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Gel phase preference of ganglioside GM1 at low concentration in two-component, two-phase phosphatidylcholine bilayers depends upon the ceramide moiety

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Abstract

In two-component phosphatidylcholine bilayers with coexisting liquid and $P_{\beta'}$ gel phases, the distribution between phases of low concentrations of glycosphingolipids can be determined by freeze-etch electron microscopy after labeling the glycolipid with a suitable protein. We have found that the distribution depends upon the glycosphingolipid species (Rock, P. et al., (1991) Biochemistry 30, 19–25). Using this technique with cholera toxin as the protein label and bilayers formed from dipalmitoyl- and dielaidoylphosphatidylcholine (1:1) containing < 1 mol% GM1, we have studied the distribution of a family of GM1 homologues differing in the acyl chain and sphingoid base moieties. The GM1 preference for the $P_{\beta'}$ ripple phase decreases with decreasing acyl chain length and increasing unsaturation. GM1 with either a C18:1 or C20:1 sphingoid base shows similar distributions in liquid and gel phases. When the molecules are preferentially found in the $P_{\beta'}$ phase, they are positioned along unique loci in both Λ and $\Lambda/2$ forms of the ripple structure. This localization and acyl chain dependence reflect the volume, shape and localization of molecular packing defects in the $P_{\beta'}$ phase. The ganglioside inclusions stabilize the $P_{\beta'}$ phase and form compositional domains of unique topography.

Keywords: Glycosphingolipid; Ganglioside; Liposome; Freeze-etch; Electron microscopy

1. Introduction

Although glycosphingolipids are a ubiquitous component of the outer leaflet of biological membranes, their organization and function in membranes is only beginning to be understood. Along with glycoproteins and glycosylphosphatidylinositol (GPI)-anchored proteins, membrane glycosphingolipids present on the outer cell surface are the primary interactants with molecules in the extracellular medium or on the surfaces of other cells. Glycosphingolipids have been shown to play a role in cell–cell interactions and to serve as receptors for some hormones, toxins, and viruses. The types and relative amounts of glycosphingolipids on the cell surface change during development, differentiation, and oncogenesis [1–4]. Recently, it has been postulated that glycosphingolipid-rich microdomains form in the *trans*-Golgi network and that GPI-anchored proteins associate with these microdomains and are transported to the apical surface of polarized epithelial cells [5,6]. Experimental evidence for this hypothesis includes the isolation of detergent-insoluble complexes of GPI-anchored proteins and glycosphingolipids from MDCK epithelial cells [7,8].

We have been investigating the molecular organization of glycosphingolipids in bilayer systems under the assumption that an understanding of their organization in simple bilayers of defined molecular composition will help to

Abbreviations: AGM1, asialo-GM1; DEPC, dielaidoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; FRAP, fluorescence recovery after photobleaching; GM1, Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeu5Ac)β1 → 4Glcβ1 → 1Cer; GPI, glycosylphosphatidylinositol; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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elucidate their functional properties in the bilayers of cellular membranes. We have been especially interested in determining the propensity of glycosphingolipids to form domains when incorporated into phospholipid bilayers. Domains consisting of one or more types of glycosphingolipids might function as receptors or cell recognition sites on cell surfaces and could in Golgi membranes serve to target GPI-linked proteins to specific sites on the surface of the cell, as noted above. We have utilized freeze-etch electron microscopy to visualize molecular markers that bind specifically to glycosphingolipids on the surface of phospholipid bilayers to evaluate the molecular organization of underlying glycosphingolipids. Using markers such as Fab fragments of IgG molecules, lectins, and cholera toxin, we have shown that negatively charged glycosphingolipids (gangliosides) are molecularly disbursed in one-component liquid-phase phospholipid bilayers whereas neutral glycosphingolipids we have examined under the same conditions are organized into microdomains [9,10].

In order to more closely simulate the complex lipid composition of biological membranes, we have begun to examine the distribution of glycosphingolipids in two-component phosphatidylcholine bilayers under conditions where both gel and liquid phases coexist. We have found that both the ganglioside GM1 and the neutral tetrahexosylceramide prepared from it, AGM1, when present in concentrations of less than one mole percent in two-component phospholipid bilayers consisting of both liquid and gel phases, show a marked preference for the $P_{\beta'}$ rippled gel phase. In contrast, Forsmann antigen, a neutral pentahexosylceramide, shows no differential preference for either gel or liquid phases in the same bilayer system [11]. The results suggest that the phase preference is not a function of the polysaccharide moiety or the molecular charge of the glycosphingolipid, but rather that the chemical structure of the ceramide portion of the molecule determines whether it inserts into the fluid or gel phase of the bilayer.

To investigate further the role of the ceramide moiety of glycosphingolipids in determining their distribution in phospholipid bilayers, we synthesized a homologous series of GM1 species that differed in acyl chain length, degree of saturation and in the sphingoid base. We especially wanted to determine how these molecular parameters influenced segregation of glycosphingolipids into coexisting liquid and gel phases of two-component phosphatidylcholine bilayers. We also were interested in relating our observation [11] of the distinctive distribution of glycosphingolipids in rows that are parallel to the gel-phase ridge system to the underlying structure of the phosphatidylcholine bilayer with the aim of better understanding why certain homologues of GM1 prefer to associate with this phase rather than to insert preferentially into the liquid portion of the bilayer, as reported for proteins added to bilayer systems [12].

2. Experimental procedures

2.1. Materials

Dielaidoylphosphatidylcholine (DEPC) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids, Pelham, AL, and used without further purification. Other commercially available compounds were of the highest grade. Common solvents were redistilled in glass apparatus. Tetrahydrofuran (THF) was depleted of peroxides by passage through an alumina column. The THF was dehydrated by refluxing over KOH pellets and finally refluxed over and distilled from lithium aluminum hydride. Silica gel 100 and silica gel 60 for column chromatography, high performance silica gel precoated thin-layer plates (HPTLC kiesel gel 60, 10×10 cm) and Lichrosorb RP8-HPLC columns were purchased from Merck, Darmstad, Germany. *N*-Acetylneuraminic acid and myristic (14:0), palmitic (16:0), heptadecanoic (17:0), oleic (18:1), linolenic (18:3), arachidic (20:0), and behenic (22:0) anhydrides were from Sigma, St. Louis, MO. Cholera toxin was also obtained from Sigma.

Ganglioside GM1 (Galβ1 → 3GalNAcβ1 → 4Gal(α2 ← 3Neu5Ac)β1 → 4Glcβ1 → 1Cer) a native GM1, was extracted from calf brain [13], purified and structurally characterized [14]. The native GM1 was over 99% pure based on the oligosaccharide portion. Its sphingoid base composition was: C18:1, 36%; C18:0, 2.5%; C20:1, 60%; C20:0, 1.7%, and the fatty acid composition was 16:0, 2.8%; 18:0, 94%, 20:0, 2%, others 1.2%. Native GM1 was maintained under high vacuum in the presence of dehydrating agents before use.

Removal of the fatty acid moiety from GM1 to give lyso-GM1 was carried out in a one-pot alkaline reaction [15]. After purification, which was carried out by silica gel 100 column chromatography (60×1.5 cm/ 50 mg of starting ganglioside) using chloroform/methanol/27 mM aqueous $CaCl_2$/100 mM aqueous KC1 (50:50:4:8, by vol.) as the equilibrating and eluting solvent system, the lyso-GM1 was 99% pure.

2.2. Methods

The procedure for the preparation of GM1 ganglioside molecular species having homogeneous acyl and sphingoid base moieties is as follows:

A methanol solution of lyso-GM1 (0.78 mM) and triethylamine (20 mM) was mixed with THF solution containing one of the following anhydrides (18 mM): myristic, palmitic, heptadecanoic, oleic, linolenic, arachidic and behenic. The final concentration of lyso-GM1 was 0.55 mM. The reaction mixture was shaken and warmed for 5 min at about 40° C using hot air. The acylation reaction was monitored by thin-layer chromatography (TLC) and the yield determined by densitometry [16]. The reaction mixture was dried, dissolved in 5 ml of redistilled water and

dialyzed against 0.05 N NaOH, to remove traces of O-acylation, and then dialyzed against distilled water.

Each GM1 ganglioside, homogeneous in the acyl moiety, was purified from the dialyzed reaction mixture by silica gel 60 column chromatography (50 × 1 cm/20 mg of starting ganglioside) using chloroform/methanol/water (60:35:5, by vol.) as the equilibrating and eluting solvent, the elution profiles were monitored by TLC. Reverse-phase high-performance liquid chromatography (HPLC) was used to prepare molecular species homogeneous also in the sphingoid base. These were either C18- or C20-sphingosine (sphingenine) [17]. To do this, 4 mg of each ganglioside, solubilized in 1 ml of the same solvent system, were injected into a Lichrosorb RP8-column (40 × 250 mm) and eluted with the solvent system acetonitrile/5 mM, pH 7 phosphate buffer (55:45, by vol.) at a flow rate of 12.5 ml/min. The elution profile was determined by UV absorbance at 195 nm; collection of the material started and ended at 10% of the UV full scale absorbance. After drying, each GM1 molecular species was dissolved in the minimum volume of distilled water and submitted to dialysis and lyophilization. A final further purification to remove contaminants derived by bleeding of the RP8-HPLC column was performed by silica gel 100 column chromatography (20 × 1 cm/5 mg of starting ganglioside) using chloroform/methanol/water (60:35:5, by vol.) as the equilibrating and eluting solvent system. The elution profile was monitored by TLC (see below).

2.3. Analytical procedures

Thin-layer chromatography of GM1, lyso-GM1, GM1 molecular species and of the ganglioside reaction mixture was performed on HPTLC plates at room temperature using the following solvent systems: (a) chloroform/methanol/30 mM aqueous $CaCl_2$/100 mM aqueous KCl (50:50:4:8, by vol.); (b) chloroform/methanol/0.2% aqueous $CaCl_2$ (50:42:11, by vol.). Ganglioside and ganglioside-derivatives were made visible by treatment with a *p*-dimethylaminobenzaldehyde and/or an anisaldehyde spray reagent followed by heating at 120° C for 15 min. Quantification of ganglioside spots was carried out by total densitometry of the plates [16].

The fatty acid and long chain base contents of the synthesized ganglioside molecular species were determined by gas chromatography-mass spectroscopy [18,19]. Ganglioside-bound sialic acid was assayed by the resorcinol-hydrochloric acid method [20] with pure Neu5Ac as the reference standard.

2.4. Multilamellar liposomes

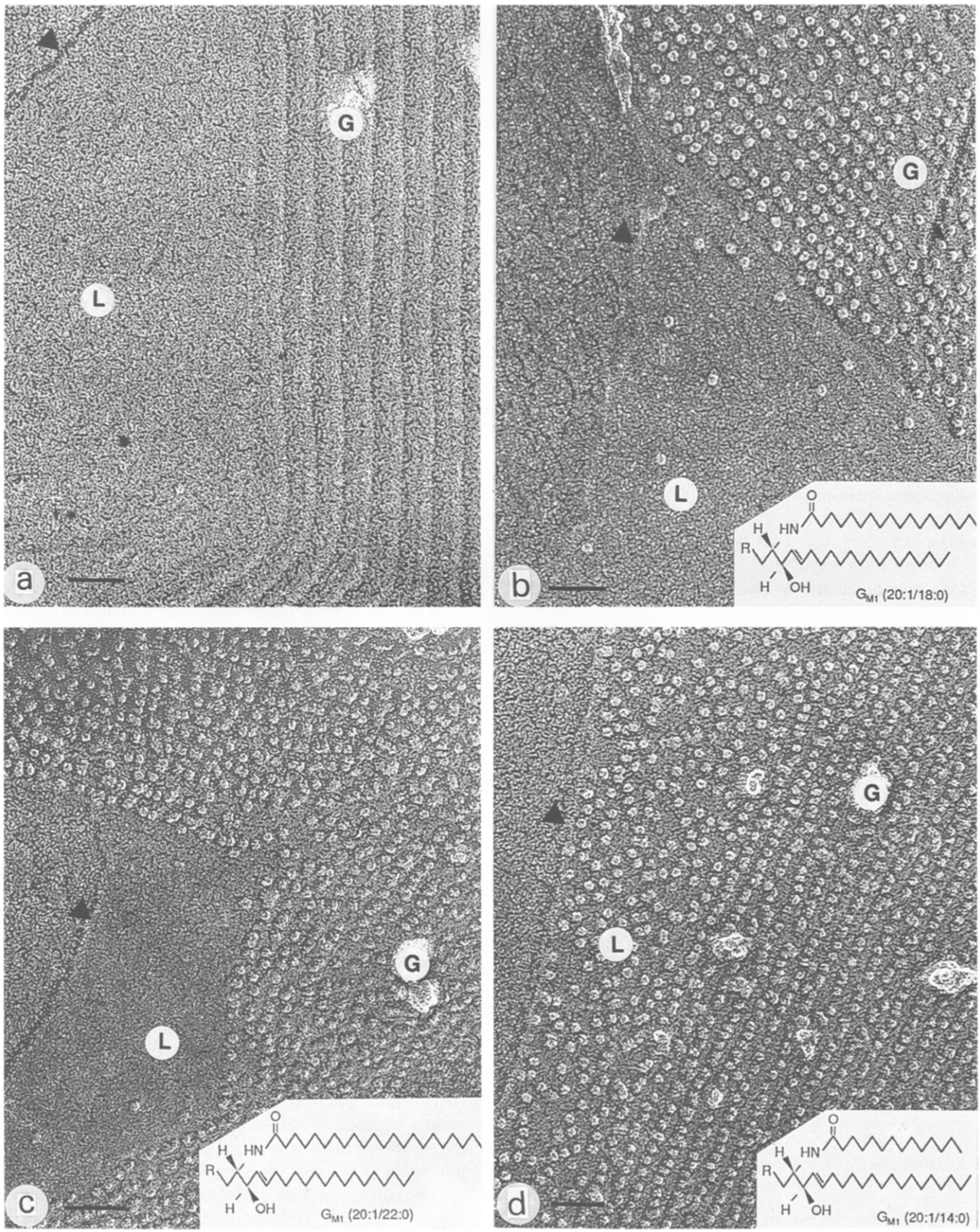
Multilamellar liposomes were prepared by spraying a chloroform solution of the mixed lipid onto a glass plate as described earlier [9]. Liposomes were hydrated and incubated for 1 h at 50° C in a pH 7.4 buffer containing 10 mM Tris and 10 mM NaCl. Prior to labeling, liposomes were assayed to determine the molar concentration of lipid phosphorus and the mole fraction of GM1 using a sphingosine analysis [21,22]. For labeling experiments, 400 nmol total lipid was incubated with a 5-fold excess of cholera toxin for 30 min to bind to the available GM1 in the liposomes. The labeled liposomes were gently pelleted, the supernatant discarded and the pellet resuspended in 20–30 μl of buffer. A 10 μl aliquot of the suspension was applied to the sample plunger of a cryopress and ultra fast frozen with liquid helium, followed by freeze-fracturing and shadowing with a Balzers BAF-300 freeze-etching apparatus [9,11,23]. Replicas were examined with a Zeiss 902 transmission electron microscope.

3. Results

3.1. Ganglioside semi-synthesis

Ganglioside molecular species containing different fatty acids were previously synthesized by means of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide activated acyl chain and deacetyl-deacyl-GM1 (Galβ1 → 3GalNAcβ1 → 4Gal(2α ← 3Neu)β1 → 4Glcβ1 → 1)-long chain bases [24]. The N-acylation reaction occurred very slowly but with good regiochemistry, the sialic acid amino group remaining free at the end of the reaction and available for a final acetylation. Other authors [25] starting from the same deacetyl-deacyl-GM1, prepared the lyso-GM1 by a four step synthesis and condensed it with *N*-succinimidyl activated fatty acid.

Following the availability of a new one-pot procedure for the preparation of lyso-GM1 [15], we now present a simple and rapid procedure for the preparation of GM1 molecular species with a defined acyl moiety. The method makes use of commercially available anhydrides. The reaction between lyso-GM1 and myristic, palmitic, heptadecanoic, oleic, linolenic, arachidic and behenic anhydride was very rapid. A slight warming of the reaction tube was required immediately after mixing of the reagents in order to avoid precipitation of the less soluble anhydrides in methanol. The final yield of the reaction, determined by TLC and densitometry, was for all the GM1 molecular species preparations, 98–99%. A minor contaminant, not characterized but less polar than GM1 by TLC, was removed by column chromatography. The combination of this synthesis with a reversed-phase HPLC fractionation procedure [17] made it possible to separate molecular species containing long chain bases of different chemical structure and allowed the preparation of sixteen GM1 molecular species containing a defined ceramide moiety.


a
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G_{M1} (20:1/22:0)
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3.2. Distribution in coexisting liquid-gel phase bilayers of GM1 molecules containing a C20:1 sphingoid base with different acyl chain lengths and degrees of unsaturation

As previously reported [11,26], liposomes composed of a 1:1 mixture of DEPC/DPPC when incubated at 20° C show lateral separation of a gel phase rich in DPPC from a liquid phase rich in DEPC, and freeze-etch electron microscopy of these liposomes shows a gel phase characterized by the presence of $P_{\beta'}$ ripples and a liquid phase that has a relatively smooth appearance (Fig. 1a). As predicted by the phase diagram for this phospholipid mixture, the rippled gel phase and the smooth liquid phase are about equal in proportion at this temperature and composition [12].

DEPC/DPPC liposomes containing approximately 0.5 mol% of a series of GM1 homologues with a C20:1 sphingoid base were prepared that differed in saturated acyl chain length, ranging from 14:0 to 22:0. Preparations containing the unsaturated acyl chains 18:1 (oleoyl) and 18:3 (α-linolenoyl) were also examined. Representative chemical structures are shown with the appropriate micrographs. After labeling the liposomes with cholera toxin at 20° C, freeze-etch electron microscopy was performed to visualize the distribution of this specific marker for GM1 on the surfaces of the liposomes. Cholera toxin appears as a distinct 80-Å diameter particle on freeze-etch replicas of liposomes containing GM1 and as shown previously, accurate quantitation of the amount of label per unit area is readily accomplished [9,11]. As shown in Fig. 2, the amount of cholera toxin label per square micron in gel and liquid phases of GM1-containing liposomes depended markedly on the acyl chain length of the GM1 and on its degree of unsaturation. Liposomes containing GM1 with 16:0-22:0 saturated acyl chains showed a marked preference of the GM1 to incorporate into the gel phase (Figs. 1b and 1c). Liposomes containing GM1 with 14:0 acyl chains showed much greater amounts of cholera toxin label in the liquid phase, although the gel phase also contained label (Fig. 1d).

DEPC/DPPC liposomes containing approximately 0.5 mol% GM1 with 18:1 and 18:3 unsaturated acyl chains showed a preference for the liquid phase when labeled with cholera toxin (Figs. 2 and 3a). This is in marked contrast to the distribution of the saturated 18:0 acyl chain-containing GM1, as noted above and in a previous report [11], where most of the GM1 was found to be in the gel phase.

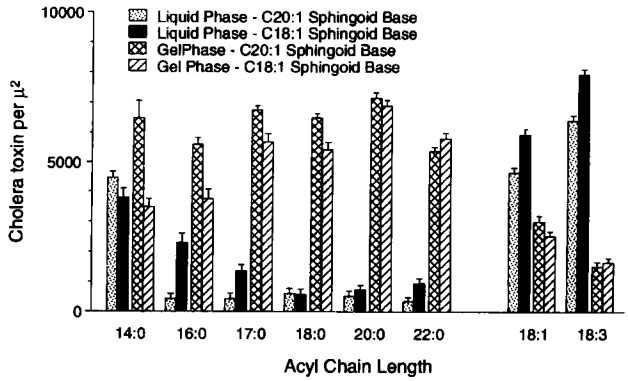

Fig. 2. Bar graph showing amount of cholera toxin per μm^2 in liquid and gel phases of 1:1 DEPC:DPPC liposomes containing 0.5 mol% GM1 with C20:1 and C18:1 sphingoid bases and different saturated (left side) and unsaturated (right side) acyl chain lengths. The standard error of the mean is indicated for each preparation.

3.3. Distribution in coexisting liquid-gel phase bilayers of GM1 molecules containing a C18:1 sphingoid base with different acyl chain lengths and degrees of unsaturation

DEPC/DPPC liposomes containing approximately 0.5 mol% of a series of GM1 homologues with a C18:1 sphingoid base, that were identical in acyl chain length and degree of saturation to those reported above for C20:1 sphingoid base-containing GM1 homologues, were labeled with cholera toxin and examined by freeze-etch electron microscopy. As shown in Fig. 2, the amount of cholera toxin label per μm^2 in gel and liquid phases of C18:1 GM1-containing liposomes depended on the GM1 acyl chain length and on its degree of unsaturation, corresponding closely to the distribution found for the C20:1 sphingoid base-containing GM1 homologues. However, slightly more cholera toxin label was found in the liquid phase of the liposomes containing GM1 with 16:0 and 17:0 acyl chains than was seen in the C20:1 sphingoid base-containing GM1 preparations. The marked preference of both C18:1 and C20:1 sphingoid base-containing GM1 with 18:0, 20:0, and 22:0 acyl chains for the gel phase is shown in Fig. 2. Also, both C18:1 and C20:1 sphingoid base-con-

Fig. 1. (a) Freeze-etch electron micrograph of a 1:1 DEPC/DPPC liposome containing 0 mol% GM1 and quenched from 20° C. There is lateral separation of the rippled gel phase (G) and the liquid phase (L). The arrow head indicates the border of the bilayer fracture face and the external surface of the liposome. Magnification is 250 000 ×. In all micrographs the bar equals 38 nm. (b) 1:1 DEPC/DPPC liposome containing 0.5 mol% GM1 with C20:1 sphingoid base and 18:0 acyl chain (see inset), labeled with cholera toxin and quenched from 20° C. The cholera toxin is localized almost exclusively over the ridged (gel) regions. G = gel phase; L = liquid phase; arrow head = fracture line. Magnification is 250 000 ×. (c) 1:1 DEPC/DPPC liposome containing 0.5 mol% GM1 with C20:1 sphingoid base and 22:0 acyl chain (see inset), labeled with cholera toxin and quenched from 20° C. The cholera toxin is localized almost exclusively over the ridged (gel) regions. G = gel phase; L = liquid phase; arrow head = fracture line. Magnification is 250 000 ×. (d) 1:1 DEPC/DPPC liposome containing 0.5% GM1 with C20:1 sphingoid base and 14:0 acyl chain (see inset), labeled with cholera toxin and quenched from 20° C. The cholera toxin is distributed over both the liquid (L) and ridged gel (G) regions in approximately equal density. Arrow head = fracture line. Magnification is 250 000 ×.

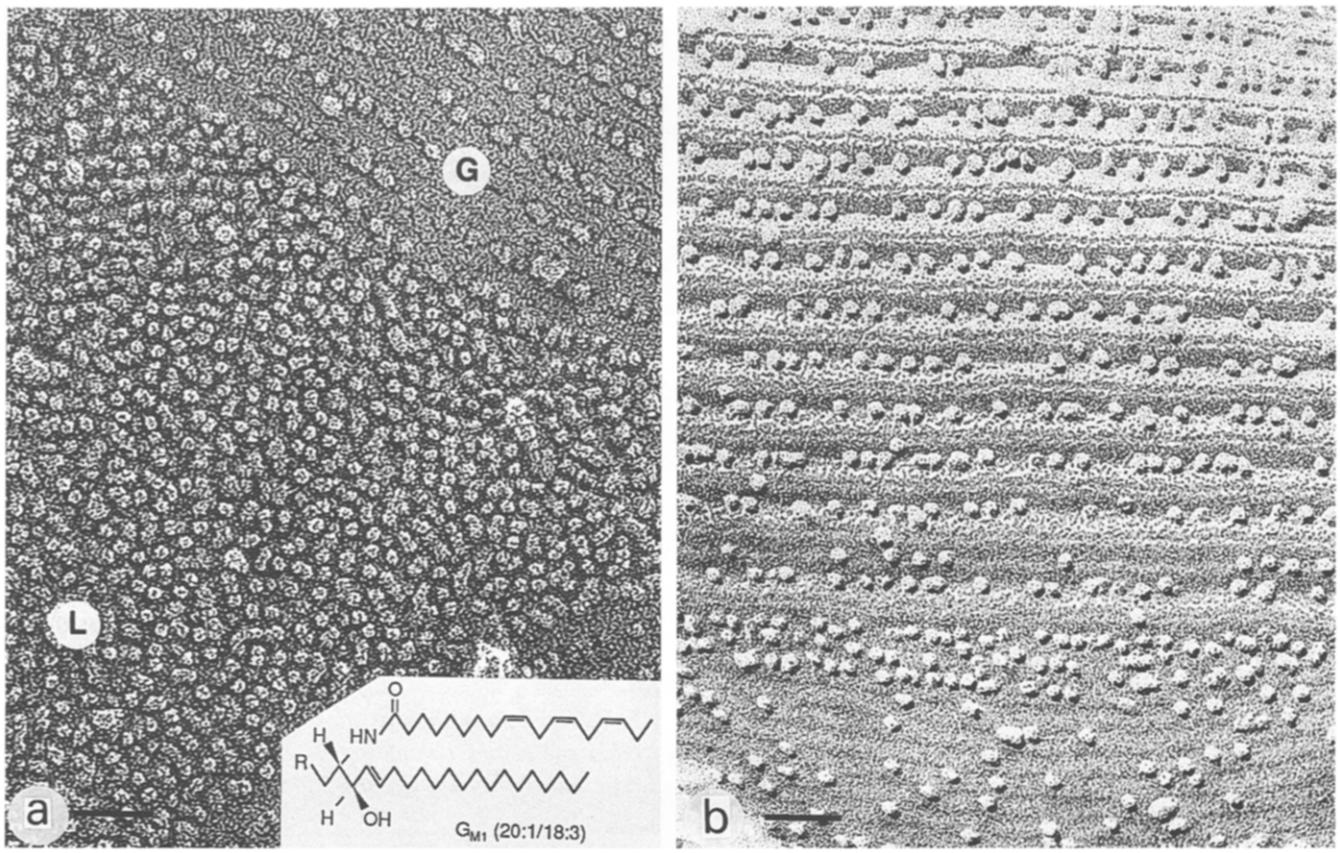

Fig. 3. (a) 1:1 DEPC/DPPC liposome containing 0.5 mol% GM1 with C20:1 sphingoid base and 18:3 acyl chain (see inset) labeled with cholera toxin and quenched from 20° C. The cholera toxin is localized predominantly over the liquid (L) regions of the bilayer, but some label is present in the gel phase (G). Magnification is 250 000 ×. (b) Freeze-etch electron micrograph of the $P_{\beta'}$ gel phase of a 1:1 DEPC/DPPC liposome containing 0.5 mol% GM1 with C18:1 sphingoid base and 18:1 acyl chain, labeled with cholera toxin and quenched from 20° C. This preparation was unidirectionally shadowed. The cholera toxin molecules are located in the valleys of the $P_{\beta'}$ ripples for the $\Lambda/2$ ridges (lower portion of photograph) and in the major trough of the Λ ridge (upper portion). Magnification is 250 000 ×.

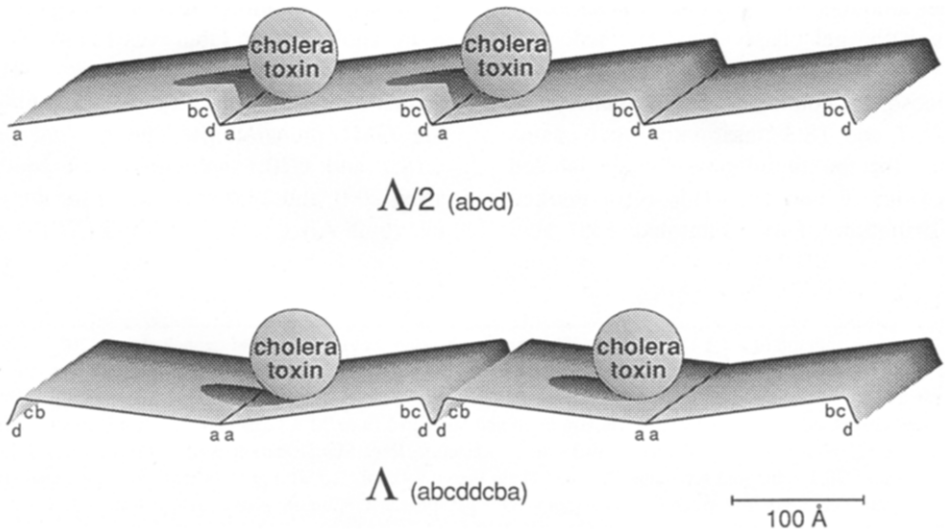

Fig. 4. Sketch, roughly to scale, depicting the surface appearance of the two types of $P_{\beta'}$ ripple seen in freeze-etch electron micrographs. Shown also is the location of cholera toxin on the bilayer surface.

taining GM1 preparations with unsaturated acyl chains showed an increasing preference for the liquid phase with increasing numbers of double bonds.

3.4. Localization of Cholera toxin in GM1-containing $P_{\beta'}$ phases

It is apparent in Figs. 1b–1d, 3a that the cholera toxin marker in the $P_{\beta'}$ phase is arranged in rows that are parallel to the ridge system exhibited by this phosphatidylcholine phase. This organization, seen in all systems examined in this work, was noted in an earlier study of the phase preference of GM1 and AGM1 in two-component, two phase phosphatidylcholine bilayers [11]. We take this arrangement of cholera toxin molecules to reflect the underlying localization of gangliosides in the bilayer. In order to examine this localization in more detail, freeze-fracture specimens were unidirectionally shadowed rather than the usual rotary shadowing. When the ridge system axes are perpendicular to the direction of shadowing, the electron micrographs can be used to locate accurately the position of the cholera toxin in the ridge system and to determine the characteristic features of the $P_{\beta'}$ ripple. An example of such a photograph is shown in Fig. 3b. A diagram of the ridge structure is shown in Fig. 4. It is apparent that the parallel structure is not a sinusoidal ripple but is an asymmetric, but regular, saw-tooth structure consisting of a long ramp (*a-b*) and a short abrupt ramp (*c-d*). Ramp *a-b* occupies about 87.5% of the bilayer surface and ramp *c-d* about 12.5%. Two repeating arrangements of ramps are observed in most preparations. The lower sketch in Fig. 4 labeled Λ shows a basic repeat unit *abcddcba*, while the upper sketch in this figure ($\Lambda/2$) shows a basic repeat unit *abcd*. These arrangements for the $P_{\beta'}$ ripple have been observed in multilamellar bilayers formed from a single saturated diacyl phosphatidylcholine [27]. They were first noted in two-component, two-phase DEPC/DPPC bilayers by Grant et al. [26] and subsequently in other two-component systems [11]. Fig. 4 also illustrates the fact that the cholera toxin label in all systems is almost always found close to the *a* end of the *a-b* ramp in both the Λ and $\Lambda/2$ configurations. We conclude that the ganglioside molecules to which the toxins are bound are localized in this region. However, because of the bulky nature of cholera toxin, there may be other regions of the complex $P_{\beta'}$ surface that contain GM1, but are inaccessible to cholera toxin. It was not possible to deduce the orientation of the cholera toxin molecule on the bilayer surface. It is thus represented in Fig. 4 as a sphere of the approximately correct relative dimensions [28].

4. Discussion

Our earlier work had demonstrated a marked preference of the negatively charged ganglioside GM1 and the neutral glycosphingolipid AGM1 for the $P_{\beta'}$ gel phase rather than the coexisting liquid phase in two-component phosphatidylcholine bilayers [11]. Gas chromatography/mass spectroscopy analysis of this commercial preparation of GM1 and the AGM1 derived from it showed 80% 18:0 fatty acid composition and a 70:30 ratio of C-18:C-20 sphingoid bases. In contrast, another neutral glycosphingolipid, Forssman glycosphingolipid, showed no preference for the $P_{\beta'}$ phase in identical phosphatidylcholine bilayers, but distributed equally in liquid and gel regions. Analysis of the chemical composition of the Forssman glycosphingolipid showed marked heterogeneity in the fatty acid composition, with acyl chains ranging from 16:0 to 24:0 and a predominance of C-18 sphingosine. These experiments suggested that the length of the acyl chain moiety of the glycosphingolipid molecule may be a critical determinant in the partitioning of glycosphingolipids between the liquid and gel phases in two-component phosphatidylcholine bilayers. In order to test this hypothesis, we prepared a series of GM1 homologues that differed in acyl chain length and examined their distribution in phosphatidylcholine bilayers with equal amounts of coexisting liquid and $P_{\beta'}$ gel phases. We also prepared GM1 homologues differing in sphingoid base composition and degree of unsaturation to examine the effect of these parameters on glycosphingolipid distribution in two phase phosphatidylcholine bilayers.

The results of our studies with GM1 homologues differing in acyl chain length clearly indicate the dominance of this parameter in determining the preference of these glycosphingolipids for the gel or liquid regions of the bilayer. Decreasing the length of the acyl chain of the GM1 molecule caused a shift in distribution of the glycosphingolipid from almost exclusive localization in the gel phase for the longer acyl chain-containing molecules to approximately equal distribution in the gel and liquid phases for molecules with shorter acyl chains. GM1 molecules with one or three unsaturated acyl chains showed a marked preference for the liquid phase, in contrast to identical GM1 species with saturated acyl chains. The nature of the sphingoid base in the GM1 molecules did not appear to have much effect on the gel/liquid phase preference of the molecules, although the sphingoid base of the homologues tested differed by only two carbon atoms in length.

The surprising preference of the longer acyl chain-containing GM1 molecules for the $P_{\beta'}$ gel phase must be due to structural features of the $P_{\beta'}$ phase. Although there are a number of studies of this phase as it occurs in one-component phospholipid bilayers, there is no general agreement about the molecular arrangement in the structure [29–35]. It seems clear, however, that the parallel ridges are the result of an ordered system of disclinations which delineate parallel strip domains. Strip domain systems have also been observed in thin films and monolayers formed from a variety of molecular types. In general the periodicity of these structures is determined by a balance between a short-range domain boundary energy and a long-range

repulsive interaction that may be electrostatic, magnetostatic or elastic in origin [36–38]. In monolayers the repulsive interaction is generated by the molecular dipoles which lie along the molecular axis [36]. In the $P_{\beta'}$ bilayer phase it seems reasonable to assign also an electrostatic origin attributable to the repulsive interaction of the molecular dipoles. However, in the bilayer, because of the mirror-plane symmetry of the structure, there is almost, but not complete, cancellation of the dipole moment of each monolayer in the bilayer by that of the apposing monolayer. In contrast to the thin film systems mentioned above in which the strip period is of the order of 10^{-3} cm, in $P_{\beta'}$ phases this period is of the order of 10^{-6} cm. The difference in period is most probably due to the difference in the magnitude of the dipolar interactions.

In the $P_{\beta'}$ structure the edges of the ramps are regions in which molecular packing defects may be expected. This is consistent with evidence for some disorder in the $P_{\beta'}$ phase obtained by low-angle X-ray diffraction [31]. We suggest that the GM1 molecules at low concentration are localized in these defects, or at least in a class of them, in the *a-b* ramp near the *a* line (Fig. 4). In general impurities are expected to localize in defect regions and in so doing relieve the strain energy and stabilize the global structure [27].

The strong partitioning at low concentration of a class of GM1 molecules into the $P_{\beta'}$ gel phase in preference to the coexisting liquid phase must then be due to the energetically and sterically favorable localization of GM1 in solid phase defects. As the system concentration of GM1 is increased, the defects saturate and the excess GM1 distributes preferentially in the coexisting liquid phase [11]. It is clearly demonstrated by the data in Fig. 2 that the $P_{\beta'}$ preference depends strongly on the structure of the ceramide portion of the molecule. The methylene chain of the acyl group must be longer than the methylene chain of the sphingoid base, and in addition, the acyl chain cannot be motionally restricted by the presence of double bonds.

This dependence on ceramide structure must then reflect the size and shape of the defect free-volume elements that exist or that can be recruited by the ceramide portion of GM1 molecules in the *a* region of the $P_{\beta'}$ phase.

The following considerations lead to the conclusion that each cholera toxin is associated with one GM1 molecule, and hence the size and shape of each individual defect must match that of the ceramide portion of a single GM1 molecule. For each ganglioside system shown in Fig. 2, the total GM1 mole percent is 0.5, but the surface density of cholera toxin in liquid and gel phases is different. However, the sum of the cholera toxin surface density in the coexisting gel and liquid phases is roughly the same for each ganglioside shown in Fig. 2. This can only be the case if the average number of molecules of GM1 associated with each cholera toxin molecule in the gel phase is equal to the corresponding number in the coexisting liquid phase. In an earlier communication we have shown that in the liquid phase each cholera toxin is associated with one GM1 molecule [9]. Therefore, the GM1-cholera toxin stoichiometry in the coexisting $P_{\beta'}$ phase for each ganglioside species must also be 1 to 1. Each defect must then accommodate a single GM1 molecule and the $P_{\beta'}$ preference for specific GM1 species must reflect the size and shape of the defect. It is interesting to note that the GM1 preference for the $P_{\beta'}$ phase is exhibited also by ganglioside molecules added by desorption from their micelles to the outer leaflet of preformed DEPC/DPPC two-phase bilayers [11]. Since the $P_{\beta'}$ structure is a solid phase, this result suggests, but does not prove, that defects of the shape and volume suitable for accommodation of individual GM1 molecules already exist in the $P_{\beta'}$ phase and that the presence of GM1 in the phosphatidylcholine mixture hydrated to form liposomes (the usual method of preparation) is not a necessary condition for the preferential partitioning of GM1. In addition, this observation rules out any requirement for transbilayer interdigitation of methylene chains by ganglioside molecules in opposite faces of the bilayer, because addition of GM1 by desorption from micelles necessarily adds ganglioside only to the outer surface of the external-most bilayer of each liposome.

The localization of GM1 molecules in a distinctive linear pattern associated with the $P_{\beta'}$ ripple structure illustrates a special type of domain formation in membranes. We have previously reported that DPPC bilayers containing less than 10 mol% of various glycosphingolipids take a far longer time than pure DPPC bilayers for ripple disappearance when the bilayers are temperature quenched from the $P_{\beta'}$ gel phase to the solid $L_{\beta'}$ phase [39]. The explanation for this finding almost certainly is that the glycosphingolipids occupy defects in the $P_{\beta'}$ ripple structure, thus stabilizing the ripples that would ordinarily disappear rapidly on lowering the temperature below the pretransition. Cholesterol had a similar stabilizing effect on the ripple structure of the $P_{\beta'}$ phase of DPPC bilayers [39]. In phosphatidylcholine two-phase monolayers formed at an air-water interface, the phase structure can be visualized by fluorescence microscopy if a suitable fluorophore is included. Cholesterol in the concentration range of 1–4 mol% included in a DPPC two-phase monolayer appeared to be localized in the defect regions at the interface between phases [40].

The results of this study show that minor membrane components, such as glycosphingolipids, localize in gel-phase defect regions to form a domain system. In the bilayer structure, the gel-phase defects have a long lifetime. It seems possible, however, that more transient defects in fluid bilayers caused by other membrane components, such as proteins, could be stabilized by minor lipid components to give the same type of domain structure. The domains in this case might be of shorter life-times than in gel phases. The existence of gel phase domains in some prokaryotic organisms has been established [41]. There is at present little evidence for the existence of gel phase

domains in the cell membranes of higher organisms, probably in part because of the high concentrations of cholesterol in most of these membranes [42]. There is, however, considerable evidence to support the existence of microdomains in many eukaryotic cell membranes in which cholesterol and sphingomyelin are important lipid components [2]. Although nothing is known about the origins of this domain structure, the possibility exists that they may be due to phase structure in the lipid bilayer [43]. This possibility is strengthened by the known propensity for cholesterol in conjunction with sphingomyelin to form microdomains [44].

Considerable evidence is accumulating that supports the spatial association of glycosphingolipids and GPI-linked proteins in the sorting machinery and surface membranes of polarized epithelial cells. Membrane complexes isolated from MDCK cells on the basis of their insolubility in Triton X-100 are enriched in glycosphingolipids and GPI-anchored proteins [7]. These membrane complexes also are rich in caveolin, a transmembrane protein associated with caveolae [45], and gangliosides have been shown to preferentially localize to caveolae by ultrastructural labeling techniques [46,47]. FRAP measurements of lateral diffusion and fluorescence resonance energy transfer experiments have demonstrated that a GPI-anchored protein is clustered and immobile after arriving at the apical surface of MDCK cells [48]. Clustering of glycosphingolipids into microdomains in cellular membranes could be an important determinant in the correct sorting of GPI-linked proteins to the apical surface of cells, where the concentration of glycosphingolipids has been shown to be enriched in polarized MDCK cells [6,49]. Since the concentration of the PI anchor of GPI-linked proteins associated with glycosphingolipid gel-like domains must be small due to the large cross-sectional area of the protein moiety relative to that of PI, it may be that the PI portions of the molecules occupy infrequently occurring defects in the gel-like glycosphingolipid domains. These defects would be analogous to the defects occupied by glycosphingolipid in the $P_{\beta'}$ phase, as discussed in this paper and by Rock et al. [39].

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