Interaction of Cholesterol with Sphingomyelin in Mixed Membranes Containing Phosphatidylcholine, Studied by Spin-Label ESR and IR Spectroscopies. A Possible Stabilization of Gel-Phase Sphingolipid Domains by Cholesterol†

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ABSTRACT: The ESR spectra from different positional isomers of sphingomyelin and phosphatidylcholine spin-labeled in their acyl chain have been studied in sphingomyelin(cerebroside)-phosphatidylcholine mixed membranes that contain cholesterol. The aim was to investigate mechanisms by which cholesterol could stabilize possible domain formation in sphingolipid-glycerolipid membranes. The outer hyperfine splittings in the ESR spectra of sphingomyelin and phosphatidylcholine spin-labeled on the 5 C atom of the acyl chain were consistent with mixing of the components, but the perturbations on adding cholesterol were greater in the membranes containing sphingomyelin than in those containing phosphatidylcholine. Infrared spectra of the amide I band of egg sphingomyelin were shifted and broadened in the presence of cholesterol to a greater extent than the carbonyl band of phosphatidylcholine, which was affected very little by cholesterol. Two-component ESR spectra were observed from lipids spin-labeled on the 14 C atom of the acyl chain in cholesterol-containing membranes composed of sphingolipids, with or without glycerolipids (sphingomyelin/cerebroside and sphingomyelin/cerebroside/phosphatidylcholine mixtures). These results indicate the existence of gel-phase domains in otherwise liquid-ordered membranes that contain cholesterol. In the gel phase of egg sphingomyelin, the outer hyperfine splittings of sphingomyelin spin-labeled on the 14-C atom of the acyl chain are smaller than those for the corresponding spin-labeled phosphatidylcholine. In the presence of cholesterol, this situation is reversed; the outer splitting of 14-C spin-labeled sphingomyelin is then greater than that of 14-C spin-labeled phosphatidylcholine. This result provides some support for the suggestion that transbilayer interdigitation induced by cholesterol stabilizes the coexistence of gel-phase and "liquid-ordered" domains in membranes containing sphingolipids.

The existence of lateral heterogeneity and the subsequent formation of domains in cell membranes is well established. In-plane heterogeneity in pure lipid bilayers has also been proposed but its existence has been more debated (*1*−*4*). Recently, the capping of ceramide-enriched domains (*5*) and the coexistence of gel and fluid phospholipid domains (*6*) have been visualized directly in giant lipid vesicles. In the past several years, the presence in cell plasma membranes of microdomains (rafts), enriched in sphingolipid and cholesterol and insoluble in detergent at 4 °C, has been proposed and supported by various types of experiment (*7*−*10*). Simons and Ikonen (*11*) suggest, without direct experimental proof, that cholesterol may complement from the inner, cytoplasmic monolayer, the length of the *N*-acyl chains of sphingolipids in the outer monolayer which is greater than the average monolayer thickness. Previously, other authors have proposed that cholesterol may stabilize bilayers by the interaction between two molecules, one in each monolayer, through their aliphatic chains (*12*−*14*). Recent data from one of our laboratories (*15*) indicate that both the OH at C3 and the aliphatic chain at C17 of the steroid nucleus are essential for the formation of detergent-insoluble sphingomyelin-cholesterol complexes. All these observations and hypotheses make studies on the ternary systems phosphatidylcholine-sphingomyelin-cholesterol of central interest in membrane biology.

In a previous paper, using spin-label electron spin resonance (ESR) spectroscopy (*16*), we have shown that in phosphatidylcholine-sphingomyelin binary mixtures, sphingomyelin-rich gel phase domains may coexist under certain conditions with phosphatidylcholine-rich fluid domains. The present study is devoted to modulation by cholesterol in ternary systems of the type phosphatidylcholine-sphingomyelin-cholesterol. ESR has been combined with IR spectroscopy to examine the interaction of the glycerolipid and

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[1] Abbreviations: n-SMSL, *N*-[n-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-sphingosine-1-phosphocholine; n-PCSL, 1-acyl-2-(n-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero-3-phosphocholine; SM, egg sphingomyelin; CB, brain cerebrosides; Gg, brain gangliosides; PC, egg phosphatidylcholine; chol, cholesterol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; IR, infrared.

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sphingolipid components, phosphatidylcholine, and sphingomyelin, with cholesterol. Sphingomyelin and phosphatidylcholine spin-labeled at different positions of the acyl chain are used to investigate the different states of lipid mobility characteristic of the various lipid phases or domains. IR spectroscopy is used to look specifically at the effects that cholesterol has on the amide I vibrational mode of the ceramide backbone of the sphingolipid. Our study supports the idea that cholesterol stabilizes the coexistence of gel-phase and liquid-ordered domains in bilayers containing sphingolipids, through transbilayer interdigitation of the sphingolipid *N*-acyl chain.

MATERIALS AND METHODS

Materials. Brain cerebrosides and egg and brain sphingomyelin were from Avanti Polar Lipids (Alabaster, AL). Egg yolk phosphatidylcholine was grade I from Lipid Products (South Nutfield, England) and brain gangliosides were a gift from Dr. G. D. Fidelio (CONICET, Universidad Nacional de Córdoba, Argentina). Cholesterol was from Sigma Chemical Co. (St. Louis, MO). Phosphatidylcholine spin-labels labeled at C5 or C14 in the stearoyl *sn*-2 chain of the lipid were synthesized as described earlier (*17*). Sphingomyelin spin-labels labeled at the C5 or C14 positions in the *N*-acyl chain were synthesized by the coupling reaction of the *N*-succinimidyl ester of spin-labeled stearic acid (*18*) with sphingosine-1-phosphocholine derived from bovine brain sphingomyelin (*19*).

Sample Preparation. For ESR measurements, 2 μmol of total lipids and 0.5% mol spin label were codissolved in chloroform or chloroform:methanol 2:1 v/v and evaporated under a stream of dry nitrogen gas. The residual solvent was removed by vacuum-drying overnight. The lipid film was then hydrated in 0.1 mL of 20 mM Hepes, 100 mM NaCl, 1 mM EDTA, pH 7.4, by incubation for 1 h at 50 °C (80 °C for samples containing brain cerebrosides) with vortexing. Samples were then pelleted into a 1-mm inside diameter (i.d.) glass capillary, the excess supernatant removed, and the capillary flame sealed.

For IR measurements, lipid mixtures were codissolved in chloroform or chloroform:methanol 2:1 v/v and evaporated under a stream of dry nitrogen gas. The residual solvent was removed by vacuum-drying overnight. The lipid film was hydrated in a D_2O buffer 20 mM Hepes, 100 mM NaCl, pH 7.4, by incubation for 1 h at 50 °C with light vortexing.

ESR Measurements. ESR spectra were recorded on a Varian Century Line 9 GHz spectrometer equipped with nitrogen gas flow temperature regulation. Samples in 1-mm i.d. glass capillaries were placed in a standard quartz ESR tube containing light silicone oil for thermal stability. Spectral data were collected digitally on a personal computer using software written by M. D. King of the Max-Planck-Institut. Outer hyperfine splittings ($2A_{max}$) were used to characterize the rotational disorder and rotational rates of the spin-labeled lipid chain segments (see, e.g., ref *20*). For rotational mobility with components in the slow-motional regime (*21*) this parameter provides a useful comparative indicator of segmental mobility in equivalent systems (e.g., ref *22*).

IR Measurements. Infrared spectra were recorded on a Nicolet Magna II 550 spectrometer, equipped with a mercury cadmium telluride detector. Samples were placed in a temperature regulated cell with CaF_2 windows and heated continuously at a rate of 60 °C/h over the 10−65 °C temperature range. Cell spacers of 50 μm were used. Spectra were taken using Rapid Scan software from OMNIC (Nicolet). For each degree of temperature interval, 306 spectra were averaged with a resolution of 2 cm^{-1}. Band maxima were determined from Fourier self-convoluted spectra (*23*); deconvolution parameters were full width at half-height (fwhh) equal to 13 cm^{-1} and band narrowing factor *k* equal to 2.

RESULTS

5-Position Spin-Labeled Lipids. The ESR spectra of 5-SMSL and 5-PCSL spin-labeled sphingomyelin and phosphatidylcholine in egg sphingomyelin, brain cerebroside, and egg phosphatidylcholine bilayer membranes in the fluid phase are compared in Figure 1 with those from the same spin labels in the corresponding sphingolipid and glycerolipid membranes containing 33 mol % cholesterol at the same temperature. All spectra contain a single axially symmetric component, corresponding to an ordered fluid environment. The molecular ordering effect of cholesterol is evident from the increased spectral anisotropy, i.e., larger outer hyperfine splitting and smaller inner hyperfine splitting, for all samples containing cholesterol (see, e.g., ref *24*). In every case, for samples both with and without cholesterol, the spectral anisotropy is greater for the 5-SMSL sphingomyelin spin label than for the 5-PCSL phosphatidylcholine spin label.

The temperature dependences of the increase in spectral anisotropy of the 5-PCSL phosphatidylcholine and the 5-SMSL sphingomyelin spin labels, on including 33 mol % cholesterol in the samples, are given in Figure 2, panels A and B, respectively. The quantity plotted in Figure 2 is the difference, $2\Delta A_{max}$, in outer hyperfine splitting between samples with and without cholesterol relative to the mean value of $2A_{max}$ for the two samples. The gel-to-fluid chain-melting phase transition of the egg sphingomyelin and brain cerebroside membranes that do not contain cholesterol is evident from the discontinuities in $\Delta A_{max}/A_{max}$ at approximately 30−35 and 50−60 °C, respectively. The difference in outer hyperfine splitting is defined such that if the spin-labeled lipid exchanges rapidly between domains rich-in and devoid of cholesterol, the resulting value will be the weighted mean of the difference between the two environments (*16*). If, on the other hand, the lipid-cholesterol system forms a homogeneous mixture, then the values of ΔA_{max} will not be related to a weighted average but will depend directly on the properties of the mixture. It is seen from Figure 2 that the values of ΔA_{max} are smaller in the ordered gel phase, but greater in the disordered fluid phase of the sphingolipids egg sphingomyelin and brain cerebroside than in the fluid phase of the glycerolipid egg phosphatidylcholine. Independent of whether the cholesterol-lipid membrane systems are homogeneous or inhomogeneous in structure, the interactions of cholesterol give rise to a greater ordering effect with the sphingolipids than with the glycerolipid, when both are in the fluid ordered phase.

The ESR spectra of the 5-SMSL and 5-PCSL spin labels in the fluid phase of equimolar mixtures of egg sphingomyelin or brain cerebrosides with egg phosphatidylcholine, both in the presence and in the absence of 33 mol % cholesterol,

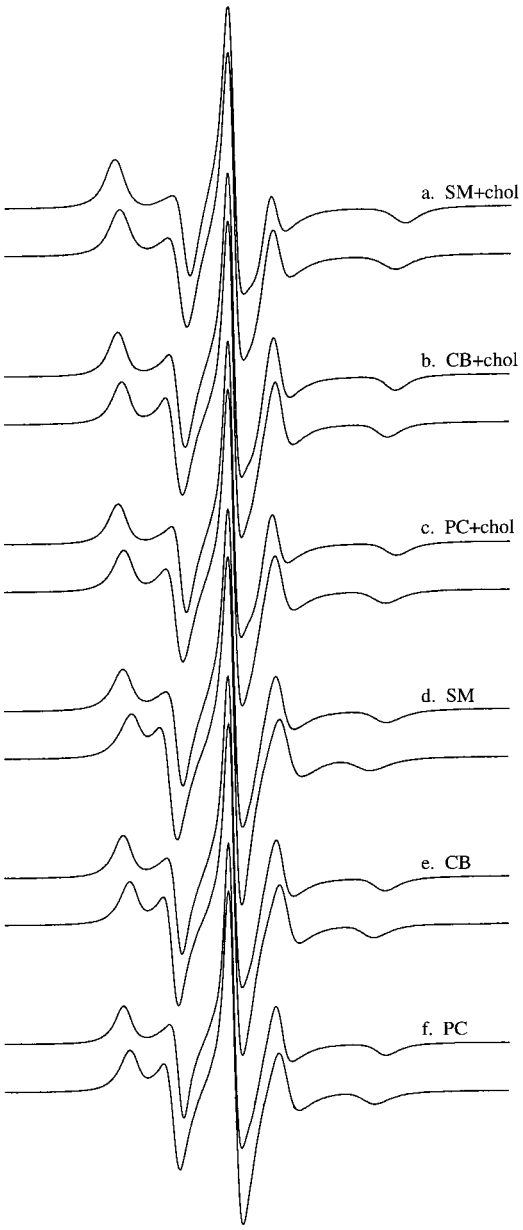

FIGURE 1: ESR spectra of the 5-SMSL sphingomyelin spin label (upper of each pair) and of the 5-PCSL phosphatidylcholine spin label (lower of each pair) in fluid bilayer membranes of (a) egg SM + chol 2:1 mol/mol at 50 °C, (b) brain CB + chol 2:1 mol/mol at 70 °C, (c) egg PC + chol 2:1 mol/mol at 37 °C, (d) egg SM at 50 °C, (e) brain CB at 70 °C, (f) egg PC at 37 °C. Total scan width = 100 G.

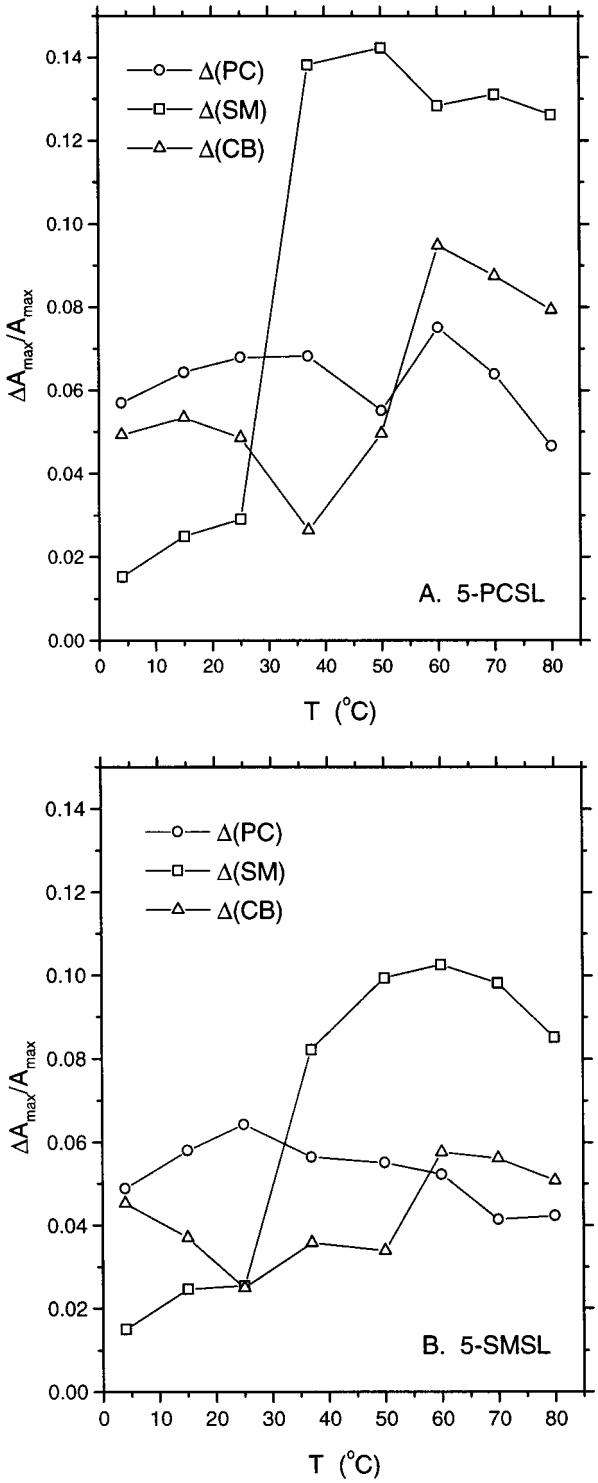

FIGURE 2: Temperature dependence of the normalized difference in outer hyperfine splitting, $\Delta A_{max}/A_{max}$, of (A) 5-PCSL phosphatidylcholine spin label and (B) 5-SMSL sphingomyelin spin label in sphingolipid or glycerolipid membranes with and without cholesterol. Differences: egg SM + chol (2:1 mol/mol) − egg SM (□); brain CB + chol (2:1 mol/mol) − brain CB (△); egg PC + chol (2:1 mol/mol) − egg PC (○).

also consist of single axially anisotropic components, qualitatively similar to those of Figure 1 (data not shown). The corresponding values of $\Delta A_{max}/A_{max}$ for the egg PC-containing mixtures are intermediate between those in the fluid and gel phase regions of the sphingolipids, SM or CB, alone. For the sake of clarity, these data points for the sphingolipid-glycerolipid mixtures are not included in Figure 2.

The differences in outer hyperfine splitting, $2\Delta A_{max}^{(i)}$, between various combinations of sphingolipids with the glycerolipid egg phosphatidylcholine and the corresponding single glycerolipid system (i.e., egg PC, indicated by the superscript *i*), all containing cholesterol, are given in Table 1 for both the 5-SMSL sphingomyelin and 5-PCSL phosphatidylcholine spin labels. In this table, the differences are normalized with respect to those between the component sphingolipid- and glycerolipid-cholesterol systems at the same total lipid/cholesterol mole ratio that are designated $2\Delta A_{max}^{(i,j)}$. The values of $\Delta A_{max}^{(i)}/\Delta A_{max}^{(i,j)}$ represent the tendency of the spin-labeled sphingolipid or glycerolipid to be associ-

Table 1: Differences in outer Hyperfine Splitting, $2\Delta A_{max}^{(i)}$, in the ESR Spectra of the 5-SMSL and 5-PCSL Spin Labels between Mixtures of Sphingolipids with the Glycerolipid Egg PC and the Single Glycerolipid, *i*, in Membranes Containing Cholesterol (unless otherwise noted, all at the same total lipid/cholesterol mole ratio)[a]

		50 °C		37 °C		25 °C		15 °C		4 °C	
membrane		5-SMSL	5-PCSL	5-SMSL	5-PCSL	5-SMSL	5-PCSL	5-SMSL	5-PCSL	5-SMSL	5-PCSL
SM + PC + chol	(1:1:1)[b]	0.21	0.31	0.23	0.19	0.13	0.29	0.37	0.32	0.50	0.42
	(1:1:1)[c]	0.40	0.38	0.46	0.43	0.47	0.54	0.66	0.62	0.76	0.67
	(2:2:1)[d]	0.37	0.23	0.28	0.25	0.22	0.14	0.18	0.17	0.37	0.29
	(2:2:1)[e]	0.00	0.14	−0.28	1.98	−0.31	−0.35	−0.47	−0.35	−0.19	−0.26
SM + Gg + PC + chol	(4:1:5:5)[f]	0.33	0.31	0.39	0.35	0.45	0.40	0.47	0.46	0.45	0.54
SM + CB + PC + chol	(1:1:2:2)[g]	0.35	0.37	0.39	0.37	0.42	0.43	0.47	0.46	0.60	0.58

		80 °C		70 °C		60 °C		50 °C		37 °C	
		5-SMSL	5-PCSL	5-SMSL	5-PCSL	5-SMSL	5-PCSL	5-SMSL	5-PCSL	5-SMSL	5-PCSL
CB + PC + chol	(1:1:1)	0.35	0.33	0.33	0.26	0.29	0.27	0.30	0.35	0.37	0.31

[a] The differences are normalized to those $[2\Delta A_{max}^{(i,j)}]$ between the two single lipid systems, *i* and *j* (i.e., glycerolipid or sphingolipid alone), that contain cholesterol. All ratios are mole ratios; values for the complementary differences, $\Delta A_{max}^{(j)}/\Delta A_{max}^{(i,j)}$, e.g., (SM + chol)−(SM + PC + chol), are simply one minus the values given, i.e., $[\Delta A_{max}^{(i)} + \Delta A_{max}^{(j)}]/\Delta A_{max}(i,j) = 1$. [b] Difference from PC+chol (2:1); the complement corresponding to 1.0 minus the values given is with SM+chol (2:1), as opposed to PC+chol (2:1), as reference state. [c] Difference from PC alone; complement SM + chol (1:1) [d] Difference from PC alone; complement SM + chol (2:1) [e] Difference from PC + chol (2:1); complement SM alone [f] Difference from PC+chol (2:1); complement SM + Gg + chol (8:2:5) [g] Difference from PC + chol (2:1); complement SM + CB + chol (1:1:1).

ated with a phase more like the sphingolipid-cholesterol system alone in the absence of glycerolipid (see ref *16*). A value of unity represents complete identity and a value of zero represents identity with the glycerolipid-cholesterol system. In the case of mixtures of egg sphingomyelin and egg phosphatidylcholine, values of the differences are also given with respect to values from the glycerolipid alone, or sphingolipid alone, at different cholesterol contents. These latter values of $\Delta A_{max}^{(i)}/\Delta A_{max}^{(i,j)}$ represent the tendencies of the spin-labeled sphingolipid or glycerolipid to associate with phases of different putative cholesterol/lipid stoichiometries (see footnotes to Table 1 and later Discussion).

The temperature dependences of the outer hyperfine splittings, $2A_{max}$, from cholesterol-containing sphingolipid-phosphatidylcholine mixtures and the corresponding single lipids with cholesterol are given in Figure 3, panels A and B, for 5-PCSL and 5-SMSL, respectively. The values of $2A_{max}$ for the sphingolipid-PC mixtures lie closer to those for the PC component than to those for the sphingolipid component, for both labeled lipids. They also lie below the mean values predicted from the values of the component single-lipids, for both spin labels (lines without symbols in Figure 3). This suggests either that the sphingolipid and glycerolipid components mix reasonably well in the cholesterol-containing membranes, or that the differences between the lipid environments are too small to be resolved by the 5-position spin labels.

14-Position Spin-Labeled Lipids: Gel Phase. The ESR spectra of the 14-SMSL sphingomyelin spin label and the 14-PCSL phosphatidylcholine spin label in bilayer membranes of egg SM or an egg SM + egg PC 1:1 mol/mol mixture, with and without 33 mol % cholesterol, are given in Figure 4. The spectra were recorded at low temperature for which egg SM membranes not containing cholesterol are in the gel phase and those containing 33 mol % cholesterol are predominantly in a liquid-ordered phase. Formation of the liquid-ordered phase is seen from the reduction in outer hyperfine splitting, $2A_{max}$, relative to the gel phase, on incorporation of cholesterol (compare Figure 4, panels a and c). The sphingolipid-glycerolipid mixture without cholesterol (i.e., egg SM + egg PC) is in a two-phase region at these temperatures, as is seen by the two-component ESR spectra of the 14-position spin labels (Figure 4d). Incorporation of cholesterol thus has a considerably greater effect on the lipid mobility in the mixtures than in egg SM alone, producing a pronounced chain ordering of 14-SMSL and an intermediate effect with 14-PCSL (compare Figure 4, panels b and d).

The relative effects of cholesterol on the 14-SMSL and 14-PCSL spin labels is therefore of central interest. Values of the outer hyperfine splittings, $2A_{max}$, of the 14-SMSL and 14-PCSL spin labels for the spectra of Figure 4 and also for the low-temperature phases of various other sphingolipids and their mixtures with egg PC, with and without cholesterol, are given in Table 2. With the exception of certain of the cerebroside-containing samples, the values of A_{max} are smaller for the 14-SMSL sphingomyelin spin label than for the 14-PCSL phosphatidylcholine spin label in membranes without cholesterol. This unusual, but highly characteristic, situation is reversed in all cholesterol-containing samples studied (see Table 2).

The outer hyperfine peaks of the cholesterol-containing samples in Figure 4, panels a and b, are asymmetric. For the egg SM + cholesterol mixture, the outer peaks have inner shoulders, while for the egg SM + egg PC + cholesterol mixture outer shoulders are present in the spectra (seen more clearly at 1 °C for 14-PCSL). The positions of these shoulders are indicated by the arrows for the high-field peaks in Figure 4, panels a and b. Although the resolution is not so pronounced as in the sphingolipid-glycerolipid mixture without cholesterol (i.e., Figure 4d), the spectra consist of two components that are characteristic of phase coexistence. Further examples of two-component spectra obtained from cholesterol-containing samples in the low-temperature regime are given in Figure 5. These correspond to mixtures of brain cerebrosides with egg sphingomyelin and/or egg phosphatidylcholine. Again, the outer hyperfine splittings of the two components (indicated by the arrows) are both large, unlike the situation for sphingolipid-glycerolipid mixtures without cholesterol. The reason for this is that the phase with higher lipid chain mobility corresponds to a liquid-ordered phase, rather than a liquid-disordered phase (see later Discussion).

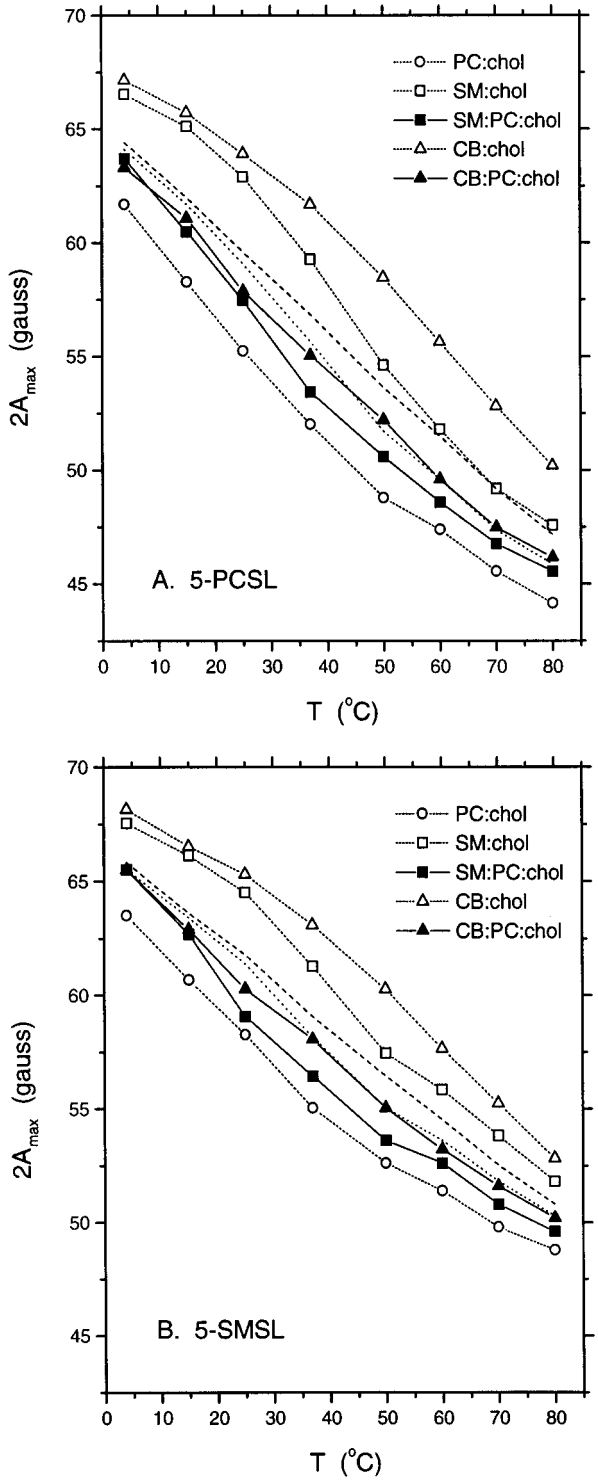

FIGURE 3: Temperature dependence of the outer hyperfine splittings, $2A_{max}$ of (A) 5-PCSL phosphatidylcholine spin label and (B) 5-SMSL sphingomyelin spin label, in bilayer membranes of egg SM + egg PC + chol 1:1:1 mole ratio (■), brain CB + egg PC + chol 1:1:1 mole ratio (▲), egg SM + chol 2:1 mol/mol (□), brain CB + chol 2:1 mol/mol (△), egg PC + chol 2:1 mol/mol (○). The lines without symbols give the mean values of $2A_{max}$ for egg SM + chol (2:1 mol/mol) and egg PC + chol (2:1 mol/mol) (dotted line) and for brain CB + chol (2:1 mol/mol) and egg PC + chol (2:1 mol/mol) (dashed line), respectively.

IR Spectroscopy. Fourier self-deconvoluted IR spectra of the sphingolipid amide I band from bilayer membranes of egg SM and its mixtures with cholesterol are given in Figure 6. At temperatures both above (55 °C) and below (18 °C)

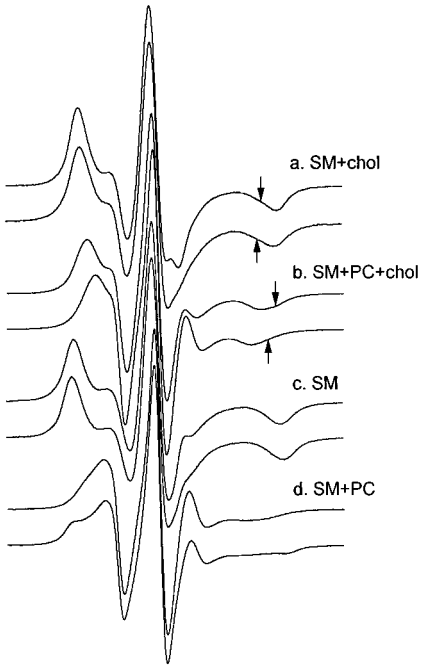

FIGURE 4: ESR spectra of the 14-SMSL sphingomyelin spin label (upper of each pair) and of the 14-PCSL phosphatidylcholine spin label (lower of each pair) in gel/liquid ordered bilayer membranes of (a) egg SM + chol 2:1 mol/mol, (b) egg SM + egg PC + chol 1:1:1 mol/mol/mol, (c) egg SM, and (d) egg SM + egg PC 1:1 mol/mol, all at 4 °C. Total scan width = 100 G. Arrows indicate the approximate position of the high-field hyperfine peak of a minority second component in the cholesterol-containing samples.

that of the chain-melting of egg SM membranes (38 °C), incorporation of cholesterol induces a progressive shift of the amide I band maximum to lower wavenumbers. These shifts are indicative of a change in hydrogen bonding pattern of the sphingolipid amide group with water, possibly involving direct hydrogen bonding to cholesterol. In contrast, incorporation of cholesterol has very little effect on the position of the carbonyl stretching bands of the glycerolipid egg PC in bilayer membranes, at either temperature (data not shown).

The temperature dependence in position of the sphingolipid amide I band maximum is given in Figure 7. Data are presented for bilayer membranes of egg SM alone, and its 1:1 mol/mol mixture with the glycerolipid egg PC, with and without 50 mol % cholesterol. For egg SM alone, there is an abrupt decrease in amide I band frequency on chain-melting at a temperature of ca. 38 °C. Incorporation of 50 mol % cholesterol abolishes this transition and shifts the amide I band maximum to considerably lower wavenumbers. Equimolar admixture with the glycerolipid egg PC decreases the amide I band frequency at temperatures corresponding to the gel phase of egg SM and increases it at temperatures corresponding to the fluid phase. No abrupt change in the amide I frequency is seen in egg SM + egg PC 1:1 mol/mol mixtures at temperatures above 18 °C. Incorporation of 50 mol % cholesterol in the egg SM + egg PC 1:1 mol/mol mixture uniformly decreases the amide I frequency throughout the temperature range studied, but to a considerably lesser extent than for egg SM alone. The relative shifts in amide I frequency on incorporation of cholesterol are given in Table 3.

Table 2: Outer Hyperfine Splittings, $2A_{max}$ (gauss), in the ESR Spectra of the 14-SMSL Sphingomyelin and 14-PCSL Phosphatidylcholine Spin Labels in Sphingolipid Membranes and in Mixed Sphingolipid-Glycerolipid Membranes, with and without Cholesterol[a]

		4 °C		15 °C	
membrane	mole ratio	14-SMSL	14-PCSL	14-SMSL	14-PCSL
SM + chol	2:1	58.9	57.7	56.5	55.0
SM	1:0	61.7	63.1	56.7	60.9
SM + PC + chol	1:1:1	56.1	46.2	45.2	41.7
SM + PC	1:1	53.6	62.9	38.1	63.7
CB + chol	2:1	58.9	57.3	57.7	(48.2)
CB	1:0	61.1	59.9	60.1	57.5
CB + PC + chol	1:1:1	55.2	56.1	46.8	43.6
CB + PC	1:1	51.0	53.2	-	-
CB + SM + chol	1:1:1	59.3	56.9	56.5	54.6
CB + SM	1:1	62.5	60.9	59.5	58.5
CB + SM + PC + chol	1:1:2:2	57.5	53.8	46.8	44.0
CB + SM + PC	1:1:2	51.6	53.6	56.9	35.5
SM + Gg + chol	8:2:5	59.3	58.5	57.3	55.2
SM + Gg	4:1	61.9	63.3	57.1	62.3
SM + Gg + PC + chol	4:1:5:5	57.3	48.0	45.8	42.7
SM + Gg + PC	8:2:5	53.4	62.3	–	–

[a] Measurements are made in the gel/liquid ordered phase at the temperatures indicated.

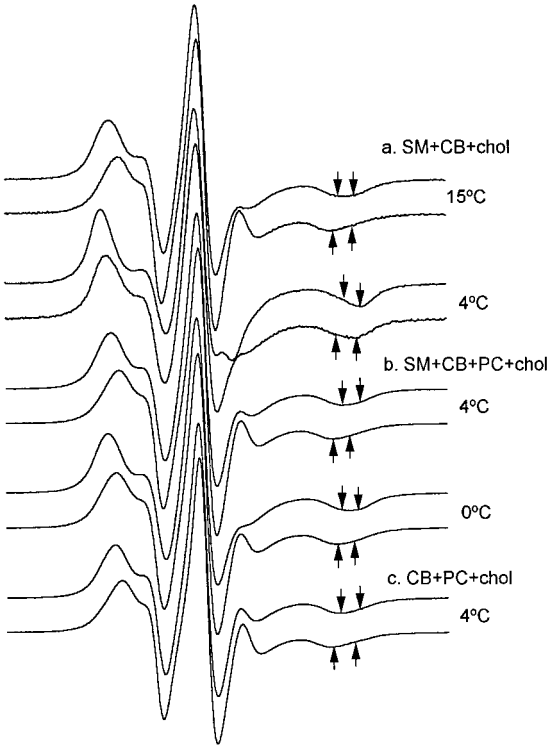

FIGURE 5: ESR spectra of the 14-SMSL sphingomyelin spin label (upper of each pair) and of the 14-PCSL phosphatidylcholine spin label (lower of each pair) in membranes of (a) egg SM + CB + chol 1:1:1 mol/mol/mol at 15 and 4 °C, (b) egg SM + CB + egg PC + chol 1:1:2:2 mole ratio at 4 and 0 °C, and (c) CB + egg PC + chol 1:1:1 mol/mol/mol at 4 °C. Spectra are recorded at the temperatures indicated. Approximate positions of the two components of the partially split high-field peaks are indicated by the arrows (see text). Total scan width = 100 G.

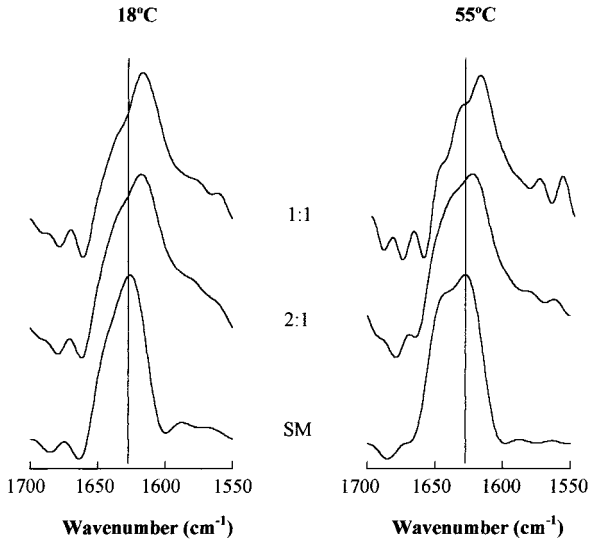

FIGURE 6: Fourier self-deconvoluted spectra of the amide I band of egg sphingomyelin in bilayer membranes of egg SM alone and mixtures at 2:1 and 1:1 mole ratios with cholesterol (bottom to top). Spectra are given at temperatures below (18 °C) and above (55 °C) the chain-melting transition.

DISCUSSION

Unlike the situation for the 14-position labels at low temperature (see later), the ESR spectra of sphingomyelin and phosphatidylcholine spin-labeled on the 5-position of the acyl chain consist of a single anisotropic component throughout the temperature range studied. This may be because the spectra are insufficiently sensitive to the mobility differences registered by the 14-position labels. At higher temperatures, the spectra may not register phase coexistence directly, if exchange of the spin-labeled lipids between two environments or domains, *i* and *j*, is rapid relative to the difference in spectral splittings, $\Delta A_{max}^{(i,j)} = A_{max}^{(i)} - A_{max}^{(j)}$, between the two environments. Then a single spectral component is obtained with mean hyperfine splitting (*25*):

$$A_{max} = f_i A_{max}^{(i)} + f_j A_{max}^{(j)} \quad (1)$$

where f_i and f_j are the fractional populations in the two environments ($f_i + f_j = 1$). In this situation, the values given in Table 1 should represent the fractional populations in environments identical to the single-lipid reference states used in calculating the difference in splittings (see Appendix in ref *16*). In situations not corresponding to fast exchange, the values given in Table 1 are a more empirical measure of similarity to the reference states, as already noted in Results.

The first feature to be deduced from Table 1 is that, by and large, the values from the spin-labeled sphingolipid

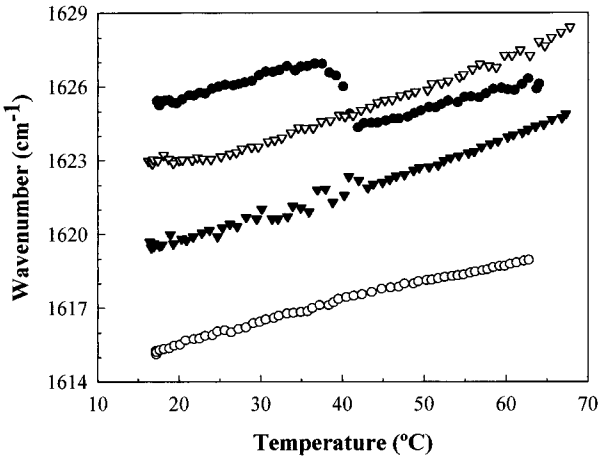

FIGURE 7: Temperature dependence of the position of the amide I band maximum of egg sphingomyelin in bilayer membranes of sphingomyelin alone and 1:1 mol/mol mixtures with egg phosphatidylcholine, with and without cholesterol. Egg SM alone (●); egg SM + chol 1:1 mol/mol (○); egg SM + egg PC 1:1 mol/mol (▽); egg SM + egg PC + chol 1:1:2 mol/mol/mol (▼).

5-SMSL do not differ drastically from those for the spin-labeled glycerolipid 5-PCSL. To the extent that the spin-labeled species reflect those of the parent unlabeled lipids, there is not an extremely strong tendency to demixing between the two lipid types in the cholesterol-containing membranes. For the mixtures SM + PC + cholesterol, alternatives with the superscripts *b* and *c*, and *d* and *e* in Table 1, depict different scenarios for the possible preferential association of cholesterol with either the sphingomyelin or phosphatidylcholine lipids. For the all-or-none extreme situations *d* and *e* with the 2:2:1 mol/mol/mol SM:PC:chol mixtures, association of cholesterol with sphingomyelin (i.e., option *d*) is clearly preferred because option *e* leads to extreme and negative values that are not easily rationalized in physical terms. Also for option *d*, spin-labeled sphingomyelin shows a preference mostly for the putative sphingomyelin-rich phase, relative to spin-labeled phosphatidylcholine. For the 1:1:1 mol/mol/mol SM:PC:chol mixture, the option *c* of preferential association of cholesterol with sphingomyelin is preferred over option *b* (i.e., equiprobable association of cholesterol with sphingomyelin and phosphatidylcholine) because, in general, it yields both a preference of 5-SMSL over 5-PCSL for association with the sphingomyelin component and higher values of the putative fraction of 5-SMSL associated with this component. On the whole, the trends for the more complex sphingolipid mixtures follow those discussed above, although conclusions are less clear-cut. The analysis presented is highly idealized and depends, of course, on the particular reference state chosen, but it does indicate a general tendency for preferential association of the sphingolipid component with cholesterol, particularly at lower temperatures. The latter region is now considered in more detail, in relation to the measurements with the 14-position spin-labeled species.

Low-Temperature Two-Phase Region. At low temperatures, the ESR spectra of the 14-position spin labels from several sphingolipid systems containing cholesterol consist of two components (see Figures 4 and 5). Unlike the situation of gel-fluid phase coexistence that is found in lipid mixtures not containing cholesterol (e.g., refs *16* and *26*), both spectral components have large outer hyperfine splittings. The reason for this difference is that, in the absence of cholesterol, the fluid phase is of the liquid-disordered type (l_d) for which the amplitude of lipid-chain segmental motion is large and consequently the outer hyperfine splittings are rather small. In the presence of cholesterol, at relatively low temperatures, however, the fluid phase is of the liquid-ordered (l_o) type, for which the outer hyperfine splittings of the spin-labeled chains are much larger. The two-component spectra of the 14-position spin labels therefore indicate a phase separation between cholesterol-rich l_o domains and cholesterol-poor gel-phase domains in the sphingolipid-containing mixed lipid membranes. Such phase separations have been found previously in cholesterol mixtures with saturated diacyl phosphatidylcholines (*12*, *27*, *28*), and also in the case of sphingomyelin-cholesterol mixtures by using ^{2}H NMR (*29*). The relative proportions of the two-phases change with temperature (see Figure 5), as is required by the phase rule. At a given temperature, the relative proportion of the two components also differs between the sphingolipid and phospholipid spin labels. Lower temperatures favor the phase with larger hyperfine splittings, as also does the 14-SMSL spin label relative to the 14-PCSL spin label. In the region that two-component spectra are resolved, the exchange of the spin-labeled sphingomyelin and phosphatidylcholine between l_o-phase and gel-phase domains, is slow relative to the difference in outer hyperfine splittings, δA_{max}, of the two spectral components (see, e.g., ref *25*). In the present case, this corresponds to frequencies in the region of $g\beta_e\delta A_{max}/\hbar \approx (4-6) \times 10^7\ s^{-1}$, which is comparable to the translational diffusion rates in fluid l_d-phases (*30*). It is therefore not surprising that lipid exchange is slow on this time scale in gel (and l_o-) phase mixtures.

Table 3: Shifts (Δ, cm^{-1}) in the Amide I Band Frequency of Egg SM Induced by Incorporation of Equimolar Cholesterol in Bilayer Membranes of Egg SM Alone and Egg SM + Egg PC 1:1 mol/mol

	Δ (cm^{-1})	
lipid	30 °C	60 °C
(SM + chol)-SM	−9.9	−7.0
(SM + PC + chol)−(SM + PC)	−3.0	−3.1
Δ/Δ_o[a]	0.30	0.44

[a] Shift for SM + PC 1:1 mol/mol relative to that for SM alone.

Cholesterol Interactions in the Gel/Liquid Ordered Phase. The most striking effect in gel phase membranes is the reversal of the relative values of the outer hyperfine splitting, $2A_{max}$, for spin-labeled sphingomyelin 14-SMSL and spin-labeled phosphatidylcholine 14-PCSL, on admixture of the sphingolipid-containing systems with cholesterol (Table 2). This is a feature restricted to labels near the end of the chain and is not observed for the 5-position labels. In the absence of cholesterol, the values of A_{max} are smaller for 14-SMSL than for 14-PCSL in the gel phase of all except certain of the cerebroside-containing systems. This has been explained in ref *16* by the difference in conformation at the point of acyl chain attachment in sphingolipids and glycerolipids that is revealed by their crystal structures (*31*). The result of this is that the spin-labeled *sn*-2 chain of phosphatidylcholine is effectively shorter by approximately 3 CH_2 groups than the partner *sn*-1 chain, whereas the spin-labeled *N*-acyl chain of sphingomyelin is effectively longer by approximately 4

CH_2 groups than the partner sphingosine chain. Hence, the outer hyperfine splitting of 14-SMSL is smaller because of the lack of intramolecular chain overlap at this position of *N*-acyl chain labeling (*16*). It was shown previously that this effect is modulated by the chain composition of the host sphingolipid, as is found here for the long-chain brain cerebroside.

In the present study, a pronounced intermolecular effect on the 14-SMSL chains, relative to those of 14-PCSL, is found with incorporation of cholesterol. The length of the entire cholesterol molecule is not greater than that of the lipid chains in the gel phase. Hence, the larger outer hyperfine splitting of 14-SMSL relative to 14-PCSL in the presence of cholesterol can be attributed to a partial interdigitation of the spin-labeled *N*-acyl chain from sphingomyelin across the bilayer mid-plane. This would be induced by the complementary presence of cholesterol in the opposing bilayer half of the membrane. Depending on relative chainlengths and their degree of asymmetry, two modes of interdigitation are possible. One, proposed previously as a mechanism for formation of liquid-ordered phases with symmetrical diacyl phosphatidylcholines by differential shortening of their chains (*12*), involves transbilayer interdigitation of cholesterol itself. The other, proposed as one of the mechanisms for stabilization of sphingolipid-cholesterol rafts in vivo (*11*), involves cholesterol filling voids in the cytoplasmic leaflet that are created by the transbilayer interdigitation of the *N*-acyl chains of sphingolipids from the exoplasmic leaflet. The present results on model systems suggest that some mechanism of this type can and possibly does take place.

Sphingolipid Amide Group. The relatively low frequency of the amide I band for membranes of egg SM alone (Figure 7) indicates that the amide group of the ceramide linkage of sphingomyelin is almost fully hydrated in both the gel and fluid states. For comparison, the amide I band from ganglioside G_{M1} micelles has a broad maximum at 1627 cm^{-1} for the amides of both the ceramide and the carbohydrate portions of the headgroup (*32*). Also, the amide I band from the ceramide portion of cerebroside sulfate in fluid-phase bilayer membranes displays a broad maximum at 1625 cm^{-1} (*33*). The relatively modest decrease in amide I frequency of approximately 2.5 cm^{-1} at the chain-melting transition of egg SM bilayers may be due to a somewhat increased degree of hydration in the fluid phase, and also to a change in polarity of the amide environment resulting from limited conformational changes (see also ref *34*).

Admixture of equimolar cholesterol produces a much greater decrease, of approximately 10 cm^{-1} (see Table 3), in the amide I frequency of egg SM than does chain melting. This suggests that direct intermolecular hydrogen bonding takes place between the 3 β-hydroxyl group of cholesterol and the amide group of egg SM. An amide I frequency of 1618 cm^{-1} has been reported for intermolecularly hydrogen-bonded cerebroside sulfate in the gel phase (*33*). This value is comparable to those found here for the egg SM + cholesterol 1:1 mol/mol mixture (see Figure 7).

Equimolar admixture of the glycerolipid egg PC with egg SM results in a downward shift of the amide I frequency at temperatures corresponding to the gel phase that is comparable to that produced by chain melting (see Figure 7). At temperatures corresponding to the fluid phase of egg SM, the amide I frequency is slightly higher for the egg SM + egg PC 1:1 mol/mol mixture. The origin of these shifts in frequency is most likely those mentioned above for chain melting of egg SM membranes. Spin label ESR data demonstrate that the egg SM + egg PC 1:1 mol/mol mixture is predominantly in a fluid phase over the temperature range given in Figure 7. Incorporation of equimolar cholesterol in the sphingolipid-glycerolipid mixture causes an appreciable shift of the amide I band to lower frequency (Figure 7). The shift relative to that for egg SM alone is given in Table 3. Clearly, the interaction between egg SM and cholesterol is attenuated by mixing with egg PC. Egg SM is not segregating with cholesterol to form a phase comparable to that of the egg SM + cholesterol 1:1 mol/mol mixture, despite the tendency for intermolecular hydrogen bonding with the sphingolipid that was discussed above. Most probably only part of the egg SM is hydrogen bonded to cholesterol in the sphingolipid-glycerolipid mixture. This intermediate situation is in agreement with the results from spin label ESR that were already discussed.

CONCLUSIONS

The present studies have revealed several important features not only of the domain formation in sphingolipid-glycerolipid mixtures with cholesterol, but also the possible mechanism by which cholesterol may stabilize such domains. By using different sphingolipids of natural origin we have attempted to establish those features of the interactions with cholesterol that are common to sphingolipids in general. Further, the *N*-acyl chain heterogeneity of the lipids used ensures that the effects observed are not highly specific to one particular chainlength, but rather are more representative of the chain composition in vivo. The biophysical results obtained here may therefore be of direct relevance to possible domain formation and membrane sorting in cellular systems.

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