A Major Fraction of Glycosphingolipids in Model and Cellular Cholesterol-containing Membranes Is Undetectable by Their Binding Proteins

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Glycosphingolipids (GSLs) accumulate in cholesterol-enriched cell membrane domains and provide receptors for protein ligands. Lipid-based “aglycone” interactions can influence GSL carbohydrate epitope presentation. To evaluate this relationship, Verotoxin binding its receptor GSL, globotriaosyl ceramide (Gb3), was analyzed in simple GSL/cholesterol, detergent-resistant membrane vesicles by equilibrium density gradient centrifugation. Vesicles separated into two Gb3/cholesterol-containing populations. The lighter, minor fraction (<5% total GSL), bound VT1, VT2, IgG/IgM mAb anti-Gb3, HIVgp120 or Bandeiraea simplicifolia lectin. Only IgM anti-Gb3, more tolerant of carbohydrate modification, bound both vesicle fractions. Post-embedding cryo-immuno-EM confirmed these results. This appears to be a general GSL-cholesterol property, because similar receptor-inactive vesicles were separated for other GSL-protein ligand systems; cholera toxin (CTX)-GM1, HIVgp120, galactosyl ceramide/sulfatide. Inclusion of galactosyl or glucosyl ceramide (GalCer and GlcCer) rendered VT1-reactive Gb3/cholesterol vesicles, VT1-reactive. We found GalCer and GlcCer bind Gb3, suggesting GSL-GSL interaction can counter cholesterol masking of Gb3. The similar separation of Vero cell membrane-derived vesicles into minor “binding,” and major “non-binding” fractions when probed with VT1, CTx, or anti-SSEA4 (a human GSL stem cell marker), demonstrates potential physiological relevance. Cell membrane GSL masking was cholesterol- and actin-dependent. Cholesterol depletion of Vero and HeLa cells enabled differential VT1B subunit labeling of “available” and “cholesterol-masked” plasma membrane Gb3 pools by fluorescence microscopy. Thus, the model GSL/cholesterol vesicle studies predicted two distinct membrane GSL formats, which were demonstrated within the plasma membrane of cultured cells. Cholesterol masking of most cell membrane GSLs may impinge many GSL receptor functions.

The availability of cell membrane receptor GSL carbohydrate for protein ligand binding is influenced by both the nature of the GSL lipid moiety and the membrane microenvironment (1), from early reports of membrane GSL “crypticity” (2–4) and lipid dependent anti-GSL binding (5–7), to recent fatty acid-dependent GSL bilayer remodeling (8, 9). The plane of the membrane bilayer, in relation to membrane GSLs, can markedly affect the conformation of the carbohydrate (10) to promote the availability of different epitopes within the same GSL sugar sequence (11). The local microenvironment of GSLs (membrane composition, type of solid phase support) can affect carbohydrate presentation for ligand binding (12–14). Protein binding can be regulated by the GSL fatty acid/ceramide content (15–19). Different protein ligands, which recognize the same receptor GSL, can bind differentially in a cell or model membrane context, and cholesterol can play a central role (20, 21). Cholesterol is key to the structural maintenance of membranes and interacts strongly with sphingolipids (22). This, combined with the hydrogen bond network between membrane GSLs (23), provides a (thermodynamically strained (1)) “interface” between the hydrophilic carbohydrate head group and the hydrophobic hydrocarbon tail, which can regulate GSL receptor activity (24). This is particularly relevant in the context of cellular GSL accumulation in cholesterol-enriched detergent-resistant membranes (DRMs) (25). Although the nature of DRM correlation to lipid rafts of model systems (26) is a matter of debate, detergent insolubility can be indicative of specific lateral interactions involving both lipids and proteins (27). In this context, we have found detergent resistance to be a useful probe of “aglycone” regulation of GSL receptor presentation in tissues (28, 29).

Escherichia coli-derived Verotoxins (VTs) are associated with hemolytic uremic syndrome and hemorrhagic colitis. With hemolytic uremic syndrome and hemorrhagic colitis.

2 The abbreviations used are: GSL, glycosphingolipid; Gb3, globotriaosyl ceramide, galactose α1–4 galactose β1–4 glucosylceramide; Gb4, globotetraosyl ceramide; Gb3, galactosyl ceramide; GaICer, galactosyl ceramide; GlcCer, glucosyl ceramide; LacCer, lactosyl ceramide; GM1, monosialogangliotetraosyl ceramide; SSEA-4, stage-specific embryonic antigen-4 (NeuAcα2–3Galβ1–3GalNAcβ1–3Galα1–4 Galβ1–4 Glic ceramide); VT, Verotoxin; CTx, cholera toxin; CTxB, cholera toxin B-subunit; DRM, detergent-resistant membrane; MJ3C, methyl β-cyclodextrin; P4, threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; IEM, immuno-EM; MES, 4-morpholineethanesulfonic acid.
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Aosyl ceramide (Gb₃, CD77, or p₅ blood group antigen) (30), but binding is affected by the lipid (aglycone) moiety (13, 17). Although VT1 and VT2 only bind Gb₃ (31), they recognize different molecular Gb₃ assemblies in the plasma membrane, which are then differentially sorted intracellularly (21). This implies cellular recognition of aglycone GSL modulation also. DRM Gb₃ is required for VT1 transmembrane signaling (32) and intracellular VT retrograde trafficking and cytotoxicity in vitro (33, 34) and potentially, in vivo (28).

Several GSLs (35), including Gb₃ (36), are bound by the HIV adhesion, gp120. Like VT, gp120/GSL binding is modulated by the lipid moiety (37). Cholesterol is important in HIV infection (38, 39), and Gb₃, and Gb₃ analogues, are HIV inhibitors (40, 41).

Elucidation of aglycone properties influencing membrane GSL carbohydrate presentation presents an experimental challenge. We developed a method to prepare simple, sucrose gradient separated, detergent-resistant GSL/cholesterol vesicles (42) with the aim of defining potential membrane components influencing GSL carbohydrate-protein ligand binding. We now find that vesicle separation has yet to equilibrate under these conditions. Prolonged centrifugation and increased gradient duration separated, detergent-resistant GSL/cholesterol vesicles were generated by a scale reduction of the described procedure (42). Briefly, a 2:1 ratio of Gb₃ (or in some cases for gp120 binding, GalCer or sulfatide (SGC), or cholera toxin binding GM1 (50 µg) and cholesterol (25 µg) in ethanol were dried together (in later unmasking studies another GSL (50 µg) was included) and dissolved in 750 µl of MES-Triton buffer (25 mM MES, 150 mM NaCl, pH 7.2, 1% (w/v) Triton X-100). The solution was vortexed (1 min), sonicated (1 min), heated at 55 °C (5 min), and vortexed again (1 min). Then 750 µl of 70% (w/v) sucrose solution in MES buffer was added, gently mixed, and allowed to stand at room temperature for ~1 h. The mixture was placed below 1 ml of 30% sucrose containing 1 µg/ml ¹²⁵I-labeled VT or unlabeled VT1, VT2, mAb anti-Gb₃, gp120, or HRP-CTB, each used at 1–4 µg/ml. This was overlaid successively with 1 ml of 30% sucrose (in later experiments 25% sucrose was used to maximize mixing of this layer with the ligand-containing layer) and 1.5 ml of 5% of sucrose. Condensed lipid species were separated by flotation ultracentrifugation using an SW55 Ti rotor at 34,000 rpm for 72 h, 20 °C. The duration of centrifugation was empirically defined for resolution of the GSL-bound ligand. Vesicle separation was not affected by temperature (4 °C or 20 °C) but was improved as compared with the original full scale method (42). From the top of the tube, 10 fractions, 500 µl each, were then collected and counted in a γ-counter to determine ¹²⁵I-VT distribution in the gradient. Unlabeled ligands were detected by dot blot as described below. In some experiments GSL/cholesterol vesicles were separated by sucrose gradient ultracentrifugation without ligand. Vesicle fraction aliquots were immobilized on nitrocellulose, and then ligand binding to the immobilized vesicles was immunodetected. In some cases fractions 9/10 were pooled.

Immunoblot Analysis of Vesicle Fractions—Equal volumes of the gradient fractions were loaded onto nitrocellulose (Whatman Protran BA85) using a dot blot apparatus (Shliecher & Schuell, Minifold I microsample filtration manifold). The samples were washed with TBS (50 mM Tris–HCl, pH 7.2, 150 mM NaCl) containing 1% skim milk or BSA blocking solution for 1 h. The membrane was rinsed with TBS and then probed for bound ligand or when ligand was not included in the gradient, used for ligand binding to the immobilized vesicles. Briefly, the immobilized vesicles were incubated with VT1, VT2, gp120, or anti-Gb₃ (1 µg/ml) for 1 h at room temperature, the blots washed, and bound ligand was detected with appropriate antibody followed by HRP-conjugated second antibody.
As an alternative to determine the distribution of VT1 in gradient fractions, equal volumes from each fraction were mixed with 1/5 volume of non-reducing 5× SDS-PAGE sample buffer and heated to 90 °C for 5 min. Ten μl of each sample were subject to 15% SDS-PAGE followed by Western blotting. The VT1-A subunit was detected with rabbit polyclonal antisera raised against the A-subunit (47). Immunoblots and TLCs were quantitated using ImageJ (48).

Post-embedding Immuno-EM of Vesicles—Gb3/cholesterol vesicles were prepared as above and processed for post-embedding cryo-immuno-EM, either before or after separation by sucrose density centrifugation. To aid in sectioning, the vesicles were stained with toluene blue dye for 1 min before excess MES buffer was added, and the solution was vortexed and centrifuged at 20,000 rpm for 30 min at 20 °C. The pelleted vesicles were then air-dried for at least 1 h at room temperature. Vesicles were infiltrated in gelatin (20% in PBS) before cryo-freeze-drying. Thin sections were cut using a Leica cryomicrotome and mounted on grids at the Advanced Bioimaging Centre, Hopital for Sick Children. For immunogold labeling, section grids were first washed by flotation on a drop of PBS. Sections were blocked with 1% BSA in PBS and then incubated with 5 μl of each sample buffer and heated to 90 °C for 5 min. Ten μl of each sample were subject to 15% SDS-PAGE followed by Western blotting. The VT1-A subunit was detected with rabbit polyclonal antisera raised against the A-subunit (47). Immunoblots and TLCs were quantitated using ImageJ (48).

Lipid Extraction—Lipids were extracted from sucrose fractions by one of two methods. Neutral lipids/glycolipids were isolated by extracting 150 μl of each fraction with chloroform/methanol 2:1 followed by Folch partition (49). The upper phase was removed, and the lower phase was washed 2× with theoretical upper phase to remove sucrose. The lower phase was dried under nitrogen gas, and ~25% of the sample was separated by gel in chloroform:methanol:water 65:25:4 (v/v). Phospholipids were detected using iodine vapor and imaged by scanning prior to Gb3 detection by VT1 overlay. Cholesterol was detected by ferric chloride spray (50). For total lipid extraction (gangliosides included), 150 μl of each fraction was mixed with an equal volume of 50% methanol in water to dissolve the membranes. The samples were loaded onto 50-mg C18 columns equilibrated in 10% aqueous methanol. Columns were washed with 50% methanol, then lipids were eluted with 2 ml of 100% methanol followed by 2 ml of chloroform:methanol (1:1). Solvent was dried, and lipids were separated using the TLC solvent chloroform:methanol:water (60:35:8). After phospholipid detection, TLC plates were probed for GM1 using biotinylated CTx B subunit and Gb3 using VT1 (51).

Preparation of Vero Cell Membranes—Cells were grown to near confluence in four 150-cm2 dishes, washed briefly, and harvested by scraping in 5 mM EDTA in PBS, and centrifugation at 4 °C. Glycosphingolipid-depleted cells were prepared by culture for 7 days in 2 μM P4 prior to harvest. The cells were suspended in 2 ml of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl containing a protease inhibitor mixture (4-(2-aminoethyl)benzenesulfonfluoride hydrochloride, E64, aprotime, leupeptin, and bestatin) and disrupted by 30 strokes in a Dounce homogenizer with a tight-fitting pestle. Nuclei and debris were removed by spinning at 800 × g for 10 min at 4 °C. The supernatant was centrifuged at 100,000 × g in an SW55Ti rotor for 30 min at 4 °C. The resulting membrane pellet was suspended gently in 1 ml of ice-cold 25 mM MES, pH 7.0, 140 mM NaCl. Protein was determined using the bicinchoninic acid method (BCA assay, Pierce). Membranes stored frozen at −80 °C before use or prepared from cell pellets frozen for up to 1 month gave identical results.

For generation of DRMs, membrane preparation equivalent to 600 μg of protein was made up to a volume of 375 μl and mixed with an equal volume of 0.5% Triton X-100 on ice. The membranes were treated for 30 min on ice and equilibrated to room temperature. The sample was mixed with an equal vol-
volume of 70% sucrose in MES buffer and allowed to sit for 1 h at room temperature, and then sucrose gradient conditions were as described for glycolipid-cholesterol mixtures. For MβCD treatment of membranes, 600 g of membrane protein was treated with 70% sucrose containing 10 mM MβCD at room temperature for 1 h following detergent extraction and prior to sucrose gradient centrifugation. Culture cells were depleted of cholesterol by treatment with 10 mM MβCD for 10 min at 37 °C in serum-free medium.

Gb3-GSL Binding—A partial survey of the potential of Gb3 to bind to other GSLs and lipids was performed using a simple TLC overlay procedure we developed. 1 g of lipid samples was applied to triplicate TLC grids and air-dried. Lipids on one grid were visualized with orcinol spray. The other two grids were blocked in TBS containing 1% BSA then washed. In a glass tube, 20 g of Gb3 (dried from ethanol under N2) was suspended in 2.5 ml of TBS containing 1% BSA by heating at 40 °C for 20 min, mixed in a bath sonicator for 1 min, and gently vortexed for 30 s. The TLC grids were incubated in “aqueous Gb3” (∼8 μM Gb3) or TBS/BSA alone for 1 h at room temperature. The grids were washed 3× with TBS, and then Gb3 was detected by VT1 binding as described above.

Fluorescence Microscopy—Prior to labeling, cells were washed with serum-free medium (HMEM: 20 mM HEPES-buffered Erhles minimal essential medium containing 0.02% BSA) and chilled on ice. Alexa488-VT1B (5 μg/ml) was added for 20 min on ice. The cells were washed twice with HMEM then fixed, for surface labeling, or pre-warmed HMEM was added, and the cells were maintained at 37 °C for 15 min to internalize bound VT1B. The medium was replaced with HMEM (control) or HMEM containing 10 mM MβCD for 10 min at 37 °C. Coverslips were washed twice with warm HMEM then cooled on ice. Unmasked surface Gb3 was labeled with Texas Red-labeled VT1B (5 μg/ml) for 20 min on ice. After washing, cells were fixed on ice with 4% paraformaldehyde in PBS, washed, and then mounted in Prolong Antifade (Molecular Probes). 5 μg/ml DAPI was included in the mounting medium for nuclear labeling. Images were obtained using a Zeiss Axioplan epifluorescence microscope (21).

RESULTS

VT1 Binding to Gradient Separated Gb3-Cholesterol Vesicles Reveals a Major Discrepancy in GSL Receptor Function—Using our method to generate detergent-resisting Gb3/cho-
HIV gp120 Binding to GSL Vesicles Mimics That of VT1 and VT2—The HIV adhesin gp120 binds several GSLs, including Gb₃ (53). As for VT1/VT2, R5 gp120 included within the gradient only bound to Gb₃ vesicle fraction A (Fig. 4A, row a). Gp120 binds galactosyl ceramide (GalCer) and 3'-sulfogalactosyl ceramide (SGC) (35). GalCer/cholesterol and SGC/cholesterol vesicles were similarly prepared and separated in a gp120-containing density gradient. Gp120, localized by immunoblot, bound GalCer and Gb₃ in the outer bilayer leaflet but not in the inner leaflet. In contrast, the fraction A vesicles were smaller and showed outer membrane VT1 binding (Fig. 3F). This suggested the gradient separates the two major vesicle formats found in the starting preparation. For fraction B vesicles, stained with rat IgM anti-Gb₃, binding to both leaflets of the outer membrane was observed (Fig. 3G), unlike VT1 (Fig. 3E).

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lesterol vesicles (42), and centrifuging to equilibrium, VT binding vesicles were separated from the bulk Gb₃ vesicles (Fig. 1). ¹²⁵I-VT1/VT2 bound only in the first (± second) fraction (vesicle fraction A) (Fig. 1A), which contained low Gb₃ levels (Fig. 1B). >95% of the Gb₃ was in higher density fractions (3,4,5-vesicle fraction B), not bound by VT1 or VT2. Thus, Verotoxin only binds a minor, more buoyant subset of Gb₃/cholesterol vesicles.

The cholesterol and Gb₃ gradient distributions coincide (Fig. 1, B and C). The Gb₃/cholesterol ratio, measured with [³H]cholesterol, was constant at ~1.4, which approximates the michele-to-vesicle transition for GM1/cholesterol mixtures (52). Triton was distributed throughout the gradient (not shown). Gb₃ distribution was unaffected by the presence of VT: the same result was obtained when VT1 was omitted from the gradient and post-bound to the vesicles immobilized on nitrocellulose after separation (Fig. 1B). In either case, only a minor subfraction (<5%) of Gb₃ vesicles at the gradient top was bound.

Other Gb₃ Ligands Only Bind the Minor Gb₃ Vesicle Fraction—An IgG mAb anti-Gb₃ showed the same restricted binding as VT1/VT2 (Fig. 2i, rows a–c). However, for an IgM mAb anti-Gb₃ (45), though vesicle fraction A remained the primary binding fraction, vesicle fraction B, the major Gb₃ fraction, was also bound (Fig. 2i, rows d–f). B. simplicifolia lectin, which binds the terminal α-galactose of Gb₃, was also restricted to vesicle fraction A (Fig. 2ii, row b).

Post-embedding VT1 Immuno-EM of fraction A and B Gb₃/Cholesterol Vesicles—Cryo-immuno-EM showed the Gb₃/cholesterol vesicle preparation largely comprises two sizes. VT1 bound outer (and inner) membranes of the smaller vesicles (Fig. 3, C and D) but only inner membranes of the larger vesicles (Fig. 3, A and B). After separation, fraction B contained only larger, multivesicular vesicles. VT1 binding was restricted within these vesicles (Fig. 3E). Gb₃ in the outer bilayer leaflet was not recognized. In contrast, the fraction A vesicles were smaller and showed outer membrane VT1 binding (Fig. 3F).
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**Cholera Toxin Binds a Minor GM1/Cholesterol Vesicle Fraction**—CXt-binding GM1 is a major tool in cell and model membrane studies (25). GM1/cholesterol vesicles were placed below a sucrose gradient containing CTxB-HRP. After centrifugation, fractions were stained with peroxidase substrate. CTxB was bound only in vesicle fraction A (Fig. 5, panel 1). By CTxB TLC overlay of lipid extracts, GM1 was concentrated in vesicle fraction B (Fig. 5, panel 2), as seen for Gb₃, GalCer, and SGC. Thus CTxB receptor function within GM1/cholesterol vesicles is also restricted to a minor GM1 subfraction. This was verified using a polyclonal rabbit anti-GM1 within a similar GM1/cholesterol sucrose gradient. After separation, bound antibody was only immunodetected in vesicle fraction A (Fig. 5, panel 3).

**Galactosyl or Glucosyl Ceramide Can “Expose” VT1 Undetectable Gb₃ in Fraction B Vesicles**—To study how Gb₃ is rendered undetectable in fraction B vesicles, we incorporated other lipids within the Gb₃/cholesterol matrix. Most showed no effect, but galactosyl ceramide (non-hydroxy or hydroxy fatty acid form) “unmasked” fraction B vesicles for VT1 binding (Fig. 6A). A using a VT1 TLC overlay binding assay, Gb₃ was found to bind GalCer, GlcCer, and LacCer (Fig. 6B). LysoGalCer and adamanlylGalCer were not bound, suggesting aglycone modulation (54). The binding of Gb₃ to GalCer may counter cholesterol masking of Gb₃ for VT1 in fraction B vesicles. GalCer and LacCer were tested to unmask fraction B vesicles for VT1 binding (Fig. 6C), but only GlcCer was active.

**VT1 Binding Cell-derived Gb₃ Vesicles: Resolution of Undetectable GSL**—Vero cell post-nuclear supernatant membranes were extracted with Triton X-100 at 4 °C and subjected to VT1/sucrose-density gradient centrifugation at ambient temperature. As for the model GSL/cholesterol vesicles, VT1-bound vesicles were found only in a light fraction (vesicle fraction A; Fig. 7, A and B, row a), separate from the major GSL-containing fractions (vesicle fraction B; Fig. 7A, panel d). Vesicle fraction B also contained most phospholipids (Fig. 7A, panel c). For Gb₃-depleted DRM vesicles, from cells grown with the glucosyl ceramide synthase inhibitor, P4 (46), no VT1 binding was detected (Fig. 7A, rows b and f). Selective depletion of GSLs by P4 was confirmed by TLC of gradient fraction extracts (not shown).

Caveolin, a cholesterol binding (55) DRM marker protein (56), accumulated together with GM1/Gb₃ in fractions 4 and 5. Lower levels were also in the VT1-bound fraction (Fig. 7A, row g).

Thus, the majority of the cellular Gb₃ in these membrane vesicles of complex lipid/protein composition (the standard “DRM” fraction), was “masked” from VT1 binding, and a lighter, VT1-reactive, minor Gb₃-containing fraction detected, in a manner similar to the model Gb₃/cholesterol vesicle system. This shows ligand-undetectable GSL is a property shared by cell-derived membranes. MβCD cholesterol extraction of cell-derived DRMs resulted in the loss of VT1 binding in fraction 2 (i.e. vesicle fraction A) and a gain of binding to fractions 4 and 5 (vesicle fraction B) (Fig. 7B, row b), indicating a key role for cholesterol in this aglycone reg-
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A model membrane system was developed (42) to study whether variability of GSL receptor function according to lipid structure/microenvironment (aglycone) modulation and the accumulation of GSLs within cholesterol-enriched microdomains were linked. The ~1:1 GSL:cholesterol molar ratio optimized VT1-Gb3 binding, but similar ratios are found in some natural membranes (60). By density gradient equilibrium centrifugation, we have separated a ligand binding, minor GSL/cholesterol vesicle population from the major vesicle population not recognized by most GSL-binding proteins. This novel invisible GSL/cholesterol format surprisingly predicted a property of cell membranes: the major fraction of cellular GSLs is prevented from protein binding, largely by cholesterol masking.

Undetectable Model Membrane GSLs—Only 5–10% of the total GSL within our model GSL/cholesterol vesicles (fraction A), could bind ligands (VT1,VT2,gp120 lectin, CTxB, or anti-GSL antibodies). The vesicle fraction B at the 5–30% sucrose interface) contains 90% of the GSL but is refractory to ligand binding.

DISCUSSION

A model membrane system was developed (42) to study whether variability of GSL receptor function according to lipid structure/microenvironment (aglycone) modulation and the accumulation of GSLs within cholesterol-enriched microdomains were linked. The ~1:1 GSL:cholesterol molar ratio optimized VT1-Gb3 binding, but similar ratios are found in some natural membranes (60). By density gradient equilibrium centrifugation, we have separated a ligand binding, minor GSL/cholesterol vesicle population from the major vesicle population not recognized by most GSL-binding proteins. This novel invisible GSL/cholesterol format surprisingly predicted a property of cell membranes: the major fraction of cellular GSLs is prevented from protein binding, largely by cholesterol masking.

Undetectable Model Membrane GSLs—Only 5–10% of the total GSL within our model GSL/cholesterol vesicles (fraction A), could bind ligands (VT1,VT2,gp120 lectin, CTxB, or anti-GSL antibodies). The vesicle fraction B at the 5–30% sucrose interface) contains 90% of the GSL but is refractory to ligand binding.
Although much of the GSL is unavailable due to multilamellar structure, the binding of mAb IgM anti-Gb₃ to vesicle fraction B, both by vesicle immunostaining and to the outer membrane leaflet by IEM, shows the Gb₃ of these vesicles is surface-available. Because this property was shared by seven protein ligands and four receptor GSLs (Gb₃, GalCer, SGC, and GM1), this could be a general GSL effect. IgM mAb anti-Gb₃ binding to both vesicle fraction A (preferentially) and vesicle fraction B suggests a carbohydrate conformation change may mask Gb₃ in vesicle fraction B. Molecular simulation predicts such a cholesterol-mediated change (61).

A difference between the Gb₃ carbohydrate hydroxyl groups required for IgM mAb anti-Gb₃ and VT1 VT2 Gb₃ binding (20) involved hydroxyl groups which restricted rotation around the glycosidic linkages. If cholesterol restricts rotation around these glycosidic bonds within vesicle fraction B, a selective effect on VT1 VT2 compared with IgM mAb anti-Gb₃ binding might result. Thus GSL mobility in these vesicles may be necessary for (most) ligand binding. The fatty acid dependence of ligand-Gb₃ binding for fraction A vesicles led to a similar conclusion (19). Hydrogen bonds between cholesterol and sphingolipids (63) may restrict GSL motion.

Gradient resolution, promoted by reduced tube size (see supplemental material) permitted complete separation of ligand-detectable and invisible GSL/cholesterol vesicles. Without detergent, Gb₃ accumulates mainly in the denser gradient fractions. Solubilizing weaker interactions results in separation of two vesicular fractions, in only the minor of which is the GSL available for ligand binding. Detergent as a tool in cell biology has been criticized (64), largely in relation to the often overextrapolated connection between membrane detergent insolubility and the cellular existence of the nanoscale sphingolipid-cholesterol-related membrane heterogeneity of lipid rafts (25, 65). Although detergent-resistant membranes do not reflect the native membrane organization, insolubility can reflect specific cellular lipid and protein interactions (57).

Two Cell Membrane GSL Pools—The principle defined with our simple GSL/cholesterol membrane model, that GSL bilayers have a major fraction unavailable for ligand binding, is largely recapitulated within cell plasma membrane vesicles, and cells themselves. The VT1 binding fraction within the Vero cell DRMs (vesicle fraction A) was separated from the bulk Gb₃-containing vesicle fraction, at the 5/30% sucrose interface. Without detergent, Gb₃ accumulates mainly in the denser gradient fractions. Solubilizing weaker interactions results in separation of two vesicular fractions, in only the minor of which is the GSL available for ligand binding.

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containing fraction B, unbound by VT1 (or CTx), shows that invisible GSL vesicles are readily prepared from cell membranes. Because this discord in GSL receptor activity was seen for vesicles prepared from VT1/CTx-treated cells, most plasma membrane Gb3/GM1 is also invisible.

Cholesterol depletion of Vero cell membrane DRMs after preparation, and Vero cells prior to extraction, resulted in the same loss of VT1 (and CTx) binding to vesicle fraction A and the induction of VT1/CTx binding to vesicle fraction B. Thus, cholesterol is central in cell membrane GSL masking. Cholesterol masking of GM1 may compromise CTx as a lipid raft marker. Some cholesterol is required for optimal ligand binding (vesicle fraction A) but cholesterol can also prevent GSL recognition (vesicle fraction B). VT1 binding in higher density cell membrane fractions near the bottom of the gradient is Gb3-dependent and may represent unresolved and detergent-soluble Gb3. Differential Gb3 fatty acid content did not explain the different fractional A versus B ligand binding or the effect of cholesterol depletion (supplemental material).

Unmasking Invisible Membrane Gb3—Although cholesterol depletion increased ligand binding in cell membrane vesicle fraction B, the GSL content of fraction B was unaltered. Cholesterol depletion of cell membranes did not render ligand binding to fraction B vesicles proportional to GSL content, indicating other components restrict fraction B GSL receptor function. Although the mechanism of cholesterol-GSL masking is not yet defined, our finding that inclusion of GalCer or GlcCer in the model vesicles can reverse cholesterol-Gb3 masking provides a probe. Gb3 binding GalCer, GlcCer, and LacCer may be new examples of GSL carbohydrate-carbohydrate interaction (54). In the novel VT1-TLC overlay assay devised to screen for GSL-GSL binding, the aqueous Gb3 sample likely contains micelles, and therefore it is unclear as yet, whether VT1 can bind Gb3 bound to another GSL. Although no Gb3-cholesterol binding was seen, hydrophobic interactions may still play a significant role. These monohexosides may counter aglycone masking of membrane Gb3 receptor function. GalCer/GlcCer-Gb3 binding in fraction B vesicles may cluster Gb3 or increase fluidity to allow multivalent VT1 binding (9). Gb3 bound to LacCer may be too large or immobile to promote VT1 fraction B vesicle binding. Reduced GlcCer levels can decrease VT1 binding to cell DRMs (34), which may be a function of the GlcCer/GalCer-Gb3 binding and unmasking we observe. Interestingly, intracellular Gb3-dependent VT1 trafficking was found to be selectively dependent on C16 ceramide monohexoside (66).

Cell Physiology of Invisible GSL—Disruption of the cellular actin cytoskeleton in cells with latrunculin also resulted in partial unmasking of invisible cell membrane Gb3, for VT1 binding. VT1 binding to fraction A vesicles was also increased (unlike cholesterol depletion), consistent with a cytoskeleton-lipid microdomain interaction (67, 68). A cholesterol-cytoskeletal linkage (69) might alter GSL receptor function during dynamic cell membrane-remodeling processes. Cholesterol is increased at the motile cell membrane leading edge (70) and is required (71) and selectively regulated (72, 73) during mitosis. Cholesterol synthesis inhibitors induce stem cell differentiation and anchorage-dependent tumor cell growth (74). The differential distribution of cholesterol in cellular membranes (75–77), its potential asymmetric cell surface topology (78, 79), and accumulation in GSL enriched rafts could provide extensive dynamic lateral regulation of GSL function. Such domains play many (patho)physiological roles, e.g. in signal transduction (80) and microbial pathogenesis (81, 82). GSL masking could be central to such processes and provide new bases for prophylaxis of GSL-targeted infectious disease.

SEEA-4 (stage-specific embryonic antigen 4) is a globoseries GSL (NeuAcα2−3Galβ1−3GalNAcβ1−3Galα1−4Galβ1−4Glc ceramide (83)), the major marker defining human pluripotent stem cells (84). Antibody/SEEA-4 binding is key in immuno-sorting undifferentiated cells (85) for potential therapeutic uses. Cholesterol masking SEEA-4 in fraction B Vero cell vesicles so only 10% of cell membrane SEEA-4 is available for antibody binding suggests practical relevance of invisible membrane GSL. The greater polarity of SEEA-4 ganglioside indicates cholesterol-GSL masking is independent of GSL headgroup character. Because peanut agglutinin bound glycoproteins in cellular fraction B vesicles, cholesterol masking is restricted to GSL cell glycoconjugates.

Cholesterol depletion of Vero cells unmasked invisible Gb3 to allow distinct VT1B cell staining. The two separate cell membrane Gb3 pools (ligand-available and cholesterol-masked) were clearly delineated by differential VT1B labeling. The non-uniform surface distribution of the (initially) invisible Gb3 pool, consistent with retention in lipid rafts (86), its relation to the initial, ligand-bound Gb3, and defining other factors, which restrict cell membrane GSL recognition, will provide a new arena for membrane GSL receptor studies. The topical separation of these pools implies separate regulation.

Several studies already indicate a physiological role for membrane GSL masking by cholesterol. Cholesterol depletion can unmask Gb3 in human renal glomeruli (20, 28). Strong, detergent-resistant VT1 VT2 binding was induced in VT1/VT2 unreactive glomeruli after cholesterol extraction. Thus membrane cholesterol masking of Gb3 may protect against VT-induced glomerular pathology and provide a risk factor for VT-induced hemolytic uremic syndrome. Cholesterol depletion to unmask membrane GSLs is also a key feature of capacitation of spermatozoa required for fertility (87, 88). The standard use of acetone (extracts steroids) to “unmask” GSLs for immunohistochemistry (89, 90) further attests to widespread cholesterol membrane GSL masking in cells and tissues.

Molecular modeling the cholesterol-sphingolipid complex shows the polar cholesterol hydroxyl group (63), can form an H-bond network to alter the sugar conformation around the anomeric linkage, to be parallel, rather than perpendicular, to the membrane (61). We have found similar simulation results for GM1 ganglioside.7 Thus, membrane cholesterol could alter GSL carbohydrate conformation, a potential on/off switch for protein recognition. This aglycone modulation translates “cis” interactions into “trans” effects. This could be bidirectional. Protein binding to membrane-available GSL might alter lateral bilayer interactions (86).
The GSL:cholesterol ratio was constant in model vesicle fractions A and B. The interaction of cholesterol with sphingolipids is more pronounced than glycolipids, but both vary with acyl chain length (C16 → C20) and saturation (91). Within Gb3/cholesterol vesicle fraction A, receptor activity for VT1 and gp120 is a function of the Gb3 fatty acid chain length in this range. From Gb3 fatty acid isoform mixing, we proposed that reduced Gb3 fluidity could restrict ligand binding to vesicle lipid headgroup fluidity (92) and promote ligand binding (93). Increased membrane curvature can increase model membrane

The major difference between vesicle fractions A and B is size. The interaction of cholesterol with sphingolipids for the differential ligand binding to vesicle fractions A and B. The interaction of cholesterol with sphingolipids fraction A (19). Membrane parallel GSL carbohydrate would be invisible GSLs

part) prevention of ligand recognition of the major cellular GSL. Although this is counter to current cell membrane paradigms, some studies have implied the presence of GSLs on the cytosolic membrane surface (62).

A remarkable discrepancy in GSL receptor function, defined in simple GSL/cholesterol vesicles, describes a new thermodynamic property of hyper- versus hypo-cell membrane GSL receptor activity, which may apply generally, i.e. cholesterol (in part) prevention of ligand recognition of the major cellular GSL fraction. This intrinsic lipid bilayer property may be modulated by other membrane components, such as the actin cytoskele-
	on, to make aglycone GSL masking a potential dynamic “cloak ing” device” in cellular physiology.

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REFERENCES

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