Effect of the Structure of Natural Sterols and Sphingolipids on the Formation of Ordered Sphingolipid/Sterol Domains (Rafts)

COMPARISON OF CHOLESTEROL TO PLANT, FUNGAL, AND DISEASE-ASSOCIATED STEROLS AND COMPARISON OF SPHINGOMYELIN, CEREBROSIDES, AND CERAMIDE

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Ordered lipid domains enriched in sphingolipids and cholesterol (lipid rafts) have been implicated in numerous functions in biological membranes. We recently found that lipid domain/raft formation is dependent on the sterol component having a structure that allows tight packing with lipids having saturated acyl chains (Xu, X., and London, E. (2000) Biochemistry 39, 844–849). In this study, the domain-promoting activities of various natural sterols were compared with that of cholesterol using both fluorescence quenching and detergent insolubility methods. Using model membranes, it was shown that, like cholesterol, both plant and fungal sterols promote the formation of tightly packed, ordered lipid domains by lipids with saturated acyl chains. Surprisingly ergosterol, a fungal sterol, and 7-dehydrocholesterol, a sterol present in elevated levels in Smith-Lemli-Opitz syndrome, were both significantly more strongly domain-promoting than cholesterol. Domain formation was also affected by the structure of the sphingolipid (or that of an equivalent “saturated” phospholipid) component. Sterols had pronounced effects on domain formation by sphingomyelin and dipalmitylophosphatidylcholine but only a weak influence on the ability of cerebrosides to form domains. Strikingly it was found that a small amount of ceramide (3 mol %) significantly stabilized domain/raft formation. The molecular basis for, and the implications of, the effects of different sterols and sphingolipids (especially ceramide) on the behavior and biological function of rafts are discussed.

There is strong evidence supporting a model of eukaryotic plasma membrane structure in which domains (rafts) rich in cholesterol and lipids with relatively saturated acyl chains (i.e. sphingolipids) co-exist with domains rich in phospholipids attached to unsaturated acyl chains (1–6). Rafts have a distinct protein composition that is especially enriched in proteins anchored to membranes by saturated acyl chains while relatively depleted of most transmembrane proteins. Rafts have been implicated in numerous cellular processes, including signal transduction, protein and lipid sorting, cellular entry by toxins and viruses, and viral budding (4, 6–15). Thus, an understanding of the principles that underlie raft formation is important with regard to the study of eukaryotic membrane function.

We have shown that sphingolipid/cholesterol domains are likely to exist in the tightly packed liquid-ordered (L0) state, whereas unsaturated phospholipid-rich domains are more likely to be in the less ordered liquid crystalline (Lc) phase even if they contain large amounts of cholesterol (16–18). The tight packing of lipids in the Lc state gives rafts their characteristic resistance to solubilization by Triton X-100 (1, 18).

Cholesterol can promote separation of lipid mixtures into co-existing Lc- and L0-domains (17, 19) and is critical for raft formation in cells (2–4, 6). We recently found that the ability of cholesterol to pack tightly with saturated lipids is a key to its ability to promote lipid domain formation and that sterols with different packing properties have different tendencies to promote domain formation (20). Some sterols that pack poorly with saturated lipids even inhibit lipid domain formation (20) and seem to disrupt raft function in cells (21).

There is some evidence from detergent insolubility that rafts exist in insect, fungal, and plant cells, although they contain sterols other than cholesterol (22–24). However, the way in which the structures of natural sterols influence domain formation and their biological function has not yet been explored. In this report, the ability of different natural sterols to promote lipid domain formation is compared. We found that natural variations in the double bond structure of the sterol rings and the structure of the aliphatic side chain can modulate domain formation by sterols. We also found that sphingolipid structure strongly influences domain formation. It seems likely that the precise ability to promote raft formation is an important aspect of sterol and sphingolipid structure and function.

EXPERIMENTAL PROCEDURES

Materials—Diphenylhexatriene (DPH)1 was purchased from Aldrich. Dipalmitylophosphatidylcholine (DPPC), dioleoylphosphatidylcholine

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1 The abbreviations used are: DPH, diphenylhexatriene; C4-sterol, 20(R)-ethyl-5-pregnen-3β-ol; C10-sterol, 20(R)-isocetethyl-5-pregnen-3β-ol; CBs, brain cerebrosides; 7-DHC, dehydrocholesterol; DPPC, dipalmitylophosphatidylcholine; DOPC, dioleoylphosphatidylcholine; LcTMADPH, 22-diphenylhexatrienyllosyltrimethylammonium
Corresponding samples and controls without quencher (ing temperatures used for the SM-, DPPC-, or CB-containing samples. Control 12SLPC mixture, 2:3 SM:12SLPC mixture, or 2:3 CBs:12SLPC mixture samples) contained a 1:1 (mol:mol) DPPC:

with or without 15 or 33 mol % sterol. Control experiments showed that interpolation did not cause a significant error. In most cases, after heating to the final temperature samples were cooled to 23 °C, and the fluorescence was then measured to confirm that the effect of temperature on quenching was reversible. Background values were subtracted before \( F/F_o \) values were calculated at each temperature. Fluorescence in samples with 33 mol % sterol was measured only at 23 °C.

Percent Solubilization Experiments—Multilamellar lipid vesicles containing 400 nmol of total lipid were prepared in 800 μl of PBS as for the quenching experiments except that lipid was dispersed in buffer at about 80 °C and then cooled to room temperature. This procedure was used to obtain more uniform dispersions at the higher lipid concentrations used in these experiments. For measuring solubilization by the loss of light scattering, the optical density of these samples was measured only at 23 °C. Generally, samples prepared at

DPPC, 1-palmitoyl-2-(12-doxystearoylphosphatidylcholine; 12SLPC), brain sphingomyelin (SM), mixed brain cerebrosides (CBs), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Ergosterol and 7-dehydrocholesterol (7-DHC) were purchased from Fluka (Rondonkoma, NY). Stigmasterol, coprostanol, and androstenedol were purchased from Steraloids (Newport, RI). Sitosterol and nonhydroxylated camellia were purchased from Sigma. Sterol melting points measured in a capillary apparatus generally agreed with the values reported by the manufacturers. An exception was sitosterol, which had a melting point 2 °C lower than expected. Sterol and lipid purity were confirmed by thin-layer chromatography of 0.04–0.08-μmol samples on silica gel plates using the solvent system 65:25:4 (v/v) chloroform:methanol:water for the phospholipids and CBs, 114:57:29 (v/v) chloroform:toluene:ethanol for ceramide, and 22:3 (v/v) hexane:ethyl acetate for the natural sterols. After spraying plates with 40% (v/v) sulfuric acid and charring, only one spot was visualized in most cases. CBs gave two spots, one for hydroxylated species and a second for nonhydroxylated species (17), and sitosterol exhibited a minor impurity with a high Rf. The fluorescent probe LcT–MADPH (22-diphenylhexatrienyl-3-decoxytrimethylammonium iodide) was synthesized as described previously (25). Synthetic cholesterol analogs with shortened or lengthened aliphatic segments, 20(R)-ethyl-5-pregnen-3β-ol (C4-sterol), and 20(R)-isooctyl-5-pregnen-3β-ol (C10-sterol) were also synthesized as described previously (26). The number in the code name, i.e. C4- or C10-sterol, refers to the total number of carbon atoms attached to C-17 of the sterol.

Fluorescence Measurements—Fluorescence intensities were measured on a Spex 212 Fluorolog fluorometer using, unless otherwise noted, a semimicro quartz cuvette with a 10-mm excitation path length and 4-mm emission path length. DPH fluorescence was measured at an excitation wavelength of 359 nm and emission wavelength of 427 nm. Narrow excitation slits (1.25 mm) were used to minimize photoisomer-
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Fig. 2. The effect of temperature on domain formation in 1:1 (mol/mol) DPPC:12SLPC mixtures with and without 15 mol % sterols assayed by the quenching of DPH fluorescence. Samples contained 1 mol % DPH bound to multilamellar vesicles dispersed in PBS at a total lipid concentration of 50 μM. The y axis (ΔF/Fo) is the difference between the fraction of DPH fluorescence unquenched in lipids and sterol (17, 20, 29).2 For simplicity, we will refer to a decreased level of quenching in the presence of sterols as a “lower Tmix” defined this way is easier to determine accurately than when defined as the temperature at which domains disappear (20, 28). A transition temperature, Tmix, can be defined as the temperature at which the domain mixing/melting is half-complete (Table I). It can be approximated by the inflection point in the curve of ΔF/Fo versus temperature (Fig. 2). Tmix is a measure of the stability of domain formation (20, 28). We find that Tmix defined this way is easier to determine accurately than when defined as the temperature at which domains disappear completely (28). Domain formation can also be assessed by the value of ΔF/Fo at a fixed temperature (Fig. 3). In general, ΔF/Fo reflects both the fraction of the bilayer in the form of sterol-containing saturated lipid-rich domains and the degree of enrichment of these domains in saturated lipid and sterol (17, 20, 29).2 For simplicity, we will refer to a decreased level of quenching in the presence of domains, i.e. a high value of ΔF/Fo, as indicative of a high “extent” of domain formation.

Fig. 2 shows the temperature dependence of domain formation in 1:1 mixtures of DPPC and 12SLPC in the absence and presence of 15 mol % sterols. At 23 °C there was less quenching (i.e. higher ΔF/Fo) in the presence of the natural sterols examined than in their absence (also see Fig. 3). This indicates all the natural sterols tested promote domain formation. The domains formed in the presence of sterol can be abolished by increasing temperature to above 60 °C. It should be noted that the temperature above which domains disappear (Tmix) was lowest in the absence of sterol (Table I). This shows that all the natural sterols also stabilize domain formation. Overall Tmix and ΔF/Fo gave a similar rank order for the effect of sterol structure on domain formation. The exact extent and stability of domain formation is a function of sterol structure. Ergosterol and 7-DHC formed domains that were significantly more stable than those formed in the presence of cholesterol. The presence of ergosterol or 7-DHC resulted in a Tmix about 5 °C higher than that obtained with cholesterol (Fig. 2 and Table I). Ergosterol and 7-DHC also promoted domain formation at 23 °C more strongly than cholesterol as judged by a higher ΔF/Fo value (Fig. 3).

Stigmasterol and sitosterol also promoted domain formation at 23 °C to a slightly higher extent than cholesterol (Fig. 3). However, at least for stigmasterol, domain formation was slightly less stable than that promoted by cholesterol with Tmix in the presence of stigmasterol 6–7 °C lower than that in the presence of cholesterol (Table I).3

Because stigmasterol differs from cholesterol only in the structure of its aliphatic chain, it is interesting to know whether domain formation is very sensitive to aliphatic chain structure. A C10-sterol analog, which has two extra carbons in the aliphatic chain relative to cholesterol (26), supported domain formation at 23 °C although not as well as cholesterol (Fig. 3). In contrast, a C4-sterol analog, which has an aliphatic

2 Quenching level will also be affected by domain size when domains are restricted to clusters of a relatively small number of molecules. Small domain size is unlikely to be a problem in samples containing 0 or 15 mol % cholesterol because the presence of a distinct thermal transition suggests the presence of domains representing large cooperative units, i.e. formed by many molecules.

3 The full thermal dependence of quenching is not shown for sitosterol because we noted a minor impurity and were concerned that this would be enough to influence the details of domain formation.

TABLE I

Relative stability (Tmix) and extent of domain formation (ΔF/Fo) in 15 mol % sterol/lipid mixtures

| Sterol               | Tmix °C | 1:1 DPPC:12SLPC | 2:3 SM:12SLPC | 2:3 CBs:12SLPC |
|----------------------|---------|-----------------|---------------|----------------|
| Ergosterol           | 44.6 ± 1 | 40.8 ± 0.3      | 46.4 ± 0.6    |                |
| 7-DHC                | 44.4 ± 0.4 | 44.0 ± 2.2      | ND            |                |
| Cholesterol          | 39.2 ± 0.4 | 36.0 ± 1.5     | 43.2 ± 1.0    |                |
| Stigmasterol         | 32.7 ± 1.1 | <23            | 42.8 ± 1.6    |                |
| None                 | <23     | <23             | 43.4 ± 2.9    |                |
| Androstrosterol      | <23     | <23             | 40.5 ± 0.5    |                |

* Average of four experiments.

* One experiment could not be easily fit to a sigmoidal curve. The average and range of two experiments are shown.

* Results from Xu and London (20).

Fig. 3. ΔF/Fo at 23 °C in 1:1 (mol/mol) DPPC:12SLPC mixtures with and without 15 mol % sterols. Samples were prepared as described in Fig. 2. The average and standard deviation of generally triplicate samples is shown. Ergos., ergosterol; Stig., stigmasterol; Choles., cholesterol; C10, C10-sterol; Sitos., sitosterol; C4, C4-sterol; None, no sterol.
chain four carbons shorter than cholesterol (26), not only failed to promote domain formation but actually inhibited it relative to that in samples containing no sterol (Fig. 2). Thus, sterol side chain structure can modulate domain formation.

Effect of Sterols on Lipid Insolubility in Triton X-100—Domains rich in saturated lipid and cholesterol can be detected by their insolubility in Triton X-100 (1, 16, 17, 20, 30). This insolubility is believed to be a result of the tight packing of lipids within such domains. It appears that lipid-lipid interactions within these microdomains are stronger than in lipid-detergent interactions (18). The amount of detergent-insoluble lipid recovered from a sample is not always precisely the same as the amount of lipid present within lipid rafts before extraction (18). Nevertheless, it has been found that the degree of insolubility in a model membrane sample is often nearly proportional to the degree of formation of tightly packed lipid domains as measured by fluorescence quenching at the same temperature (17, 20).

Fig. 4 shows the effect of sterol structure on lipid insolubility in Triton X-100. The set of natural sterols examined in the quenching experiments all strongly promoted insolubility at 23 °C as judged by the higher %OD in samples containing those sterols than that observed in the absence of sterol. This supports the conclusion that domain formation is promoted by the presence of these natural sterols. In addition, this finding indicates that the sterol-containing DPPC-rich domains formed in the presence of 15 mol % natural sterols contain lipids in a tightly packed state. Comparison of Figs. 3 and 4 shows that in general the differences in the degree of insolubility with different sterols closely parallel differences in quenching at 23 °C. In particular, it is noteworthy that ergosterol and 7-DHC gave rise to the greatest degree of insolubility, supporting the conclusion that they promote stable domain formation most strongly. Comparison of Figs. 3 and 4 shows samples with sitosterol have an anomalously low insolubility relative to that expected from the extent of domain formation (ΔF/F₀). This suggests that the DPPC-rich domains formed in the presence of sitosterol are not as tightly packed as those formed in the presence of the other natural sterols (also see below).

Domain Formation in Model Membranes with 33 mol % Sterol—Cell membranes can have sterol concentrations significantly higher than 15 mol %. To see if sterol concentration affects the dependence of domain formation on sterol structure, quenching profiles for samples containing 33 mol % sterol were compared with those for samples containing 15 mol % cholesterol. As shown by comparison of Figs. 3 and 5, the effects of sterol structure on the extent of domain formation at room temperature were very similar at these two sterol concentrations. Specifically the order of sterols in terms of most-to-least strongly domain-promoting is similar at 15 and 33 mol % sterol. Thus, it appears that the effect of sterol structure on domain formation is not strongly dependent on sterol concentration.

Fig. 5 also suggests that, as judged by DPH quenching, the extent of domain formation at 33 mol % sterol (Fig. 5) was generally less than at 15 mol % sterol (Fig. 3). However, in this case the increased sterol concentration could be decreasing ΔF/F₀ by altering domain composition and/or domain size rather than by decreasing the fraction of the bilayer in the form of DPPC-rich domains. For example, a drop in ΔF/F₀ as sterol concentration increases could mean that the DPPC-rich domains have an increased 12SLPC content. Alternately, if the domain size is very small at high sterol levels, in particular on the order of a few molecules in diameter, then DPH molecules at domain edges would give an anomalous quenching that would tend to obscure domain formation (calculation not shown).

Experiments at 33 mol % sterol were repeated using LeT-MADPH, a DPH derivative attached to a long, saturated, tri-methylamino-terminated alky chain. In contrast to DPH, which can partition roughly equally between ordered and fluid domains (27, 31), LeT-MADPH partitions preferentially into ordered domains (32). Thus, formation of an ordered domain depleted in quencher lipid should reduce quenching of LeT-MADPH fluorescence more strongly than that of DPH. As a result, LeT-MADPH should be a more sensitive probe of domain formation than DPH if the quencher-depleted DPPC-rich domains are in a more ordered state than the rest of the bilayer. The greater sensitivity of LeT-MADPH to the formation of DPPC-rich domains was confirmed by the increased ΔF/F₀ values detected when LeT-MADPH was substituted for DPH (Fig. 5). This indicates that the DPPC-rich domains formed in the presence of ergosterol, 7-DHC, cholesterol, and stigmasterol are more highly ordered than the remainder of the bilayer (which is enriched in 12SLPC). Interestingly the DPPC-rich domains formed in the presence of sitosterol did not exhibit a ΔF/F₀ with LeT-MADPH that was significantly higher than that with DPH. This suggests that the DPPC-rich domains formed...
in the presence of sitosterol are not highly ordered, consistent with their relatively loose packing as judged by solubility in Triton X-100 (see above).

It should also be noted that in control samples lacking domains, i.e. 1:1 12SLPC:DOPC, quenching of LcTMADPH and DPH was very similar (average $F/F_0$ 0.05 versus 0.043, respectively). This shows the difference by quenching of DPH and LcTMADPH in samples containing domains is not an artifact of an inherently lesser ability of 12SLPC to quench LcTMADPH relative to DPH.

**Effect of Sterols on Domain Formation by SM**—Sterol effects on domain formation by DPPC are informative because DPPC:sterol interactions have been characterized by numerous investigators (Ref. 20 and references therein). However, because sphingomyelin is the most common mammalian lipid with saturated acyl chains, the effect of sterol structure on domain formation in SM-containing mixtures was also examined. Fig. 6 and Table I show that the promotion of domain formation by sterols in SM:12SLPC mixtures was similar to that in DPPC:12SLPC mixtures. In the absence of sterol, there was no significant domain formation above 23°C. As in DPPC:12SLPC mixtures, in the presence of ergosterol and 7-DHC the SM-rich domains formed to a greater extent and were more thermally stable than those formed with cholesterol (Fig. 6 and Table I). In contrast, stigmasterol promoted domain formation to a lesser extent and imparted less thermal stability than did cholesterol (Fig. 6 and Table I).

The behavior of androstenol, a sterol that inhibits domain formation in DPPC:12SLPC mixtures (20), is also shown for comparison. As in the case of no sterol, there seemed to be little domain formation in the presence of androstenol.

**Effect of Sterols on Domain Formation by Cerebrosides**—Other than sphingomyelin, sphingolipids in mammalian cells are glycosphingolipids. We examined the effect of sterol structure on domain formation by CBs:12SLPC mixtures prepared at room temperature exhibited a decrease in $F/F_0$ on heating that was not fully reversible upon cooling. This indicated a metastable domain arrangement in CB-containing samples prepared at room temperature. For this reason, in the experiments presented in Fig. 7 the CB-containing samples were dispersed in buffer at 65°C, which did yield samples with thermally reversible changes in quenching. The overall pattern of the temperature dependence of quenching for samples prepared at 23°C was similar to that for samples prepared at 65°C (not shown). However, $T_{\text{mix}}$ for all samples was roughly 5°C higher for samples prepared at 23°C, and prior to warming larger differences between $F/F_0$ values for CB-containing and DOPC controls ($\Delta F/F_0$) were found for samples prepared at 23°C. These properties suggest that the samples prepared at 23°C contain domains that were more enriched in CBs than those formed at 65°C.

$^6$ Unlike DPPC:12SLPC and SM:12SLPC mixtures, CBs:12SLPC mixtures prepared at room temperature exhibited a decrease in $F/F_0$. This prompted us to test the effects of androstenol and coprostanol on domain formation by CBs. We previously found that these sterols have a tendency to abolish domain formation (20). Fig. 7 shows that androstenol and coprostanol both reduced the extent of, and destabilized, domain formation. However, they did not totally abolish domain formation and only decreased $T_{\text{mix}}$ by about 5–10°C (Table I, right column). Overall the domain-forming abilities of cerebrosides seemed much more weakly modulated by sterols than those of SM or DPPC. Possible implications of this behavior are considered under “Discussion.”

The Effect of Ceramide on Domain Formation—Ceramide is a sphingolipid lacking a complete polar head group. It is a key metabolic intermediate in sphingolipid signal transduction (34). Inclusion of a small amount of ceramide in lipid mixtures had striking effects on domain formation. Fig. 8 shows that as little as 3 mol% ceramide significantly stabilized domain formation in SM:12SLPC mixtures containing 15 mol% cholesterol increasing $T_{\text{mix}}$ by about 5°C. This effect was even more striking with 5.7 mol% ceramide, which induced an increase in $T_{\text{mix}}$ on the order of 15°C. The effects of 3 mol% ceramide were greater than those obtained by incorporating additional cholesterol or SM. Increasing cholesterol by 4 mol% had no detectable effect on domain formation, and increasing SM by 4 mol% only increased $T_{\text{mix}}$ by about 3°C.

**DISCUSSION**

The Relationship between Sterol Structure and Domain Formation and Its Possible Biological Implications—Most studies of sphingolipid/sterol rafts involve mammalian cells. However, detergent-resistant sphingolipid/sterol membrane fractions have been isolated from plants, fungi, and insects (which like fungi can have ergosterol-rich membranes) (22–24). This report shows that, like cholesterol, plant and fungal sterols promoted formation of ordered lipid domains that were detergent-resistant. Thus, it is likely that rafts composed of ordered lipid domains exist in plants, fungi, and insects as well as in mammalian cells.
Nevertheless, different natural sterols do not promote raft formation to the same extent. In particular, sterols that have a double bond in the B ring (between C-7 and C-8), i.e. 7-DHC and ergosterol, promote domain/raft formation more strongly than cholesterol. The structure of the aliphatic side chain of a sterol also influences the ability to form domains although generally to a lesser degree.

It should be noted that the higher propensity of ergosterol to form rafts does not necessarily mean that fungal plasma membranes will have a higher level of raft formation than those of mammalian cells. Fungal sphingolipids differ significantly from their mammalian counterparts in polar head group structure (35), and this could influence raft formation (see below). In addition, any differences between fungal and mammalian sterol/sphingolipid/phospholipid ratios and/or the degree of fatty acyl chain unsaturation of their phospholipids could significantly influence the degree of lipid raft formation in vivo.

The different extent to which sterols support domain formation may have biological implications. The difference between 7-DHC and cholesterol is intriguing in this regard. In Smith-Lemli-Opitz syndrome, there is an inability to convert 7-DHC to cholesterol, and 7-DHC levels are markedly elevated, at least in plasma (36, 37). Developmental effects of Smith-Lemli-Opitz syndrome have been explained by perturbation of signal transduction mediated by the cholesterol-linked Hedgehog protein (38). Whether the attachment of 7-DEHC to Hedgehog protein or 7-DHC perturbations of lipid raft structure contribute to this process is an interesting question for future studies.

The difference between promotion of domain formation by ergosterol and cholesterol may also have implications for the action of polyene antibiotics. The sterol selectivity of polyene antibiotics is believed to be a crucial factor in their specificity for fungi. Relative to cholesterol, ergosterol exhibits an increased ability to complex with certain polyene antibiotics (39). This raises the possibility that the molecular interactions involved in sterol-saturated lipid interaction are the same as those involved in polyene antibiotic-sterol complex formation. Another intriguing possibility yet to be explored is whether the apparent disruptive effects of polyene antibiotics on rafts account for some of their antibiotic action (6).

Effect of Sphingolipid Structure on Sterol-promoted Domain Formation: Difference of Sphingomyelin and Cerebroside Behavior—Domain formation was also dependent on the structure of the sphingolipid component. Sterol effects on $T_{\text{mix}}$ show that SM domain formation was very strongly promoted by natural sterols. In contrast, domain formation by CBs was surprisingly insensitive to sterols. One possible interpretation is that SM-rich domains accommodate higher amounts of sterol than CBs-rich domains.

It should be noted that previous results have shown that cholesterol can have relatively small effects on domain formation by cerebrosides or CBs/SM mixtures when the temperature is below the gel to fluid phase melting temperature ($T_m$) of pure cerebrosides in the absence of sterol (17, 40). However, such experiments only indicate that sterols have little effect on domain formation under conditions in which ordered phase lipid would exist in the absence of sterol. They do not define the effect of sterol on the stability of domain formation.

The difference between SM and CBs in terms of domain formation behavior suggests the possibility that SM and glycosphingolipids have distinct roles in the maintenance of lipid rafts in cells. However, in B16 melanoma cells, which normally have a roughly 1:1 SM:glycosphingolipid ratio, SM can be substituted for glycosphingolipid (in a glycosphingolipid-negative mutant) without appreciable effects on the amount of detergent-resistant membranes that can be isolated from the cells (41). This suggests the substitution of SM for glycosphingolipid does not affect the amount of rafts present prior to detergent addition. In addition, substitution with SM does not affect the sensitivity of detergent-resistant membranes to cholesterol depletion or detergent-resistant membrane protein composition (41). These results suggest that the details of sphingolipid structure and composition do not modulate raft formation in these cells. However, cellular lipid raft formation is often evaluated at low temperature, which might obscure differences between SM and glycosphingolipid behavior. In addition, the wild type cells membranes contained a 1:1 SM:glycosphingolipid mixture not pure glycosphingolipid. It is conceivable that the presence of SM obscures the effects of glycosphingolipids on raft behavior. The possibility that the details of the both the head group and acyl chain structure of sphingolipids may affect raft structure and function under some conditions cannot be ruled out.

Effect of Sphingolipid Structure on Sterol-promoted Domain Formation: Effect of Ceramide—One final striking observation in this report is that ceramide can strongly stabilize domain formation in sphingolipid/sterol/phospholipid mixtures. This is not totally surprising because ceramide can promote phase separation when mixed just with phospholipids (42, 43). In addition, the very recent comprehensive studies of Massey (44) have shown that relatively small amounts of ceramide (5–10%) can significantly increase the gel to fluid $T_m$ of SM. In other words, ceramide greatly stabilizes the ordered gel state. Because relatively little effect of ceramide upon SM/cholesterol mixtures was found, Massey (44) proposed that high ceramide levels (e.g. metabolically induced by a sphingomyelinase) might have the greatest impact in the formation of gel phase lipid in cholesterol-poor bilayers.

Our data suggest that domain behavior may be significantly impacted by ceramide in cholesterol-rich membranes. In support of this, it is noteworthy that the effects of ceramide upon the stability of ordered domains in the SM/phosphatidylycholine/cholesterol mixtures studied here appear to be greater than the effects of ceramide on ordered domain formation by SM alone. Assuming this difference does not result from variations in structures of the ceramides used in these two studies, the increase in $T_{\text{mix}}$ per percentage of ceramide (see Fig. 8) is roughly 2–3-fold greater than the ceramide-induced increase in
the $T_m$ for the gel to fluid transition in pure SM (44). The effect of ceramide on mixtures of SM and disorder-favoring phospholipids may be more relevant than its effects on pure SM because such mixtures more closely approximate biological membranes. The ability of ceramide to strongly promote domain formation could mean it can partially substitute for cholesterol under some conditions in vivo. On the other hand, the observation that the addition of ceramide greatly boosted $T_m$, under conditions in which additional cholesterol had little effect (Fig. 8) suggests the effects of cholesterol and ceramide on domain formation are not identical. Furthermore, the ordered phase formed by mixtures of saturated lipids and ceramide is likely to have properties quite different from that of the liquid ordered phase formed by saturated lipid and cholesterol. In any case, ceramide behavior is of great interest because SM turnover during cell signal transduction generates ceramide, which itself is a signaling molecule (34, 45).

The ability of ceramide to stabilize the formation of SM-rich domains is also important because it suggests a strong affinity of ceramide for sphingolipid/cholesterol rafts. Thus, it would be expected that ceramide would likely be concentrated within sphingolipid/sterol rafts, perhaps to an even greater degree than other raft components. This by itself may have important physiological consequences. It is possible that the ability of ceramide to participate in raft formation, stability, and/or organization may be important in signaling, as indeed has been proposed in very recent studies (46, 47).

In summary, these studies show that the structure of natural sterols and sphingolipids can greatly influence the tendency of lipid bilayers to form lipid domains/rafts. Such behavior may play a key role in regulating raft properties and/or dynamics and thus be one important reason for the diversity of sphingolipid and sterol structures in nature.

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Effect of the Structure of Natural Sterols and Sphingolipids on the Formation of Ordered Sphingolipid/Sterol Domains (Rafts): COMPARISON OF CHOLESTEROL TO PLANT, FUNGAL, AND DISEASE-ASSOCIATED STEROLS AND COMPARISON OF SPHINGOMYELIN, CEREBROSIDES, AND CERAMIDE

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