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Topical Review

Structure and Origin of Ordered Lipid Domains in Biological Membranes

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Introduction

The clearest function of membrane lipids is to form amphipathic bilayers that surround cells and organelles and block leakage of hydrophilic compounds while housing membrane proteins. However, the wide variety of lipids observed in biological membranes would not be required for a simple barrier function. Phospholipids alone display a variety of headgroup and acyl chain structures, and eukaryotic cell membranes often contain sphingolipids and sterols as well. Functional consequences of this lipid heterogeneity are starting to emerge.

One such consequence is the possibility of nonrandom mixing in the bilayer and the formation of lipid microdomains. It is clear that microdomains can form in artificial bilayers [55]. However, despite much interest in the subject, convincing evidence that lipids can cluster in cell membranes has been slow to emerge.

This review summarizes evidence that one type of microdomain may exist in cell membranes. Most of this evidence has come from studies of membrane fragments that are insoluble in cold non-ionic detergents such as Triton X-100. These detergent-resistant membranes (DRMs) are rich in cholesterol and sphingolipids, and may exist in membranes in the liquid-ordered (l_o) phase or a phase with similar properties. These studies may provide some of the first evidence for phase separation in biological membranes. Readers are also referred to our recent reviews of DRMs and the ordered-domain model [5, 6], and to two other insightful reviews of these domains [8, 82].

Increasing evidence suggests that another type of domain, formed by electrostatic interactions between membrane-associated components, may exist in membranes [44]. One intriguing example is the ability of a membrane-associated positively charged peptide derived from the MARCKS protein to organize domains rich in phosphatidylinositol bisphosphate [19]. Though potentially very important, formation of these domains will not be covered here.

Lipids Can Undergo Phase Separation in Membranes; Description of the l_o Phase

Phospholipid bilayers usually exist in a ''frozen,'' ordered gel phase at low temperatures. Above a melting temperature (T_m) that is characteristic of each lipid, the bilayer is present in a phase, termed liquid-crystalline (l_c) or liquid-disordered (l_d), in which the lipid acyl chains are fluid and disordered. Binary mixtures of lipids with different T_m can be examined at temperatures between the T_m of the two lipids. When one component is present at low levels, the mixture is uniform, and generally remains in the phase favored by the major component. Above a threshold concentration of one component, cooperative phase separation occurs, and gel and l_c phase domains coexist.

Eukaryotic cell membranes contain mixtures of glycerolipids (in mammalian cells, all phospholipids except sphingomyelin), sphingolipids, and sterols. Biological glycerolipids generally have very low T_m, while sphingolipids (especially glycosphingolipids) have much

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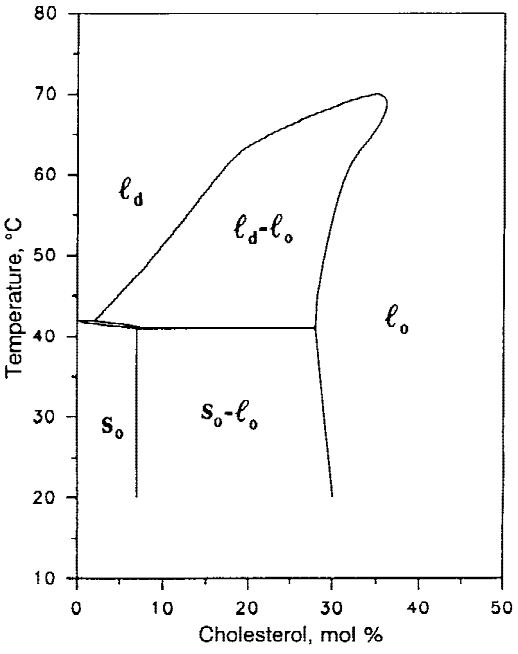

Fig. 1. Temperature-composition phase diagram of the DPPC-cholesterol system. Reproduced with permission from [72].

higher T_m. This disparity suggests that phase separation between glycerolipid- and sphingolipid-rich domains might occur. Consistent with this possibility, indications of glycosphingolipid clustering in the plasma membrane have sometimes been observed morphologically [94].

Phase separation may explain the observation that cellular membrane lipids are not completely solubilized by nonionic detergents such as Triton X-100 [7, 104]. Low-density detergent-resistant membranes (DRMs) that are enriched in sphingolipids [7, 47, 53] and cholesterol [7] and have a clear bilayer appearance [7] were isolated from mammalian cell lysates several years ago [7, 23, 48, 104]. Two clues suggested that DRMs might originate from ordered lipid domains. First, model membrane studies showed that the high T_m of sphingolipids was an important determinant of detergent insolubility [77, 78]; reviewed in [6]. Second, dipalmitoyl phosphatidylcholine (DPPC) was found to be Triton insoluble when it was in the gel phase, but soluble when in the l_d phase [65, 78].

Although phase separation between l_c and gel phases has been well characterized in model membranes, the gel phase does not appear to exist in biological membranes except in unusual cases [59]. However, phase separation between two fluid phases in model membranes containing binary mixtures of saturated-chain lipids (which have high T_m) and cholesterol has been described [25, 26, 36, 63, 71, 72, 99]. In this case, above the T_m of the lipid, a liquid-ordered (l_o) phase can separate from the l_d phase as the amount of cholesterol is increased (Fig. 1). (Similarly, the l_o phase can separate from the gel phase below the T_m.) Acyl chains of lipids in the l_o phase have properties that are intermediate between those of the gel and l_d phases. They are extended and ordered, as in the gel phase, but have high lateral mobility in the bilayer, as in the l_d phase [54]. Phase separation between the l_d and l_o phases also occurs in ternary mixtures containing high- and low-T_m lipids and cholesterol [1, 81].

Several lines of evidence suggest that DRMs are in the l_o phase. First, model membranes that are similar to DRMs in terms of being rich in sphingolipids and cholesterol are in the l_o phase [1]. Second, model membrane studies reveal a good correlation between the presence of the l_o phase and detergent insolubility [1, 78]. Increasing the concentration of sphingolipids and cholesterol in model membranes can promote both l_o phase formation [1, 78, 81] and detergent insolubility [1, 78], and detergent insolubility is not observed unless the l_o phase is present. Third, depletion of sphingolipid and cholesterol in cells reduces detergent insolubility [9, 21]. Finally, the acyl chain ''fluidity'' of DRMs, as measured by the fluorescence polarization of diphenylhexatriene, is similar to that in l_o phase bilayers [77].

However, it should be emphasized that the existence of l_o phase domains in cell membranes has not been directly demonstrated. Furthermore, if these domains are present, they may be similar but not identical to the l_o phase described in model membranes. For simplicity, we will use the terms ''l_o phase'' or ''ordered phase'' to describe these sphingolipid and cholesterol rich membrane domains.

Certain Proteins Prefer l_o Phase Domains

The physiological significance of phase separation is likely to stem from the preferential partitioning of certain proteins into one of the two phases. It has been known for several years that proteins anchored in membranes by glycosyl phosphatidylinositol (GPI) are detergent-insoluble [35] and are present in DRMs isolated from cells [7] and liposomes [77]. GPI-anchored proteins generally contain saturated acyl chains [43], which might be expected to prefer an ordered lipid environment and are likely to target the proteins to l_o phase domains. Several other acylated proteins linked to two or more saturated acyl chains are also present in DRMs [6]. It is likely that the acyl chains target many of these to ordered domains, as has been shown for Src-family kinases [80]. Caveolin, a structural protein of caveolae (*see below*) may be an interesting exception, as both the wild type and a nonpalmitoylated mutant are present in DRMs [11].

Does Detergent Affect Domain Structure?

Because l_o phase liposomes are Triton-insoluble, while those in the l_d phase are soluble [77], and because assays

of phase separation are difficult to apply to cells, detergent-insolubility is one of the most powerful probes of phase behavior in cell membranes. However, the specter of detergent-induced artifact has loomed over the study of DRMs. Do DRMs correspond to domains in cell membranes, or are the domains altered or even created by detergent? This section describes several possible artifacts and experiments that have addressed some of them.

One major early concern was that detergent could create insoluble lipid domains from uniform l_d phase membranes. This might occur by selective extraction of low-T_m phospholipids, leaving the remaining sphingolipids to pack tightly with cholesterol to form DRMs in an l_o-like phase. Two studies showed that this does not occur [1, 78]. In these studies, lipids that favored l_d and l_o phases were mixed in various ratios and subjected to detergent extraction. Comparison with results obtained from independent detergent-free methods showed whether the membranes were purely l_d or l_o phase, or whether both l_d and l_o phase domains were present. In both studies, detergent insolubility was found only when the l_o phase was present prior to detergent addition, showing that detergent did not reorganize lipids in the l_d phase to create insoluble domains.

Another concern is that insolubility might reflect slow solubilization, and that lysates containing insoluble membranes might not have reached equilibrium. However, detergent solubilization was shown to plateau and stabilize even when insoluble membranes remained [1, 77].

Detergent might also affect the distribution of lipids between membranes. For instance, order-preferring lipids present at low concentration in a l_d phase membrane might ''hop'' into l_o phase domains in separate membranes in the same lysate. The following experiment suggested that this does not occur [78]. Two sets of vesicles, one in the l_d phase and the second in the insoluble l_o phase, were mixed and extracted with Triton. As expected, a trace amount of radioactive sphingomyelin incorporated into the l_o phase vesicles was insoluble, as was the bulk unlabeled sphingomyelin in the same vesicles. However, the radioactive sphingomyelin was fully solubilized if it were incorporated instead into the l_d phase vesicles, although abundant insoluble membranes derived from the l_o phase vesicles were present in the lysate. A similar approach showed that a GPI-anchored protein in detergent-soluble liposomes mixed with protein-free insoluble liposomes prior to detergent lysis did not ''hop'' into the insoluble membranes [78], as has also been shown in cells [7].

These studies address some of the most serious questions about DRMs. Most importantly, detergent does not create insoluble membranes from fully l_d phase membranes. However, some concerns remain. For one, temperature effects must be considered. Formation of ordered domains is strongly favored as the temperature is lowered. Detergent insolubility experiments have generally been performed on ice, and most DRM proteins are solubilized at temperatures above 10–20°C [45]. Thus, data from detergent insolubility studies do not show that l_o domains are present in cell membranes at physiological temperatures. (Of course, the domains may exist, but may be solubilized by detergent at 37°C.) However, morphological studies (*see below*) suggest that domains can exist even at elevated temperatures. In addition, the finding that the l_o phase forms at 37°C in liposomes roughly modeled on the plasma membrane lipid composition [1] shows that phase separation in biological membranes is plausible.

Another concern stems from the observation that when two-phase liposomes containing both l_o and l_d phase domains were extracted with Triton, some of the membrane in the l_o phase was solubilized [78]. This suggests that detergent insolubility may underestimate the amount of the l_o phase in membranes that contain both l_o and l_d phase domains. This effect could be selective; certain lipids that associated with l_o phase domains might be preferentially extracted, and not be detected in DRMs.

The control experiments described above focused mainly on possible detergent effects on organization of lipids in domains. Possible detergent effects on the association of proteins with domains have received much less attention. One concern is that proteins, like lipids, present in l_o phase domains might be selectively extracted by detergent. Thus, the absence of a protein from DRMs cannot show that it is not present in l_o domains. Another concern was raised by a study in which cell remnants were examined by electron microscopy after Triton extraction *in situ* [40]. A GPI-anchored protein that was uniform in the membrane appeared more clustered after detergent extraction, suggesting that detergent may alter the distribution of the protein [40].

Finally, as detailed in the next section, inspection of phase diagrams shows that single-phase bilayers can exist in a state intermediate between the l_d and l_o phases. The detergent solubility of such membranes is not known, but they could easily show partial insolubility similar to that observed for two-phase membranes [78].

In summary, although important questions about the fidelity of detergent resistance in reporting on phase behavior remain, many of the most serious concerns have been put to rest. Detergent insolubility appears to be a useful and powerful, although imperfect, indicator of membrane phase state.

Estimating the Phase Behavior of Lipids in Cellular Membranes

As we have seen, detergent insolubility points to the existence of interesting phase behavior in cellular mem-

branes, but does not show precisely what phases are present there. Phase diagrams derived from model membrane studies can provide some further clues. An unusual feature of the phase behavior of cholesterol-containing mixtures that may be relevant to cellular membranes also emerges from examination of these diagrams.

The phase behavior of mixtures of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol has been fairly well characterized. A phase diagram based on work of several groups [72] is reproduced in Fig. 1. The phase or phases present as a function of lipid composition (increasing cholesterol at the expense of DPPC along the X axis) and temperature are shown. Focusing first on temperatures between about 41°C (the T_m of DPPC) and 70°C, it is seen that mixtures are entirely present in the l_d phase at low cholesterol concentrations. As cholesterol is increased, mixtures contain both l_d and l_o phase domains. Finally, above about 30% cholesterol, mixtures are entirely l_o.

In contrast, in some cases it is possible to move from an l_d to an l_o state without undergoing phase separation. This can be observed in Fig. 1 at temperatures above about 70°C. As more cholesterol is added, the membrane changes gradually from the l_d to the l_o state, without phase separation. Thus, uniform lipid mixtures can have properties intermediate between the l_d and l_o states.

Because DPPC:cholesterol mixtures are very nonphysiological, it would be useful to have a phase diagram for lipids in cell membranes. Unfortunately, the complexity of real membranes makes this virtually impossible. Nevertheless, useful information can be obtained from model membranes of appropriate lipid composition. For the purposes of studying phase separation, biological membrane lipids can be grouped in three classes; glycerolipids (low T_m), sphingolipids (high T_m), and cholesterol. Thus, determining the phase behavior of ternary mixtures containing high and low-T_m lipids and cholesterol is a reasonable starting point. Such a phase diagram is available from the pioneering work of Silvius and colleagues [81].

This diagram (reproduced in Fig. 2) shows the phases present at 25°C in mixtures of 12-bromo phosphatidylcholine (12BrPC; a low T_m lipid), DPPC (with a high T_m of 41°C), and cholesterol. The exact lipid composition at any point on the diagram can be determined from the labeled axes. Along the horizontal axis, DPPC increases at the expense of 12BrPC. The amount of cholesterol, as mol% of the total, is shown on the left axis. Depending on the lipid composition, the bilayer can be present in l_d, l_o, or P_β' (essentially gel) phases, or can contain coexisting domains in two or even three phases. Note that the phases present in binary mixtures can be determined by moving along the axes. The left axis is of particular interest. By moving along this axis, it is possible to go from the l_d phase to the l_o phase without a cooperative phase transition (as was seen in Fig. 1 at high temperatures). In this diagram, such a gradual transition occurs at 25°C in mixtures of the low T_m lipid and cholesterol. This observation reinforces the important idea that uniform lipid mixtures with properties intermediate between l_d and l_o can exist. (It should be cautioned that this situation is highly dependent on the exact properties of the components. For example, if a low T_m lipid with a slightly higher T_m were substituted, then a cooperative phase separation instead of a gradual transition from l_d to l_o might be observed [81]).

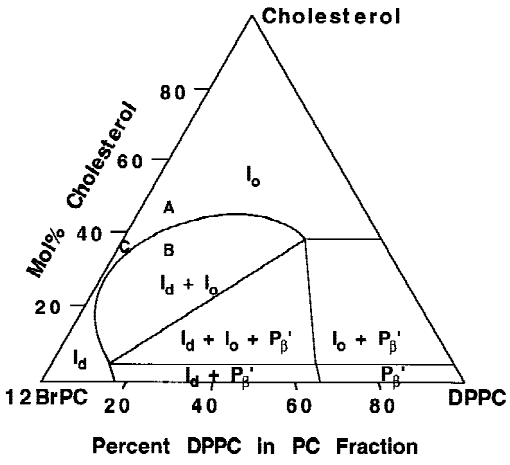

Fig. 2. Ternary phase diagram for the 12BrPC/DPPC/cholesterol system at 25°C. Adapted with permission from [81].

We can use this phase diagram to estimate the phase behavior of mixtures of biological phospholipids, sphingolipids, and cholesterol, substituting ''phospholipids'' for 12BrPC and ''sphingolipids'' for DPPC on the X axis. The rationale for doing so is that T_ms of the components are similar; the T_m of DPPC and of the most abundant cellular sphingolipid, sphingomyelin, (37–40°C [37]) are close, and 12BrPC and biological phospholipids both have very low T_m. In further support of the similarity of the two systems, we have used a fluorescence quenching technique to study the phase behavior of mixtures of the low-T_m lipid 12SLPC and either DPPC or sphingomyelin, with or without 33% cholesterol. The phase behavior of the DPPC-containing mixtures was quite similar to that of the sphingomyelin-containing mixtures, both with and without cholesterol [1]. In both cases, clear evidence of phase separation between the l_d and l_o phases was observed in cholesterol-containing membranes. Thus, as will be discussed in the next section, the phase diagram gives a rough idea of what phases are likely to be present in various eukaryotic cell membranes, with no reliance on detergent-insolubility data.

What Cellular Membranes are Likely to Have l_o Phase Domains?

Plasma Membrane

The cholesterol and sphingolipid-rich plasma membrane seems the most likely to display complex phase behavior. Although estimates vary, plasma membranes have been reported to contain 30–40 mol% sterol [83, 100] and generally 10–20% sphingomyelin [28, 98]. Sphingolipids are enriched in the outer leaflet of the plasma membrane, raising the effective concentration there [10, 96]. Although the phase diagram shown in Fig. 2 is only a crude approximation, it shows that a membrane with the lipid composition of the plasma membrane is unlikely to be in the l_d phase. However, there are several other possibilities. The plasma membrane may be entirely in the l_o phase (for instance, as marked *A* in Fig. 2), or in a two-phase region of mixed l_d and l_o domains (*B* in Fig. 2). Alternatively, it might be in a uniform phase with properties intermediate between l_d and l_o (*C* in Fig. 2). In any case, the plasma membrane may be poised at a composition close to the phase separation boundary, where the smallest change could have large effects on phase behavior. As detailed below, this would open up the interesting possibility of regulated phase separation.

As detergent insolubility correlates with the presence of the l_o phase, the fraction of the plasma membrane that is detergent insoluble offers a clue to its phase behavior. Thus, it is interesting that a substantial fraction of the plasma membrane was found to be detergent insoluble in three studies. 41% of erythrocyte plasma membrane phospholipids [104] and 35% of phospholipids in mastocytoma plasma membranes [47] were insoluble in Triton. In a study of brain plasma membranes, the lipid compositions of plasma membrane and of plasma membrane-derived DRMs were determined, but the amount of each lipid that was insoluble was not [53]. However, this quantity can be estimated by assuming there here, as in other studies of plasma membrane [47, 104], and whole cells [7, 105], most sphingomyelin was insoluble. Simple calculations show that if 75% of brain plasma membrane sphingomyelin were insoluble [53], then 45% of the total plasma membrane phospholipids would have been insoluble. Together, these studies show that a high fraction of plasma membrane lipids are detergent-insoluble. Consistent with this result, a large fraction of the plasma membrane appeared to remain intact morphologically after Triton extraction of fibroblasts [40, 79].

Does the detergent resistance of the plasma membrane reveal its phase behavior? Unfortunately, the data do not distinguish between the possibilities outlined earlier. The observed partial insolubility might reflect either coexisting l_o and l_d domains, a uniform l_o state (as detergent can partially solubilize l_o phase model membranes), or a uniform state intermediate between l_o and l_d, as the detergent solubility behavior of such membranes is not known. However, insolubility of the plasma membrane certainly reinforces the idea that it is not in the l_d phase.

Secretory Pathway Organelles

The endoplasmic reticulum (ER) is very low in cholesterol and sphingolipids [97], and is probably present in the l_d phase, unless cholesterol and sphingolipids are somehow concentrated in highly restricted ER subdomains. The fact that a newly synthesized GPI-anchored protein was Triton soluble while it was in the ER in mammalian cells [7] supports the idea that the ER is in the l_d phase. However, lowering sphingolipid synthesis specifically retarded ER to Golgi transport of a GPI-anchored protein in yeast, possibly reflecting a role for ordered domains [24, 84, 92]. In another study, acute inhibition of sphingolipid synthesis in fibroblasts retarded transport of vesicular stomatitis virus G protein (which is not in DRMs) through both the ER and Golgi [67]. It should be kept in mind that inhibition of sphingolipid synthesis can lead to accumulation of bioactive intermediates [46]. These compounds, rather than the loss of ordered domains, may be responsible for the observed effects.

Membranes such as the Golgi that contain significant levels of sterol and sphingolipids may also show interesting phase behavior. In one study, a GPI-anchored protein first became insoluble in the early Golgi during biosynthetic transport, suggesting that the early Golgi can support DRM formation [7]. In an intriguing hypothesis, it was suggested that cholesterol-rich domains in the Golgi might be selectively transported forward through the secretory pathway after being sorted from cholesterol-poor domains [3]. This could explain the observed gradient of cholesterol through the Golgi [58]. Domain segregation was also proposed to explain the retention of resident Golgi proteins. Golgi proteins tend to have shorter membrane spanning domains than plasma membrane proteins [3], and should partition preferentially into the thinner cholesterol-poor domains. In support of this proposal, a protein containing a 17 Leu transmembrane span was retained in the Golgi, while the same protein with a 23 Leu transmembrane span was delivered to the plasma membrane [56]. In this regard, it is interesting to note that in vitro studies show that the ratio of transmembrane helix length to bilayer width can strongly modulate helix structure [64, 101].

It was proposed several years ago that apical pro-

teins in polarized epithelial cells are sorted by associating with glycolipid ''rafts'' in the *trans*-Golgi network (TGN) [83]. Later studies suggested that these rafts might be DRMs [7]. In agreement with this proposal, sorting of a GPI-anchored protein to the apical surface of polarized epithelial cells was disrupted by inhibiting sphingolipid synthesis [42]. In another study, however, inhibition of cholesterol synthesis in epithelial cells retarded transport of a GPI-anchored protein and decreased its Triton insolubility, but did not affect sorting [22]. For this and other reasons [5, 102], the role of these ''rafts'' in epithelial cell sorting is not yet clear.

Endocytic Pathway Organelles

Ordered domains may also function in the endocytic pathway [39]. After internalization from the cell surface, a GPI-anchored protein was found to recycle to the cell surface more slowly than bulk membrane. This retardation of recycling was prevented by depletion of cholesterol [39] and sphingolipids (S. Mayor, *unpublished data*), suggesting that the protein may normally be sorted to an ordered domain in an endocytic compartment.

Other Membranes

When both cholesterol and high-T_m lipid are present at low concentrations (i.e., the lower left-hand corner of the phase diagram in Fig. 2), only the l_d phase is present. Thus, because sterols and sphingolipids are generally restricted to the plasma membrane and organelles of the secretory and endocytic pathways, other membranes, such as those surrounding mitochondria, should be entirely present in the l_d phase.

Organization of Domains in the Plasma Membrane

The biophysical studies described above have shown that detergent-insolubility can be explained by phase separation and formation of l_o domains, but have not revealed the size, shape, or stability of the domains in cells. In the following sections, we will discuss complementary morphological studies that have provided further insights into these questions and also into other aspects of the organization of ordered domains in cells. All of these studies have been done on the plasma membrane, and we will focus on this membrane for the rest of the review. It remains to be determined whether conclusions reached from these studies apply to intracellular cholesterol and sphingolipid-rich membranes as well. Two lines of investigation have been especially useful. First, several studies show that order-preferring membrane components have an affinity for caveolae. Second, the plasma membrane distribution of proteins and lipids expected to associate with l_o domains has been determined morphologically.

Lipid Domains in Caveolae

Caveolae, or plasmalemmal vesicles, are 50–70 nm plasma membrane invaginations found in a variety of mammalian cells. Caveolae have been implicated in transcytosis [50], lipid trafficking [14, 86], and signal transduction [30, 33, 34, 49, 51]. (For recent reviews, *see* [29, 31, 61]. They are surrounded by a striated coat, visible in freeze-etch electron microscopy, that contains the 22 kDa protein caveolin [68]. Caveolin forms homooligomers [52], binds cholesterol tightly [57], and is likely to be an important structural component of caveolae, as expression in cells that lack the protein induces formation of caveolae [16]. A connection between caveolae and DRMs became apparent when caveolin was found in DRMs [12, 73]. This led to the idea that all DRMs are derived from caveolae.

Several observations show that DRMs are not restricted to caveolae. First, DRMs can be derived from cells that do not contain caveolae or caveolin [15, 20]. Second, as detailed above, a significant fraction of the plasma membrane may be detergent-resistant, while only a small fraction is in caveolae. In addition, large regions of flat detergent-resistant plasma membrane were observed in detergent-extracted cells by electron microscopy [40, 79], and many vesicles observed in DRMs prepared from cells that contain caveolae are much larger than caveolae [7]. Thus, an important distinction must be made between caveolae, which have been best defined morphologically and by their caveolin-rich coats, and DRMs, whose formation reflects their lipid composition and phase state.

The distinction is important because there may be significant differences between domains in and out of caveolae. As an example, the fact that GPI-anchored proteins [17, 62, 69, 90], sphingolipids [17], and even cholesterol [18] can be targeted to caveolae under appropriate conditions suggests that the organization of lipids in caveolae may be unique. Preferential caveolar localization of these molecules occurs despite the fact that large regions of the membrane outside caveolae are detergent insoluble, and should have at least some l_o-like character. Thus, the domains in caveolae may be ''more l_o-like,'' or may be stabilized in some way. As will be discussed below, this may result from the effect of caveolin on lipid organization.

Morphological Clues to Domain Organization

Several morphological approaches have been used to search for domains regardless of whether or not they are in caveolae. One is to simply examine the distribution of

those proteins and lipids expected to be in the domains by fluorescence and electron microscopy. As will be seen, domains have often proven very difficult to detect this way. It has generally proven easier to visualize domains after experimentally clustering proteins or lipids. These findings must be taken into account in models of domain structure.

In early studies, several GPI anchored proteins appeared to be clustered in the membrane [69, 103]. A disproportionate number of these clusters localized to caveolae. However, later work suggested that the fixation protocol used in these studies did not immobilize the GPI-anchored proteins in the membrane [41]. Although still controversial [85], it appears that the antibodies used to detect the proteins crosslinked them, causing their clustering and caveolar localization [17, 41, 62; reviewed in 38]. Several GPI-anchored proteins appeared uniformly distributed in the membrane when antibodies were added after more stringent fixation in cells that contained [17, 41] or lacked [15] caveolae. In contrast, the GPI-anchored urokinase-type plasminogen activator receptor (uPAR) was detected in caveolae even after stringent fixation, suggesting a constitutive localization there [90]. Two GPI-anchored proteins also showed a clustered distribution in a promonocyte cell line even after prolonged fixation [95]. The divergent nature of these results may reflect a variability in the affinity of different GPI-anchored proteins for caveolae. The affinity may often be increased by clustering the proteins, as discussed in a later section.

Intriguingly, in one study GPI-anchored proteins were localized to a ring surrounding the caveolar neck, and not within caveolae [76]. In contrast, the ganglioside GM_1 was in caveolae themselves. The significance of this difference in localization is not yet known, but it should be kept in mind when we refer to a ''caveolar'' localization of GPI-anchored proteins in this review.

Lipid distribution has also been examined. Sphingomyelin and several neutral glycosphingolipids [17] and cholesterol [18] appeared randomly distributed on the cell surface by electron microscopy. All of these lipids concentrated in caveolae after experimental clustering with crosslinking antibodies (for sphingolipids) [17] or after avidin-mediated clustering of a modified biotinylated perfringolysin O toxin that bound to cholesterol [18]. In another study, detection with gold-labeled cholera toxin showed that the ganglioside GM_1 was enriched in caveolae [60]. However, cholera toxin is pentavalent [74], and could have caused GM_1 clustering. Finally, two gangliosides, GM_3 and GD_3, in T lymphocytes (which do not contain caveolae) were examined after fixation under conditions shown earlier [41] to immobilize GPI-anchored proteins [87]. (It is not known if lipids are immobilized under these conditions.) GM_3 appeared clustered, while GD_3 did not [87].

Thus, although clustering of GPI-anchored proteins and sphingolipids in caveolae or other sites has sometimes been detected, they appear randomly distributed in the membrane in a number of other studies. A striking concentration of these markers in caveolae is generally observed after clustering in cells that contain caveolae. Implications for the association of these molecules with l_o domains will be discussed below.

The membrane dynamics of a GPI-anchored proteins and a ganglioside were examined by single-particle tracking, in which the lateral diffusion of gold particles bound to cell-surface molecules was examined [79]. (Gold particles were linked to target molecules through antibodies, so that although the valency was kept as low as possible, clustering may have occurred.) About one-third of the molecules of Thy1 (a GPI-anchored protein) and of the ganglioside GM_1 were transiently confined to 300 nm domains in the bilayer. Inhibition of glycolipid synthesis reduced both the fraction of each marker in the confinement zones and the size of the zones, suggesting that they might be ordered domains. Significantly, the markers remained in these zones for only a few seconds. If the confinement zones are ordered domains, then the domains, or the association of proteins or lipids with them, may be very short-lived.

A second morphological approach has been used to detect domains that might be too small to visualize directly. As an indirect way of determining whether two different molecules are in the same domain, one species is experimentally clustered in the membrane. If the two molecules are associated, then the second could be ''dragged along'' with the first, as the whole domain might be moved.

In one such study, lymphocyte gangliosides were labeled with either rhodamine or Lucifer yellow [88]. Both markers initially appeared to be uniformly distributed on the cell surface. However, addition of anti-rhodamine antibodies resulted in capping of both populations. Thus, the two gangliosides interacted with each other, at least after the rhodamine-labeled population was clustered.

In another study, either a GPI-anchored protein or a ganglioside was intentionally clustered on the surface of unfixed lymphocytes, using antibody- or toxin-mediated crosslinking respectively [15]. Clustering of either component did not lead to redistribution of the other. Similarly, antibody-mediated crosslinking of one GPI-anchored protein did not lead to redistribution of another in fibroblasts [41]. However, in this case, the effect of clustering both proteins independently was also determined. Clusters of the two proteins colocalized, demonstrating an affinity between them. This would be expected if the clusters were present in ordered domains, as these should have an affinity for each other. Many but not all of the clusters were present in caveolae, as de-

tected by colocalization with caveolin in immunofluorescence microscopy. Interestingly, the clustering of GPI-anchored proteins is strongly inhibited by perturbation of the membrane with cholesterol-binding agents [70]. This supports the idea that the clusters have a l_o-like character.

A study of the fluorescent lipid DiI in the membrane of RBL 2H3 basophilic leukemia cells provided striking visual evidence of ordered lipid domains in cell membranes [93]. Though not a glycosphingolipid, DiI has saturated acyl chains and prefers an ordered environment [89]. DiI was initially distributed uniformly after being incorporated into the plasma membrane. However, when the cell-surface IgE receptor was clustered, DiI colocalized with the clusters [93]. DiI also co-clustered with antibody-induced clusters of an unusual ganglioside expressed in these cells [93]. Of several fluorescent probes examined, regardless of charge, only those that might be expected to partition preferentially into an ordered environment co-clustered with the receptor [93]. In addition, the IgE receptor was partially recruited into DRMs when it was clustered. This important study provides the best evidence to date that the clusters represent an ordered lipid environment. Similar conclusions were reached in a second study, in which IgE-bound IgE receptor clustered and colocalized with clusters of GM_1 (visualized with cholera toxin) after addition of antigen [91].

It should be noted, however, that in another study, monomeric GM_1 did not associate with clusters of Thy1 on lymphocytes [15]. Co-clustering might have been predicted from the behavior of DiI and GM_1 in co-clustering with the IgE receptor [91, 93]. This difference may reflect cell-type differences.

A further study suggested a functional role for ordered domains in basophils [13]. As noted above, some of the IgE receptor associated with DRMs after clustering. Remarkably, only the receptor molecules in DRMs were tyrosine phosphorylated by Lyn, the receptor-associated kinase that is also in DRMs [13], suggesting that both receptor and kinase may need to be in ordered domains for signaling to occur.

Inner Leaflet Domains

An important question concerning the organization of l_o phase domains is their disposition between the two leaflets of the bilayer. Very little is known about how ordered domains form in the sphingolipid-poor inner leaflet, and how such domains might be coupled with outer leaflet domains. It is possible that sphingolipids in the outer leaflet may aid in coupling of domains in the two leaflets, possibly through interdigitation of the long sphingolipid acyl chains with lipids in the opposite leaflet [75]. Many cytoplasmically localized molecules, such as Src-family kinases, associate with DRMs through a dual acylation motif [80] and can only interact with the cytoplasmic bilayer leaflet. As these proteins appear to be functionally coupled to GPI-anchored and transmembrane proteins in l_o domains [4, 13], understanding how inner leaflet domains are organized is an important topic for further research. However, except in the context of caveolae, this poorly understood topic will not be discussed further here.

Models of Domain Structure in the Plasma Membrane

Detergent-insolubility studies suggest that the plasma membrane is likely to be at least partially in an l_o-like phase, and bring up the possibility that l_d and l_o phase domains coexist. However, proteins and lipids expected to have the highest affinity for the l_o phase often appear uniformly distributed in the membrane unless they are experimentally clustered. These results put important constraints on models of domain structure and suggests that domains (if they exist) may be altered substantially by clustering. Several alternate models of l_o domain organization in the ''unclustered'' plasma membrane are outlined next. The possible effects of clustering on domains structure will then be discussed. Finally, we will consider the special case of caveolae.

Model 1: Domains for Which Proteins and Lipids Have a Weak Affinity

Domains may be large enough to be detectable by microscopy, but may be difficult to visualize as individual components interact with them only weakly and transiently. The single-particle tracking experiment discussed above [79] is consistent with this model, as individual molecules remained in transient confinement zones for only a few seconds. If individual components actually have such a low affinity for l_o domains, then their concentration there may be only a few fold higher than in surrounding l_d domains.

Support for this possibility comes from a study of the phase behavior of liposomes containing sphingomyelin, dioleoyl PC (DOPC), and cholesterol at 37°C [1]. The mixtures were present in either a uniform l_d phase, a mixture of l_d and l_o phases, or a uniform l_o phase, depending on the relative amounts of sphingomyelin and DOPC. Throughout the two-phase region (i.e., all liposomes that have undergone phase separation and contain both l_d and l_o phase domains), the lipid composition of each phase is expected to remain relatively constant. The concentrations of sphingomyelin at the beginning and the end of the two-phase region provide an estimate of the lipid composition of each phase. Only a

crude estimate can be obtained in a ternary lipid mixture, but the data obtained [1] suggest that the sphingomyelin concentration in the l_o phase might have been only 4–5 fold higher than that in the coexisting l_d phase. Such a small difference in the sphingolipid concentration between domains of unspecified size might be extremely difficult to detect by microscopy, especially if the examination were focused on searching for small clusters. It should be pointed out that even such weak favorable partitioning could have important functional implications. For example, a molecule that partitions even modestly into an ordered domain could interact more easily with a protein constitutively localized in that domain than could a molecule that was excluded.

Another possible explanation for the difficulty of detecting domains is that they may be very large, constituting a substantial fraction of the cell surface, and irregularly shaped. If so, individual components might appear uniformly distributed in the membrane, even if their concentration were higher than in the l_d domains. It should be noted, though, that models postulating large fixed domains cannot easily explain localization of proteins and lipids in much smaller, easily visualized domains after clustering.

Model 2: ''Microdomains'' Containing Only a Few Molecules

l_o domains may be very small, including only a few lipids and proteins. In this case, they would be below the level of resolution by microscopy. The fact that a GPI-anchored protein did not associate with antibody-mediated clusters of a second protein [41] or a toxin-induced ganglioside clusters [15] suggests that GPI-anchored proteins are not present in ordered domains before clustering, unless the domains are very small. (Note that antibody-mediated redistribution of a GPI-anchored protein did not affect the distribution of a second protein [41]. This shows that the two proteins did not interact with each other without clustering, suggesting that each microdomain is too small to contain molecules of both proteins.) In accord with this model, very small clusters of glycosphingolipids have been observed in model membranes [66]. It is true that large DRMs are often observed after extraction of cells [7, 40]. However, these might arise from detergent-induced coalescence of small domains.

In order for such small domains to be stable, they would have to exhibit strong internal interactions between components, but ordinarily have only a weak tendency to coalesce with each other. As one example, specific proteins might have multiple high-affinity binding sites for saturated lipids and/or cholesterol. In this case, a ''domain'' would consist of one protein and the surrounding lipids, serving as a valid case of the more general annulus or boundary lipid models of membrane structure [27]. As another example of how microdomains might be stabilized, the fatty acid composition of some lipids might give them a strong affinity for l_o domains on one side and a strong affinity for l_d domains on the other [32]. Such lipids might prefer the boundaries between l_d and l_o domains. If present at high levels, these lipids would have a tendency to increase the amount of lipid at domain boundaries, resulting in a reduction in domain size.

Model 3: Induction of Domain Formation by Clustering

It is also possible that stable domains do not exist constitutively, except possibly in caveolae, but are induced during clustering of order-preferring components. This might be possible because cholesterol-containing lipid mixtures can exist in a uniform state with a significant degree of l_o phase behavior and a lipid composition close to that at which phase separation can occur. In such a state, very small changes might be able to induce phase separation. Clustering of molecules that have an inherent affinity for ordered domains might do so, for instance by raising the local concentration of order-preferring acyl chains. Such a concentration might recruit other order-preferring lipids and lead to phase separation. The affinity of several order-preferring molecules for caveolae suggests that caveolae may serve a similar function.

The observations that order-preferring molecules appear dispersed in the membrane, but that ordered domains may be present after aggregation of proteins or lipids, provide the strongest support for this model. Two of the experiments discussed above are especially significant in this regard. First, the association between order-preferring lipids and IgE receptor clusters [91, 93] strongly suggests that these clusters exist in an ordered lipid environment. The second important experiment is the colocalization of two independently clustered GPI-anchored proteins [41]. This colocalization would be expected if both proteins were present in ordered lipid domains, as such domains should have an affinity for each other. Colocalization is more difficult to explain without invoking lipid domains, especially as GPI-anchored proteins are restricted to the outer leaflet of the bilayer and cannot interact directly with the cytoskeleton.

Clustering May Alter the Affinity of Molecules for Ordered Domains

Models 1 and 3 propose that clustering of order-preferring molecules alters the domain structure of the membrane. Clustering might either increase the affinity of these molecules for pre-existing domains (Model 1), or induce the domains to form (Model 3). How could

clustering have this kind of effect? Clustering of GPI-anchored proteins or sphingolipids would increase the local concentration of saturated acyl chains in the membrane. This could increase the affinity of the grouped molecules for an ordered domain, as proposed in Model 1. A crude calculation shows how important such an effect could be. Consider a weakly favorable acyl chain interaction in which partitioning into the ordered phase is favored 5-fold (i.e., K_p, the coefficient of partition favoring the ordered phase, is 5). Since $\Delta G = -RTlnK_p$, this corresponds to a ΔG of about 1 kcal/mole. A clustering event resulting in a species with just four such interactions, i.e., $\Delta G = 4$ kcal/mole, would increase K_p to close to 800. (Such cumulative effects have been postulated to be important for the regulation of electrostatic interactions between membrane components [2]).

Clustering of molecules that prefer an ordered environment might nucleate or stabilize domains in a similar manner, as proposed in Model 3, promoting recruitment of additional order-preferring lipids. Clustering might thus provide the ''trigger'' required for phase separation in a uniform membrane of a lipid composition that was poised on the brink. This could be physiologically significant because although clustering has been induced experimentally through antibody-mediated crosslinking, it may easily occur in vivo as well. An example is receptor clustering during signaling through the IgE receptor [13].

Caveolae as Constitutive Lipid Clusters

The fact that so many order-preferring species localize to caveolae, at least after they are aggregated in the membrane, suggests that the caveolar membrane has unusual properties. It is very likely that one or more proteins in caveolae influence the organization of the lipids there, possibly through the ''clustering effect'' as described above. Caveolin is an attractive candidate for this role. Caveolae are surrounded by homo-oligomers of caveolin [52, 68]. Each caveolin monomer is linked to three palmitate chains, and caveolin-linked palmitate chains are likely to make up a significant fraction of the total lipid acyl chains in caveolae [6]. Caveolae are thus rich in constitutively clustered acylated proteins. This high concentration of saturated acyl chains, possibly in combination with caveolin-bound cholesterol [57], may affect the phase behavior of the membrane. This could increase the affinity of sphingolipids and GPI-anchored proteins for caveolae, especially after clustering of these molecules to further increase their affinity for an ordered lipid environment.

It should be noted that caveolin-linked palmitate chains are likely to insert into the cytoplasmic leaflet of the bilayer, opposite from clustered GPI-anchored proteins and sphingolipids. These chains might be important in establishing ordered domains in the sphingolipid-poor inner leaflet. It is not yet known how such domains might be coupled to outer-leaflet domains.

Summary

Detergent insolubility studies point to the possibility of phase separation and formation of an l_o-like phase in cell membranes. Sphingolipids, GPI-anchored proteins, and other multiply-acylated proteins, whose saturated acyl chains would predict an affinity for ordered domains, tend to be detergent insoluble. Complementary model membrane studies that do not involve detergent resistance support the plausibility of phase separation in cell membranes. However, domains are difficult to detect in cell membranes. Instead, putative marker components often appear to be randomly distributed in the plasma membrane. Nevertheless, the distribution of these markers can be dramatically altered after clustering, when more convincing evidence of their presence in ordered lipid domains appears.

Several models for the structure of ordered domains in cells that incorporate these observations can be imagined. The domains might be either very small, or very large, and components might associate with them only weakly and transiently. Alternatively, domains might only form upon clustering of order-preferring proteins or lipids. The membrane in caveolae may be more ordered than the rest of the membrane. If so, caveolar proteins (possibly caveolin) may affect the caveolar lipid environment, favoring the localization of other order-preferring molecules there. Further increases in the affinity of these molecules for an ordered environment by clustering may sometimes be required for their stable localization in caveolae. Whatever model is correct, it now appears that the phase behavior of cell membranes is an important aspect of their structure and function.

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