The Size of Lipid Rafts: An Atomic Force Microscopy Study of Ganglioside GM1 Domains in Sphingomyelin/DOPC/Cholesterol Membranes

Chunbo Yuan, Jennifer Furlong, Pierre Burgos, and Linda J. Johnston
Steacie Institute for Molecular Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

ABSTRACT Atomic force microscopy has been used to study the distribution of ganglioside GM1 in model membranes composed of ternary lipid mixtures that mimic the composition of lipid rafts. The results demonstrate that addition of 1% GM1 to 1:1:1 sphingomyelin/dioleoylphosphatidylcholine/cholesterol monolayers leads to the formation of small ganglioside-rich microdomains (40–100 nm in size) that are localized preferentially in the more ordered sphingomyelin/cholesterol-rich phase. With 5% GM1 some GM1 microdomains are also detected in the dioleoylphosphatidylcholine-rich phase. A similar preferential localization of GM1 in the ordered phase is observed for bilayers with the same ternary lipid mixture in the upper leaflet. The small GM1-rich domains observed in these experiments are similar to the sizes for lipid rafts in natural membranes but considerably smaller than the ordered bilayer domains that have been shown to be enriched in GM1 in recent fluorescence microscopy studies of lipid bilayers. The combined data from a number of studies of model membranes indicate that lateral organization occurs on a variety of length scales and mimics many of the properties of natural membranes.

INTRODUCTION

The role of membrane organization, and in particular the questions of the existence and role of sphingolipid/cholesterol-rich rafts in natural membranes, are currently central issues in membrane structural biology (Brown and London, 1998a, 1998b, 2000; Jacobson and Dietrich, 1999; Jacobson et al., 1995; Simons and Ikonen, 1997, 2000). Although phase separation to give domains with different lipid compositions is well documented in model membranes, it is much more difficult to obtain direct evidence for the existence of lipid domains or rafts in cellular membranes (Jacobson and Dietrich, 1999). The original model for the organization of lipid rafts suggested by Simons and Ikonen proposed that membranes contain microdomains or rafts enriched in sphingolipids (both sphingomyelin and glycosphingolipids) and cholesterol (Simons and Ikonen, 1997). Proteins may be selectively included or excluded from these microdomains, which are postulated to have important roles in membrane transport and signal transduction (Simons and Ikonen, 1997; Simons and Toomre, 2000). Much of the original evidence supporting membrane domains with specialized functions came from the isolation of detergent-resistant membrane fractions (DRMs) that are rich in cholesterol and saturated lipids such as sphingomyelin (Brown and London, 1997). Model membranes with similar compositions to DRMs exist in a liquid-ordered phase that is characterized by tightly packed acyl chains (and hence detergent insolubility) but a high degree of lipid mobility (McMullen and McElhaney, 1996).

Although the affinity of glycolipids and proteins for DRMs has been widely used to provide evidence for membrane rafts, the conditions used for the isolation of DRMs may be responsible for some of the clustering and localization of components (Brown and London, 1997). As a result, a variety of microscopic and spectroscopic studies have attempted to provide more direct evidence for the formation of lipid rafts in plasma membranes. In many cases, the studies have concluded that small microdomains ($<$100 nm) do exist in cell membranes, although their small size is beyond the resolution of conventional optical microscopy, thus presenting considerable limitations to their direct detection (Jacobson and Dietrich, 1999). Many of these studies rely on demonstrating colocalization of specific raft markers such as gangliosides and glycosylphosphatidylinositol (GPI) anchored proteins on the cell surface and frequently use cross-linking experiments with antibodies. For example, some of the most direct evidence for raft formation comes from fluorescence experiments that indicate that the folate receptor, a GPI-anchored protein, is clustered in small domains that are $<$70 nm in size and is colocalized with other GPI-linked proteins (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). Similarly, diffusion of GPI-anchored proteins as small stable rafts (26 nm in size) has also been observed (Pralle et al., 2000). Despite the conclusions of these and related studies demonstrating the colocalization of GPI-anchored proteins and ganglioside GM1 (Harder et al., 1998; Stauffer and Meyer, 1997), another recent study has concluded that rafts are either present as transient structures or comprise a minor component of the cell surface (Kenworthy et al., 2000).

Although there is uncertainty concerning the existence and size of rafts in natural membranes, phase separated

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Address reprint requests to Dr. Linda J. Johnston, Steacie Institute for Molecular Sciences, National Research Council Canada, 100 Sussex Dr., Ottawa, Ontario K1A 0R6, Canada. Tel.: 613-990-0973; Fax: 613-952-0068; E-mail: linda.johnston@nrc.ca.

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domains in bilayer membranes have been directly observed using several microscopic techniques (Bagatolli and Gratton, 2000; Dietrich et al., 2001; Dufrene et al., 1997; Hollars and Dunn, 1998; Korlach et al., 1999; Samsonov et al., 2001). Two recent studies have shown that ganglioside GM1 is enriched in the more ordered domains of phase separated lipid mixtures that mimic raft compositions (Dietrich et al., 2001; Samsonov et al., 2001). The localization of the ganglioside in large (10 μm) domains (Dietrich et al., 2001) is in contrast to the above results in cells and is also inconsistent with the hypothesis that rafts in cell membranes are small and dynamic structures (Jacobson and Dietrich, 1999; Simons and Ikonen, 1997). It has been postulated that the membrane-associated cytoskeleton may play a role in regulating raft size and, thus, account for the apparent discrepancy between the size of lipid microdomains in cell membranes and in model lipid mixtures (Dietrich et al., 2001; Jacobson and Dietrich, 1999). An alternate possibility is that small GM1 microdomains may not be readily detectable with the spatial resolution attainable with fluorescence microscopy. This hypothesis is consistent with atomic force microscopy (AFM) studies of the distribution of GM1 in model monolayers and bilayers. Others and we have recently shown that GM1 is heterogeneously distributed in small microdomains in phosphatidylcholine (PC) and PC/cholesterol monolayers and bilayers under a variety of conditions (Milhiet et al., 2001b; Vie et al., 1998; Yuan and Johnston, 2000, 2001).

AFM is an ideal method for obtaining high-resolution images of supported biological samples under physiological conditions (Bustamante et al., 1993; Dufrene and Lee, 2000; Engel, 1991; Engel et al., 1997; Hoh and Hansma, 1992; Rinia and de Kruijff, 2001). Recent AFM studies have demonstrated the feasibility of carrying out in situ direct visualization of supported bilayers in an aqueous environment and of detecting submicron-sized domains in these model membranes (Dufrene et al., 1997; Giocondi et al., 2000, 2001; Grandbois et al., 1998; Hollars and Dunn, 1998; McKiernan et al., 2000; Mou et al., 1995; Muresan and Lee, 2001; Reviakine et al., 2000; Rinia et al., 1999; Tamm et al., 1996). Supported lipid bilayers provide excellent models for studying membrane assembly and dynamics and have the advantages of being compatible with asymmetric lipid compositions in the two membrane leaflets and retaining much of the fluidity of natural membranes (Boxer, 2000; Sackmann, 1996). We have now used AFM to examine the distribution of GM1 in supported monolayers and bilayers of ternary lipid mixtures that mimic the composition of lipid rafts. Our results indicate that GM1 is heterogeneously distributed in small submicron-sized domains within the more ordered phase of SPM/DOPC/cholesterol monolayers and bilayers.

MATERIALS AND METHODS

Materials

L-α-Dioleoylphosphatidylcholine (DOPC), cholesterol, and L-α-dipalmitoylphosphatidylethanolamine (DPPE) were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-Palmitoyl-D-sphingomyelin (SPM) from bovine brain (97%), monosialoganglioside-GM1 from bovine brain and cholera toxin B subunit were from Sigma (St. Louis, MO). DOPC and cholesterol were dissolved in chloroform (1 mg/mL). SPM, DPPE, and GM1 were dissolved in chloroform/methanol (v/v, 4:1) (1 mg/mL for SPM, DPPE, and 0.5 mg/mL for GM1).

Planar supported membranes

The planar supported monolayers and bilayers were prepared by the Langmuir-Blodgett (LB) or Langmuir-Schaeffer techniques. First, a monolayer of DPPE, SPM/DOPC (1:1), SPM/DOPC/cholesterol (1/1/1), or SPM/DOPC/cholesterol (1/1/1) with small amounts of GM1 was spread on a Langmuir-Blodgett trough (NIMA 611, Coventry, UK) using Milli-Q water as the subphase. After 10-min evaporation of the solvent, the monolayer was compressed at 50 cm^2/min to the required surface pressure. The surface pressure was measured with a precision of 0.1 mN/m using a Wilhelmy balance. The monolayer was annealed twice before transferring to freshly cleaved, hydrophilic mica at a preset surface pressure by vertical deposition at a dipping speed of 5 mm/min. To deposit bilayers, a second monolayer was transferred to DPPE-coated mica (Yuan and Johnston, 2001a). After transferring the first layer of DPPE, the resulting monolayer was dried for 30 min and a new monolayer of SPM/DOPC (1/1), SPM/DOPC/cholesterol (1/1/1), or SPM/DOPC/cholesterol (1/1/1) with different amounts of GM1 was spread on the water surface. This monolayer was also compressed, annealed twice, and then transferred to the DPPE-coated mica at a preset surface pressure either by vertical (5 mm/min) or horizontal deposition. The resulting bilayers were kept under the subphase in a preset small container and then transferred to a larger container full of water. Finally, the supported bilayers were transferred under water to the AFM liquid cell (Molecular Imaging Inc, Phoenix, AZ).

AFM measurements

AFM measurements for monolayer samples were carried out on a multimode nanoscope III atomic force microscope (Digital Instruments, Santa Barbara, CA) in the repulsive mode in air. The J scanner (maximal scan area of 120 μm $\times$ 120 μm) and 200-μm-long soft cantilevers with integrated pyramidal silicon nitride tips (spring constant of 60 mN/m) were used for all measurements. The imaging force was approximately 2 to 4 nN, and the scan rate was typically 1 Hz. AFM measurements for bilayer samples were carried out on a Mac mode Picoscan atomic force microscope (Molecular Imaging Inc.) in the repulsive mode. Silicon nitride tips with a spring constant of 60 mN/m were used. Normally, the imaging force was minimized to $\sim$1 nN, and the scan rate was 1 Hz. The bilayers prepared from LB transfer were imaged in Milli-Q water. Two or three independently prepared monolayers or bilayers were prepared and imaged for each lipid composition, and several areas were scanned for each sample.

RESULTS

Surface pressure-area isotherms

Isotherms for SPM, DOPC, SPM/DOPC (1:1), and SPM/DOPC (1:1) with varying amounts of cholesterol are shown in Fig. 1. Pure DOPC forms a fluid expanded monolayer with a collapse pressure of 45 mN/m, whereas SPM forms a more

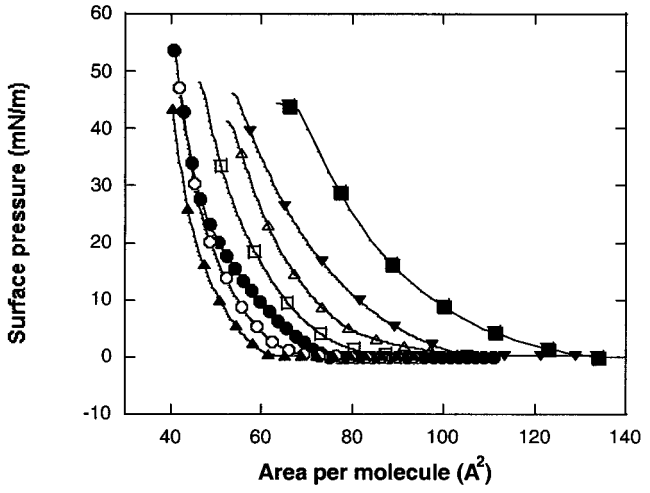

FIGURE 1 Surface pressure-area isotherms for SPM (●), DOPC (■), SPM/DOPC (1:1) (▼), SPM/DOPC (1:1) mixtures with 10% (△), 20% (□), and 33% (○) cholesterol, and a SPM/DOPC/cholesterol (1:1:1) mixture with 5% GM1 (▲).

condensed monolayer with a collapse pressure of 58 mN/m. The SPM isotherm does not show the clear phase transition from the liquid-expanded state to the liquid-condensed state that has been observed for a number of SPMs with saturated acyl chains of varying lengths (Kuikka et al., 2001; Li et al., 2001, 2000). The lack of a distinct phase transition is analogous to previous results for egg and bovine SPM and is indicative of significant acyl chain heterogeneity (Li et al., 2000). The isotherm for SPM/DOPC (1:1) shows a collapse pressure of ~45 mN/m, suggesting that SPM is not miscible with DOPC at this molar ratio. The addition of 10%, 20%, and 33% cholesterol to the SPM/DOPC (1:1) mixture leads to a gradual condensation of the monolayers, similarly to results obtained upon addition of cholesterol to both PC and SPM monolayers (Li et al., 2001; Slotte, 1995a,b; Smaby et al., 1994; Worthman et al., 1997). Addition of 5% GM1 to a SPM/DOPC/cholesterol (1:1:1) mixture slightly condenses the monolayer, probably as a result of electrostatic interactions between the negatively charged sialic acid residue in the polar headgroup of GM1 and the positively charged choline groups of SPM and DOPC.

A range of surface pressures has been used for preparation of the monolayer and bilayer samples studied. A surface pressure between 30 and 35 mN is generally considered appropriate to model a biological membrane (Feng, 1999; Nagle, 1976). However, in some cases, the phase separation behavior of the initial monolayers is more easily understood by comparing both low (10–15 mN) and higher surface pressures.

SPM/DOPC (1:1) monolayers

Monolayers of SPM/DOPC (1:1) were transferred to mica at surface pressures of 10, 15, and 30 mN/m and imaged with AFM (Fig. 2 *a–c*). Phase separation is observed at each surface pressure. At 10 mN, there are a large number of small domains that are several hundred nanometers in size and ~1 nm above the surrounding matrix. The bright (higher) domains are assigned to the SPM phase, whereas the low regions correspond to a DOPC-rich phase; the fraction of the higher phase for monolayers with variable fractions of SPM and DOPC is in agreement with this assignment. The domains cover ~23% of the total surface area for a 1:1 SPM/DOPC monolayer (Fig. 2 *a*), which is significantly lower than would be expected if all the SPM molecules were in the higher condensed phase. This suggests that at a surface pressure of 10 mN/m, some SPM molecules are in the lower DOPC-rich phase. Consistent with this, AFM images of SPM monolayers transferred at 10 mN/m show a mixture of coexisting liquid expanded and liquid condensed phases (Fig. 2 *d*); similar results have been observed recently by epifluorescence of sphingomyelin monolayers at the air water interface (Kuikka et al., 2001). By contrast, AFM images of pure DOPC monolayers show only flat, featureless monolayers for a range of surface pressures (figures not shown). SPM/DOPC monolayers transferred at 15 mN show a mixture of small and large (3–5 μm) domains of the higher phase, whereas at 30 mN the larger domains predominate and have further increased in size (Fig. 2, *b* and *c*). The height difference between the two phases is ~0.5 nm. At 30 mN/m the higher phase accounts for ~45% of the total area, which is close to what would be expected based on the area/molecule if all the SPM was in the higher condensed phase. These results for 1:1 DOPC/SPM monolayers are in agreement with a recent AFM study of monolayers with a range of PC/SPM ratios (Milhiet et al., 2001a).

SPM/DOPC/cholesterol monolayers

Varying amounts of cholesterol were added to equimolar binary SPM/DOPC mixtures, and monolayer samples were deposited at low surface pressure to study the initial stages of phase separation (Yuan and Johnston, 2002). Large scale AFM images of 1:1 SPM/DOPC monolayers transferred at 7 mN/m were flat but numerous small islands of a higher phase were evident in small scale images (Fig. 3, *a* and *inset*). This indicates that the start of the liquid-expanded to liquid-condensed phase transition occurs at ~7 mN/m. Addition of 10% cholesterol to the SPM/DOPC (1:1) mixture (Fig. 3, *b* and *inset*), interestingly, did not alter the monolayer very much other than eliminating the small dots seen in Fig. 3 *a*. However, addition of 20% cholesterol changed the monolayer significantly (Fig. 3 *c*). Clear phase separation is observed with the formation of both large and small round domains that are assigned to an SPM/cholesterol-rich liquid-ordered phase (Dietrich et al., 2001). The size and number of the large domains increase for a monolayer containing 33% cholesterol, and there are still a few small

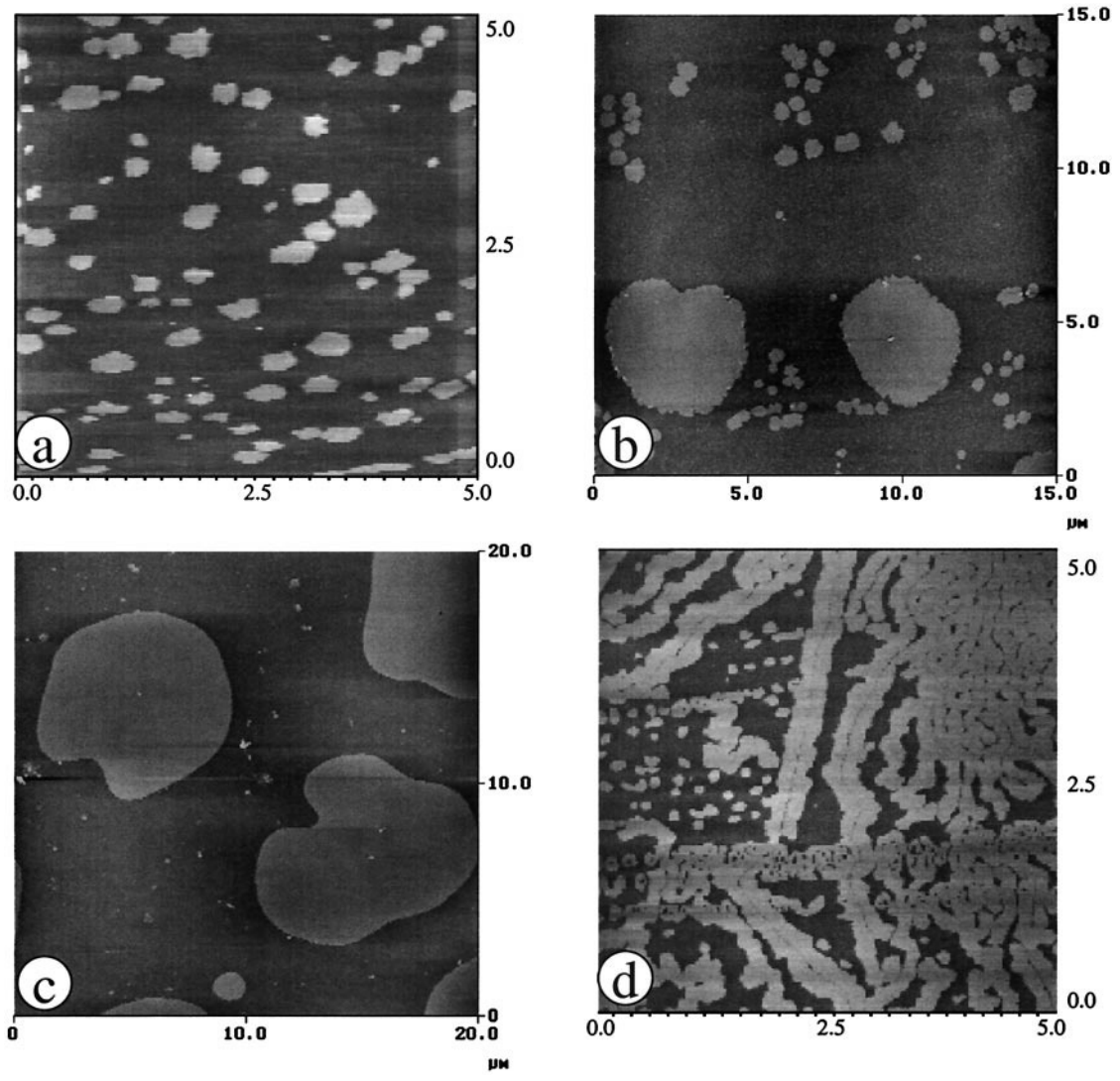

FIGURE 2 AFM images for SPM/DOPC (1:1) monolayers at a surface pressure of 10 mN/m (*a*), 15 mN/m (*b*), and 30 mN/m (*c*) and a SPM monolayer deposited at a surface pressure of 10 mN/m (*d*). The *z* scale is 5 nm for images *a* and *d* and 10 nm for *b* and *c*.

islands of the higher phase (Fig. 3 *d*); a modest increase in pressure gives a significant increase in the amount of the higher phase with many of the smaller domains now having coalesced into larger ones (10 mN/m; Fig. 3 *e*). The height difference between the two phases is ~1 nm. Monolayers deposited at a surface pressure of 30 mN/m still showed clear phase separation, although now the higher phase predominates and there are only occasional micron-sized domains of the lower phase (Fig. 3 *f*). Further increases in pressure give uniformly flat monolayers. This is in contrast to SPM/DOPC mixtures with 10% and 20% cholesterol where phase separation remained at 40 mN/m (figures not shown). Similar variations in the phase separation behavior of monolayers of ternary lipid mixtures have been reported recently; in this work the domain morphology also changed significantly with changes in cholesterol concentration (Milhiet et al., 2001a).

Addition of GM1 to SPM/DOPC/cholesterol monolayers

Monolayers of SPM/DOPC/cholesterol (1:1:1) containing 1% and 5% GM1 were deposited at low and high surface pressures and imaged with AFM (see Fig. 4). At low surface pressures, large round or elliptical domains that are similar to those observed for the ternary lipid monolayers in the absence of ganglioside were still detected (see Fig. 4, *a* and *c* for 1% and 5% GM1). The large domains (high phase), however, were no longer homogeneous. In the presence of 1% GM1, there are numerous bright dots that are randomly distributed in the higher phase (Fig. 4 *b*). The dots range from 40 to 150 nm in diameter, are approximately 1.0 nm higher than the surrounding phase (see the section analysis in Fig. 4 *b*), and are localized predominantly in the higher phase. The distance between the small microdomains of the

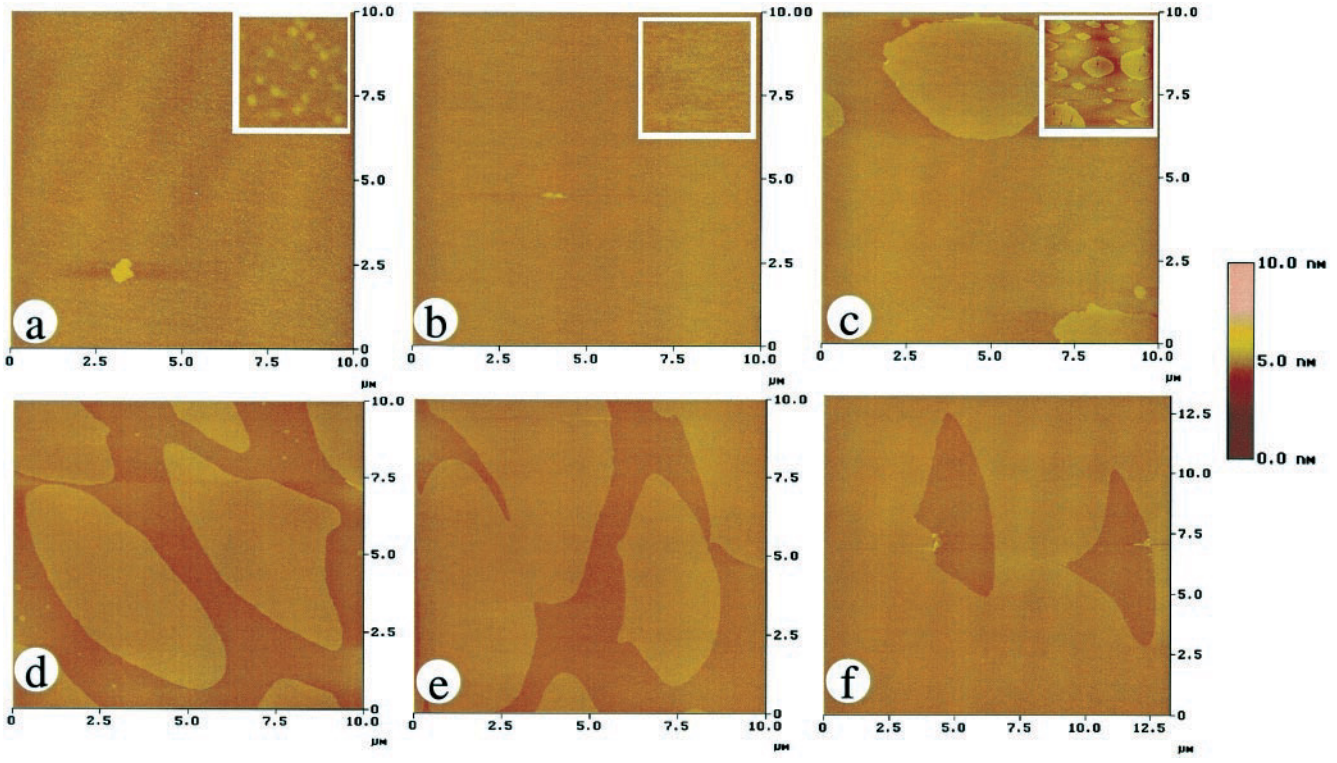

FIGURE 3 AFM images for SPM/DOPC and SPM/DOPC/cholesterol monolayers. (*a*) SPM/DOPC (1:1) monolayer deposited at 7 mN/m. The inset is a small-scale image (500 × 500 nm^2). (*b*) SPM/DOPC (1:1) monolayer with 10% cholesterol transferred at 7 mN/m. The inset is a small-scale image (500 × 500 nm^2). (*c*) SPM/DOPC (1:1) monolayer with 20% cholesterol at 7 mN/m. The inset is a large-scale image (40 × 40 μm^2). (*d*) SPM/DOPC (1:1) monolayer with 33% cholesterol at 7 mN/m. (*e*) SPM/DOPC (1:1) monolayer with 33% cholesterol at 10 mN/m. (*f*) SPM/DOPC (1:1) monolayer with 33% cholesterol at 30 mN/m. The *z* scale is 10 nm for all images (see color bar) except image *a*, which is 5 nm.

higher phase varies between 100 to 400 nm. Upon addition of 5% GM1 to the ternary lipid mixture, most of the bright dots coalesce to form a complex network of filaments (Fig. 4 *d*), although a few residual dots remain in some areas. The filaments are ~100 nm in width and ~1.3 nm above the high phase (inset of Fig. 4 *d*). At the higher GM1 concentration a significant number of small dots are also evident in the lower DOPC phase.

Monolayers of the ternary mixture with 1% GM1 were also deposited at high surface pressure (30 mN/m) and imaged with AFM (Fig. 4 *e*). The large condensed domains show many small dots of a higher phase that are similar in size and distribution to those found at low surface pressure. At higher surface pressures (40 mN/m) the large condensed domains are no longer observed, consistent with the results in the absence of GM1; rather there is a single phase that contains a large number of small islands, ranging in size from 40 to 100 nm in diameter with heights that vary between 0.5 to 1.2 nm (see the section analysis in Fig. 4 *f* for a sample with 5% GM1). The small microdomains and filaments that appear in the presence of ganglioside are assigned to a GM1-rich phase, by analogy with results in other monolayers (Yuan and Johnston, 2000; Vie et al., 1998).

Hybrid bilayers of SPM/DOPC/cholesterol (1:1:1) mixtures with and without GM1

Monolayers of ternary lipid mixtures with or without GM1 were transferred to DPPE-coated mica (Yuan and Johnston, 2001) to prepare hybrid bilayers via either Langmuir-Blodgett transfer at 30 to 35 mN/m or Langmuir-Schaeffer transfer at 10 mN/m. AFM images of these bilayers are shown in Fig. 5. At both surface pressures, AFM images showed that the ternary mixture without GM1 formed reasonably flat bilayers (Fig. 5, *a* and *b*). However, the bilayer at high surface pressure was more uniform and tightly packed with fewer pinhole defects, whereas the bilayer at low surface pressure was more heterogeneous with some tightly packed areas (marked with arrows in Fig. 5 *a*) similar to those seen at high surface pressure. Most areas of the bilayer were loosely packed with many pinholes. It is interesting to compare Fig. 5, *a* and *b*, with the monolayer images shown in Figs. 3 and 4. Two phases that differ in height by ~1 nm are very clearly evident in the monolayers for a range of pressures and cholesterol concentrations; by contrast, no height difference can be detected in the bilayer, although there are both tightly packed regions of the bilayer and areas that are loosely packed with many pinholes.

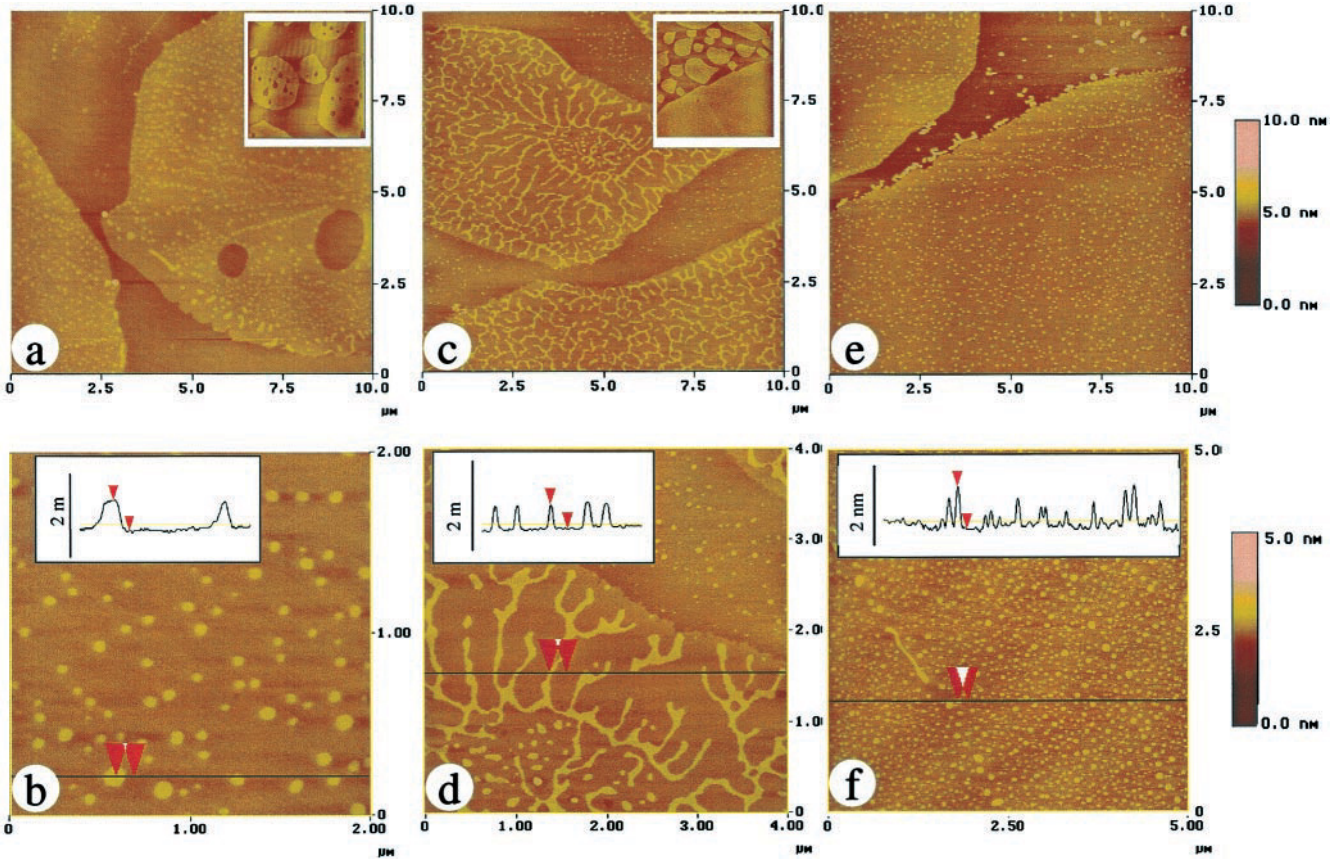

FIGURE 4 AFM images for monolayers of SPM/DOPC/cholesterol (1:1:1) with 1% GM1 (*a*, *b* at 10 mN/m, and *e* at 30 mN/m) and 5% GM1 (*c*, *d* at 10 mN/m, and *f* at 40 mN/m). The insets in *a* and *c* are large-scale images (40 × 40 μm^2) for the same sample. The *z* scale (see color bar) is 5 nm for *b*, *d*, and *f* and 10 nm for *a*, *c*, and *e*. Section analyses (for the solid line on the images) are shown as insets in *b*, *d*, and *f*; the *x* scale for the inset is the same as that for the image.

Incorporation of 1% GM1 in a SPM/DOPC/cholesterol bilayer deposited at 10 mN/m gave a heterogeneous sample with many small irregularly shaped microdomains that are ~2 nm (Fig. 5 *c*) higher than the surrounding bilayer. The small GM1-rich microdomains do not cover the entire bilayer surface but are confined to large circular domains, one of which is outlined in black in Fig. 5 *c*. The observation of these large domains is similar to the monolayer results, except that there is no apparent height difference between the large domains in which the small microdomains are localized and the surrounding uniform bilayer matrix. The microdomains range in size from 100 to 400 nm and are larger and more irregularly shaped than those observed for similar monolayers. The distance between microdomains is also more variable.

The results for a bilayer containing 5% GM1 in an upper SPM/DOPC/cholesterol leaflet deposited at 10 mN were similar in that there were some areas of the bilayer that contained a heterogeneous distribution of a higher phase surrounded by uniform bilayer (Fig. 5 *d*). In this case both the small microdomains and the domains in which they are localized are larger than for a monolayer containing 1% GM1.

Hybrid SPM/DOPC/cholesterol bilayers containing GM1 and transferred at higher surface pressures are also shown in Fig. 5, *e* and *f*. The image shown in Fig. 5 *e* for 5% GM1 (30 mN/m) still shows some regions of bilayer that do not contain small GM1-rich microdomains (e.g., top right corner of the image). However, the image in Fig. 5 *f* for 5% GM1 transferred at 35 mN/m shows only a random distribution of small islands of a higher phase; the islands are 200 to 300 nm in diameter and ~2 nm above the bilayer matrix. The differences in the apparent heights measured for GM1-rich aggregates in monolayers and bilayers are related to variations in electrostatic interactions between tip and sample as a function of bilayer composition, as discussed in detail in an earlier paper (Yuan and Johnston, 2001).

The height differences and the fact that the microdomains only appear in the presence of GM1 make it likely that these are ganglioside-rich aggregates. We have also used selective binding of cholera toxin B subunit to the bilayers to confirm this assignment, as in previous work for PC and PC/choles-

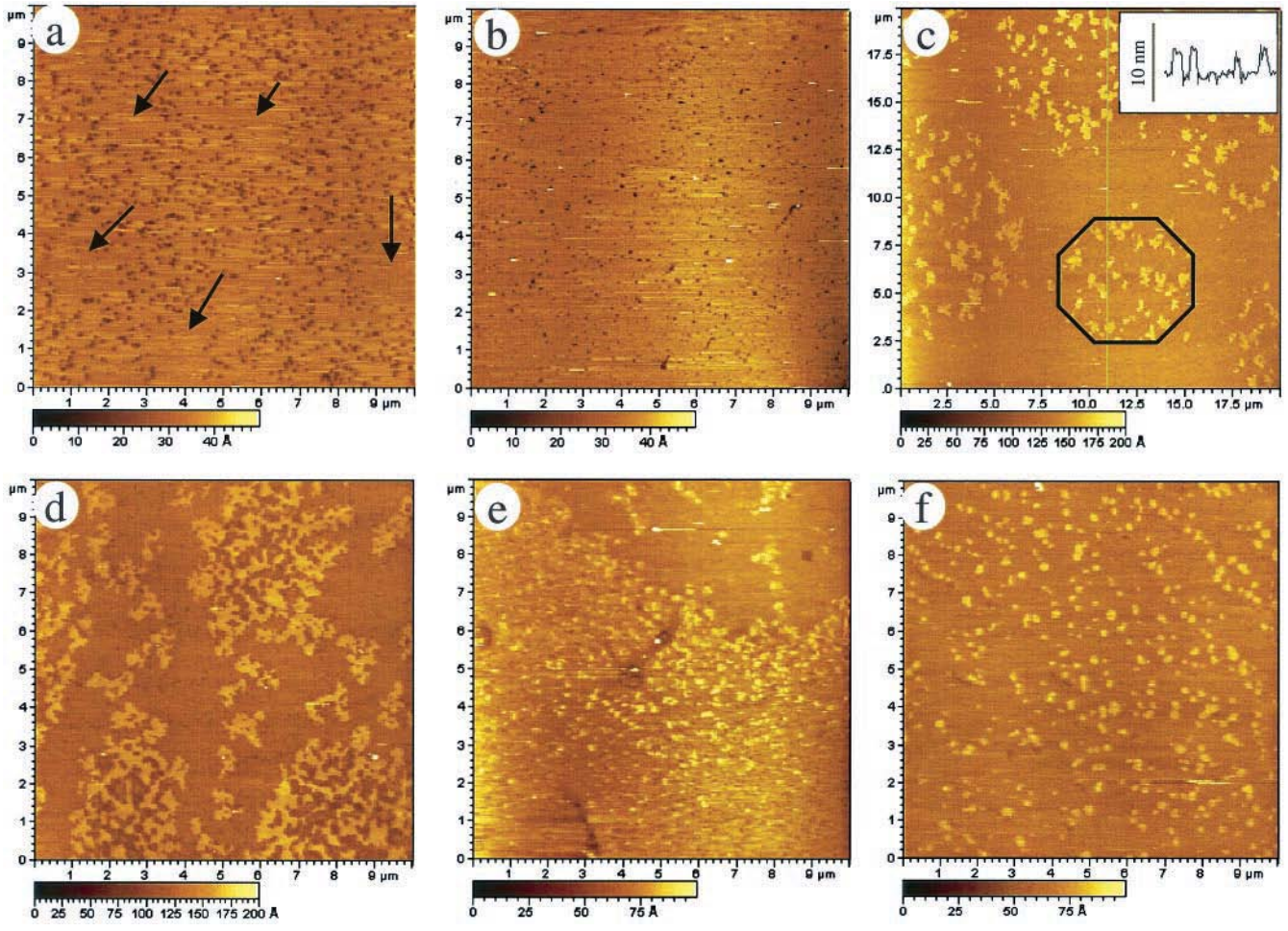

FIGURE 5 AFM images for hybrid bilayers of SPM/DOPC/cholesterol (1:1:1) with and without 5% GM1. (*a* and *b*) SPM/DOPC/cholesterol (1:1:1) bilayers deposited at 10 mN/m (*a*) and 35 mN/m (*b*). The arrows indicate more tightly packed areas of the bilayer. (*c*) SPM/DOPC/cholesterol (1:1:1) bilayer with 1% GM1 deposited at 10 mN/m with one of the large domains containing small GM1-rich microdomains outlined in black; the *x* scale for the section analysis shown in the insert is the same as the image. (*d–f*) SPM/DOPC/cholesterol (1:1:1) bilayers with 5% GM1 deposited at 10 mN/m (*d*), 30 mN/m (*e*), and 35 mN/m (*f*).

terol bilayers (Yuan and Johnston, 2001). Imaging the protein on bilayers of ternary lipid mixtures proved considerably more problematic than in one and two components bilayers. However, there is clear evidence for protein domains that are 6 to 8 nm in height and are heterogeneously distributed in a similar fashion to the initial GM1-rich microdomains for SPM/DOPC/cholesterol bilayers containing 1% and 5% GM1. Note that it is difficult to image exactly the same area after protein addition and there is considerable variability of domain size and shape within a single sample. Despite this, it appears that the protein aggregates are somewhat larger than the initial GM1-microdomains, a result that has also been observed with PC and PC/cholesterol bilayers.

DISCUSSION

A number of studies have demonstrated that model membranes of ternary lipid mixtures similar to those found in natural raft-containing membranes show phase separation to give a condensed SPM/cholesterol-rich phase and an expanded PC-rich phase (Dietrich et al., 2001; Samsonov et al., 2001; Rinia et al., 2001). The present study has focused on the distribution of GM1 in such phase separated mixtures in an attempt to resolve the inconsistencies between postulated raft sizes in natural and synthetic membranes. Our results clearly demonstrate that incorporation of 1% GM1 in ternary SPM/DOPC/cholesterol mixtures leads to localization of the ganglioside in small microdomains that are distributed throughout the SPM/cholesterol-rich ordered phase. Small GM1-rich islands that range in size from 40 to 150 nm and are ~1 nm higher than the ordered phase are observed in the condensed phase of monolayer samples deposited at both low and high surface pressures. Similarly, a heterogeneous distribution of GM1 is observed in bilayers with the same ternary lipid mixture in the outer leaflet. Note that phase separation between the ordered SPM/cholesterol-

rich and the disordered DOPC-rich phases in the bilayer is difficult to detect in the absence of GM1 because there is little height difference between the two phases; however, the domains are visible as differences in the packing density of the upper leaflet. Upon addition of 1% GM1 there are clearly large domains in which small ganglioside aggregates are preferentially localized as well as areas of uniformly flat bilayer. These presumably correspond to the same SPM/cholesterol-rich and DOPC-rich phases that are observed in the corresponding monolayers. Other recent results for cholesterol containing PC/SPM bilayers made by vesicle fusion indicate that apparent height differences between the two phases decrease significantly as more cholesterol is added (Rinia et al., 2001). These results also demonstrated that the lipid composition in the two leaflets is coupled for bilayers made by vesicle fusion. The height differences would be expected to be smaller in hybrid bilayers as the ternary lipid mixture is only in the upper leaflet as is consistent with our inability to detect the two phases before addition of GM1.

The distribution of GM1 in both monolayers and bilayers is also heterogeneous for samples with 5% ganglioside. However, there are two important differences for monolayer samples with higher GM1 concentrations. First, the images show a network of filaments of a GM1-rich phase, which appears to form by coalescence of the small islands observed at lower concentrations. Both the small islands (1%) and the network of filaments (5%) are analogous to results obtained earlier for GM1 in PC/cholesterol monolayers (Yuan and Johnston, 2000). The second point of interest is the detection of a significant number of GM1 islands in the less ordered (lower) DOPC-rich phase (Fig. 4 *d*). Bilayers containing 5% GM1 have small round GM1-rich domains that are localized in larger domains at low surface pressure, whereas at higher pressure the GM1 microdomains appear to be randomly distributed throughout the entire sample. The latter result is consistent either with a lack of phase separation into cholesterol/SPM-rich and DOPC-rich phases under these conditions or with little preference of GM1 for either of the two phases. The monolayer images at various pressures and ganglioside loadings do not allow one to distinguish between these possibilities since both surface pressure and GM1 concentration affect the localization of the ganglioside. Clearly there is a delicate balance in partitioning of GM1 between the two phases with both higher pressure and concentration increasing the amount of ganglioside in the DOPC-rich phase. Interestingly, one can still distinguish areas of bilayer that do not contain GM1-rich microdomains in samples with 5% GM1.

As noted above the present results are analogous to earlier studies that showed similar small GM1-rich domains in PC and PC/cholesterol monolayers and bilayers (Milhiet et al., 2001b; Vie et al., 1998; Yuan and Johnston, 2000, 2001). In fact the potential for glycosphingolipids to form domains has been known for some time (Thompson et al., 1985). However, the present results differ from our earlier work in PC/cholesterol membranes in which a random distribution of GM1-rich microdomains in a single homogeneous lipid matrix was observed (Yuan and Johnston, 2001). We have now shown conclusively that GM1 is heterogeneously distributed in small submicron-sized islands within the larger domains of the condensed SPM/cholesterol-rich phase. The preferential localization of GM1 in the ordered phase of ternary lipid mixtures was observed previously by fluorescence microscopy (Dietrich et al., 2001; Samsonov et al., 2001), although these results did not provide any evidence for phase separation within the ordered phase as we have observed in the AFM studies. There are a number of possible explanations for the differences between the results in model membranes. For example, it is possible that differences in the method of sample preparation or composition for the supported lipid bilayers lead to changes in the GM1 distribution. Alternately, in at least some cases the size and distribution of the small GM1 domains may make it difficult to detect them by conventional fluorescence microscopy. In this regard it is worth noting that the fluorescence studies require labeling the ganglioside with cholera toxin, which under our conditions appears to lead to larger domains. One must also consider the possibility that the distribution of GM1 reported by fluorescence and AFM is different; for example, the relative sensitivity of the two methods to aggregates and individual ganglioside molecules may not be the same. Regardless of the explanation for the differences between our results and those of Dietrich et al., it is clear that small GM1-rich raft-like islands are found within larger liquid-ordered domains, under at least some conditions. A combination of AFM and near-field fluorescence microscopy measurements for the same samples is in progress to investigate further the relationship between the size and distribution of GM1 domains in a variety of monolayers and bilayers.

The distribution of GM1 in ternary SPM/cholesterol/DMPC monolayers has also recently been studied by epifluorescence using both dye-labeled lipids and cholera toxin to visualize domains (Radhakrishnan et al., 2000). These experiments provide evidence for three separate phases that are identified as a phospholipid-rich phase, a cholesterol-rich phase, and a condensed phase containing cholesterol/phospholipid complexes (Radhakrishnan et al., 2001; Radhakrishnan and McConnell, 1999a,b). The ganglioside is found exclusively in the complex-rich phase, which is favored by lipid compositions similar to those reported for lipid rafts (Radhakrishnan et al., 2000). These results are somewhat analogous to those presented herein in that the GM1 is heterogeneously distributed within specific domains of a phase-separated monolayer, although our results do not provide any evidence for prior existence of a third complex-rich phase before GM1 addition. A number of studies have examined the basis for the strong interaction between sphingomyelins and cholesterol (Kuikka et al., 2001; Li et al., 2001; Radhakrishnan et al., 2001; Smaby et al., 1994). The

increased interaction of cholesterol with sphingomeylin as compared with PCs is not simply due to the preponderance of saturated chains in SPMs but also involves the increased amount of hydrogen bonding due to the presence of both hydrogen bond donors and acceptors in the sphingolipid (Li et al., 2001).

Implications for natural membranes

The size of the GM1-microdomains observed in our studies is comparable with estimates for the size of lipid rafts in natural membranes (Jacobson and Dietrich, 1999). Many of these studies have examined the localization of glycolipids or GPI-linked proteins, suggesting that the estimated size of the "rafts" reflects the organization of these species within a larger liquid-ordered SPM/cholesterol-rich phase. In this context it is interesting to note that the putative functional role of lipid rafts seems to require them to be small dynamic platforms, similar to what is observed here. The role of glycolipid aggregation in determining raft size and function also nicely accommodates the problem of understanding the size, location, and stability of such small domains in plasma membranes that contain relatively large amounts of both SPM and cholesterol. Previous work had suggested that the cytoskeleton might play a significant role in regulating raft size in natural membranes (Dietrich et al., 2001; Jacobson and Dietrich, 1999). Whereas the present results do not preclude this, they do indicate that glycolipid aggregation may in fact be sufficient to account for many of the observed phenomena. For example, glycolipids have been shown to be enriched in isolated signaling domains (Iwabuchi et al., 1998; Prinetti et al., 1999) and are also the natural receptors for a variety of membrane proteins, leading to lipid-protein complexes that are key intermediates in signal transduction across membranes (Harder and Simons, 1999). The small size of these domains is important in providing the necessary mobility for their involvement in a range of dynamic membrane processes. It is obviously important to demonstrate the generality of the results on GM1 microdomains to other glycolipids, such as glycosylphosphatidylinositol. In this context it is noteworthy that other modified lipids also form small aggregates within a bilayer matrix (Cuccia and Johnston, unpublished results).

It is now clear from a combination of results for model membranes that lipid properties alone are sufficient to direct the formation of complex lateral organization in membranes. This organization can occur on a variety of length scales (small glycolipid aggregates versus large liquid ordered phases) and does not require the presence of proteins, although they may also affect the lateral membrane organization. There are still many unresolved questions concerning the existence and role of lipid microdomains/rafts in natural membranes. However, the observation of small GM1-rich rafts in model membranes provides important insight on the ability of glycolipids to organize within a liquid-ordered phase. The factors that trigger organization in cellular membranes and recruit the proteins that determine the function of a particular microdomain remain to be established.

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