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BREAKTHROUGHS AND VIEWS

Structure of Detergent-Resistant Membrane Domains: Does Phase Separation Occur in Biological Membranes?

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Detergent-resistant membrane domains (DRMs) can be isolated from a variety of eukaryotic cells. DRMs are of interest because of their potential importance in processes such as intracellular membrane sorting, and signal transduction at the cell surface. One type of DRM is also present in caveolae, non clathrin-coated plasma membrane pits with proposed roles in endocytosis, lipid transport, and signal transduction. Here we review recent advances in understanding the structure of these domains, and explore the possibility that DRMs are present in a phase separate from the surrounding bilayer. DRMs are rich in sphingolipids and cholesterol. The long saturated acyl chains and high acyl chain melting temperature of sphingolipids mediate their association in detergent resistant domains. These sphingolipid and cholesterol-rich domains have the properties of the liquid-ordered phase previously described in model membranes. Several lines of investigation support the idea that DRMs are not detergent-induced artifacts, but exist as domains in cell membranes. A striking feature of the proteins in DRMs is that many of them are linked to lipids. These include both GPI anchored proteins, and acylated proteins such as Src-family kinases. The linkage of these proteins to saturated acyl chains may help in targeting them to ordered membrane domains. Caveolin, the major structural protein of caveolae, is multiply palmitoylated. The presence of a high concentration of palmitate chains in DRMs in caveolae may help stabilize ordered domains.

Lipids and proteins in mammalian cell membranes are often organized into domains. In some cases, such as the apical and basolateral domains of epithelial cells (1), the axon and cell body of neurons (2), and the head and tail of sperm (3), compositionally distinct plasma membrane domains are easily distinguishable morphologically. The question of whether smaller microdomains containing clustered lipids and associated proteins also exist in biological membranes has vexed membrane biologists for many years (4).

It is now clear that such domains can exist in simple model membranes (5). The best understood example is gel-fluid lipid phase separation. Pure phospholipids are characterized by a melting temperature (T_m) at which they change from a solid-like gel state to the fluid liquid crystalline state that is generally present in biological membranes. The gel state is characterized by an ordered and tightly packed structure, and the fluid state by its lesser order, looser packing and more rapid molecular motion. Co-existing gel and fluid phases (or domains) are often observed in mixtures of two different phospholipids at temperatures between the T_ms of the individual components (6). In addition to gel-fluid phase separation, other types of phase separation have also been observed. For example, separation of two liquid phases based on differences in acyl chain length (7), and separation of liquid-crystalline and liquid-ordered domains in mixtures of saturated-chain phospholipids and cholesterol (see below), have been reported. In addition, it has been found that interactions with membrane proteins, including electrostatic interactions between lipids and charged peptides (8), can induce domain formation.

The idea that domains such as these are present in biological membranes has remained attractive (5). For the most part, however, it has been difficult to unambiguously apply the physical techniques used in model membranes to cells. For this reason, the existence of such domains has not been widely accepted until recently.

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SPHINGOLIPIDS AND PHASE SEPARATION

Sphingolipids are relatively rich in long and saturated fatty acyl chains, which allow tight packing and confer much higher T_m than unsaturated chains. The T_m of sphingomyelin is about 37-41°C, and some glycosphingolipids have T_m of 60-70°. Sphingolipids are unique among mammalian cell membrane lipids in having such high T_m; most biological phospholipids have T_m below 0°C (9). Hydrogen bonding between polar head groups may also contribute to tight packing of sphingolipids. For these reasons, many investigators have looked for gel phase sphingolipid domains in model and natural membranes (10). However, with few exceptions strong evidence for such domains in natural membranes has not been obtained. Clustering of glycosphingolipids has been observed (10-12), although its basis has not been determined. Simons and van Meer postulated that glycosphingolipid domains (of undefined phase) might be important in apical sorting in epithelial cells, and that polar headgroup interactions might drive such phase separation (13,14).

DETERGENT-RESISTANT MEMBRANE DOMAINS

Strong evidence now suggests that sphingolipid-rich domains do exist in cell membranes, and can be isolated by a surprising property; insolubility in non-ionic detergents such as Triton X-100. Yu et al. first showed that a sphingolipid-rich detergent-resistant membrane-like fraction could be isolated from erythrocytes, and speculated that it might contain lipid microdomains formed by tight interactions between sphingolipid molecules (15). They suggested that these interactions might help the lipids associate with the detergent resistant protein matrix of erythrocytes.

Skibbens et al. showed that influenza hemagglutinin, an apical protein, was not fully solubilized from epithelial cell membranes. They speculated that insolubility might stem from association with glycosphingolipid lipid clusters, which might be important in sorting as postulated by Simons and van Meer (16). More direct evidence for the proposal that membrane proteins can become detergent insoluble by associating with lipid domains came from the finding that glycosyl phosphatidylinositol (GPI) anchored proteins are present in detergent-resistant membranes (DRMs) with a distinct bilayer structure (17).

DRMs have now been isolated from a wide variety of mammalian cell types, including several types of epithelial cells (18-23), fibroblasts (24-27), a variety of hematopoietic cells (28-36), endothelial cells (37-40), adipocytes (41,42), muscle cells (43), neuroblastoma cells (44,45), neurons (46,47), and melanoma cells (48). DRMs have also been isolated from the yeast *Saccharomyces cerevisiae* (49). [DRMs have also been called GEMs, glycolipid-enriched membranes (50); DIGs, detergent-insoluble glycolipid-enriched domains (51); TIM, Triton-insoluble membranes (52); LDTI, low-density Triton-insoluble fractions (34); and TIFF, Triton-insoluble floating fraction (53)].

DRMs have been postulated to function in processes as diverse as sorting in the secretory pathway and signal transduction, and readers are referred to recent reviews (54-56). DRMs are also likely to be important in the functioning of caveolae, membrane pits that may function in endocytosis (57-59), signal transduction (60-62), and/or lipid transport (63). (For recent reviews of caveolae, see Refs. 59, 64, and 65.) This review will focus on the structure of DRMs, and evidence that they have properties of the liquid-ordered phase. Isolation of DRMs may provide evidence for phase separation in biological membranes.

DRMS ARISE FROM LIPID-LIPID INTERACTIONS: CORRELATION OF ACYL CHAIN STRUCTURE, T_m, AND DETERGENT INSOLUBILITY

The studies cited above suggest that DRMs owe their insolubility to lipid-lipid interactions. However, early studies of low-density insoluble material isolated from cells suggested that it consisted of a scaffold of protein, with which some lipids associated (15,66). To further investigate the origin of DRMs, model membranes of various compositions were extracted with Triton X-100 (9). Detergent insolubility was observed even in the absence of protein, supporting the idea that DRMs arise from lipid-lipid interactions. As was observed in cells, sphingolipids were much more resistant to solubilization than phospholipids (9). Intriguingly, cholesterol was also partially detergent-resistant in mixtures that contained sphingolipids. DRMs isolated from cells are also rich in cholesterol. The role of cholesterol in detergent insolubility will be considered further below.

The fact that sphingolipids are more insoluble than phospholipids suggests that the tight packing ability and unusually high T_m of sphingolipids are important in insolubility. Consistent with this view, the saturated-chain, high-T_m phospholipid dipalmitoyl phosphatidylcholine is Triton insoluble in DRM-containing liposomes, while the low T_m, unsaturated-chain phospholipid dioleoyl phosphatidylcholine is mostly solubilized from the same liposomes (9). This result is not consistent with alternative models suggesting that lipid clustering and detergent insolubility arise primarily from glycosphingolipid head-group interactions (55). The finding that DRMs can be isolated from a mutant melanoma cell line that lacks glycosphingolipids (67) (K. Ivarson and D. A. B., unpublished observations) is similarly inconsistent with such models.

THE LIQUID-ORDERED PHASE

The properties of sphingolipids described above suggest that detergent-resistant phase rich in tightly packed sphingolipids might separate from a phase enriched in loosely packed phospholipids. Consistent with this idea, pure dipalmitoyl phosphatidylcholine is insoluble in Triton X-100 when it is present in the gel phase (68). However, the high amount of cholesterol present in DRMs argued against their being in the gel phase. Instead it is likely DRMs are more similar to the liquid-ordered (l_o) phase than the gel phase. The l_o phase was first identified in binary mixtures of saturated phosphatidylcholines and cholesterol (69-74), and requires cholesterol to form. Mixtures of sphingomyelin and cholesterol also form the l_o phase (72). The l_o phase has properties that are intermediate between those of the gel and fluid phases. Like the gel phase, the l_o phase is characterized by tight acyl-chain packing and relatively extended acyl chains. This probably explains why this phase can be detected primarily in mixtures of cholesterol and phospholipids with saturated (or largely saturated, in the case of sphingomyelin) acyl chains (70-74). However, unlike the gel phase, acyl chains in the l_o phase have rapid lateral mobility in the bilayer (75).

DRMS AND THE LIQUID-ORDERED PHASE

We wondered whether DRMs are present in the l_o phase, or a phase with similar properties. Unfortunately, the l_o phase can difficult to distinguish from fluid lipid phases, probably explaining the fact that its existence has not been more widely appreciated. Scanning calorimetry, which is the primary tool for detecting transitions between the fluid liquid-crystalline (l_c) and solid gel phases, is not sensitive enough to detect similar transitions between l_c and l_o phases. Methods such as ESR (72), NMR (73), and fluorescence polarization (74), have been used to detect the l_o phase in simple binary mixtures of phospholipids and cholesterol, but their application to cells would face significant technical barriers.

Nevertheless, several pieces of evidence support the idea that DRMs are present in the l_o phase or a phase with similar properties. Artificial l_o phase bilayers, like those in the gel phase (68), are insoluble in Triton X-100 (9). Also, separation of fluid and l_o phases can be demonstrated in mixtures of phospholipids, sphingolipids, and cholesterol, under physiologically reasonable conditions of temperature and lipid composition, using a detergent-free fluorescence-quenching technique (76). This striking result strongly suggests that phase separation can occur in biological membranes as well. Finally, the "fluidity" or degree of acyl chain mobility of lipids in DRMs isolated from cells is very similar to that of lipids in the l_o phase (9).

LIPID COMPOSITION OF DRMS

This model predicts that DRMs should always be rich in high T_m species like sphingolipids and cholesterol. Indeed, the insoluble fraction isolated from erythrocytes was found to be enriched in sphingolipids (both sphingomyelin and glycosphingolipids) at the expense of glycerophospholipids (15). This was also observed in DRMs from MDCK canine kidney epithelial cells (17) and FRT rat thyroid cells (20). Sphingomyelin is also enriched in a detergent-insoluble fraction isolated from mastocytoma cells (77) and neurons (46). (Glycosphingolipids were not analyzed.) In both cases, (particularly in neurons) but not in MDCK or FRT cells (17,20), an enrichment in phosphatidylcholine was also observed (46,77). The significance of this is not yet clear. Where tested, [MDCK cells (17) and neurons (46)], DRMs have also been found to be rich in cholesterol. Thus, enrichment in l_o forming lipids appears to be a consistent feature of DRMs. Although DRMs are sometimes called "glycosphingolipid rafts", it should be noted that these glycosphingolipids are not absolutely required for DRM formation; sphingomyelin- and cholesterol-rich DRMs can be isolated from cells that do not contain glycosphingolipids (K. Ivarson and D. A. B., unpublished observations).

WHAT CELL MEMBRANES CONTAIN DRMS?

As outlined above, sphingolipids and cholesterol are probably essential for formation of DRMs from biological membranes. The plasma membrane, which is rich in both these lipids, is clearly an important source of DRMs.

However, certain other organelles of the secretory and endocytic pathways also contain sterols and sphingolipids, and may contain ordered, detergent-resistant domains This is particularly true of the trans-Golgi network (TGN), which is rich in cholesterol (78). Formation of DRMs in the Golgi was also suggested by a pulse-chase study showing that a GPI-anchored protein became detergent-insoluble while in the Golgi (17). In contrast, the endoplasmic reticulum (ER) and intermediate compartment between the ER and the Golgi have very little sterol, and are unlikely to house DRMs. As expected, a GPI-anchored protein was Triton-soluble while present in these membranes during biosynthetic transport (17). It is not known whether DRMs form in other membranes of the endocytic pathway or lysosomes. Mitochondria and other membranes that do not contain sterols or sphingolipids are not expected to contain DRMs.

LIPID-LINKED PROTEINS IN DRMS

A disproportionate number of the known proteins in DRMs are linked to saturated-chain lipids, which are likely to insert readily into tightly packed membrane domains. This suggests that such acyl chains may aid in DRM targeting of these proteins. The first proteins to be identified in DRMs were those anchored by GPI. The GPI-anchor is required for these proteins to associate with DRMs (50,79). Purified GPI-anchored proteins in liposomes of the appropriate lipid composition are detergent-insoluble, showing that they interact directly with DRM lipids. GPI-anchored proteins generally have saturated acyl chains, consistent with an association with ordered domains (80) .

Several other DRM proteins are linked directly to saturated acyl chains. These include Src-family kinases (24), heterotrimeric G protein α subunits (81), GAP 43 (82), endothelial cell nitric oxide synthase (52), CD44 (83), influenza hemagglutinin (16), CD36 (33), and caveolin (22). Src-family kinases are known to require dual acylation for DRM association (24). Further studies will show whether this is a general property of lipid-linked DRM proteins. Caveolin, which does not require acylation for DRM association (22), may be an exception.

ARE DRMS DETERGENT ARTIFACTS?

It has been suggested that DRMs themselves, or the association of specific proteins with DRMs, might be detergent-induced artifacts. Detergent might preferentially extract phospholipids from homogeneous bilayers, creating insoluble aggregates from the remaining lipids. Similarly, detergent insolubility might overestimate the amount of lipid or protein in domains that are actually present. This could occur if lipids or proteins that are not in domains, but have the potential to partition into them, are driven into co-existing DRMs during detergent extraction. We have performed the following control experiments to address these possibilities.

One way of determining whether detergent can reorganize lipids into insoluble domains is to compare liposome insolubility with domain formation detected by independent techniques. Formation of the l_o phase in mixtures of DPPC and cholesterol has been very well characterized, and a phase diagram has been published (84). By comparing the detergent insolubility of various DPPC:cholesterol liposomes to the phase diagram, we found that DRMs were only formed when domains in the l_o phase were present (85). Thus, detergent did not create insoluble domains where there were no l_o-phase domains present. On the contrary, some of the membrane in the l_o-phase was solubilized.

The fluorescence quenching technique mentioned in the last section was used to demonstrate that phase separation occurs in mixtures of lipids similar to those present in biological membranes (76). Detergent-extraction experiments performed in parallel demonstrated a good correlation between phase separation and detergent insolubility, as in the DPPC:cholesterol experiments.

Finally, we used another approach to show that exposure to detergent does not cause lipids that are present in fluid membranes before lysis to partition into DRMs formed from ordered domains present in the same lysate. Fluid (non-DRM containing) liposomes containing ^{3}[H]sphingomyelin were mixed with unlabeled DRM-containing liposomes. All the ^{3}[H]sphingomyelin in the mixture was Triton soluble, showing that it did not "jump" into DRMs during extraction (85). Similarly, when fluid liposomes containing a GPI-anchored protein were mixed with DRM-containing liposomes before detergent extraction, the protein was fully solubilized (85).

Together, these studies argue strongly that detergent does not induce artifacts in the organization of DRM lipids. Since fewer studies have been performed on DRM proteins, the question of how proteins associate with DRMs will bear further investigation in the future.

Except for DRMs in caveolae (see below), the size of ordered domains and their distribution in membranes are not known. DRMs isolated from cells can reach 1 μm in diameter (17). It is possible that ordered domains are smaller than this, and that smaller domains may coalesce into larger DRMs during detergent extraction. Alternatively, DRMs may be quite large. There is some evidence that palmitoyl oleoyl phosphatidylcholine (POPC), one of the most abundant biological phospholipids, may form an l_o-like phase with cholesterol under some circumstances (74). It is possible that in some cases, plasma membranes rich in sphingolipids, cholesterol and POPC or similar lipids may exist in a uniform, essentially l_o phase.

THE INNER LEAFLET QUESTION

The fact that sphingolipids are highly concentrated in the outer leaflet of the plasma membrane would suggest that domains capable of forming DRMs would be concentrated there. However, several observations suggest that DRMs are derived from the inner leaflet as well. First, DRMs clearly have a bilayer appearance (17). In addition, proteins such as Src-family kinases, which can only interact with the inner leaflet, can be recovered in DRMs. These observations suggest that domains in the inner leaflet are present in DRMs, although their structure is not clear. As discussed above, POPC may aid in formation of DRMs, and may contrib-

ute to domain formation in the inner leaflet in a way that is not yet understood. As detailed below, palmitate chains linked to caveolin may affect domain formation in the inner leaflet of caveolae. In addition, it is possible that the long sphingolipid acyl chains present in outer-leaflet domains may help organize and stabilize phospholipid domains in the inner leaflet. Such sphingomyelin-induced monolayer coupling has been observed in model membranes (86).

RELATION BETWEEN DRMS AND CAVEOLAE

Caveolae are non-clathrin coated invaginations in the plasma membrane. Caveolin, a 22 kDa homo-oligomeric protein and the best marker of caveolae, is present in DRMs. This finding led to the proposal that DRMs are caveolae. Support for this idea came from the observation that under some circumstances, DRMs have the same size and shape as caveolae and can be heavily labeled with anti-caveolin antibodies (43).

It is now clear, however, that not all DRMs are caveolae (53). First, DRMs can be isolated from cells that do not contain caveolae (30,44). In addition, DRMs prepared without sonication (even from cells that contain caveolae) can be several hundred nm to 1 μm in diameter (17,66,81,87). This is not consistent with the smaller and more uniform size of caveolae, and suggests that only a subset of DRMs are present in caveolae.

It is not yet clear why the caveolar membrane is detergent-resistant. As described earlier, detergent-resistance of membranes stems from lipid composition and organization. Thus, a caveolar protein (or proteins) probably interact(s) with lipids that can be detergent-insoluble and either organize or stabilize them in the form of ordered domains.

Caveolin is an attractive candidate for a protein that organizes such domains. Caveolin plays an important structural role in the formation of caveolae. Expression of this protein in lymphocytes, which lack caveolae, induces their formation (88). Each monomer of caveolin binds tightly to one molecule of cholesterol (89), and is covalently linked to three palmitate chains (22). Both these lipids favor formation of the l_o phase. The following very rough calculation suggests that a high concentration of caveolin-linked palmitate chains is present in caveolae. Caveolin forms homo-oligomers, each containing approximately 14 monomers (90). These may represent the bead-like structures that make up the caveolar "coat" (91). Estimating from nystatin-treated caveolae in (91), about 40 bead-like structures containing 1,680 caveolin-linked palmitate chains may be present on each caveola. If the cross-sectional area of each phospholipid is 70 $Å^2$, then each leaflet of a 70 nm diameter protein-free vesicle contains 44,000 acyl chains. All of the caveolin-linked palmitate chains are likely to be present in the cytoplasmic leaflet. These numbers predict that caveolin-linked palmitate could make up 1 out of 26 acyl chains present in a 70 nm caveola (or 1 out of 13 chains in a 50 nm caveola). The actual number is likely to be higher, as the membrane contains proteins and cholesterol in addition to phospholipids, reducing the total number of acyl chains.

Thus, the lipid composition in the caveolar membrane is probably very unusual, in that it contains a high concentration of palmitate chains. Linkage to caveolin would prevent lateral mobility of these palmitate chains in the bilayer. This high concentration of "fixed" palmitate chains could help recruit other order-preferring lipids, and stabilize ordered domains in caveolae. Caveolin-bound cholesterol may also affect the structure of the caveolar membrane, although the ability of this cholesterol to interact with other lipids is not known. In sum, caveolin-bound lipids could have important effects on the formation, stability, and biophysical properties of ordered membrane domains in caveolae.

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