Commentary

Seeing is believing: Visualization of rafts in model membranes

Deborah A. Brown*

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215

Do membrane lipids mix uniformly or are they arranged in discrete microdomains? This question has vexed membrane biochemists since the structure of the bilayer was first elucidated. The last few years have seen the emergence of interest in one type of microdomain: lipid rafts, or sphingolipid-rich domains in the liquid-ordered phase. Although significant evidence for the existence of rafts has been presented, compelling proof has remained elusive. Additional avenues of investigation are sorely needed. In a paper in this issue of PNAS (1), together with a paper that appeared in *Biophysical Journal* in March 2001 (2), Dietrich *et al.* have pioneered a new approach that allows direct microscopic visualization in model membranes of domains with properties expected of rafts. The results support the tenets of the raft hypothesis to a remarkable degree.

In its present form, the raft hypothesis states that separation of discrete liquid-ordered (l_o) and liquid-disordered (l_d) phase domains occurs in membranes containing sufficient amounts of sphingolipid and sterol (3, 4). The l_o phase domains are proposed to have properties similar to domains described in binary mixtures of a single order-preferring phospholipid and cholesterol (5–10). First, they are fluid, and lipids in them have a relatively high diffusion rate. Second, the acyl chains are tightly packed and highly ordered. Lipids and proteins that prefer such an ordered environment (including both glycosphingolipids and glycosylphosphatidylinositol-anchored proteins) are proposed to partition favorably into the l_o-phase domains and thus to be enriched in rafts.

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The theoretical basis of this model does not yet enjoy extensive experimental support from model membranes, in which phase separation can be analyzed with far more precision than in cells. The model outlined in the last paragraph represents an extension of the "classical" case of l_o/l_d phase separation (5–10). The l_o state has been best described in binary mixtures of an order-preferring phospholipid and cholesterol. Increasing the amount of cholesterol in these mixtures leads to formation of cholesterol-rich l_o phase domains, which coexist with l_d domains that are composed primarily of phospholipid.

In contrast, it has been proposed that, in cholesterol-rich membranes, sphingolipid-rich l_o-state domains separate from phospholipid-rich l_d-state domains (11, 12). The separation is driven by the greater tendency of sphingolipids, with their saturated acyl chains, than biological phospholipids, which contain unsaturated chains, to form an ordered state in conjunction with cholesterol. This system is quite different from that in which the l_o phase was originally described. Experimental support for this model has come from only two approaches: raft detergent insolubility [which results from the tight acyl chain packing of raft lipids (13)] and fluorescence quenching assays (12, 14). Although there is no reason to doubt the conclusions of these experiments, confidence in such a novel model is significantly bolstered by complementary evidence by using an independent approach, especially one that allows direct visualization of domains that have properties predicted by the model.

Dietrich *et al.* have now developed such a method and can visualize rafts in model membranes by examining fluorescent lipid probes expected to partition preferentially into either raft or nonraft domains (1, 2). Micrometer-sized domains were detectable in both monolayers and supported bilayers containing phospholipids, sphingolipids, and cholesterol (2). Further characterization showed that the domains had properties expected of rafts. Texas red (TR)-dipalmitoyl phosphatidylethanolamine (DPPE) and GM1 [visualized by using fluoresceinated (Fl) cholera toxin] partitioned into opposite domains in both systems. This result mirrored behavior seen in preliminary studies by using a well-characterized gel-fluid system, in which TR- and Fl-DPPE preferred the fluid phase and GM1 the gel phase. Further studies elucidated the physical properties of the two phases. The same particle diffused faster in the Fl-DPPE-rich domains, showing that these were less ordered than the Fl-DPPE-depleted domains. Nevertheless, several lines of evidence showed that the more-ordered domains were fluid. Most tellingly, individual molecules diffused relatively freely between the domains (2). Together, these results fit the behavior expected of the fluid-yet-ordered l_o phase. Lipids isolated from brush border membranes (BBM), expected to be a rich source of rafts, also formed separate domains with similar properties to those in the simpler lipid system (1, 2).

A concern with experiments on supported bilayers and monolayers is the effect of the support on lipid behavior. For this reason, complementary studies by using giant unilamellar vesicles [GUVs; liposomes whose large (>50 μm) size allows visualization of domains] were especially important (2). The GUVs contained the fluorescent probe LAURDAN, taking advantage of the fact that this probe fluoresces more intensely in disordered than ordered domains. Temperature-sensitive 10-μm-sized domains were seen in both simple phospholipid-sphingomyelin-cholesterol GUVs and in GUVs made of BBM lipids (2). The domains were fluid but had the degree of order expected of the l_o phase. Importantly, domains in both leaflets of the bilayer were always perfectly coincident. This be-

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*E-mail: deborah.brown@sunysb.edu.

havior, reported previously in cholesterol-free GUVs (15, 16), suggests that rafts in the two leaflets of cell membranes may be similarly coupled, although the mechanism is not known.

The domains identified by Dietrich *et al.* showed additional properties expected of rafts. First, monolayer experiments showed that the raft domains were detergent-insoluble, whereas the nonraft domains were completely solubilized (2). Remarkably, detergent extraction did not grossly affect raft size, and the domains remained separate. This behavior probably reflected the association of the monolayer with the support. By contrast, detergent extraction of cell membranes may cause small rafts to coalesce into larger ones (17). In addition, the rafts were cholesterol dependent. The cholesterol effect was first demonstrated by using BBM lipid GUVs (2). Rafts first appeared in these membranes when the temperature was lowered to 45°. However, when BBM lipids were depleted of cholesterol before formation of GUVs, domains appeared only when the temperature reached 24°. This result showed that rafts did not form as readily as in the cholesterol-containing lipid mixtures. Although rafts in the cholesterol-containing GUVs had the round shape and other properties characteristic of the l_o state, rafts in the cholesterol-free GUVs had the irregular shape characteristic of gel-phase domains. These results agree with previous findings, by using detergent insolubility and fluorescence quenching, that cholesterol promotes both detergent insolubility and phase separation (12, 18).

In the more recent paper (1), the authors extended their analysis of raft cholesterol dependence by showing that removal of cholesterol from raft-containing monolayers by using methyl-β-cyclodextrin disrupted the domains (1). They also found that a GPI-anchored protein, Thy-1, partitioned preferentially into raft domains in BBM lipid monolayers (1). Interestingly, Thy-1 had only a slight preference for rafts in these membranes and actually had a slight preference for nonraft domains in dioleoyl phosphatidylcholine (DOPC)/sphingomyelin/cholesterol mixtures (2). This sphingomyelin may reflect similar behavior in cells. Several studies suggest that the affinity of individual GPI-anchored proteins for rafts may be quite low, although this affinity can be increased by antibody-mediated clustering (19–21). Surprisingly, addition of 1 mol% of the raft-enriched GM1 to both mixtures reduced the partitioning of Thy-1 into raft domains. Similar behavior was observed earlier in cell membranes, as exogenously added GM1 displaced a GPI-anchored protein from cholesterol-dependent raft-like clusters (22). Dietrich *et al.* (1, 2) suggest that this baffling behavior may result from competition between the two molecules for raft occupancy. Alternatively, GM1 may alter the properties of rafts sufficiently to affect Thy-1 partitioning. Finally, the authors showed that clustering of the saturated-chain lipid Fl-DPPE by using anti-Fl antibodies converted it into a raft-preferring lipid. Similar clustering of the unsaturated-chain lipid Fl-DOPC with the same antibodies did not affect its preference for nonraft domains. This result supports the idea that clustering of individual molecules can affect their raft partitioning (19, 20).

Seeing is believing. The work of Dietrich *et al.* (1) provides important new support for the raft model and argues persuasively that l_o domains can form in phospholipid/sphingolipid/cholesterol mixtures, at least in model membranes. Much of the behavior of these domains corresponds reassuringly well to that seen in earlier work by using other techniques. But how do these results relate to rafts in cells? The existence of rafts in cholesterol- and sphingolipid-rich cell membranes has not yet been conclusively demonstrated, and putative raft markers such as individual GPI-anchored proteins often appear to be relatively uniformly distributed in the plasma membrane (21, 23). It has not yet been possible to see micrometer-sized domains enriched in GPI-anchored proteins or gangliosides, as detected by Dietrich *et al.* in model membranes, in cell membranes.

Nevertheless, several approaches suggest that cell membranes do contain rafts. These methods include cholesterol-dependent detergent insolubility and colocalization of independently clustered proteins and lipids in patches on the cell surface (reviewed in ref. 4), as well as biophysical techniques such as fluorescence resonance energy transfer between GPI-anchored proteins (24), single-particle tracking (25), and single-molecule microscopy (26). Estimates of raft size vary from a few 10s of nanometers to 100s of nanometers or even microns (refs. 24–27, reviewed in ref. 28). This state of affairs suggests that rafts in cells are more complex than those in model membranes—even model membranes made from BBM lipids. The next challenge will be to determine how the basic principles of raft formation, for which the experiments of Dietrich *et al.* (1) provide such clear support, are applied to make rafts in cells.

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