Plasma membrane organization and function: moving past lipid rafts

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ABSTRACT **"Lipid raft" is the name given to the tiny, dynamic, and ordered domains of cholesterol and sphingolipids that are hypothesized to exist in the plasma membranes of eukaryotic cells. According to the lipid raft hypothesis, these cholesterol- and sphingolipid-enriched domains modulate the protein–protein interactions that are essential for cellular function. Indeed, many studies have shown that cellular levels of cholesterol and sphingolipids influence plasma membrane organization, cell signaling, and other important biological processes. Despite 15 years of research and the application of highly advanced imaging techniques, data that unambiguously demonstrate the existence of lipid rafts in mammalian cells are still lacking. This Perspective summarizes the results that challenge the lipid raft hypothesis and discusses alternative hypothetical models of plasma membrane organization and lipid-mediated cellular function.**

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INTRODUCTION

Plasma membrane research was once dominated by a protein-centric view in which proteins executed most membrane functions and the lipids served as a solvent that permitted protein diffusion (Singer and Nicolson, 1972). Although the idea that lipids self-assemble into compositionally and functionally distinct domains within the plasma membrane was not new (Karnovsky *et al.*, 1982), the lipid raft hypothesis popularized the idea that lipids modulate membrane organization and function (Simons and Ikonen, 1997). The hypothesis postulated that favorable molecular interactions induce the formation of cholesterol- and sphingolipid-enriched domains, referred to as lipid rafts, within the plasma membrane (Simons and Ikonen, 1997). Then the difference in each membrane protein's affinity for raft and nonraft lipid species regulates the protein's location within the plasma membrane, and, consequently, its proximity to potential binding partners. Lipid rafts were initially proposed to function as sorting platforms that mediate membrane traffic and cell signaling (Simons and Ikonen, 1997). Since then, raft involvement in additional cellular functions has been suggested, and the definition of a lipid raft has been revised (Simons and Gerl, 2010). Lipid rafts are defined as small, dynamic, ordered assemblies of cholesterol, sphingolipids, and proteins that may combine into larger structures due to lipid–lipid, lipid–protein, and protein–protein interactions (Lingwood and Simons, 2010; Simons and Gerl, 2010). The self-organizing potential of sphingolipids and cholesterol remains a key tenet in the raft hypothesis.

The potential importance of lipid rafts has attracted many new investigators to biological membrane research. After more than 15 years of study, the lipid raft hypothesis seems to be largely accepted and is presented in several current textbooks. Yet the evidence that supports the lipid raft hypothesis is primarily indirect, and alternative explanations for these results have not been thoroughly investigated. Although cellular cholesterol and sphingolipid levels clearly influence protein function, the question remains, is the mechanism for this lipid-modulated protein function accurately described by the lipid raft hypothesis? Here I summarize results that are relevant to this question and present the argument that data support alternative hypothetical models of plasma membrane organization. I also discuss alternative mechanisms for lipid-mediated cellular signaling and function that warrant further investigation.

ATTEMPTS TO DETECT LIPID RAFTS IN THE PLASMA MEMBRANES OF MAMMALIAN CELLS

The isolation of glycosylphosphatidylinositol (GPI)-anchored proteins in a low-density, detergent-insoluble fraction enriched with cholesterol and glycosphingolipids (Brown and Rose, 1992)

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Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; ERK, extracellular signal-regulated kinase; GPI, glycosylphosphatidylinositol; OSBP, oxysterol-binding protein; SIMS, secondary ion mass spectrometry.

supported the idea that GPI-anchored protein transport to apical membranes is modulated by preferential lipid–cholesterol–protein interactions. This finding contributed to the expectation that GPI-anchored proteins reside in rafts, and it also led to the extensive use of detergent extraction for lipid raft "detection" (Simons and Ikonen, 1997; Simons and Gerl, 2010). However, detergent-resistant membranes are artifactual structures that were not present before cell disruption, so such studies cannot confirm or exclude lipid raft existence (Schuck *et al.*, 2003; Lichtenberg *et al.*, 2005).

A more direct way to determine whether lipid rafts exist would be to simultaneously image the cholesterol, sphingolipids, and putative raft proteins in the plasma membrane and assess whether they are colocalized within domains. Unfortunately, the direct imaging of cellular cholesterol and sphingolipids in parallel is very difficult, especially without using labels that might alter the interactions or intracellular trafficking that might influence native component distribution. Decisive testing of the lipid raft hypothesis is further complicated by the argument that lipid rafts are too small and dynamic to be resolved with most optical approaches (Harder *et al.*, 1998); this argument was elicited by the early findings that affinity-labeled glycosphingolipids and GPI-anchored proteins were evenly distributed in the plasma membrane (Mayor *et al.*, 1994; Mayor and Maxfield, 1995; Fujimoto, 1996).

Subsequent studies yielded data that support the existence of nanoscale lipid rafts. Assessment of the proximities of putative raft markers with fluorescence resonance energy transfer or the use of improved affinity labels for component detection revealed the presence of submicrometer-scale domains of GPI-anchored proteins and glycosphingolipids in the plasma membrane (Varma and Mayor, 1998; Sharma *et al.*, 2004; Fujita *et al.*, 2007; Janich and Corbeil, 2007; Chen *et al.*, 2008). Clustering of these putative raft components is reduced by cholesterol depletion (Varma and Mayor, 1998; Sharma *et al.*, 2004; Fujita *et al.*, 2007), which is consistent with the prediction that cholesterol depletion disperses lipid rafts by eliminating the cholesterol-sphingolipid self-organizing potential that drives their formation. The presence of nanoscale lipid rafts was also suggested by the cholesterol-dependent trapping of GPI-anchored proteins and sphingolipids that was detected by single particle/molecule tracking performed with advanced fluorescence microscopy techniques (Dietrich *et al.*, 2002; Eggeling *et al.*, 2009).

Although these results are consistent with the lipid raft hypothesis, other findings indicate that they can be explained by alternative hypothetical models of plasma membrane organization. Studies of putative raft marker organization after treatment with drugs that disrupt the actin cytoskeleton show that the clustering of GPI-anchored proteins and sphingolipids is more directly dependent on cortical actin organization than association with cholesterol (Goswami *et al.*, 2008; Umemura *et al.*, 2008; Fujita *et al.*, 2009; Gowrishankar *et al.*, 2012). Moreover, cholesterol depletion perturbs cortical actin organization via a mechanism that involves changes in phosphatidylinositol 4,5-bisphosphate availability (Kwik *et al.*, 2003). Single-molecule studies of sphingolipid diffusion indicate that cholesterol-dependent sphingolipid trapping also involves the actin cytoskeleton and not the favorable cholesterol–lipid interactions that produce ordered domains in model membranes (Mueller *et al.*, 2011). Finally, numerous studies indicate that sphingolipid subspecies are organized into separate domains that differ in size (Fujita *et al.*, 2007, 2009; Janich and Corbeil, 2007; Chen *et al.*, 2008; Tyteca *et al.*, 2010; D'Auria *et al.*, 2013; Frisz *et al.*, 2013b), which suggests a more active mechanism of plasma membrane organization than cholesterol-sphingolipid self-organizing potential.

These reports support alternative hypotheses in which cortical actin and its associated proteins compartmentalize the plasma membrane into multiple types of compositionally distinct domains (Figure 1) by either indirectly associating with the membrane components (Gowrishankar *et al.*, 2012) or forming a diffusion barrier that sustains the concentration gradients produced by vesicle traffic (Tang and Edidin, 2001; Kusumi *et al.*, 2005; Lavi *et al.*, 2007). These findings also stimulated the incorporation of a role for cortical actin in modulating lipid rafts into the raft model (Lingwood and Simons, 2010; Simons and Gerl, 2010). Consequently, the extent that cholesterol and sphingolipid self-organizing potential contributes to plasma membrane organization must be evaluated to discriminate between these hypothetical models.

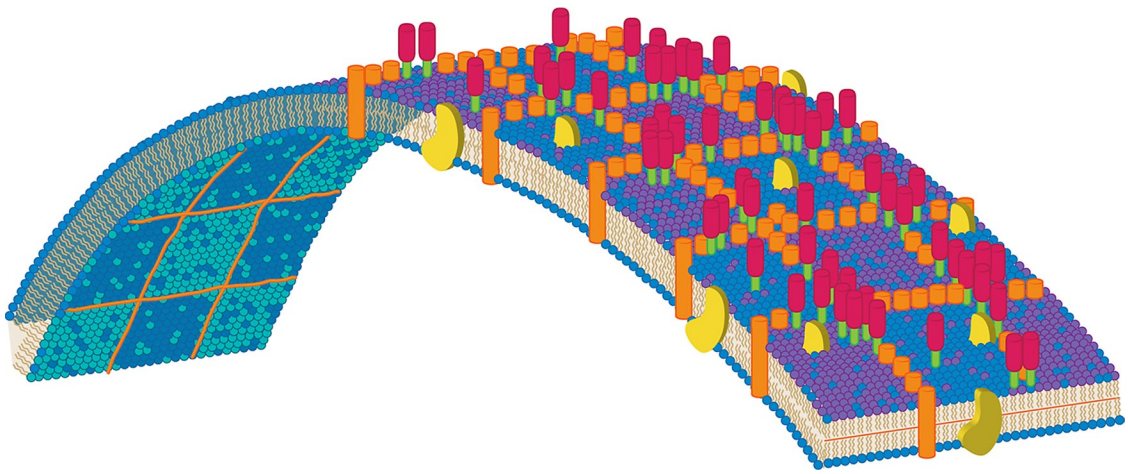

FIGURE 1: Hypothetical model in which the plasma membrane is compartmentalized by a network of transmembrane proteins (orange) that interact with the underlying cytoskeleton and obstruct the lateral diffusion of free transmembrane proteins (yellow), GPI-anchored proteins (green and red), and lipids. In this model, compositionally distinct domains may result from the association of the cytoskeleton with membrane components. Alternatively, the diffusion barrier established by the cytoskeleton and its associated proteins may maintain the concentration gradients produced by vesicle traffic.

DIRECT CHEMICAL IMAGING OF CHOLESTEROL AND SPHINGOLIPIDS IN CELL MEMBRANES

High-resolution secondary ion mass spectrometry (SIMS) was recently used to more directly assess the existence of cholesterol- and sphingolipid-enriched plasma membrane domains in fibroblast cells (Frisz *et al.*, 2013a). High-resolution SIMS performed with a Cameca NanoSIMS 50 reveals the elemental and isotope composition in a sample with a lateral resolution as high as 50 nm (Boxer *et al.*, 2009). Thus distinct stable isotopes can be used to label the lipid species of interest, which circumvents the use of complex labels (i.e., antibodies or fluorophores) that may alter the distribution of the labeled molecule within the plasma membrane.

Living fibroblast cells were metabolically labeled to selectively incorporate the distinct stable isotopes ^{15}N and ^{18}O into cellular sphingolipids and cholesterol, respectively (Frisz *et al.*, 2013a). After chemical fixing of the cells, the lipid-specific isotope enrichments on their surfaces were mapped with a lateral resolution of ~90 nm by using a Cameca NanoSIMS 50. Large (1–2 µm in diameter) sphingolipid patches consisting of clusters of sphingolipid microdomains were detected in the plasma membranes of the fibroblast cells (Frisz *et al.*, 2013a,b). Numerous control experiments and the complementary fluorescence imaging of metabolically generated fluorescent sphingolipids in living cells and during fixation ruled out the possibility that these domains were artifacts caused by fixation or the detection of intracellular membranes (Frisz *et al.*, 2013b). The sphingolipid domains were not enriched with cholesterol; instead the cholesterol was evenly distributed within the plasma membrane (Frisz *et al.*, 2013a). Note that this even cholesterol distribution is consistent with the previous report that the uneven florescence from the naturally fluorescent cholesterol analogue dehydroegosterol in the plasma membranes of mammalian cells is due to cell surface protrusions and not lateral variations in sterol concentration (Wustner, 2007). High-resolution SIMS imaging of the sphingolipid distribution after various drug treatments showed that depolymerizing the actin cytoskeleton with latrunculin A eliminated the sphingolipid domains, whereas cholesterol depletion more moderately reduced their abundance (Frisz *et al.*, 2013a,b).

The lack of cholesterol enrichment in the sphingolipid domains indicates that the self-organizing potential of cholesterol and sphingolipids was not responsible for plasma membrane organization in the fibroblast cells, which conflicts with a major tenet of the lipid raft model. The absence of cholesterol-enriched domains in the plasma membranes of these fibroblast cells also argues against the existence of lipid rafts. The possibilities, however, that the domains were smaller than the lateral resolution of the NanoSIMS analysis, their enrichment was too low to be detected, or the lipid distribution in fibroblast cells differs significantly from other cell types cannot be excluded. Nonetheless, these data support the aforementioned alternative model in which lipid organization is actively established by cortical actin and its associated proteins (Kusumi *et al.*, 2005) and cholesterol abundance influences the actin organization (Kwik *et al.*, 2003).

ALTERNATIVE MECHANISMS FOR LIPID-MEDIATED CELLULAR FUNCTION

Although the cytoskeleton-based model may accurately describe the determinants of plasma membrane organization, it does not explain how cellular levels of cholesterol and sphingolipids influence protein function. Functional assays that use drugs or chelating agents to alter cellular cholesterol or sphingolipid levels clearly demonstrate that cellular signaling and protein function are influenced by cholesterol and sphingolipid abundance (Zhao *et al.*, 2006; Lasserre *et al.*, 2008; Lingwood *et al.*, 2011). These data are often cited as support for the lipid raft model because they are consistent with the hypothesis that cholesterol and sphingolipid depletion disperses lipid rafts and eliminates the functions that they perform. The sensitivity of cell signaling to sphingolipid levels, however, is already explained by the known role of sphingolipid metabolites as ligands that selectively bind to and activate or inhibit several kinases, phosphatases, and membrane receptors involved in cell signaling (Bartke and Hannun, 2009). Changes in the cellular sphingolipid levels affect the availability of the bioactive sphingolipid metabolites (i.e., ceramide and sphingosine-1-phosphate) and the signaling pathways they regulate.

Alternative hypotheses for nonraft mechanisms of cholesterol-regulated signaling are emerging. Some data support a recently proposed model in which the direct binding of cholesterol to a scaffold protein regulates signaling complex assembly and thus the functions it performs (Sheng *et al.*, 2012). In this mechanism, either sterol binding or unbinding to the scaffold protein may serve as the activating event. Such cholesterol-dependent signaling complex formation could be modulated by the differential cholesterol concentrations present in the plasma membrane and intracellular compartments where scaffold proteins reside in the absence of lipid rafts. As an example, a cholesterol-regulated signaling complex consisting of a serine/threonine phosphatase (PP2A), a tyrosine phosphatase (HePTP), oxysterol-binding protein (OSBP), and cholesterol is involved in the extracellular signal-regulated kinase (ERK) signaling pathway (Wang *et al.*, 2005). Cholesterol binding to the OSBP scaffold protein allows OSBP to bind to PPA2 and HePTP, forming the complex that cooperatively dephosphorylates pERK; cholesterol depletion or the addition of 25-hydroxcholeserol causes the complex to disassemble and abolishes its phosphatase activity (Wang *et al.*, 2008). As a second example, cholesterol binding to the PDZ domain on the NHERF1 scaffold protein is required for sustained colocalization of NHERF1 with the cystic fibrosis transmembrane conductance regulator (CFTR) and CFTR activity (Sheng *et al.*, 2012). The cholesterol-dependent formation of other signaling complexes was also reported but often attributed to assembly in lipid rafts (Green *et al.*, 1999; Kranenburg *et al.*, 2001; Roitbak *et al.*, 2005). Might the mechanism for cholesterol depletion-induced cytoskeleton reorganization involve a cholesterol-dependent scaffold protein? Many additional studies will be required to assess the generality of this mechanism and thus the validity of this model for lipid-mediated cellular function.

The possible alternative mechanisms for cholesterol- and sphingolipid-mediated protein organization and activity discussed here are in no way comprehensive and require substantial testing. Given the complexity of the numerous events that give rise to cell signaling, the mechanisms that produce the observed lipid-mediated cellular functions may be far more elaborate than those described here or proposed to date. A substantial increase in efforts to develop and test alternative mechanisms will be essential to achieving an accurate model of lipid-mediated cellular function. Based on the growing interest in the existence of rafts and raft-like domains in the membranes of yeast (Wachtler and Balasubramanian, 2006), plant cells (Grennan, 2007), and even prokaryotic cells (LaRocca *et al.*, 2013), these efforts should not be restricted to mammalian cells.

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