24:1 and C18 Gb_3 fatty acid isoforms (which individually do not bind gp120), generated vesicles highly reactive with gp120. Similar results were obtained for VT1 binding to these Gb_3 fatty acid isoforms. (Adapted from Mahfoud et al. 2009; reprinted with permission from ASBMB Journals © 2009.)

maintain Gb_3 in VT1 detectable plasma membrane DRMs (Smith et al. 2006), required for ER retrograde transport of VT.

The demonstration that within a cholesterol-enriched membrane, GSLs interacting in combination, may present the appropriate receptor format for ligand binding, provides the molecular basis for an on/off switch behavior. Like the cross-arcs from a compass, different membrane trafficking pathways (e.g., anterograde and retrograde) could thereby define coordinates—a form of "barcode"— to induce or suppress ligand binding at specific points of membrane domain intersection (Fig. 4). This could be considered as a 2D microform of Wolperts French flag theory of cell positional information (Tickle et al. 1975; Wolpert 1989). The aggregation of lipid microdomains following membrane fusion could serve as a temporal "address" for initiation of GSL receptor function by providing the appropriate local GSL fatty acid isoform mixture or GSL mixture to promote GSL presentation from a cholesterol enriched microenvironment, and provide discrete triggers for ligand binding at different points in the intersected pathways.

Lastly, the presence of unavailable GSL in the membrane plane raises questions as to GSL membrane distribution. Short chain carbohydrate GSLs are no more polar than phospholipids and by that reasoning alone, may spontaneously flip across bilayers as frequently, yet none are seen in the cytosolic leaflet. What maintains this asymmetry? Perhaps the asymmetry is also an issue of detection.

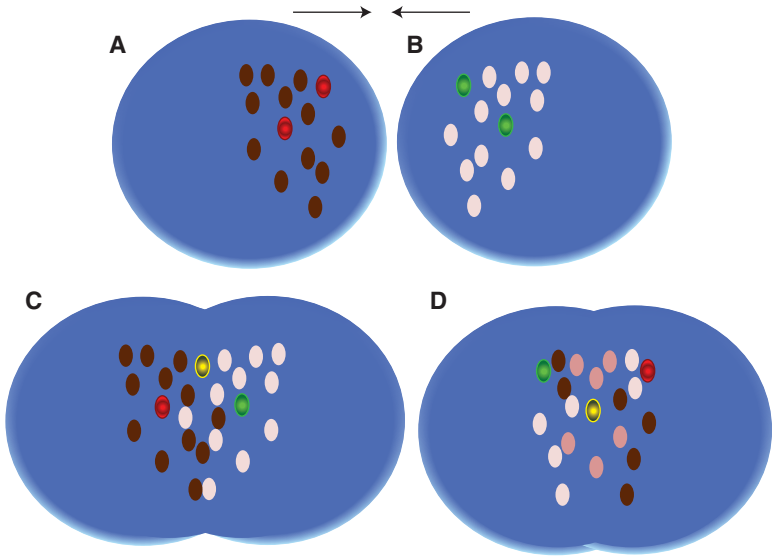

Figure 4. Composite GSL membrane receptor foci as dynamic positional "bar code" markers during vesicular transport. Differential GSL receptor function according to fatty acid isoform mix or GSL "coreceptor" presence provides a mechanism to generate precise positional foci for ligand binding during dynamic membrane processes. Complementary (which in combination, bind ligand) lipid microdomains in vesicular compartments *A* and *B* are indicated in red and green. The red domains in vesicle *A* contain GSL fatty acid isoform mixtures, which either do not bind ligand within a cholesterol matrix, or are missing a coreceptor GSL able to facilitate GSL presentation for ligand binding within a cholesterol matrix. In vesicle *B*, the green lipid microdomains contain the appropriate GSL fatty acid isoform to induce ligand binding within the red domains, or the appropriate coreceptor GSL to promote GSL receptor function in the red lipid microdomains of vesicle *A*. On vesicle fusion (*C*), the initially separate domains can now aggregate and can show a time-dependent initiation of GSL receptor competency via fusion of the red and green domains (yellow domains). At later times (*D*) these domains may separate as shown, or remain stable and additional domains of GSL receptor competency (yellow) can be formed. Although shown as domains, intersection of GSL diffusion gradients could achieve the same ends.

PERSPECTIVES

GSLs function to gate many processes in biology. When considering this function it is important to remember that for GSL, carbohydrate and lipid are chemically joined. It is therefore myopic to discuss the biology of either moiety in the absence of the other. During solvent extraction most GSL partition into the organic phase, despite the equimolarity of hydrophilic carbohydrate. At the other extreme, the large carbohydrate head group ensures that most pure GSLs form micelles in solution. Thus, at its most basic level there is a strained coupling between glycone and aglycone. In the chemical complexity of cell membranes there is evidence that this coupling remains, having had its allosteric potential optimized to passively regulate the bioactivity of these most enigmatic molecules.

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Glycosphingolipid Functions

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In the original version of this article, some of the text labels in Figure 1 were incorrect.

The publisher apologizes for these errors. The correct Figure 1 is reprinted below, and the PDF and HTML versions of the article have been corrected accordingly.

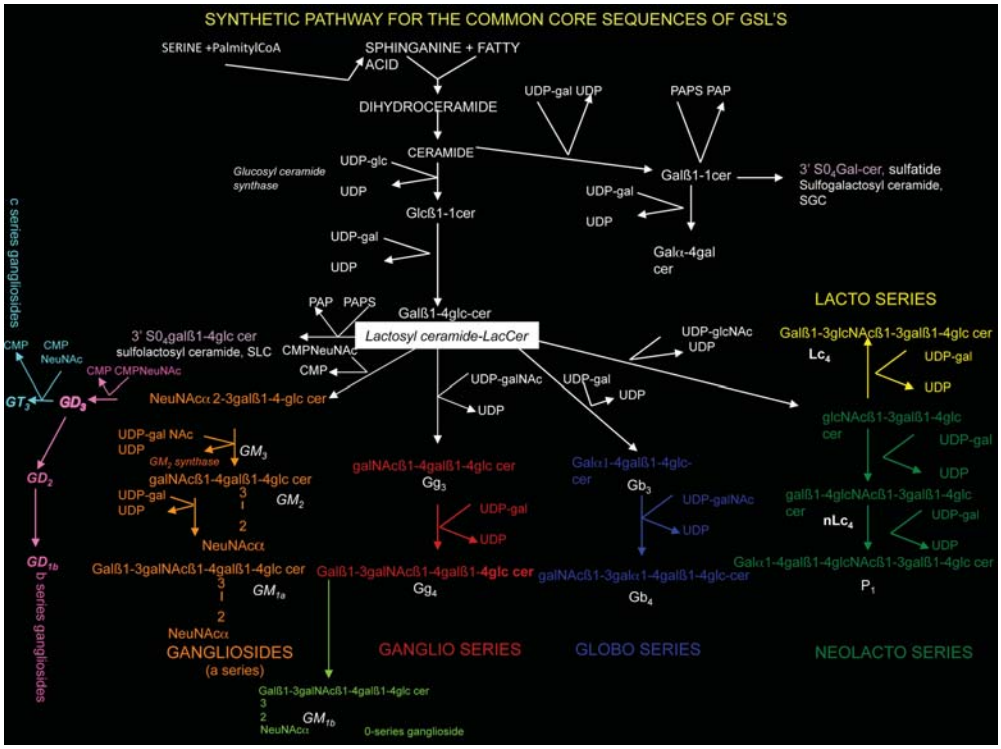

Figure 1. Synthetic pathways for the major GSL species. Glucosyl ceramide is the key precursor for most GSLs and lactosyl ceramide provides the branch point for the different GSL series.