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Review

Role of cholesterol in lipid raft formation: lessons from lipid model systems

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Abstract

Biochemical and cell-biological experiments have identified cholesterol as an important component of lipid 'rafts' and related structures (e.g., caveolae) in mammalian cell membranes, and membrane cholesterol levels as a key factor in determining raft stability and organization. Studies using cholesterol-containing bilayers as model systems have provided important insights into the roles that cholesterol plays in determining lipid raft behavior. This review will discuss recent progress in understanding two aspects of lipid–cholesterol interactions that are particularly relevant to understanding the formation and properties of lipid rafts. First, we will consider evidence that cholesterol interacts differentially with different membrane lipids, associating particularly strongly with saturated, high-melting phospho- and sphingolipids and particularly weakly with highly unsaturated lipid species. Second, we will review recent progress in reconstituting and directly observing segregated raft-like (liquid-ordered) domains in model membranes that mimic the lipid compositions of natural membranes incorporating raft domains.

Keywords: Sterol; Phospholipid; Sphingolipid; Lipid bilayer; Lipid monolayer; Membrane domain

1. Introduction

Cholesterol–lipid interactions have long been recognized as an important element in membrane structure and cholesterol homeostasis in animal cells. The nature of lipid–sterol interactions has accordingly been intensively studied, using a variety of physical methods (for reviews see Refs. [1–7]). An exciting new aspect of this field has emerged with the recognition that the plasma and other membranes of animal cells may incorporate laterally segregated domains known as 'lipid rafts.' As currently understood, rafts are considered to comprise relatively small (submicroscopic) domains that are depleted in unsaturated phospholipids and many (though not all) transmembrane proteins but enriched in cholesterol, sphingolipids and certain lipid-anchored proteins [8,9]. Rafts and related membrane microdomains such as caveolae have been proposed to play important roles in sorting of membrane molecules and in signal transduction in animal cells (reviewed in Refs. [10–20]).

Two major observations suggest that cholesterol–lipid interactions play an important role in the formation of rafts in animal cell membranes. First, as already noted, 'raft' fractions isolated from mammalian cells typically are found to be enriched in cholesterol [8,21,22]. Second, disruption or depletion of cell membrane-associated cholesterol can induce major changes in the distribution and/or function of raft-associated membrane components [23–31]. Studies of cholesterol-containing model membranes have played an important role in elucidating the physical bases for such findings and, more generally, in deepening our understanding of how cholesterol contributes to the formation and the organization of lipid rafts [32].

This review will discuss two types of studies of cholesterol–lipid interactions whose findings are particularly germane to understanding the origin and organization of lipid rafts. First, we will review the results of thermodynamic and spectroscopic studies that support longstanding suggestions that cholesterol interacts differentially with different membrane lipids and, among naturally occurring lipids, shows a particular affinity for sphingolipids. Further discussions of this aspect of cholesterol–lipid interactions can be found in the chapter by McConnell and Radhakrishnan in this issue

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[32,33]. These findings are of biological interest, not only for their potential relevance to the formation of lipid rafts but also in other physiological contexts, as for example in understanding the energetics of lipid sorting and transport between different cellular membranes. Second, we will consider recent studies demonstrating the formation of segregated liquid domains in model membranes combining cholesterol with phospho- and sphingolipids, and the important role played by cholesterol in modulating and even promoting such domain segregation. In both cases, we will focus on recent developments and unresolved questions in these areas, and on how progress in these fields is contributing to our understanding of the origin and properties of cholesterol-enriched microdomains in biological membranes. We begin with an overview of some of the particular challenges that have complicated investigation of the nature of sterol/lipid interactions.

2. Special challenges in studying cholesterol–lipid interactions

The interactions of lipids and sterols in lipid bilayers have been studied extensively, using a variety of spectroscopic and thermodynamic methods, for over 40 years. In spite of such intensive efforts, fundamental gaps remain in our understanding of the nature and consequences of cholesterol–lipid interactions. This situation in part reflects some distinctive features of cholesterol–lipid systems that complicate their analysis.

Characterization of the interactions between various phospho- and sphingolipids in lipid bilayers has been facilitated by the broadly similar (and relatively straightforward) phase equilibria of different species and by their tendencies to form large, coexisting domains in phase-separated lipid mixtures (for reviews see Refs. [34–36]). Cholesterol, by contrast, forms crystals rather than bilayers when dispersed in aqueous media, strongly broadens the phase transitions of phospho- and sphingolipids with which it is intermixed and can adopt complex patterns of lateral organization in mixtures with other lipids (reviewed in Refs. [1–7]). Such factors greatly complicate the analysis and modeling of the thermotropic properties of lipid–cholesterol mixtures. While significant advances have been made in evaluating these questions at both a theoretical and an experimental level, at present our picture of the microscopic organization of lipid/cholesterol bilayers is far from complete.

Ipsen et al. [37,38] provided an important conceptual tool for understanding lipid–cholesterol interactions by postulating, on the basis of theoretical simulations, that cholesterol and saturated phospholipids could form a 'liquid-ordered' phase that could coexist with other lipid phases such as the familiar 'fluid' (liquid-disordered) and gel phases formed by pure phospholipids. Subsequent NMR studies of lipid–cholesterol bilayers [39–42] provided experimental support for this hypothesis. These studies provided two important elements that have shaped much subsequent thought concerning the organization of cholesterol-containing membranes. First, in the concept of a 'liquid-ordered' phase, Ipsen and colleagues focussed and extended previous conclusions that cholesterol promotes a state of 'intermediate fluidity' between the familiar gel and fluid phases formed by pure phospho- and sphingolipids [2–7]. Specifically, these workers noted that liquid-ordered bilayer domains could exhibit a degree of *translational* freedom (crudely, lateral mobility) of the lipid molecules that was similar to that of a conventional fluid bilayer state, while at the same time the *configurational* freedom (order) of the lipid hydrocarbon chains more closely resembled that of the gel state. Second, the studies just described provided evidence that the liquid-ordered state could, at least in some circumstances, constitute a distinct *phase* that could coexist with other, more conventional gel or fluid lipid phases. These studies thereby offered theoretical support for the possibility that liquid-ordered domains might coexist with other lipid domains in sterol-containing biological membranes. While other workers have proposed phase diagrams for lipid/cholesterol binary mixtures that are more complex than that originally proposed by Ipsen et al. (see for example Ref. [43]), there is fairly broad agreement that such mixtures can exhibit at least one phase with liquid-ordered characteristics, and that liquid-ordered and liquid-disordered domains can coexist in these systems.

While the idea of a 'liquid-ordered phase' has proven very powerful for conceptualizing the possible nature of 'lipid rafts' and related domains in biological membranes, it is important to note that the origin and the detailed structure of the condensed phases favored by cholesterol remain to be fully clarified, even in some very fundamental aspects. Based on extensive monolayer studies (reviewed in Ref. [33] in this volume), McConnell and colleagues have proposed that cholesterol cooperatively forms condensed molecular complexes with certain lipid species (e.g., longer-chain saturated phospholipids), and that this phenomenon plays an important role in promoting fluid–fluid phase separations in biological membranes [44–46]. Other workers have presented evidence that cholesterol may adopt a regular distribution in mixtures with other lipids [47–50] and have suggested that segregated domains, exhibiting different lattice arrangements of sterol and lipid molecules, may coexist in cholesterol-containing bilayers. Huang and Feigenson [51] have shown by computer modeling that highly regular distributions of cholesterol and lipids can in principle arise without the formation of specific complexes (in a chemical sense) between the lipid and sterol molecules. A common feature of the above proposals is their emphasis on the importance of multibody, as opposed to strictly pairwise, interactions between lipid and sterol molecules in determining the organization and physical properties of lipid/cholesterol bilayers. Inclusion of such effects will enrich, but also inevitably complicate, theoretical modeling

of the organization of lipid–cholesterol systems, since in principle the relevant multibody interactions can take many different forms.

Alongside the theoretical complexities discussed above, it is worth noting that the preparation of homogeneous lipid–cholesterol samples can pose a significant experimental challenge in certain circumstances. Most conventional methods of lipid-vesicle preparation, in which the lipids are first dried down together from a solvent, entail the risk that lipid and sterol components may dry down at different rates as the solvent evaporates, yielding an inhomogeneous final sample. The problem can be mitigated somewhat by judicious choice of solvents and sample drying conditions [41; D. del Giudice and J. Silvius, unpublished results] but (at least in our experience) remains a challenge for samples with elevated cholesterol contents (>ca. 40 mol%). Buboltz and Feigenson [52] have recently described a novel method, based on rapid solvent removal in an aqueous medium, that provides homogeneous vesicle preparations with cholesterol contents ranging up to the apparent thermodynamic (saturation) limit. It will be of great interest to compare the physical properties of cholesterol-rich dispersions prepared using this or related methods (e.g., rapid dilution from ethanol [53]) to those previously reported for analogous dispersions prepared by conventional solvent-drying/hydration protocols.

3. Experimental evidence for differential interactions of cholesterol with different lipid species

A variety of methods has been used to examine the physical consequences of cholesterol incorporation into lipid bilayers [1–7]. While cholesterol produces qualitatively similar effects on the behavior of diverse types of phospho- and sphingolipids (e.g., ordering and condensing fluid-phase bilayers and 'fluidizing' gel-state bilayers), more detailed comparisons reveal significant differences in the interactions of cholesterol with different lipids. In the following sections we will review some of the experimental evidence for such differential interactions, particularly those that may occur in the biologically important fluid state.

3.1. Calorimetric studies

Early indications that cholesterol interacts differently with different lipid species were provided by results from calorimetric experiments (for reviews see Refs. [1–7]). Cholesterol strongly broadens the thermotropic gel-to-liquid-crystalline phase transitions of a variety of phospho- and sphingolipids [54,55]. However, careful analysis reveals quantitative and even qualitative differences in the effects of cholesterol on the phase transitions of phospholipids that differ in their acyl chain and/or headgroup structures (see for example Refs. [56–60]). Most dramatically, it has been reported that the gel-to-liquid-crystalline phase transitions of phospholipids with two polyunsaturated chains are not significantly perturbed by even equimolar proportions of cholesterol in the bilayer [61], while much lower bilayer concentrations of cholesterol strongly broaden the sharp phase transitions of saturated phospholipids [55].

Further suggestions that cholesterol may show differential interactions with different lipid species were provided by calorimetric studies of ternary (lipid/lipid/cholesterol) mixtures [62–64]. These workers noted that in mixtures of lipids exhibiting very extensive phase separation, cholesterol in some cases perturbed differentially the endothermic transition components associated with the different lipid species. From systematic studies of a variety of such ternary mixtures, van Dijck and colleagues concluded that cholesterol interacts with different headgroup classes of phospholipids with different affinities, in the order sphingomyelin>phosphatidylserine>phosphatidylcholine>phosphatidylethanolamine. While these conclusions are intriguing, it is difficult to deduce from such findings whether cholesterol interacts differentially with different phospholipid components in a given *single* phase (e.g., the fluid phase). Complicating further the interpretation of the findings just discussed, subsequent studies reported that the thermotropic behavior of other lipid mixtures, combining cholesterol with phospholipids with more nearly ideal miscibilities, did not suggest preferential interactions of the sterol with one type of lipid headgroup over another [65,66]. Nonetheless, as discussed in the following sections, two additional techniques, monolayer measurements and direct assays of cholesterol partitioning between different bilayer environments, have yielded results that support (at least qualitatively) the conclusions of the calorimetric experiments discussed above.

Estimates of the maximum solubility of cholesterol in different lipid bilayers have also suggested marked differences in the interaction of cholesterol with lipids with different headgroups, at least at high levels of cholesterol (for further discussion see the article by Bach and Wachtel [67] in this volume). Huang et al. [68] found that the maximum solubility of cholesterol in bilayers of various saturated and monounsaturated phosphatidylcholines was 67 mol%, as compared to only 51 mol% in phosphatidylethanolamine bilayers. In a subsequent theoretical analysis [69], these workers argued that this difference in solubility limits reflects an important difference in the nature of the interactions of cholesterol with the two types of phospholipids, dictated by the different effective sizes of the phospholipid headgroups. Other studies have suggested that the maximum solubility of cholesterol in phosphatidylserine bilayers may be substantially lower than that in bilayers of phosphatidylcholine or phosphatidylethanolamine with the same acyl chains [67], although divergent findings have been reported [58,70]. Brzustowicz et al. [71] have reported that the maximum solubility of cholesterol in bilayers of highly unsaturated phosphatidylcholines is substantially lower than that in bilayers composed of saturated or monounsaturated species, again suggesting that highly unsatu-

rated lipids may exhibit particularly weak interactions with cholesterol.

3.2. Monolayer measurements

Monolayer methods have long played an important role in the study of cholesterol–lipid interactions, providing among other insights the first demonstration of the 'condensing effect' that cholesterol exerts upon lipids in the fluid state (reviewed in Refs. [1,72]). At a given surface pressure, mixtures of cholesterol with various phospho- and sphingolipids typically show average molecular areas that are smaller than the weighted average of the molecular areas of the lipid and sterol components. The magnitude of this area-condensing effect (particularly at surface pressures thought to approximate those prevailing in bilayers) has been used as a rough indicator of the relative strength of interaction of cholesterol with diverse phospholipid species. Such studies have suggested for example that phospholipids bearing two unsaturated chains show markedly weaker area condensation than do species bearing at least one saturated chain [73,74].

Recent work has suggested that cholesterol-induced reductions in interfacial elasticity provide a more sensitive index than do area-condensation measurements to compare the strength of interactions between cholesterol and different lipid species in monolayers. In a study of phosphatidylcholine/cholesterol monolayers spread at a surface pressure considered to approximate that prevailing in biological membranes, Smaby et al. [75] showed that the extent of the interfacial elasticity reduction produced by cholesterol varies strongly with the structures of the acyl chains. The magnitude of this effect was found to be greatest for disaturated phosphatidylcholines, substantially less for species bearing one mono- or diunsaturated chain, still smaller for species bearing two such chains or one polyunsaturated chain, and very small for species bearing two polyunsaturated chains. The results obtained from both area-condensation and elasticity-reduction measurements thus suggest that highly unsaturated lipid species interact relatively weakly with cholesterol. This conclusion is supported by calorimetric results discussed earlier [61] and by fluorescence- and NMR-based findings that cholesterol exerts relatively weak ordering effects on highly polyunsaturated phospholipids in bilayers [76,77]. Interfacial-elasticity measurements suggest more generally that over a wide range of lipid acyl-chain compositions, species with increasing saturation show progressively stronger interactions with the sterol. As discussed later, these conclusions agree with those derived from studies of bilayer systems using other techniques.

Several recent monolayer studies have examined the interactions of cholesterol with different sphingolipids compared to phospholipids with similar physical properties. Initial monolayer studies based on area-condensation measurements, particularly using a lateral pressure thought to approximate that prevailing in biological membranes (30 mN/m), showed little difference in the interaction of cholesterol with sphingomyelins (or galactosylcerebrosides in the liquid state) compared to phosphatidylcholines with similar hydrocarbon chains [74,78]. In other aspects, however, such as the kinetics of cholesterol desorption or oxidation by cholesterol oxidase, cholesterol/sphingomyelin monolayers behave quite differently from similar monolayers incorporating chain-matched phosphatidylcholines [79–82]. In agreement with these latter findings, recent studies [83], measuring the elasticity-reducing rather than the area-condensing effect of cholesterol in monolayers, have suggested a significantly stronger interaction of cholesterol with sphingomyelins compared to phosphatidylcholines with similar hydrocarbon chain lengths. Studies of cholesterol interactions with sphingomyelin analogues in monolayers (and lipid vesicles) have suggested that this difference is mainly determined by the presence of an amide linkage in sphingomyelin [84,85].

3.3. Cholesterol-partitioning measurements

Cholesterol can exchange spontaneously between different fluid lipid vesicles much faster than do most membrane lipids, requiring a few hours or less to redistribute to equilibrium in some systems. By examining the equilibrium distribution of cholesterol between different lipid vesicles (or biological membranes), it has thus been possible to compare directly the affinities of the sterol for bilayers with different compositions (Table 1). Using this approach, Yeagle and Young [86] concluded that cholesterol associates with greater affinity with unsaturated phosphatidylcholines than with unsaturated phosphatidylethanolamines. Using a similar approach, Lange et al. [87] showed that cholesterol partitions with roughly twofold greater affinity into vesicles prepared from a saturated phosphatidylcholine or sphingomyelin than into vesicles prepared from an unsaturated (egg yolk) phosphatidylcholine. These studies suggested that in fluid bilayers cholesterol interacts differentially with different membrane lipids, with affinity varying in the order sphingomyelin>PC>PE, and can discriminate as well between species with different acyl chains. Studies of cholesterol partitioning between lipid vesicles with more complex compositions, including mixtures of lipids extracted from biological membranes, gave results entirely consistent with these conclusions [88–90].

Most of the cholesterol-partitioning studies just described were carried out using small unilamellar vesicles, which allow faster equilibration of cholesterol than do larger vesicles. We have recently shown, however, that β- or γ-cyclodextrins, acting catalytically, can strongly accelerate intervesicle cholesterol transfer, allowing ready measurement of the partitioning of cholesterol between large unilamellar vesicles with widely varying compositions [91]. Such measurements showed that the affinity of cholesterol for different phospholipids varies significantly with polar headgroup and 'backbone' structure, in the order sphingo-

Table 1
Partition coefficients (K_p) for cholesterol distribution between different donor and acceptor vesicles at 37 °C

Lipid composition A	Lipid composition B	K_p(B/A)[a]	Conditions[b]	Reference
Egg PC	Brain sphingomyelin	2.0	SUV, 46 °C	[87]
Egg PC	N-16:0 sphingomyelin	1.9	SUV, 46 °C	[87]
Egg PC	16:0/16:0 PC	1.9	SUV, 46 °C	[87]
16:0/181 PC	14:0/14:0 PC	1.8	LUV, 37 °C	[86]
16:0/181 PC	16:0/18:1 PC/PE (1:1)	0.8	LUV, 37 °C	[86]
16:0/181 PC	16:0/18:1 PC + 18:1/18:1 PE (1:1)	0.6	LUV, 37 °C	[86]
18:0/18:1 PC	18:2/18:2 PC	0.31	LUV, 37 °C	[91]
18:0/18:1 PC	18:0/20:4 PC	0.45	LUV, 37 °C	[91]
18:0/18:1 PC	18:1/18:1 PC	0.51	LUV, 37 °C	[91]
18:0/18:1 PC	18:0/18:2 PC	0.66	LUV, 37 °C	[91]
18:0/18:1 PC	18:0/18:1 PE + 18:0/18:1 PS (85:15)	0.67	LUV, 37 °C	[91]
18:0/18:1 PC	18:0/18:1 PS	1.45	LUV, 37 °C	[91]
18:0/18:1 PC	18:0/18:1 PC + 16:0/16:0 PC (50:50)	1.67	LUV, 37 °C	[91]
18:0/18:1 PC	18:0/18:1 PC + brain sphingomyelin (50:50)	2.56	LUV, 37 °C	[91]

[a] K(B/A) = relative preference for vesicles of composition B compared to composition A, calculated from data reported in the indicated references.
[b] SUV/LUV—small/large unilamellar vesicles.

myelin>PS>PC>PE. This order of affinities agrees well with that deduced from the earlier cholesterol-partitioning studies described above, and strikingly, with that deduced from calorimetric experiments as discussed earlier [62–64]. The affinity of cholesterol for phospholipids was also found to decrease strongly with increasing unsaturation of the lipid acyl chains. As a result of these combined factors, cholesterol for example associates roughly sixfold more avidly with (largely saturated) sphingomyelin than with 1-stearoyl-2-arachidonoyl phosphatidylcholine in fluid bilayers [91]. This result suggests that sphingolipid-enriched liquid-ordered microdomains will be relatively enriched in cholesterol, consistent with the compositional data reported for detergent-insoluble 'raft' fractions isolated from model and mammalian cell membranes [8,21,22].

3.4. Spectroscopic studies

As discussed earlier, ^{2}H-NMR studies of the behavior of bilayers combining cholesterol with saturated phosphatidylcholines played an important role in shaping our current picture of the 'liquid-ordered' state. These findings naturally raised questions whether other types of phospho- and sphingolipids can also adopt a liquid-ordered state in the presence of cholesterol. Thewalt and Bloom [92] concluded from ^{2}H-NMR measurements that mixtures of cholesterol and monounsaturated phosphatidylcholines can adopt a liquid-ordered state, and that the overall phase diagrams for these systems are broadly similar to those determined earlier for mixtures of saturated phosphatidylcholines and cholesterol [39–42], though shifted downward on the temperature axis in accord with the lower transition temperatures of the monounsaturated lipids. Fluorescence-lifetime measurements using *trans*-parinaric acid-labeled cholesterol/POPC bilayers have also suggested the formation of domains with liquid-ordered properties in this system [93]. In spite of this apparent qualitative similarity in the behavior of saturated and monounsaturated phospholipids in the presence of physiological levels of cholesterol, electron spin resonance experiments have suggested that the lateral interactions between lipid and cholesterol molecules are in fact significantly different in the two types of lipid–sterol mixtures [94–96]. NMR and fluorescence-spectroscopic studies have also suggested that bilayers composed of cholesterol and highly unsaturated phospholipids differ sharply in their lateral organization from bilayers combining cholesterol with more saturated lipid species [76,77].

3.5. Synthesis

The various approaches described above yield a broadly consistent picture of the differential interactions of cholesterol with different lipid species. First, the strength of such interactions, as assessed by a variety of measures, *gradually* decreases as the extent of lipid unsaturation increases. The common perception that cholesterol interacts preferentially with saturated lipid species in membranes, while technically correct, should thus not be extended to imply that all unsaturated lipid species behave identically in cholesterol-containing membranes. Using a fluorescence-quenching assay, we have found for example that monounsaturated, but not polyunsaturated, lipid species can partition to a significant extent into liquid-ordered domains in cholesterol-containing bilayers [97]. A second consistent finding from various physical studies is that the nature and affinity of lipid–cholesterol interactions can also vary significantly with the structure of the lipid polar headgroup and backbone. The preferential interaction of cholesterol with 'typical' membrane sphingolipids, compared to 'typical' membrane phospholipids, thus reflects differences in the structures of both the polar portions and the acyl chains of these two lipid classes. As discussed below, these differential interactions

can promote formation of segregated fluid lipid domains in bilayers combining cholesterol with sphingolipids and unsaturated phospholipids.

4. Evidence for lateral inhomogeneity in fluid bilayers containing cholesterol

4.1. Detergent-fractionation and fluorescence studies

While solid/fluid phase separations were demonstrated many years ago in lipid model membranes, the potential formation of laterally segregated lipid domains in mammalian cell membranes was long regarded as a strictly theoretical possibility, since gel-phase lipid domains are not observed in most such membranes at physiological temperatures. Cholesterol was moreover often considered to promote homogenization of lipid properties, and hence to suppress the tendency of lipids to form segregated domains, in biological membranes. Evidence to modify this view was first provided by a combination of fluorescence-microscopic studies of lipid monolayers [98,99] and, as discussed earlier, theoretical and NMR studies of cholesterol–phosphatidylcholine bilayers [37–42], which suggested that cholesterol could promote fluid–fluid phase separations. As noted earlier, this latter concept gained particular interest following the isolation and characterization of membrane 'raft' fractions from mammalian cells [8,9].

Studies like those just noted, suggesting the possible existence of lipid microdomains in the plasma (and possibly other) membranes of mammalian cells, received strong support from parallel studies of lipid vesicles constructed to mimic the lipid component of these membranes [100]. Schroeder et al. [21] demonstrated that when mixtures of sphingolipids, unsaturated phospholipids and cholesterol were treated in the cold with nonionic detergents such as Triton X-100, the lower-melting phospholipids were readily solubilized while the higher-melting sphingolipid species, and to a lesser extent cholesterol, were largely recovered in an unsolubilized, sedimentable fraction. These results strongly resembled those observed when mammalian cell membranes were treated with the same detergents under comparable conditions. Similar results were obtained using analogous lipid mixtures without cholesterol, or in which long-chain saturated phospholipids replaced the sphingolipid component. Measurements of diphenylhexatriene fluorescence polarization suggested that the existence of a detergent-insoluble fraction was correlated with the presence of ordered (gel or liquid-ordered) lipid domains in the original bilayers.

Direct evidence that cholesterol can *promote*, rather than simply support, the formation of segregated domains in fluid lipid bilayers was first obtained using fluorescence-quenching assays [32,97,101–104]. These assays demonstrated first, that at physiological temperatures mixtures combining cholesterol, unsaturated phospholipids and saturated phospho- or sphingolipids form laterally segregated domains and, second, that cholesterol can actually *induce* such domain segregation in bilayers containing relatively low levels of saturated lipids. Related fluorescence-quenching measurements have demonstrated that in such systems saturated phospholipids and sphingolipids, as well as peptides modified with multiple saturated acyl chains, partition with substantial affinities into domains enriched in the saturated lipid species. By contrast, multiply unsaturated species show essentially no affinity for such domains under the same conditions [97,105,106]. These findings provide direct support for previous hypotheses concerning the physical bases of 'raft' association of various membrane lipids and lipid-modified proteins [12,13,15,21]. Interestingly, however, mixtures combining physiological levels of cholesterol with lipids resembling those found at the inner surface of the plasma membrane do not appear to form coexisting lipid domains [107].

4.2. Microscopic studies

The fluorescence-quenching studies just noted provide direct evidence for the formation of laterally segregated domains in lipid bilayers with compositions mimicking that of the plasma membrane. However, fluorescence-quenching methods can in principle detect the segregation of even very small lipid domains, with dimensions as small as several nanometers or tens of nanometers [108]. Fluorescence and atomic force microscopy have recently been used in an effort to assess more directly the dimensions and morphologies of coexisting segregated domains in cholesterol-containing mono- and bilayers.

For technical reasons, epifluorescence microscopy was first used to examine monolayers combining cholesterol with phospho- or sphingolipids. These studies revealed that cholesterol-containing monolayers could exhibit segregated liquid-phase domains with micron dimensions under certain conditions of composition, temperature and surface pressure [44–46,98,99,109–114]. Such monolayers, however, typically show increasing intermingling (and a reduction in the average dimension) of the coexisting domains, and ultimately formation of a visually homogeneous phase, as the surface pressure increases toward a threshold value determined by the lipid composition [44,114,98]. For many monolayer compositions the threshold surface pressure falls below the values thought to prevail in bilayer membranes (30–35 mN/m), and it is thus unclear whether segregation of large domains would occur in bilayers with such compositions. Significantly, however, coexisting micron-sized liquid domains have been observed even at 'physiological' surface pressures in monolayers prepared from mixtures of unsaturated phospholipids, sphingomyelin and cholesterol [46]. Monolayer studies thus demonstrate, first, that cholesterol-containing systems can form large (micron-dimension) coexisting liquid domains under certain conditions (including, potentially, physiological conditions) and, second, that

the size, the morphology and even the existence of such domains can vary strongly in response to modest changes in variables such as composition or temperature.

Several recent microscopic studies have extended the conclusions just noted from mono- to bilayer systems [115–118]. These studies have demonstrated that mixtures of unsaturated phosphatidylcholine, sphingolipids and cholesterol, in supported bilayers, giant unilamellar vesicles or black lipid membranes, can exhibit coexistence of micron-sized segregated liquid domains. Qualitatively similar domains are observed in ternary mixtures in which the sphingolipid component is replaced by a long-chain saturated phospholipid, or using a lipid mixture extracted from renal epithelial brush-border membranes [115,117]. Atomic force microscopy (AFM) has provided a similar picture of the organization of mica-supported bilayers spread from sphingomyelin/unsaturated phospholipid/cholesterol mixtures at 'physiological' surface pressures (30–32 mN/m [119,120]). The coexisting domains in these systems appear fluid and dynamic, as indicated by their smooth, rounded borders and their ability to coalesce rapidly after contact [117]. Interestingly, fluorescence microscopy and AFM both indicate that low-temperature Triton X-100 extraction of supported bilayers selectively removes regions of the bilayer identified as liquid-disordered, while leaving essentially intact those identified as liquid-ordered [115,119,120].

Additional findings support the conclusion that the coexisting domains observed in cholesterol/unsaturated phospholipid/sphingolipid bilayers provide a useful, if approximate, model for 'raft' vs. 'non-raft' domains in mammalian cell membranes. First, the domains identified as liquid-ordered in the lipid model systems preferentially accumulate ganglioside GM_1 and the glycosylphosphatidylinositol-anchored (GPI-) protein Thy-1, both of which also associate with 'lipid rafts' in mammalian cells [115,117]. Second, antibody-mediated cross-linking of a saturated lipid probe, which as a monomer shows only a modest affinity for liquid-ordered domains, enhances partitioning into liquid-ordered domains by severalfold, consistent with findings that cross-linking markedly enhances association of certain membrane components with 'raft' fractions in mammalian cells [116]. Finally, and rather curiously, incorporation of as little as 1 mol% GM1 into the model lipid bilayers substantially reduced the partitioning of the GPI-anchored Thy-1 into liquid-ordered domains [116]. A similar reduction of GPI–protein partitioning into 'raft' domains was reported after incorporation of exogeneous gangliosides into the plasma membrane of mammalian cells [121]. The origin of this intriguing effect remains unknown, although other studies also raise the possibility that gangliosides, even at low concentrations, may exhibit rather complex behavior in membranes incorporating liquid-ordered domains [122,123].

The results of the above and other studies indicate that cholesterol can modulate the organization of bilayers combining saturated and unsaturated lipids in at least three ways. First, of course, as already discussed, in bilayers that exhibit coexisting (solid/fluid) domains even in the absence of cholesterol, the addition of cholesterol can transform solid-phase domains into liquid-ordered domains. Second, and also as discussed earlier, in some cases cholesterol can induce or enhance the formation of segregated domains under conditions where such segregation does not occur in the absence of sterol (see for example Ref. [116]). Finally, the presence of cholesterol can alter markedly the dimensions and morphology of segregated domains in lipid bilayers. Perhaps most interesting in this regard are observations suggesting that under certain conditions, cholesterol may promote the formation of very small domains that cannot be observed by microscopy but whose presence can be detected by fluorescence-quenching approaches. Dietrich et al. [115] thus observed, for example, that in giant cholesterol/sphingomyelin/dioleoyl phosphatidylcholine vesicles no microscopically visible segregated domains were present above ca. 26 °C. By contrast, fluorescence-quenching measurements, which monitor lipid lateral distributions on much smaller distance scales (nm), indicate that bilayers with such compositions continue to exhibit domain segregation even at physiological temperatures [102]. Similar evidence for the formation of segregated liquid domains smaller than the optical resolution limit has been obtained in other systems incorporating physiological proportions of cholesterol (compare for example Refs. [101] and [124]). Varma and Mayor [124] have proposed that under some conditions, such lipid mixtures may not undergo true phase separation but rather exhibit a 'nanocomposite' organization, with more and less ordered domains intimately intermingled on a submicron scale of distances. These findings are of interest in the light of observations that in unperturbed mammalian cell membranes, 'lipid rafts' are submicroscopic domains [125,126] and may have dimensions as small as a few tens of nanometers [126]. Interestingly, depletion of cholesterol from the plasma membranes of CHO cells has been reported to increase the size of segregated lipid domains to micron dimensions [127].

Results obtained using both fluorescence and atomic force microscopy indicate that the segregated domains in sphingolipid/unsaturated phospholipid/cholesterol bilayers are aligned between the two leaflets of the bilayer [115,117,119,120], as has been demonstrated previously for cholesterol-free bilayers exhibiting gel/liquid-crystalline phase separation [128]. It is thus clear that in fluid, cholesterol-containing lipid bilayers, correlation of physical properties between the two bilayer leaflets is possible. This finding is noteworthy in view of the apparent transmembrane character of 'rafts' in biological membranes, as evidenced for example by correlations in the distributions of 'raft' markers between the inner and outer leaflets of the plasma membrane in some systems [129–132]. However, since the cytoplasmic and extracytoplasmic leaflets of the plasma membrane differ greatly in composition [133,134], it

is not yet clear whether the physical factors that promote alignment of liquid-ordered domains in symmetrical model membranes are the same as those that confer a transmembrane character to 'rafts' in biological membranes.

4.3. Synthesis

Observations that cholesterol can actually enhance, rather than merely support, the tendency of saturated phospho- and sphingolipids to segregate from unsaturated phospholipids in bilayer membranes are consistent with findings discussed in earlier sections that cholesterol interacts with greater affinity with saturated membrane lipids. Observations that in some cholesterol-containing bilayers segregated domains of submicroscopic dimensions may be present, and that the positions of liquid-ordered domains can be correlated between the two bilayer leaflets, have evident potential implications for understanding the organization of 'lipid rafts' in mammalian cell membranes.

Results from studies of model membranes also raise, but do not resolve, intriguing questions concerning other key aspects of raft organization. The classic image of a lipid raft is a small, closed patch of liquid-ordered lipids surrounded by a continuous liquid-disordered phase. The results of studies of cholesterol-containing model systems, however, suggest other possible geometries for lipid rafts under certain conditions, ranging from large domains to an extended (stripe) or reticular arrangement of domains to a 'nanocomposite' organization of very small, intimately intermingled liquid-ordered and liquid-disordered domains [76,115,117,124,135]. It is an open question which of these alternatives may provide the most appropriate description of raft organization in a given cell membrane. Similarly, to date, little is known to define to what degree the geometries and sizes of rafts may be dictated (and regulated) by factors such as membrane proteins [135,136] or whether changes in these aspects of raft organization play important roles (physiologically or pathologically) in cellular function. In the latter regard, however, the examples of T-lymphocyte and mast cell activation suggest that changes in at least the distribution of raft domains on the cell surface may play important roles in cellular signaling [130,137,138].

5. Future directions

Studies of lipid model systems continue to shape our understanding of the physical principles governing the formation and organization of lipid rafts, and of the key role played by cholesterol in determining raft organization and behavior. Recent successes in applying microscopic approaches to study cholesterol-containing model systems underscore the potential of such systems to provide further important insights into the origins and properties of lipid rafts in biological membranes. Increasingly sophisticated model systems, for example incorporating membrane proteins [139,140], or 'asymmetric' bilayers whose two leaflets differ in their lipid composition will allow us to better assess how these important features of biological membrane organization can influence the formation of raft structures. Studies of the dynamics and stability of segregated lipid domains in cholesterol-containing bilayers [115,117] will continue to provide useful information to understand the analogous properties of rafts and raft-associated molecules in intact cell membranes [141]. Finally, novel microscopic methods such as near-field scanning microscopy [142] and atomic force microscopy [118–120] may allow us to investigate directly the sizes, the morphologies and even the existence of lipid domains whose dimensions fall below the normal resolution limits of optical microscopy.

Much work remains to define fully the organization and dynamics of raft domains in biological membranes. Model system studies like those just noted promise to continue to provide important insights and novel approaches toward this objective.

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