On the Origin of Sphingolipid/Cholesterol-Rich Detergent-Insoluble Cell Membranes: Physiological Concentrations of Cholesterol and Sphingolipid Induce Formation of a Detergent-Insoluble, Liquid-Ordered Lipid Phase in Model Membranes

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ABSTRACT: Detergent-insoluble membrane fragments that are rich in sphingolipid and cholesterol can be isolated from both cell lysates and model membranes. We have proposed that these arise from membranes that are in the liquid-ordered phase both *in vivo* and *in vitro* [Schroeder et al. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12130−12134]. In order to detect formation of the liquid-ordered phase while avoiding possible detergent artifacts, we have now used fluorescence quenching to examine the phase behavior of mixtures of phosphatidylcholines, sphingolipids, and cholesterol. Phase separation was found in binary mixtures of either dipalmitoylphosphatidylcholine (DPPC) or sphingomyelin (SM) and a nitroxide-labeled phosphatidylcholine (12SLPC). A DPPC- or SM-enriched solidlike gel phase coexisted with a 12SLPC-enriched liquid-disordered fluid phase at 23 °C. As expected, phase separation was not seen at low concentrations of DPPC or SM. Instead, only a uniform fluid phase was present. Including 33 mol % cholesterol in model membranes greatly promoted phase separation. Phase separation was seen at higher temperatures and/or at lower concentrations of DPPC or SM in the presence of cholesterol than in its absence. Mixtures of DPPC or SM and cholesterol are known to form the liquid-ordered phase. Therefore, the fact that phase separation was observed in the cholesterol-containing membranes shows that liquid-ordered and liquid-disordered phase domains coexist. At 37 °C, the SM-enriched liquid-ordered phase was first seen at a SM/PC ratio of close to 0.25, when SM made up 17% of the total lipid including cholesterol. (This is similar to or less than the SM concentration of the plasma membranes of mammalian cells.) Furthermore, the detergent insolubility of cholesterol-containing model membranes correlated well with the amount of liquid-ordered phase as detected by fluorescence quenching. Thus, the detergent-insoluble membranes isolated from cells are likely to exist in the liquid-ordered phase prior to detergent extraction. The promotion of liquid-ordered phase formation may be an important function of cholesterol and sphingolipids in cells and may be a major distinction between the cholesterol- and sphingolipid-rich plasma membrane and most other cellular membranes.

According to the simplest version of the fluid-mosaic model of biological membrane structure, bilayer lipids form a uniform and homogeneous fluid mixture. Strong evidence has now accumulated, however, that specialized lipid domains can exist in membranes. Sphingolipids are attractive candidates for lipids that might form such domains. Sphingolipids, especially glycosphingolipids, have much higher acyl chain melting temperatures (T_m) than phospholipids and can form separate gel-phase domains when mixed with phospholipids (Thompson & Tillack, 1985; Johnson & Chapman, 1988). Some evidence suggests that glycosphingolipids can also self-associate in plasma membranes (Thompson & Tillack, 1985). However, except in special cases such as myelin (Ruocco & Shipley, 1984), direct evidence for the existence of gel-phase domains in mammalian membranes is lacking.

Yu et al. (1973) showed that sphingolipids are more resistant to detergent extraction from erythrocyte membranes than phospholipids and speculated that this might reflect phase separation. We showed that detergent-insoluble membranes (DRMs),[1] with a distinct bilayer appearance and a unique protein composition, can be isolated from epithelial cell lysates (Brown & Rose, 1992). DRMs are rich in cholesterol as well as sphingolipids. Further work by several groups has shown that DRMs can be isolated from a number of eukaryotic cell types.

A special subset of membrane proteins is present in DRMs. The first proteins to be identified in DRMs were glycosylphosphatidylinositol-(GPI-) anchored proteins (Brown & Rose, 1992). Since then a number of other proteins have been identified in DRMs (Cinek & Horejsi, 1992; Sargia-

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[1] Abbreviations: CB, bovine cerebrosides; DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; DRM, detergent-resistant membrane; L_d, liquid-disordered phase; L_o, liquid-ordered phase; PC, phosphatidylcholine; 12SLPC, 1-palmitoyl-2-(12−doxyl)stearoylphosphatidylcholine; SM, sphingomyelin; Tempo PC, 1,2-dioleoylphosphatidyl[4-[*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium]-2,2,6,6,-tetramethylpiperidine-1-oxyl].

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como et al, 1993; Cerneus et al., 1993; Fiedler et al. 1993; Anderson, 1993; Draberova & Draber, 1993; Arreaza et al., 1994; Lisanti et al., 1994a,b; Chang et al., 1994; Schnitzer et al., 1995; Hanada et al., 1995; Gorodinsky & Harris, 1995; Liu & Anderson, 1995; Pike & Casey, 1996; Kubler et al., 1996; Field et al., 1995; Garcia-Cardena et al., 1996). Many of these are involved in signal transduction, suggesting that the concentration of interacting proteins and lipids into small, confined regions of the bilayer is an important function of these domains. The membrane of caveolae (plasma membrane invaginations implicated in signal transduction, lipid trafficking, and transcytosis) is also detergent-insoluble (Sargiacomo et al., 1993, Chang et al., 1994; Lisanti et al., 1994a,b; Anderson, 1993; Schnitzer et al., 1995).

As key physiological roles for DRMs are emerging (see Discussion), it is important to determine their structure and physical origin. On the basis of our studies of cholesterol- and sphingolipid-rich model membranes, we recently proposed that DRMs exist in cells as domains different than the fluid liquid-disordered (L_d, L_α) phase that makes up most cellular membranes (Schroeder et al., 1994). This is not the solidlike gel (L_β) phase, however, but rather is a phase similar or identical to the liquid-ordered (L_o) phase.

The L_o phase was first identified in binary mixtures of saturated phosphatidylcholines and cholesterol (Ipsen et al., 1987, 1990; Vist & Davis, 1990; Sankaram & Thompson, 1990; Mateo et al., 1995). Mixtures of sphingomyelin and cholesterol also form the L_o phase (Sankaram & Thompson, 1990). The L_o phase has properties that are intermediate between those of the gel and fluid phases. Like the gel phase, the L_o phase is characterized by tight acyl-chain packing and relatively extended acyl chains. This probably explains why this phase has been detected in mixtures of cholesterol and phospholipids with saturated (or largely saturated, in the case of sphingomyelin) acyl chains (Ipsen et al., 1987, 1990; Vist & Davis, 1990; Sankaram & Thompson, 1990; Mateo et al., 1995). We found that L_o phase bilayers, like those in the gel phase (Ribiero & Dennis, 1973) are Triton X-100-insoluble (Schroeder et al., 1994). On the other hand, like lipids in the L_d phase, lipids in the L_o phase exhibit relatively rapid lateral mobility within the bilayer (Almeida et al., 1992).

Several pieces of evidence support our model that DRMs are present in an L_o-like phase. Both the lipid composition (rich in cholesterol and sphingolipids) and physical behavior (detergent insolubility) of DRMs suggest a close relationship with the L_o phase. We found that model membranes with a lipid composition similar to that of cellular DRMs were insoluble in Triton X-100, as was a purified GPI-anchored protein inserted into these membranes (Schroeder et al., 1994). We also found that lipids in DRM-like model membranes have a motional state close to that of the L_o phase, as judged by fluorescence polarization (Schroeder et al., 1994). These results strongly suggest that lipids themselves determine the detergent insolubility of both the lipids and proteins in DRMs.

So far, detergent extraction has been used to isolate DRMs from cells. Although our results strongly suggest that insoluble lipids exist in L_o-like domains in cells, it has been possible that detergent might introduce artifacts into the analysis. We were concerned that detergent could alter the lipid composition of the domains or even induce their formation from previously uniform lipid mixtures. For this reason, it is important to use other, non-detergent-based techniques to detect formation of an L_o-like phase in complex lipid mixtures. In this report, a fluorescence quenching assay that we developed previously (London & Feigenson, 1981) is used to detect phase separations in the presence of cholesterol. This assay has been used earlier to detect a variety of phase separations (Huang et al., 1988; Florine-Casteel & Feigenson, 1990; Spink et al., 1990; Silvius, 1990, 1992). In this study, the quenching assay is used to demonstrate that L_o phase formation occurs in membranes with a lipid composition similar to that of the plasma membrane. In addition, it is shown that formation of an L_o-like phase correlates well with detergent insolubility. These results strongly suggest that L_o-like phase domains are likely to be present in plasma membranes. Induction of L_o phase formation by cholesterol and sphingolipids may be one of the more important roles of these lipids in cell membrane function.

EXPERIMENTAL PROCEDURES

Materials. Phospholipids, porcine brain sphingomyelin (SM), porcine brain cerebrosides (CB), TempoPC, and 12SLPC were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid concentrations were confirmed by dry weights. Diphenylhexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). All the chemicals used are reagent grade. The purity of the lipids was verified by thin-layer chromatography (Macala et al., 1983; Chattopadhyay & London, 1987). All the lipids gave a single spot at a loading of 4−7 μg with the exception of brain CB, which gave a double spot because it is a mixture of hydroxylated and nonhydroxylated species. The manufacturer's analysis showed the sphingolipids have predominantly saturated acyl chains, with the remainder being very long singly unsaturated acyl chains. Lipids and DPH were dissolved in ethanol and stored at −20 °C before use.

Vesicle Preparation. Multilamellar vesicles (MLV) were prepared as follows. To the desired mixture of phospholipid, sphingolipid, and/or cholesterol, a constant amount of DPH (0.5 mol %) was added. The samples with cholesterol had a (phospholipid + sphingolipid):cholesterol composition of 2:1 mol/mol. Lipid and DPH were dried under N_2, dissolved in $CHCl_3$, redried under N_2, and further dried under high vacuum for 1 h. MLVs were prepared by vigorously mixing (vortexing) the dried samples in PBS buffer (10 mM sodium phosphate and 150 mM NaCl, pH 7), for 60 s at room temperature (23 °C). Test tubes containing the MLV samples to be prepared at elevated temperatures were hydrated in PBS buffer warmed to the desired temperature. The tubes were then placed in a small beaker containing water warmed to the appropriate temperature and vortexed for 15 s. The samples were then incubated for 5 min at the elevated temperature and finally vortexed more vigorously for 30 s (which required brief exposure to 23 °C) to ensure more uniform dispersion of the vesicles. The samples were returned to the elevated temperature bath and incubated in the dark for a minimum of 40 min prior to fluorescence or light scattering experiments. For fluorescence quenching experiments the total lipid concentration (including cholesterol) in each sample was 50 μM, and for light scattering the total lipid concentration in each sample was 150 μM.

SM/12SLPC samples were prepared at 65 °C; CB/SM/12SLPC at 81 °C; DPPC/12SLPC and DPPC/12SLPC/DOPC at 50 °C; and DOPC/12SLPC at 23 °C; and then the samples were incubated at these temperatures for 40 min−3 h. These temperatures were chosen to exceed the T_m of the lipid in the sample with the highest T_m. Fluorescence measurements of each preparation were made at up to three different temperatures. In some experiments, fluorescence was measured at 23 °C and then at up to two higher temperatures. In other experiments, samples were measured at their preparation temperature and then at up to two lower temperatures. When changing temperature, the samples were allowed to incubate at least 1 h (and up to 3 h for samples cooled to 23 °C) before measurement of fluorescence. The thermal history had no significant effect on the results obtained, and the results of measurements made at the same temperature were averaged.

Samples with cholesterol were prepared either at or higher than the final experimental temperature (usually at 81 °C) and were then incubated before reading fluorescence for as long as the samples without cholesterol. In general, differences in thermal history did not affect the results significantly. However, protocols involving measuring fluorescence after reheating cholesterol-containing samples were avoided, because of the hysteresis sometimes observed. In these cases, phase separation that occurred at low temperature was not abolished upon subsequent incubation at higher temperatures.

Fluorescence Measurements. The fluorescence experiments were performed on a Spex 212 Fluorolog spectrophotometer. Fluorescence was measured in a 1 cm excitation, 4 mm emission path length quartz cuvette. Excitation and emission slits of 1.25 mm were used. The temperature in the cuvette was regulated by a Neslab RTE 100 temperature bath and verified with an electronic thermometer. After the sample was placed in the fluorometer, it was incubated in the dark for 1 min at the appropriate temperature and then fluorescence was read over a 3 s interval, recording in the ratio mode. The excitation/emission wavelength settings were 358 and 430 nm, respectively.

For each experiment duplicate samples (triplicate for samples lacking quencher) were prepared. The results shown are the average of 1−7 such experiments. A background sample lacking quencher lipid and DPH was also prepared. The fluorescence of this background, which was approximately 0.1% of the fluorescence in the DPH-containing sample without quencher, was subtracted from the fluorescence of each sample.

To determine the points of phase separation, the ratio of fluorescence in the presence of the quencher 12SLPC (F) to that in its absence (F_0) was calculated. Because F_0 can depend on the lipid used, this ratio can give different values for fluorescence at 100% 12SLPC. To avoid this the F/F_0 values were also normalized to a value of 0 at 100% 12SLPC (not counting cholesterol) using the formula $F/F_{0(\text{corrected})} = [F/F_0 - F/F_{0(100\%12SLPC)}]/[(1 - F/F_{0(100\%12SLPC)}]$. This normalization has only a small effect on curve shape as residual fluorescence at 100% 12SLPC was only 2−6% of F_0.

Detergent Solubilization Determined by Light Scattering. Light scattering was measured on the spectrofluorometer using both an excitation and emission wavelength of 500 nm with 0.2 mm excitation and emission slits. Light scattering was measured in a 1 cm excitation and 4 mm emission path length quartz cuvette.

Detergent insolubility experiments were performed at 23 °C using MLV at a total lipid concentration of 150 μM. Samples containing DPPC/12SLPC/cholesterol, DPPC/DOPC/cholesterol, or SM/12SLPC/cholesterol were prepared at 81 °C, as described above, and then incubated at 23 °C. The DOPC/12SLPC/cholesterol samples were prepared at 23 °C. Duplicate samples were incubated overnight in the dark at 23 °C. In most cases, DPH was also included, so that the phase behavior of the mixtures could be checked by quenching prior to the solubilization experiment. (Control experiments demonstrated that DPH had no effect on the results.) No significant differences in phase behavior as detected by the quenching assay were seen relative to samples used only in quenching experiments.

The light scattering of the samples was first measured prior to solubilization. Three individual 1 s readings were made and averaged. Then 14 μL of 10% (w/v) Triton X-100 was added to 800 μL of the MLV sample. After vortexing, the samples were incubated 20 min prior to remeasuring light scattering. Solubilization was calculated from the ratio of light scattering after treatment with Triton X-100 to that before Triton X-100 addition. The background light scattering of Triton X-100 in PBS was subtracted from the sample values.

Model Quenching Calculations. The quenching behavior of a binary system was modeled using the equations of London and Feigenson (1981). The fluorescence quenching of a fluorescent probe in a uniform phase with a random lateral distribution of quencher (12SLPC) mixed with an unlabeled lipid was assumed to be given by $F/F_0 = \exp(-\pi R_{c(\text{effective})}{}^2 C)$ with $R_{c(\text{effective})} = (R_c{}^2 + z^2)^{1/2} = 12$ Å, where R_c is the critical quenching distance, z is the difference in depth of fluorophore and quencher, and C is 12SLPC concentration in units of molecules/per square angstrom (Chattopadhyay & London, 1987). For mixtures forming two phases over some range of compositions and a uniform single phase in others, F/F_0 was assumed to be the same as for the uniform binary mixture in the one-phase region. In the region where two phases are present, F/F_0 was calculated from $F/F_0 = F/F_0' + (F/F_0'' - F/F_0')[X/(K_p - K_pX + X)]$, where F/F_0' is the fluorescence at the composition at the boundary between the one-phase and two-phase regions at which the 12SLPC-enriched phase saturated with the nonquenching lipid is predominant; F/F_0'' is the fluorescence at the other boundary between the one-phase and two-phase regions at which the 12SLPC-depleted phase saturated with 12SLPC is predominant; K_p is the partition coefficient of the fluorescent probe between the two phases, given by K_p = [probe in quencher-enriched phase]/[probe in quencher-depleted phase]; and X is the fraction of the total bilayer that is in the quencher-depleted phase, given by $X = ([12SLPC]' - [12SLPC])/([12SLPC]' - [12SLPC]'')$, where [12SLPC] is the 12SLPC concentration and [12SLPC]′ and [12SLPC]″ are the concentrations of 12SLPC at the phase boundaries. See London and Feigenson (1981) for details.

RESULTS

Fluorescence Quenching Assay of Phase Behavior. A fluorescence quenching method that we developed previously was used to examine the phase behavior of lipid mixtures

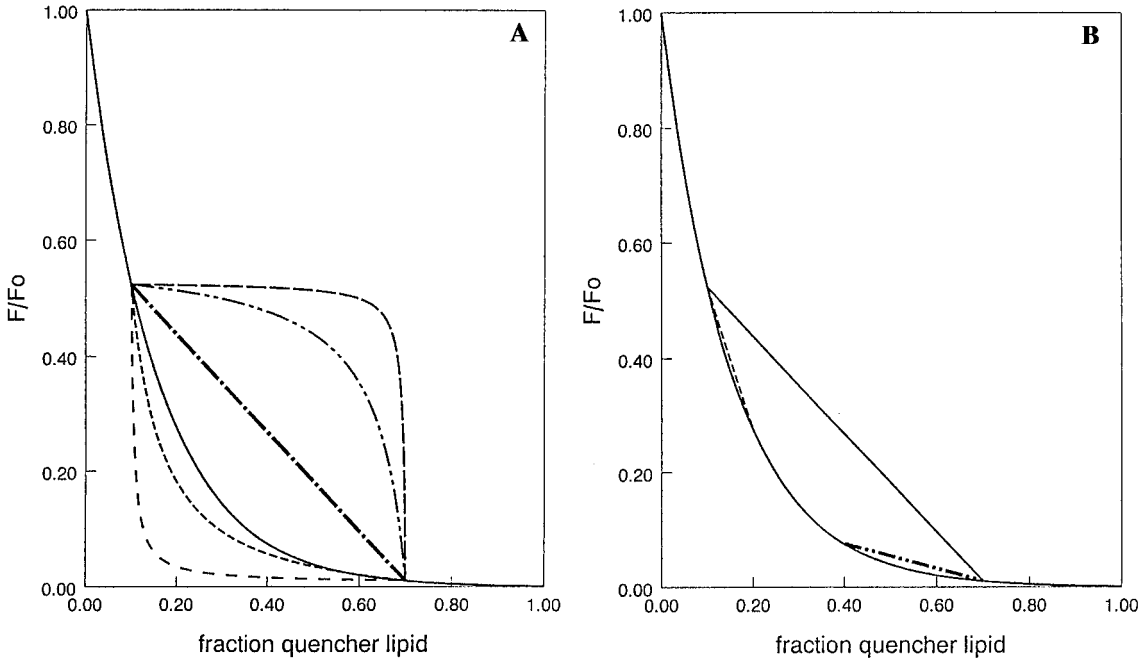

FIGURE 1: Theoretical quenching patterns showing the effect of probe partition coefficient and phase separation compositions on quenching curves. (*y*-axis) Ratio of fluorescence in the presence of quencher to that in its absence. (*x*-axis) Fraction of quencher lipid in a binary mixture with a nonquenching lipid. (A) Fluorescence vs mol fraction quencher lipid for a mixture forming a uniform phase at all compositions (—) or mixtures forming two phases between 10% quencher lipid (fraction 0.1) and 70% quencher (fraction 0.7) lipid with the fluorescent probe having a partition coefficient (K_p) of (— — —) 100, (- - -) 10, (—·—) 1, (—··—) 0.1 and (————) 0.01. (B) Effect of phase separation points on fluorescence. Data were calculated for a fluorescent probe with $K_p = 1$ and phase boundaries at (- - -) 10% and 20% quencher lipid, (—) 10% and 70% quencher lipid, and (—··—) 40% and 70% quencher lipid, and (curved solid line) for a uniform phase at all compositions. See Experimental Procedures for equations used for these calculations.

(London & Feigenson, 1981). In this method, a series of liposomes containing graded mixtures of two or more lipids is prepared. In a binary lipid system, one lipid is linked to a fluorescence quencher, and the identity of the other unlabeled lipid is varied as desired. A membrane-bound fluorescent probe is also incorporated into the samples. The fluorescence of each sample is measured and normalized to that of a sample without quencher. The amount of fluorescence declines as the amount of quencher in the mixture increases. If both lipids mix to form a single uniform phase, then quenching has a smooth (Yeager & Feigenson, 1990), nearly exponential (Chattopadhyay & London, 1987) dependence on the membrane concentration of the quencher-labeled lipid (solid line, Figure 1A). However, some lipids can separate into two phases (i.e., exhibit lipid−lipid immiscibility) when combined. Such lipids usually form a single phase (i.e., are miscible) when the concentration of either component is low but undergo phase separation at other concentrations. As the two phases have different lipid compositions, one phase is enriched and the other is relatively depleted in quencher.

This behavior affects quenching: the quenching of probe molecules that partition into the quencher-rich phase is greater than for those that partition into the quencher-poor phase. As a result, there can be striking changes in the dependence of fluorescence on quencher concentration when two phases appear (Figure 1A). The boundaries of the two-phase region are given by the lipid compositions at which the quenching diverges from that for a lipid mixture forming a uniform phase (Figure 1A).

The exact shape of the quenching curve in the two-phase region depends on the partitioning of the fluorescent probe between the two phases (Figure 1A). For example, if the probe partitions preferentially into the quencher-rich phase, then fluorescence will usually decline more rapidly as the concentration of quencher is increased in the two-phase region than in the one-phase regions ($K_p = 10$ and 100 curves, Figure 1A). Conversely, if the probe partitions preferentially into the quencher-depleted phase, then fluorescence will decline more slowly than in the one-phase regions ($K_p = 0.1$ and 0.01 curves, Figure 1A). If the probe partitions evenly between the two phases, then fluorescence will decline linearly with increasing quencher ($K_p = 1$ curve, Figure 1A). These behaviors have been observed experimentally in a number of systems (see introduction).

The usefulness of quenching for detecting phase separations is strongly influenced by the range of lipid compositions over which the phase separation occurs. When phase separation occurs over a wide range of concentrations, for instance, between 10% and 70% quencher lipid, the phase separation is easily detected by the divergence of quenching from that in a uniform phase (Figure 1B, solid lines). However, the divergence is much more difficult to detect when phase separation occurs over only a narrow range of lipid compositions: for example, when the two-phase region occurs only between 40% and 70% quencher (Figure 1B, - . -), or between 10% and 20% quencher (Figure 1B, - - -).

Quenching Behavior in DOPC/12SLPC Mixtures. The quenching of diphenylhexatriene (DPH) in binary mixtures of dioleoylphosphatidylcholine (DOPC) mixed with the nitroxide-labeled quencher 12SLPC was examined first. DPH was chosen as the fluorescent probe due to its strong fluorescence and its tendency under many conditions to partition nearly equally between different phases (Lentz et al., 1976; London & Feigenson, 1981; Florine-Casteel & Feigenson, 1988). DOPC was chosen as a lipid that, like

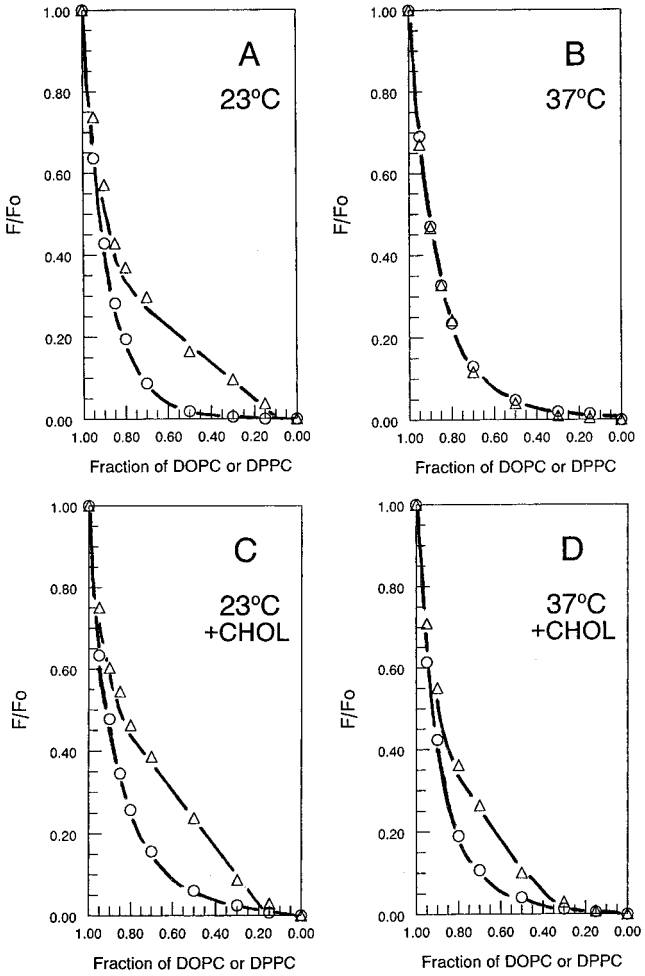

FIGURE 2: Comparison of quenching curves for mixtures of DOPC and 12SLPC to that for mixtures of DPPC and 12SLPC. Data are shown for (○) DOPC/12SLPC mixtures and (△) DPPC/12SLPC mixtures at (A, C) 23 °C or (B, D) 37 °C. (A, B) No cholesterol; (C, D) with 33 mol % cholesterol. The *x*-axis shows DOPC or DPPC as a mol fraction of total phospholipid. In the cholesterol-containing samples, phospholipid is 67% of total lipid including cholesterol.

most natural phospholipids, has a very low gel to fluid melting transition [T_m about −20 °C (Jain, 1988)] and as a result would remain in the fluid L_d phase under all experimental conditions. 12SLPC was chosen as it is an efficient quencher of DPH fluorescence and also because (due to the bulky nitroxide-bearing doxyl group on the 12 carbon of one fatty acyl chain) it remains in the fluid L_d phase to below 0 °C (Chen & Gaffney, 1978).

The quenching of DPH fluorescence in mixtures of DOPC and 12SLPC at 23 and 37 °C is shown in Figure 2A,B (○). As previously seen for single-phase mixtures of fluid lipids, quenching increased as a smooth function of 12SLPC concentration at both temperatures. This indicates that DOPC and 12SLPC mixtures form a single liquid-disordered (L_d) phase. This was expected, as both lipids have T_m less than 0 °C. The quenching of mixtures of DOPC and 12SLPC with cholesterol is shown in Figure 2C,D (○). Again, quenching is given by a smooth function, strongly suggesting that a single L_d phase is present. Our earlier results (Schroeder et al., 1994) also suggested that an L_d phase is present in similar mixtures of low T_m phopsholipids and choleserol.

Quenching Behavior in DPPC/12SLPC Mixtures. The behavior of mixtures of dipalmitoylphosphatidylcholine (DPPC) with 12SLPC was examined next. DPPC was chosen because its detailed phase behavior is relatively well understood (Jain, 1988). DPPC occurs in the gel phase below its T_m of 41 °C, in the fluid (L_d) phase above 41 °C, and in the liquid-ordered (L_o) phase under appropriate conditions in the presence of cholesterol.

The behavior of DPPC/12SLPC (Figure 2A, △) is quite different from that of DOPC/12SLPC at 23 °C. Pure DPPC is in the gel phase at this temperature, whereas 12SLPC is in the fluid L_d phase. Therefore, a phase separation would be expected in some DPPC/12SLPC mixtures. This is reflected in the quenching data. The divergence of the DPPC/12SLPC curve (△) from the DOPC/12SLPC curve (○), shows that one boundary of the two-phase region (the boundary on the "right" or low DPPC concentration side) is about 10 mol % DPPC. Below 10% DPPC (i.e., above 90% 12SLPC), a single phase is present. As pure 12SLPC is in the L_d phase, this single phase must also be the L_d phase. The other boundary (that on the "left" or high DPPC concentration side) of the two-phase region can be estimated from the change in the shape of the DPPC/12SLPC curve to be about 80% DPPC. This means that bilayers containing more than 80% DPPC (i.e., less than 20% 12SLPC) are in a single phase. This phase must be the same as that of pure DPPC at 23 °C (i.e., the gel phase).

It is difficult to estimate the precise phase boundary on the "left" side of the two-phase region. This results from the fact that even at the lowest 12SLPC concentrations, at which a single phase should be present, we consistently observed less quenching of DPH in DPPC/12SLPC than in DOPC/12SLPC. This is likely to reflect the fact that the DOPC is in the L_d state, whereas the DPPC is in the gel state under these conditions. DPH quenching is probably slightly reduced in the gel and L_o states due to decreased DPH motion (Schroeder et al. 1994). In any case, it should be emphasized that we are primarily concerned with the right-side boundary of the two-phase region, which gives the minimum concentration of lipid at which an ordered phase begins to separate from the 12SLPC L_d phase. This information is pertinent to the question of whether ordered domains are likely to be present in cellular membranes.

Between 10% and 80% DPPC, a DPPC-rich gel phase and a 12SLPC-rich fluid L_d phase must coexist. The fact that fluorescence declined in a relatively linear fashion with increasing 12SLPC concentration reflects the nearly equal partition of DPH between the quencher-poor gel phase and quencher-rich fluid phase (Figure 1).

It was necessary to establish that a difference in quenching in DPPC/12SLPC mixtures relative to that in DOPC/12SLPC mixtures did not reflect a change of DPH depth. Movement of DPH away from the deep doxyl quenching group on 12SLPC and toward the membrane surface could potentially explain the weaker quenching in DPPC/12SLPC relative to DOPC/12SLPC. This possibility was tested by preparing samples with TempoPC, in which the quencher is attached to the polar headgroup (Abrams & London, 1993). If DPH were closer to the membrane surface in the presence of DPPC, there would be more DPH quenching in DPPC/TempoPC than in DOPC/TempoPC. However, this was not observed. Instead, there was less quenching of DPH in

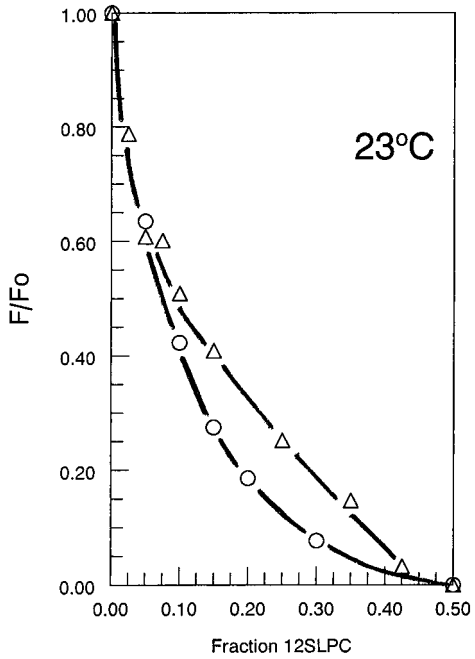

FIGURE 3: Comparison of quenching curve for mixtures of DOPC and 12SLPC to that for mixtures of DPPC with 1:1 (mol/mol) DOPC/12SLPC. Data are shown for (○) DOPC/12SLPC mixtures and (△) DPPC/DOPC/12SLPC mixtures at 23 °C.

DPPC/TempoPC than in DOPC/TempoPC (not shown), in agreement with the results using 12SLPC.

At 37 °C, DPPC/12SLPC mixtures (Figure 2B, △) did not exhibit a discernable two-phase region, as shown by the coincidence with the DOPC/12SLPC curve. This indicates miscibility of DPPC and 12SLPC lipids at 37 °C over a wide range of compositions. This is not surprising since, at 37 °C, pure DPPC is close to its T_m (41 °C). Therefore, a single fluid L_d phase should predominate for most DPPC/12SLPC compositions. However, a small two-phase region over a narrow range of compositions close to 100% DPPC would have been expected. As noted in Figure 1B, such narrow phase separations are difficult to detect by the quenching assay.

Phase Separation Is Not Restricted to Mixtures with 12SLPC. It was desirable to confirm that the phase behavior of 12SLPC, with its unnatural doxyl ring, was similar to that of an unmodified unsaturated chain. For this reason, the experiment shown in Figure 2A was repeated, substituting a 1:1 (mol/mol) DOPC/12SLPC mixture for 12SLPC. If DOPC and 12SLPC have very similar phase behavior, it would be predicted that DPPC would phase-separate from 12SLPC and from the 1:1 mix of DOPC/12SLPC at the same concentrations. Figure 3 shows this was indeed the case. The boundaries of the two-phase region were close to 10−15% DPPC and 80−85% DPPC, very similar to the results for DPPC mixed with 12SLPC alone.

Quenching Behavior in DPPC/12SLPC/Cholesterol Mixtures: Promotion of Phase Separation by Cholesterol. The behavior of mixtures containing 33 mol % cholesterol, similar to the amount of sterol reported to be present in plasma membranes (Gennis et al., 1989), was also examined. Previous studies have shown that a mixture of 33% cholesterol and DPPC is in the L_o phase over a wide range of temperatures (Ipsen et al., 1987; Vist & Davis, 1990; Sankaram & Thompson, 1990), whereas similar mixtures of DOPC and cholesterol appear to remain fluid (Schroeder et al., 1994).

Quenching behavior in DPPC/12SLPC/33% cholesterol mixtures is shown in Figure 2C,D (△). Comparison to quenching in DOPC/12SLPC/33% cholesterol mixtures (○) shows that phase separation occurs in DPPC/12SLPC/33% cholesterol. At 23 °C (Figure 2C), the right-side boundary between the one-phase and two-phase region occurred at a DPPC concentration of 15% of total phospholipid (10% of total lipid). The two-phase region persisted to 85−90% DPPC. At higher DPPC concentrations only one phase was present. This must be the L_o phase, as this phase is known to be formed by DPPC with 33% cholesterol (Sankaram & Thompson, 1990). Between these boundaries, a DPPC-rich L_o phase coexists with the 12SLPC-rich L_d phase. Thus at 23 °C, the phase separation between DPPC and 12SLPC (Figure 2A) is maintained upon addition of cholesterol.

Cholesterol had a striking effect on phase separation in DPPC/12SLPC/33% cholesterol mixtures at 37 °C. In contrast to the situation without cholesterol (Figure 2B), a pronounced phase separation was detected over a wide range of compositions (Figure 2D). Thus, cholesterol promotes phase separation at this temperature. As expected, the amount of DPPC necessary for phase separation was higher than at 23 °C, with 30−40% DPPC needed for the separation to occur at 37 °C.

The phase separation in DPPC/12SLPC/33% cholesterol mixtures appeared to persist up to 65 °C, but was almost totally abolished at 75 °C. Cooling samples from 75 to 23 °C restored the phase separation (not shown).

Quenching Behavior in SM/12SLPC Mixtures. Because sphingomyelin (SM) is found in relatively high concentrations in plasma membranes, its phase behavior was of particular interest. The quenching of SM/12SLPC mixtures at 23 °C is shown in Figure 4A. As in the case of DPPC, phase separation was observed, although the phase separation occurs over a narrower range of concentrations of SM than was seen for DPPC. Divergence of the SM/12SLPC and DOPC/12SLPC quenching curves shows that at 23 °C phase separation requires SM concentrations above about 30%. In contrast, only 10% DPPC was required for phase separation in DPPC/12SLPC mixtures (Figure 2A). Therefore, more SM than DPPC is needed to achieve the onset of phase separation. The left boundary between one-phase and two-phase regions is hard to discern for SM/12SLPC mixtures.

At 37 °C, no phase separation could be observed for SM/12SLPC (Figure 4B). This is similar to the behavior of DPPC/12SLPC mixtures at 37 °C, as would be predicted by the similarity in the T_m of SM and DPPC (see Discussion).

Quenching Behavior of SM/12SLPC/Cholesterol Mixtures. Inclusion of 33% cholesterol in SM/12SLPC mixtures strongly influenced their phase behavior (Figure 4C,D). Cholesterol promoted phase separation at both 23 and 37 °C. At 23 °C, phase separation appeared when about 15% of total phospholipid was SM in the presence of cholesterol. By contrast, 30% SM was required for phase separation in the absence of cholesterol. It is especially noteworthy that at 37 °C (physiological temperature for mammalian cells) the separation of a SM-rich phase appeared at a SM

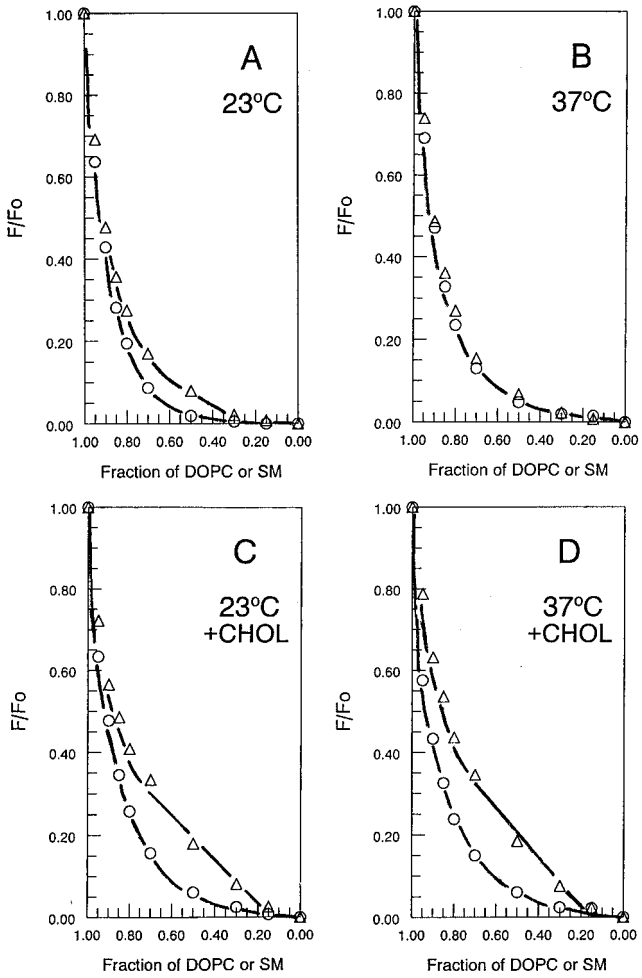

FIGURE 4: Comparison of quenching curves for mixtures of DOPC and 12SLPC to those for mixtures of SM and 12SLPC. Data are shown for (○) DOPC/12SLPC mixtures and (△) SM/12SLPC mixtures at (A, C) 23 °C or (B, D) 37 °C. (A, B) No cholesterol; (C, D) with 33 mol % cholesterol. The *x*-axis shows DOPC or SM as a mol fraction of total phospholipid. In the cholesterol-containing samples, total phospholipid is 67% of total lipid including cholesterol.

concentration of 15−20% of total phospholipid (10−13% of total lipid).[2]

As was true in the absence of cholesterol, the highest SM concentration at which two phases persisted (left phase boundary) was again difficult to determine from quenching. However, it appeared that phase separation may have been present up to almost 80% SM. Because SM with 33% cholesterol is in the L_o phase (Sankaram & Thompson, 1990) the bilayers must be in this phase above 80% SM. Therefore, the phases present in the two-phase region of lipid compositions are a SM-rich L_o phase and a 12SLPC-rich L_d phase. As in the case of DPPC/12SLPC/33% cholesterol, this phase separation persisted up to 65 °C but was abolished at 75 °C, where complete or almost complete miscibility was observed. Phase separation reappeared upon cooling (not shown).

Effect of Substitution of 1:1 Cerebroside/SM for SM. In some specialized cell membranes, such as the apical membranes of epithelial cells, glycosphingolipids can make up a considerable fraction of the total sphingolipid (Simons & van Meer, 1988). Pure glycosphingolipids have a very high T_m (Ruocco & Shipley, 1984), and so could significantly alter the phase behavior of lipid mixtures. To see if glycosphingolipids affect phase separation, the phase behavior of a 1:1 mixture[3] of SM with cerebrosides (CB, simple glycosphingolipids found in many plasma membranes) was examined (Figure 5). In contrast to the behavior of SM/12SLPC mixtures (Figure 4B), phase separation was seen at 37 °C (Figure 5A). Thus, CB promotes overall sphingolipid phase separation, in agreement with earlier studies examining detergent insolubility (Schroeder et al., 1994). This is not surprising, as the T_m of CB is in the 60−80 °C range (Ruocco & Shipley, 1984), much higher than that of SM. Therefore, a CB-rich gel phase would be expected to form over a wide range of CB concentrations in the membrane.

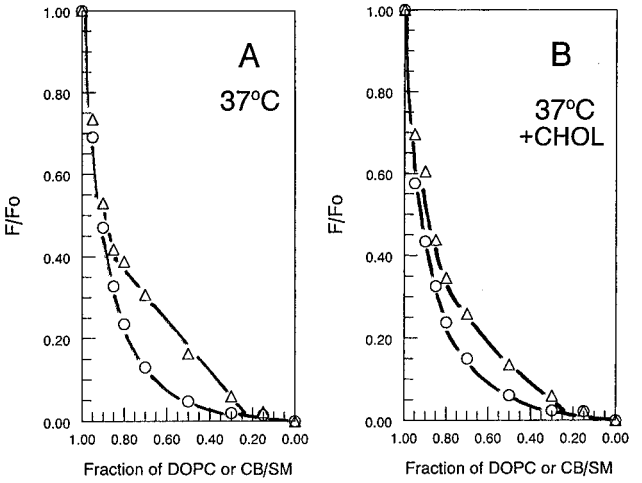

FIGURE 5: Quenching curves for 1:1 (mol/mol) CB/SM mixed with 12SLPC with and without 33 mol % cholesterol. (A) Comparison of quenching curve for (○) DOPC/12SLPC mixtures to that for (△) (1:1) CB:SM/12SLPC mixtures at 37 °C. (B) Comparison of quenching curves for (○) DOPC/12SLPC/33 mol % cholesterol to that for (△) (1:1) CB:SM/12SLPC/33 mol % cholesterol at 37 °C. The *x*-axis shows percent DOPC or CB/SM as a mol fraction of total phospholipid.

The inclusion of 33% cholesterol in the CB/SM/12SLPC mixtures did not promote total sphingolipid phase separation relative to the sample without cholesterol (Figure 5B). Phase separation was first seen at about 25% total sphingolipid in both cases.[4] This behavior is consistent with the relative lack of a cholesterol effect on the behavior of CB/PC mixtures previously observed by Silvius (1992) (see Discussion). In addition, the presence of CB did not result in a lower sphingolipid threshold for phase separation in cholesterol-containing mixtures compared to samples containing SM as the only sphingolipid (compare Figures 5B and 4D).

Phase Behavior Assayed by Detergent Solubility Agrees with Quenching. Insolubility in detergent (usually Triton X-100) has been widely used to isolate sphingolipid/cholesterol-rich membranes from cells (see introduction). We previously found that L_o phase DPPC/cholesterol model membranes are detergent-insoluble (Schroeder et al., 1994),

[2] In another preparation of 12SLPC we found a slightly higher separation point (SM = 20% of total lipid). This may reflect small variations in the measurement of lipid concentrations.

[3] We originally based this composition on the commercially reported molecular weight of 700. Based on the presence of a C24:0 chain, the molecular weight is 812, and the ratio of CB/SM is 0.9:1.

[4] Part of the difference in the shape of the CB/SM/12SLPC curves with and without cholesterol may reflect a change in DPH K_p (Figure 1A).

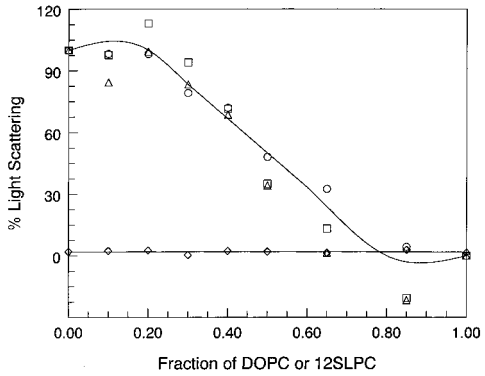

FIGURE 6: Effect of lipid composition on detergent insolubility at 23 °C as assayed by change in light scattering. (△) DPPC/12SLPC/cholesterol mixtures; (□) SM/12SLPC/cholesterol; (○) DPPC/DOPC/cholesterol; (◇) DOPC/12SLPC/cholesterol. The *x*-axis is the fraction of total phospholipid. For all cases except DOPC/12SLPC/cholesterol, values shown are normalized to 100% in DPPC/cholesterol and SM/cholesterol and 0% in 12SLPC/cholesterol and DOPC/cholesterol (see Results). The cholesterol concentration was 33 mol % in all cases.

suggesting that cell membranes resistant to detergent solubilization are in the L_o phase.[5] To see if detergent insolubility reflected the amount of L_o phase present, the Triton X-100 insolubility of DOPC/12SLPC/cholesterol, DPPC/12SLPC/cholesterol and SM/12SLPC/cholesterol liposomes was compared to the phase behavior determined by quenching at 23 °C. The decrease in light scattering that occurs when multilamellar vesicles dissolve in detergent was used to monitor solubilization (Partearroyo et al., 1992). As shown in Figure 6, DOPC/12SLPC/cholesterol mixtures, which form a single L_d phase (Figure 2C), were fully dissolved.[6] In contrast, most DPPC/12SLPC/cholesterol, DPPC/DOPC/cholesterol, and SM/12SLPC/cholesterol mixtures were not fully dissolved. Mixtures containing more than 80% DPPC or SM, shown by the quenching assay to be in a single L_o phase, exhibited maximum insolubility. Mixtures containing less than 20% SM or DPPC, shown by the quenching assay to be in a uniform L_d phase, exhibited maximum solubility. Between 20% and 80%, there was an intermediate amount of solubilization, which declined in a roughly linear fashion as DPPC or SM concentrations were decreased. The solubilization levels appeared to represent equilibrium values, as extending the incubation of DOPC/DPPC/cholesterol samples from 20 min to 24 h did not increase solubilization.

These results show that detergent insolubility is strongly correlated with the amount of detergent-resistant L_o phase present. Thus, the amount of detergent insolubility reflected the amount of L_o phase(s) present before detergent was added. This strongly suggests that the insoluble membranes isolated from cells exist as L_o phase before detergent extraction.

DISCUSSION

Phase Behavior Can Be Determined by Quenching. We have shown that fluorescence quenching by nitroxide-labeled lipids can be used to detect phase separations in complex mixtures containing cholesterol.[7] An advantage of the quenching method is that it does not require a large difference in the physical properties of the two phases to detect phase separation. Since the L_d and L_o phases have similar physical properties, quenching is especially useful for studying the L_o phase, which is often difficult to detect by other methods. This was also shown by the recent study of Silvius et al. (1996), using a brominated lipid quencher in a variant of this assay.

SM and DPPC Show Similar Behavior. It is noteworthy that DPPC and SM showed similar phase separation behavior. This probably reflects their similar T_m values. Presumably, the two lipids share a tendency for tight acyl chain packing. Whatever differences were observed between DPPC and SM behavior may reflect the somewhat more complex melting behavior of SM, which exhibits a relatively broad multistep series of transitions centered around 37 °C, even in a synthetic chemically pure form (Maulik & Shipley, 1995).

Cholesterol Effects on Phase Separation. The most striking result of this study was that addition of cholesterol to a lipid mixture promoted separation of a DPPC- or SM-rich phase from the L_d phase at 37 °C. In contrast, at 23 °C, which is significantly below T_m for DPPC and SM, cholesterol either had no effect (for DPPC) or more moderately promoted phase separation (for SM). It may be a general rule that cholesterol has little or no effect on phase separation at temperatures far below T_m, though few data on this point are available. However, the results of Silvius (1992) are highly suggestive. He found that cholesterol had, at most, only modest effects on the phase separation between a fluid phase PC and CB at temperatures well below the T_m of CB.

Lipid Phase Behavior and Detergent Insolubility Suggest That L_o Phase Occurs under Physiological Conditions in Cells. The phase behavior seen in this report strongly supports the proposal that L_o phase domains occur in cells. In addressing the question of whether phase separation is likely to occur in cell membranes, one must first ask: is the lipid composition of the cell membrane consistent with L_o phase formation? Answering this question is easiest for the cholesterol-rich plasma membrane. Many plasma membrane phospholipids have highly unsaturated acyl chains and low T_m. As the L_o phase is generally formed by high T_m lipids with saturated chains and cholesterol, most biological phospholipids would not be expected to participate in the formation of an L_o phase *in vivo*. Sphingolipids, which contain highly saturated acyl chains, are the only major lipids

[5] Gel phase is also resistant to detergent solubilization (Ribiero & Dennis, 1973) but is unlikely to occur in the cholesterol-rich plasma membrane. Interestingly, we found that SM alone was detergent-soluble at 23 °C despite being in the gel phase. This solubility is not a surprise, as insolubility of gel phases can require temperatures well below T_m (Ribiero & Dennis, 1973). We found that pure SM was insoluble at 4 °C (data not shown).

[6] The absolute decrease in light scattering when detergent was added was slightly variable, within the range 0−20% for DPPC/cholesterol and SM/cholesterol and 80−100% for DOPC/cholesterol and 12SLPC/cholesterol. This variation appears to reflect an artifact in light scattering measurements. Centrifugation experiments previously demonstrated the total insolubility of 2:1 (mol/mol) DPPC/cholesterol and total solubility of 2:1 DOPC/cholesterol (Schroeder et al., 1994). In addition, centrifugation and filtration experiments showed that 12SLPC/cholesterol and DOPC/cholesterol were equally detergent-soluble (unpublished observations).

[7] It should be cautioned that the phase behavior of ternary and higher order mixtures examined in this report is more complex than that of binary mixtures. In binary mixtures where two phases are present, the amount and composition of each phase is fixed by the compositions at which the phase separation begins and ends. In more complex mixtures the composition of each phase may vary.

in eukaryotic cell membranes with high T_m. Therefore, they are the biological lipids most likely to participate in an L_o phase. (However, see below.) The question now becomes: is there sufficient sphingolipid in cells to support L_o phase formation?

To answer this, it must be noted that the lipids used in this study are likely to be distributed more or less symmetrically between the inner and outer leaflets of the bilayer. In plasma membranes, however, sphingolipids are highly concentrated in the outer leaflet (Verkeij et al., 1973). The total plasma membrane concentration of sphingolipids can be as high as 15−20% (Ruocco & Shipley, 1984; Gennis, 1989). Thus, the amount of SM in the outer leaflet is likely to exceed the 17% we found to be necessary for phase separation in the presence of cholesterol.

More direct evidence supporting the existence of L_o phase domains in cell membranes comes from the many studies showing the isolation of detergent-resistant sphingolipid/cholesterol-rich membranes (see introduction). We have shown here that detergent insolubility is a good indicator of the amount of L_o phase present in mixtures containing SM and cholesterol. This greatly strengthens the proposition that insoluble membranes derived from cells arise from a L_o phase present *in vivo*. Studies showing that lowering cholesterol and/or sphingolipid concentrations *in vivo* lessens detergent insolubility also argue for the existence of a separate phase that is dependent on both of these lipids (Hanada et al., 1995; Cerneus et al., 1993).

Other Lipids May Modulate the L_o Phase Content in Cell Membranes. Several factors limit the ability to predict exact lipid phase behavior *in vivo* from phase separation *in vitro*. First, little is known about the phase behavior of natural PC species in cholesterol-containing systems. Highly unsaturated natural PCs have very low T_m and are unlikely to participate in L_o phase formation. On the other hand, some natural PCs may promote L_o formation. For example, there is evidence that 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), one of the most common species of PC in mammalian cells, may form an L_o-like phase with cholesterol under some circumstances (Mateo et al., 1995). It is possible that our study *underestimates* the degree of L_o phase formation in biological membranes containing large amounts of POPC. In fact, under some conditions, it is possible that the mixture of sphingolipids, cholesterol, and almost saturated lipids like POPC may cause the plasma membrane to exist in a uniform, essentially liquid-ordered phase.

Cholesterol content must also be considered. Recent studies by Silvius et al. (1996) show that modulation of L_o phase separation by cholesterol is strongly dependent on cholesterol concentration. The behavior of mixtures of sphingolipids with phospholipids other than PC must also be characterized. Finally, membrane proteins may affect lipid phase behavior. Further investigation of these factors will enhance our ability to understand and predict phase behavior in cell membranes.

Thus, it should be cautioned that our study was intended only to demonstrate that an L_o phase is likely to exist in cell membranes. Questions of the size and connectivity of domains in a particular phase, and how differences between cytoplasmic and outer leaflets of the membrane affect phase behavior, remain open.

Possible Significance in Cells. Several recent studies suggest that L_o phase membranes, usually isolated as DRMs, are important in cell function. Two areas that have received the most attention are sorting in the secretory pathway and signaling at the cell surface. It has been proposed that apical proteins are sorted into glycosphingolipid-rich domains predicted to be in the L_o phase during sorting in epithelial cells (Simons & van Meer, 1988). It has also been shown that sphingolipid synthesis is required for efficient transport of GPI-anchored proteins in yeast, suggesting a possible role for the L_o phase there as well (Horvath et al., 1994).

One of the best-characterized examples of the role of the L_o phase in signaling is found in the RBL-2H3 basophil cell line. Binding of antigen to IgE linked to its cell-surface receptor causes the receptor to cluster (Barsumian et al., 1981). In one of the few cases where L_o domains may be detectable by fluorescence microscopy, a saturated-chain diI was seen to cocluster with the IgE receptor (Thomas et al., 1994). As predicted, diI species with unsaturated acyl chains, which should not partition into L_o phase domains, did not cluster with the receptor. During clustering, the IgE receptor moves into DRMs (Field et al., 1997), and the amount of the associated nonreceptor tyrosine kinase, Lyn, in DRMs increases (Field et al., 1995). Tyrosine phosphorylation of the receptor by Lyn, a necessary step in signaling, is largely restricted to the fraction of the receptor in the domains (Field et al., 1997). These examples suggest that the role of the L_o phase may be to facilitate interactions between proteins or to confine interacting proteins to small regions of the membrane.

The membrane of caveolae may be in the L_o phase. In support of this idea, caveolin, a marker for caveolae, is present in DRMs (Sargiacomo et al., 1993; Chang et al., 1994). In addition, GPI-anchored proteins can be enriched in caveolae (Rothberg et al, 1990), particularly after cross-linking with antibodies (Mayor et al., 1994). Caveolin oligomerizes into units of about 14 monomers (Monier et al., 1995). These oligomers may form the building blocks of the caveolar coat. Each monomer contains three covalently linked palmitate chains (Dietzen et al., 1995) and is bound tightly to a molecule of cholesterol (Murata et al., 1995). This suggests that caveolin would have an affinity for L_o domains and that high concentrations of palmitate chains are present in caveolae. It will be interesting to determine whether caveolin with its associated lipids affects the formation or properties of the L_o phase.

Although DRMs are sometimes equated with caveolae, there is no evidence that membrane in the L_o phase is restricted to caveolae. In fact, DRMs can be isolated from cells that do not express caveolae.

CONCLUSION

Strong evidence from both *in vitro* and *in vivo* studies now indicates an important role for lipid phase behavior and the L_o phase in cell membranes. The promotion of L_o phase formation is likely to be an important function of cholesterol and sphingolipid in cell membranes. Since these lipids are enriched in the plasma membrane, the presence of the L_o phase may be a major distinction between the plasma membrane and most other cellular membranes. An interesting possibility is that transitions between a phase-separated and uniform plasma membrane state may be physiologically regulated. Further investigation of the control of phase behavior by lipid composition and protein interactions should

clarify the role of the L_o phase in cell membrane structure and function.

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