Cell, Vol. 115, 377–388, November 14, 2003, Copyright ©2003 by Cell Press

# Lipid Rafts: Elusive or Illusive?

Review

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**There has been considerable recent interest in the possibility that the plasma membrane contains lipid "rafts," microdomains enriched in cholesterol and sphingolipids. It has been suggested that such rafts could play an important role in many cellular processes including signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry. However, rafts have proven difficult to visualize in living cells. Most of the evidence for their existence and function relies on indirect methods such as detergent extraction, and a number of recent studies have revealed possible problems with these methods. Direct studies of the distribution of raft components in living cells have not yet reached a consensus on the size or even the presence of these microdomains, and hence it seems that a definitive proof of raft existence has yet to be obtained.**

The plasma membrane of eukaryotic cells, like other biological membranes, contains more lipid species than are needed to form a simple bilayer (Bretscher, 1973). This has long raised interest in the question of what purpose underlies this diversity. The notion that specific lipids could serve to organize membranes into discrete domains with different properties had received only sporadic attention over the years (Jain and White, 1977; Karnovsky et al., 1982; Miller, 1984; Thompson and Tillack, 1985). However, this changed when studies on lipid biophysics, lipid sorting, and the effects of detergents on biological bilayers were brought together to argue for the existence in the plasma membrane of a particular type of microdomain that is enriched in cholesterol and sphingolipids. These microdomains are usually called lipid rafts, and many papers have suggested that rafts play a role in a wide range of important biological processes, including numerous signal transduction pathways, apoptosis, cell adhesion and migration, synaptic transmission, organization of the cytoskeleton, and protein sorting during both exocytosis and endocytosis (Brown and London, 1998; Simons and Toomre, 2000; Harris and Siu, 2002; Tsui-Pierchala et al., 2002). In addition to roles in normal cellular function, rafts have also been suggested to be the point of cellular entry of a wide range of viruses, bacteria and toxins, as well as being a site of viral assembly and formation of both prions and Alzheimer amyloid (Naslavsky et al., 1997; Kovbasnjuk et al., 2001; Suomalainen, 2002; Ehehalt et al., 2003).

Despite this large body of work, doubts persist. In particular, the most commonly used methods for raft detection, resistance to solubilization by the nonionic detergent Triton X-100 and sensitivity to cholesterol depletion, are indirect and potentially open to alternative interpretations (Heerklotz, 2002; Pizzo et al., 2002; Edidin, 2003). In addition, rafts have proven difficult to visualize in living cells, and when evidence has emerged, the apparent properties of the rafts have varied widely (Anderson and Jacobson, 2002; Kenworthy, 2002). In this review I will initially summarize what features of the plasma membrane are currently undisputed, and how these are incorporated into the raft model. I will then discuss the limitations of the methods used to argue for the existence of rafts, and recent experiments that raise potential concerns about rafts. I will also discuss other effects that cholesterol and sphingolipids could have on the physical properties of the plasma membrane. It is not my intention to argue that the plasma membrane cannot contain rafts, but rather that current knowledge does not unequivocally support the existence of rafts, and that alternative models for the structure of the plasma membrane are equally plausible.

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## The Lipids of the Plasma Membrane

The plasma membrane has a lipid composition that is very different from that of the other membranes of the cell. All cellular membranes contain glycerophospholipids, comprising one of several head groups attached via glycerol to two acyl chains, one of which is usually unsaturated. Although glycerophospholipids are sufficient to form bilayers, most eukaryotic cells contain two additional classes of lipids: sterols and sphingolipids (Figure 1). Sphingolipids are based on ceramide and have either a phosphocholine head group (sphingomyelin) or one of a range of carbohydrate structures (glycosphingolipids). They differ further from the glycerophospholipids in that both acyl chains are often saturated, and the ceramide backbone contains both hydrogen bond donors and acceptors (Holthuis et al., 2001; Ramstedt and Slotte, 2002). The sterols are based on a rigid four-ring structure, with cholesterol being the principle form found in vertebrates. Sterols and sphingolipids are present at low levels in internal membranes and indeed are synthesized in the endoplasmic reticulum (ER) and Golgi respectively, but are at high levels in the plasma membrane and endosomes (van Meer, 1998; Hao et al., 2002). Although the precise levels of these lipids vary between different cell types, cholesterol is typically present at 30–40 mol % of plasma membrane lipids (Lange et al., 1989; van Meer, 1989). Sphingomyelin is present at 10–20 mol %, while glycosphingolipids are usually present at only low levels, but in some specialized membranes such as myelin or barrier epithelia like intestine that face the external environment they are present at levels up to, or exceeding, 30 mol % (Simons and van Meer, 1988; Le Grimellec et al., 1992; Stoffel and Bosio, 1997).

A second established property of the plasma membrane is that the lipid compositions of the two sides, or leaflets, of the bilayer are very different. Most, if not all,

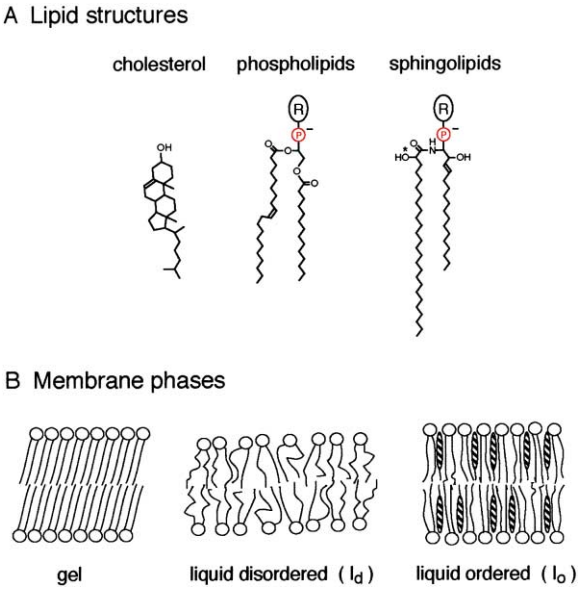


Figure 1. The Lipids of the Plasma Membrane

(A) Structures of the major lipid classes of eukaryotic cells. Sterols are based on a four-ring structure, with cholesterol being the form present in mammals, and different versions found in species such as plants and fungi (Ohvo-Rekila et al., 2002). Glycerophospholipids are based on diacylglycerol and typically carry acyl chains of 16–18 carbon atoms, one of which contains a *cis* double bond. The head group (R) is either neutral, (serine or inositol) to give a net acidic charge, or basic (ethanolamine or choline) to give a neutral, or zwitterionic lipid. Sphingolipids are based on a ceramide, and in mammals the head group is either choline (sphingomyelin), or in the case of the glycosphingolipids the phosphate is replaced with glucose, which is further elaborated to make a wide range of glycolipids. The acyl chain attached to the sphingoid base is typically saturated, varying in length from 16–26 carbons depending on the lipid and tissue (C26 is shown here). The presence of hydrogen bond donors (hydroxyl groups) and acceptors (carbonyl groups) is in contrast to the glycerophospholipids, which have only the latter (Holthuis et al., 2001). These promote interactions between sphingolipids, and in cases such as in myelin and fungi, additional hydroxyl groups are found on the acyl chains (asterisk).

(B) Structures of lipid bilayers. Simple phospholipid bilayers below their $T_m$ form a solidified gel phase, which melts above the $T_m$ to a fluid phase (liquid-disordered ($l_d$), sometimes referred to as "liquid-crystalline"). The presence of cholesterol (hatched ovals) orders the acyl chains of the latter phase, and indeed can fluidize the former phase, arriving at an intermediate state for which the term liquid-ordered ($l_o$) was coined (Ipsen et al., 1987).

of the sphingolipids are present in the outer leaflet, while some of the glycerophospholipids, i.e., phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine, are restricted to the inner leaflet (Bretscher, 1973). This latter asymmetry is maintained by active transport, and its effectiveness is illustrated by phosphatidylserine being seen in the outer leaflet only when cells undergo apoptosis (Balasubramanian and Schroit, 2003). Determining the distribution of cholesterol has proven challenging as, unlike the phospholipids, it has a high rate of spontaneous flipping between the two leaflets ($t_{1/2}$ ~1 s) (Muller and Herrmann, 2002; Steck et al., 2002). However, it is known that cholesterol preferentially interacts with sphingolipids rather than unsaturated phospholipids (Ramstedt and Slotte, 2002), and so it seems likely that it is at least as abundant in the outer leaflet as in the inner leaflet.

Given the high level of cholesterol in the plasma membrane it is important to consider how cholesterol affects the physical properties of lipid bilayers. Pure phospholipid bilayers can exist in two states, a solid or "gel" state, and above their melting temperature (Tm), a fluid or "liquid" state (Figure 1). The solid gel phase is not thought to be of physiological relevance, and indeed the *cis* double bond present in phospholipids reduces their Tm by antagonizing close packing of the acyl chains: this ensures bilayer liquidity at physiological temperatures. However, the fluid structure of liquid bilayers is profoundly altered by the addition of high levels of cholesterol (Yeagle, 1985; Ohvo-Rekila et al., 2002). The rigid sterol causes the lipid acyl chains to become closely packed or compacted, and the bilayer to be thickened. Because the long rigid cholesterol molecule is arranged perpendicular to the bilayer, this organizing effect is highly directional. This means that the lipid acyl chains cannot readily deform to allow movement of small molecules across the bilayer, but the lipids can still move freely past each other. Therefore, cholesterol has the striking property of ordering the bilayer in one dimension and hence reducing permeability, while only having a minimal effect on the rate of lateral diffusion of proteins and lipids in the plane of the bilayer (Owicki and McConnell, 1980; Smith et al., 1980). The high cholesterol bilayer is thus termed "liquid-ordered" ($l_o$), in contrast to the "liquid-disordered" state without cholesterol ($l_d$) (Figure 1).

The evidence for these two bilayer states first came from examining the physical properties of bilayers containing a range of cholesterol concentrations. Moreover, these same studies indicated that these two states could be relevant to the question of lipid microdomains. Evidence from a number of different methods indicated that the properties of the bilayer do not change in a gradual fashion as cholesterol is increased, but rather there are phase transitions at certain levels of cholesterol (Sankaram and Thompson, 1990; Vist and Davis, 1990; Thewalt and Bloom, 1992; Xiang and Anderson, 1998). This has been interpreted as meaning that above a threshold level of cholesterol, the cholesterol-rich $l_o$ and cholesterol-poor $l_d$ phases coexist within a single bilayer, with the amount of the former increasing with the cholesterol level until there is a continuous $l_o$ phase. The formation of $l_o$ phase is also promoted by lipids that contain exclusively saturated acyl chains, as these pack more readily against cholesterol. Saturation is much more prevalent in sphingolipids, and they indeed appear to promote $l_o$ phases, an effect enhanced by the capacity of sphingolipids to form intermolecular hydrogen bonds (Li et al., 2001; Ramstedt and Slotte, 2002).

## The Raft Model for the Lateral Organization of Plasma Membrane Lipids

Current knowledge therefore allows the safe conclusion that not only are cholesterol and sphingolipids abundant in the plasma membrane, but that the latter are restricted to the outer leaflet, along with at least half of the cholesterol. Moreover, in model bilayers these same two lipid types appear to have the capacity to form ordered and/

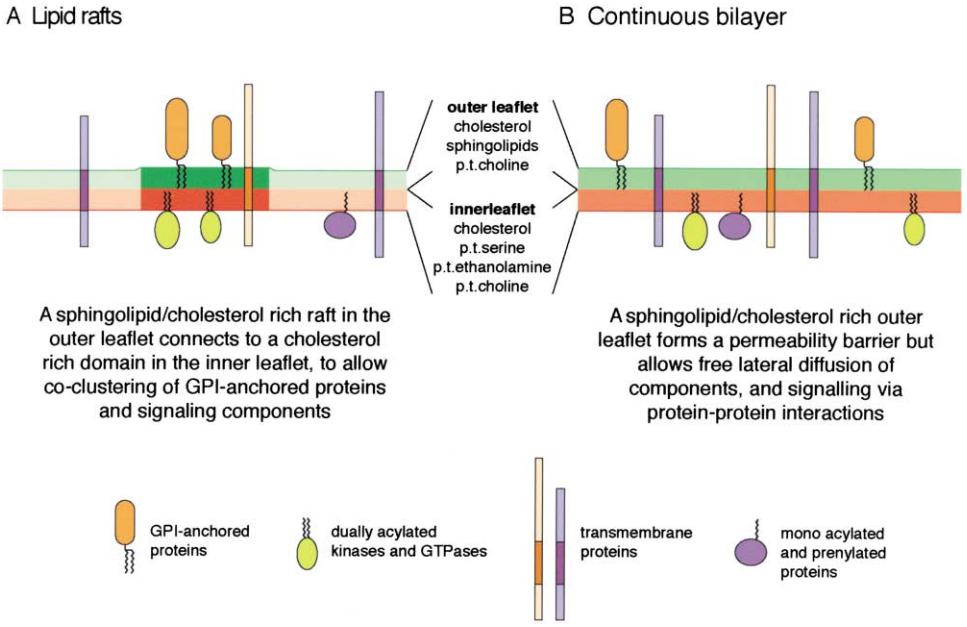


Figure 2. Models for the Organization of the Plasma Membrane

(A) The lipid raft model. In the outer leaflet of the plasma membrane there are microdomains of cholesterol and sphingolipid rich $l_o$ phase that are surrounded by $l_d$ phase. These domains are proposed to be coupled to cholesterol-rich microdomains in the inner leaflet by an as yet uncertain mechanism. The proteins of the plasma membrane partition between the raft and surrounding bilayer on the basis of their physical properties. In particular GPI-anchored proteins, dual-acylated kinases and GTPases, and some transmembrane proteins are clustered in the rafts. It is suggested that some signaling receptors can move into the rafts upon ligand engagement, or "cluster" smaller rafts into larger ones (Brown and London, 1998; Simons and Toomre, 2000). Liquid-ordered monolayers and bilayers are known to be thicker than their liquid-disordered equivalents.

(B) The continuous model. If rafts do not exist, the outer leaflet of the plasma membrane would be an essentially homogenous phase rich in cholesterol and sphingolipids. This would provide a permeability barrier to cells that remained highly fluid in the plane of the bilayer, thereby allowing proteins to move freely by lateral diffusion and participate in protein:protein interactions. The high levels of cholesterol and sphingolipids are also likely to cause the bilayer to be thicker than those of the earlier compartments of the secretory pathway (Bretscher and Munro, 1993). The inner leaflet would also be essentially homogenous, but rich in acidic and amino phospholipids, with the former serving to attract basic peripheral proteins.

or disordered domains in a fluid bilayer. The lipid raft model therefore proposes that the cholesterol and sphingolipids of the outer leaflet of the plasma membrane are not distributed evenly, but rather cluster into $l_o$ domains that float in an $l_d$ bilayer (Schroeder et al., 1994; Brown, 1998; Rietveld and Simons, 1998) (Figure 2). Transmembrane proteins in the lipid bilayer would either reside in, or be excluded from, rafts depending on partitioning imparted by their physical properties. In the case of the outer leaflet of the bilayer, it is suggested that a major resident of rafts are proteins attached to the bilayer by covalent linkage to glycosylphosphatidylinositol (GPI) lipid anchors. The functional relevance of the raft model comes from the proposal that the sphingolipid and cholesterol-rich domains in the outer leaflet are connected to lipid domains in the inner leaflet, and that components of signal transduction pathways, in particular G proteins and nonreceptor tyrosine-kinases that are anchored to the inner leaflet of the plasma membrane by virtue of multiple acyl chains, selectively partition into this inner leaflet part of the raft (Brown, 1993; Harder et al., 1998). This would allow rafts to act as "signaling platforms" that couple events on the outside of the cell with signaling pathways inside the cell, and indeed this has been suggested to be their most important function (Simons and Ikonen, 1997; Brown and London, 1998; Simons and Toomre, 2000). Rafts have been proposed to play a role in signaling by, amongst others, T cell receptors, B cell receptors, IgE receptors, neurotrophic factors, growth factors, chemokines, interleukins, and insulin (Bromley et al., 2001; Paratcha and Ibanez, 2002). It is argued that restricting these signaling pathways to microdomains would allow activated receptors enhanced access to particular downstream signaling proteins, while excluding them from others. In addition, it has been suggested that clustering of receptors might cause the clustering of small rafts into a larger microdomain and hence bring together signaling components previously isolated in the smaller domains (Simons and Toomre, 2000; Anderson and Jacobson, 2002; Harris and Siu, 2002; Subczynski and Kusumi, 2003).

Evidence to argue for the existence of such rafts has come from biophysical studies, detergent extraction, examination of the effects of cholesterol depletion, and examining the distribution of putative raft components in living cells, and I will discuss what these methods can reveal about the organization of the plasma membrane. Before doing so it is also worth noting the null hypothesis for the raft model, which is that large scale clustering of lipids does not occur in the plasma membrane, and hence the lipids form a continuous essentially uniform phase in each leaflet of the bilayer (Figure 2).

### Studies in Model Membranes

While the results from model systems indicate that the existence of $l_o$ microdomains in a fluid plasma membrane is physically possible, a number of issues need to be considered when applying this idea to living cells. There are of course the usual caveats that apply to the simplifications inherent in model systems, which in this instance is that studies are often with one or two homogeneous phospholipids, and at nonphysiological temperatures, and sometimes in monolayers where the lateral pressure may be less than in a bilayer. In contrast, biological bilayers contain lipid acyl chains with great heterogeneity in their length and saturation, and also contain a large number of diverse integral proteins, which can themselves exert an organizing effect on surrounding lipids. Because lipid phase separation is sensitive to changes in temperature, lateral pressure, lipid heterogeneity, and protein content, the results from model membranes do not at present allow a precise prediction of what is happening in the bilayers of the cell.

However, beyond these general concerns, the results from model membranes raise a number of more specific issues that need to be considered in the context of the plasma membrane. The first is to consider what proportion of the bilayer might be in $l_o$ or $l_d$ domains. Although the levels of sphingolipids and cholesterol vary between different cell types, they are both abundant components of the plasma membrane, and in the case of sphingolipids their asymmetric distribution means that they will be present in the outer leaflet at double their apparent abundance in total membrane lipid. In fact, in many model studies the amount of cholesterol required to form a continuous $l_o$ phase is 25–30 mol %, which is at the lower end of the range reported for plasma membrane cholesterol levels. Indeed one early study concluded the $l_o$ phase they had observed in vitro would be the probable state of the whole plasma membrane—i.e., the outer leaflet would be one big raft (Thewalt and Bloom, 1992).

A second issue is related to the proposal that rafts organize signaling components in the inner leaflet of the bilayer, when the lipids believed to form rafts are present in part, or in the case of sphingolipids completely, in the outer leaflet of the bilayer. To reconcile this, it has been proposed that the sphingolipid and cholesterol raft in the outer leaflet is able to organize the lipids on the inner leaflet, with the result that the raft is coupled to a cholesterol-rich domain in the inner leaflet. In particular it has been suggested that the difference in length of the acyl chains of sphingolipids would allow them to interdigitate with the lipids in the inner leaflet (Simons and Ikonen, 1997; Simons and Toomre, 2000). Although not all sphingolipids show a marked length difference, it has nonetheless been found in biophysical studies of pure lipid bilayers that when there is a large length difference then there does appear to be interdigitation between the leaflets (Schmidt et al., 1978; McIntosh et al., 1992). However, this effect is reduced by the presence of cholesterol (McIntosh et al., 1992). In addition, the inner leaflet of the plasma membrane lacks sphingolipids but is rich in acidic phospholipids and unsaturated acyl chains. A recent biophysical study of membranes with a lipid composition corresponding to that of the inner leaflet found that cholesterol does not induce $l_o$ domains, nor is the bilayer detergent-resistant (Wang and Silvius, 2001). Of course it is possible that transmembrane proteins could contribute to domain formation in vivo, but at present there is no clear explanation for how lipid rafts in the outer leaflet could organize signal-transducing molecules in the inner leaflet of the plasma membrane.

Finally, it should be noted that many studies on domains in model membranes rely on using spectroscopic methods to detect phase transitions as temperature is altered. The interpretation of the resulting phase diagrams is complex and not universally agreed upon. For instance, it has recently been proposed that some observations interpreted as phase changes might instead be caused by the formation of small short-lived "condensed complexes" containing just a few cholesterol and phospholipid molecules (McConnell and Vrljic, 2003). Given all these uncertainties, it seems reasonable to conclude that the formation of discrete $l_o$ microdomains in the plasma membrane is possible, but that other interpretations cannot be excluded, the simplest of which would be a homogenous outer leaflet with $l_o$ or intermediate properties.

### Detergent-Resistant Membranes

The most widely used assay for raft existence and residence is based on the observation that when the plasma membrane is extracted with the nonionic detergent Triton X-100 at 4°C, only a subset of the components is solubilized (Yu et al., 1973; Edidin, 2003). Typically, cells or isolated membranes are exposed to detergent, placed at the bottom of a density gradient and subjected to centrifugation to allow the detergent-resistant membrane to float away from the soluble and nonmembranous material. It has been argued that this resistant material represents raft domains based on the seminal observation that the proposed raft residents, GPI-anchored proteins, acquire resistance to Triton as they move through the secretory pathway, and that the lipids in the Triton-resistant fraction are enriched in cholesterol and sphingolipids (Brown and Rose, 1992). In addition, liposomes containing cholesterol and sphingolipids in an $l_o$ phase are resistant to cold Triton, suggesting that this extraction could allow a means to monitor the presence of $l_o$ phase domains in vivo (Schroeder et al., 1994, 1998). It was subsequently found that a number of signal transduction components, in particular acylated GTPases and nonreceptor tyrosine kinases, are at least partially insoluble in cold Triton (typically 10%–30%, but sometimes even less), and from this it has been proposed that they are associated with rafts (Montixi et al., 1998; Zhang et al., 1998; Niv et al., 2002; Foster et al., 2003). However, there are a number of reasons why it cannot be assumed that detergent resistance reflects a protein being present in a microdomain that was present before Triton was added.

Detergent solubilization involves the partitioning of individual detergent molecules into the lipid bilayer until they reach a sufficient level to induce the formation of holes, and then micellar fragments (le Maire et al., 2000). A number of nonphysiological rearrangements of the bilayer could occur during this process. Firstly, lipid phase behavior is highly temperature dependent and

so the reduction in temperature alone could potentially induce alterations in lipid organization. It also seems likely that the highly asymmetric nature of the bilayer will lead to anomalous processes during detergent solubilization. The formation of holes in the bilayer would result in mixing of the two leaflets early in the extraction process, and hence cause a large perturbation of their lipid compositions (an issue also of relevance to studies on torn sheets of plasma membrane; Prior et al., 2003). Even if such mixing did not occur, the two leaflets of the bilayer may be differentially sensitive to Triton due to their different lipid compositions, with the resulting generation of transient unstable structures such as monolayers. In vitro studies with model membranes have found that when sufficient cholesterol is present to form a continuous $l_o$ phase, i.e., if there are no microdomains, then the entire bilayer is detergent-resistant (Schroeder et al., 1994, 1998). In contrast, when the inner leaflet of the plasma membrane is reconstituted in vitro it is entirely soluble in Triton (Wang and Silvius, 2001). Thus, one limitation of the detergent solubilization approach is that partial solubilization of the bilayer need not arise from differences in the detergent sensitivity of coexisting domains, but could instead be due to differences in the detergent sensitivity of the two leaflets. Finally, it has recently been observed that Triton actually promotes the formation of ordered domains in model bilayers, by reducing further the already low levels of sphingolipids and cholesterol in the $l_d$ phase, and so the presence of the detergent may induce segregation of components of the bilayer (Heerklotz, 2002). All this suggests that detergent solubilization could involve the formation of highly nonphysiological structures from the starting membrane. Particular proteins could be extracted from the resulting structures depending on their physical properties, rather than their lateral distribution in the living cell. Indeed it has been noted that there is a general tendency for the components of the bilayer that are more hydrophobic, or have a higher Tm, to appear in detergent resistant membranes (Ferguson, 1999).

One interesting approach to understanding what actually occurs during detergent extraction has been to expose cells to cold Triton, and to then use microscopy rather than fractionation to examine the distribution of membrane components in the detergent-extracted cells. This has shown that most of the plasma membrane surface remains intact, with the removal of a subset of membrane proteins and the appearance of a few holes (Mayor and Maxfield, 1995; Hao et al., 2001) (Figure 3). Clearly such a result is not readily compatible with the notion of small rafts. Another illuminating study is a recent proteomic analysis of detergent resistant fractions prepared by flotation (Foster et al., 2003). This was performed on samples prepared in parallel from untreated cells, and from cells from which cholesterol had been depleted, a treatment that is believed to abrogate raft formation (see below). This study found that, as expected, many GPI-anchored proteins and lipid anchored G proteins showed a cholesterol-dependent appearance in the floating "raft" fraction, although there were also a large number of proteins present in this detergent-resistant fraction whose presence was unaffected by depletion of cholesterol and hence could not

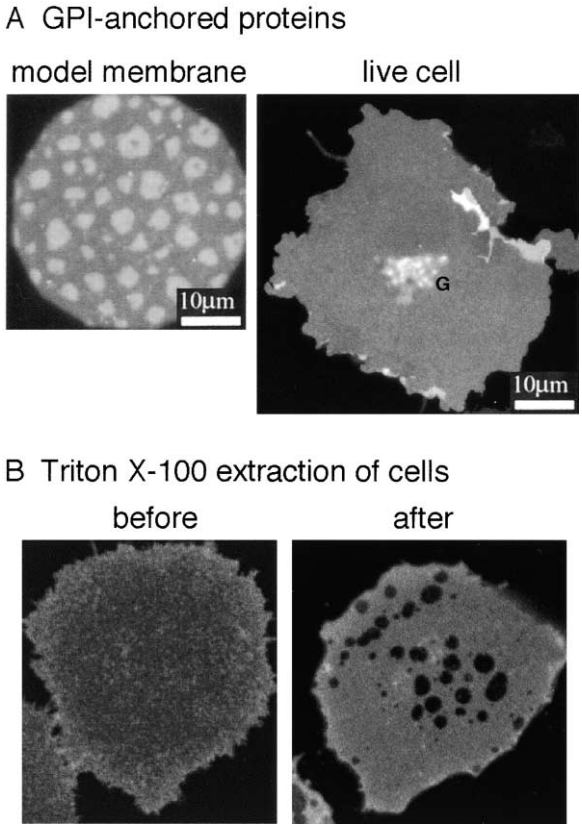


Figure 3. Looking for Rafts

(A) Microdomains can be readily visualized by light microscopy in model membranes under appropriate conditions, but they have proven more elusive in the plasma membrane of cells. Distribution of a fluorescein-labeled GPI-anchored protein (Thy1) in a monolayer formed with lipids extracted from kidney brush border membranes, imaged at 24°C (Dietrich et al., 2001b). Distribution of GFP-GPI in a transfected COS cell. The folded membranes at the edge and the Golgi apparatus (G) are bright, but otherwise the plasma membrane fluorescence appears uniform (image kindly provided by Ben Nichols; Nichols et al., 2001).

(B) Resistance to Triton solubilization is often used to identify rafts, but much of the plasma membrane is resistant to this treatment. Micrographs of cells stained with a fluorescent monoclonal antibody to a GPI-anchored protein (folate receptor), with or without extraction with Triton X-100 for 30 min at 4°C, before being rinsed and imaged (Hao et al., 2001). Note that the dark patches that appear after Triton extraction are holes, not bilayer domains that exclude the GPI-anchored protein (Mayor and Maxfield, 1995). Images provided with permission. Copyright (2001) National Academy of Sciences, U.S.A.

be present in cholesterol-dependent domains. More strikingly, a number of the proteins found to show cholesterol-dependent detergent resistance are not normally associated with the plasma membrane, such as components of the ribosome, mitochondria, and the nucleus, suggesting associations that formed upon detergent addition.

For these reasons, it seems difficult to argue with Heerklotz's conclusion from the study of the mechanism of Triton action described above, that "detergent resistant membranes should not be assumed to resemble biological rafts in size, structure, composition or even existence" (Heerklotz, 2002). This of course does not

prove that rafts do not exist, rather that detergent resistance membranes cannot be assumed to accurately report their content, or indeed demonstrate their existence. Nor is it to say that the detergent insolubility assay is without value. An increase in the detergent resistance of an individual protein during a cellular process does suggest a change in interactions, such as becoming bound to a protein that is poorly solubilized. Indeed, detergent resistance has proven a valuable means of assessing protein associations with large nonlipid containing structures such as the cytoskeleton and centrosomes.

### What Does Cholesterol Depletion Do to Cells?

Many experiments have evoked a role for rafts in signaling or other processes based on the effects of depleting cholesterol, and sometimes sphingolipids, from cells. Cholesterol is typically depleted either by inhibitors of synthesis in combination with cholesterol-free medium, or by extracting it directly with cyclodextrins. Although both may affect other molecules, it is possible to demonstrate specificity by adding back cholesterol to the depleted cells and although complete depletion of cholesterol and sphingolipids results in cell death, cells can withstand temporary reductions in the levels of cholesterol, and many processes do indeed appear to be affected by this. However, the main concern with this approach is that if cholesterol and sphingolipids were to have functions in the plasma membrane apart from forming lipid rafts, then their depletion could affect these other roles and hence any consequences would not necessarily be due to the loss of rafts. A number of nonraft roles have been suggested for cholesterol and sphingolipids, including alteration of the physical properties of cellular membranes such as permeability and fluidity, as well as interacting with specific proteins (Ohvo-Rekila et al., 2002; Ramstedt and Slotte, 2002; Kurzchalia and Ward, 2003).

Of the possible nonraft roles of cholesterol, the best characterized is the effect on bilayer permeability. Addition of cholesterol or sphingolipids to phospholipid bilayers in vitro reduces their permeability to small molecules such as ions and small nonelectrolytes like glucose and water (Finkelstein and Cass, 1967; Demel et al., 1968; Finkelstein, 1976). This is believed to be caused by the cholesterol, or the saturated acyl chains that predominate in sphingolipids, preventing the acyl chain rearrangements that accommodate small molecules in transit across the bilayer (Haines, 2001; Ohvo-Rekila et al., 2002). When cholesterol is depleted from living cells, the plasma membrane shows increased permeability to both ions and small nonelectrolytes (Grunze and Deuticke, 1974; Chen et al., 1978; Launikonis and Stephenson, 2001). Conversely, elevated levels of cholesterol and sphingolipids with long acyl chains are found in cell types with particularly impermeant plasma membranes, such as the barrier epithelia of kidney, the myelin sheath that insulates neurons, or the plasma membrane of fungi (Simons and van Meer, 1988; Le Grimellec et al., 1992; Stoffel and Bosio, 1997). Reduction in the levels of these lipids results in increased bilayer permeability (Gaber et al., 1989; Coetzee et al., 1998; Krylov et al., 2001). As well as altering permeability, cholesterol has also been suggested to prevent the formation of gel phases and hence increase the lateral fluidity of cellular membranes (Ohvo-Rekila et al., 2002; Ramstedt and Slotte, 2002). In addition, cholesterol is required for the optimal activity of some plasma membrane proteins, such as $Na^+/K^+$ ATPase, perhaps to ensure that newly synthesized molecules are not active during transit through the early parts of the secretory pathway (Sotomayor et al., 2000).

Of course a role for cholesterol and sphingolipids in maintaining the physical properties of the plasma membrane need not exclude them from also forming lipid rafts, but it does complicate the interpretation of the effects obtained by reducing their levels. One interesting approach has been to compare the sensitivity of different processes to cholesterol depletion. For instance, cholesterol depletion inhibits signaling by H-Ras but not K-Ras, and endocytosis by the clathrin-independent route is more sensitive to cholesterol removal than the clathrin dependent pathway (Roy et al., 1999; Nichols, 2003). However, the limitation with this approach is that there would appear to be no a priori reason to assume that formation of rafts is more sensitive to cholesterol depletion than the other processes that cholesterol is involved in. Indeed there is evidence that a number of cellular processes are affected by partial cholesterol depletions that do not compromise cell viability. For instance, a study on signaling in T cells found that a mild cholesterol depletion that did not inhibit TCR-induced tyrosine phosphorylation, nonetheless had an effect on $Ca^{2+}$-dependent responses due to nonspecific depletion of $Ca^{2+}$ stores and loss of membrane depolarization (Pizzo et al., 2002). In addition, sublethal cholesterol depletion has also been found to affect internal organelles (Keller and Simons, 1998; Schmidt et al., 2001). Since it has been reported that H-Ras, but not K-Ras, signals from the internal membranes of the cell such as Golgi and endosomes, the differential sensitivity of the Ras signaling pathways to cholesterol depletion could reflect differences in the sensitivity of their locations to cholesterol removal (Chiu et al., 2002).

Given that cholesterol affects a number of physical properties of bilayers, and that cellular processes are in any case likely to be differentially sensitive to changes in any one physical property of the plasma membrane, then differential effects of cholesterol depletion could have a number of causes of which loss of microdomains is only one. Interestingly, a recent study found evidence that cholesterol depletion actually induced the formation of domains in the plasma membrane that preferentially included lipid probes that prefer a disordered bilayer (Hao et al., 2001). This would be consistent with cholesterol normally being sufficiently abundant to maintain a continuous $l_o$-like phase in the outer leaflet of the plasma membrane.

### In Vivo Observation of Putative Raft Components

From the biological point of view, the only system where raft existence matters is of course in living cells unperturbed by detergent or cholesterol-depletion. Therefore the most direct and meaningful test of the existence of rafts is to examine the distribution of putative rafts residents in living cells. However, when fluorescent mi-

croscopy has been used to examine the distribution of GFP fusions to GPI proteins or acylated signaling components, or of fluorescent lipid analogs, these reporters generally show an even distribution across the plasma membrane (Mayor et al., 1994; Chen et al., 1997; Mukherjee et al., 1998). One interpretation of this is that the rafts are "too small" to be seen by light microscopy (Simons and Toomre, 2000), although strictly speaking fluorescence-based mechanisms have no lower limit of object size that can be detected, but rather limits of resolution and sensitivity. Hence, the uniform appearance of putative raft markers by light microscopy implies that if rafts exist they are too faint or too close together to be resolved. In the absence of clearly visible clustering of putative raft components, methods such as fluorescence energy transfer (FRET) and single-particle tracking have instead been used to determine if their distribution or motion is consistent with clustering closer than would be expected from a random distribution, or with confinement in small patches in the plasma membrane. These studies have produced mixed results. In some cases, both protein and lipid reporters have shown no detectable clustering and instead appeared to be randomly distributed (Chen et al., 1997; Kenworthy and Edidin, 1998; Kenworthy et al., 2000; Vrljic et al., 2002). Other studies have found evidence for local clustering or confinement, but they have reported widely varying values for the diameter of the putative rafts (25–700 nm) (Varma and Mayor, 1998; Pralle et al., 2000; Zacharias et al., 2002; reviewed in Anderson and Jacobson, 2002). This variability in the apparent size of rafts could in part be caused by the differing sensitivity and resolution of the different methods used. However, in one case, two reporters carrying different lipid modifications proposed to confer raft association both appeared to form clusters in the inner leaflet of the plasma membrane, but they did not cocluster, suggesting that the clustering observed did not reflect partioning into a shared microdomain (Kenworthy, 2002; Zacharias et al., 2002). This raises the possibility that in at least some cases what are being observed are reporter-specific interactions with other cellular components and not phenomena based on a shared mechanism such as rafts.

One interesting approach to determining if there are raft microdomains in cells is to compare the properties of putative raft residents in vivo with that shown by similar reporters in model membranes in which cholesterol-rich microdomains are known to form. When fluorescent lipids, or GPI-anchored proteins are incorporated into model membranes comprising cholesterol, sphingomyelin, and phosphatidylcholine, the probes can be seen to segregate between cholesterol-rich and cholesterol-poor domains, both of which appear to be fluid (Dietrich et al., 2001a, 2001b; Kahya et al., 2003). These domains coalesce to become very large, typically 5–20 $\mu$m across, a size that would be hard to miss in vivo (Figure 3). Evidence for domains with similar properties in living cells was then sought by following the motion of beads attached at multivalency to lipid or GPI reporters, and looking for trapping in "transient confinement zones" (Dietrich et al., 2002). Evidence for such trapping was observed, with zones of $\sim$0.2 $\mu$m diameter, consistent with previous studies showing transient trapping of proteins during their diffusion across the plasma membrane (Edidin, 1993). However, not only were these zones much smaller than the 5–20 $\mu$m lipid domains seen in vitro (indicating that if they were lipid domains, something would have to be preventing them from coalescing), but they showed different physical properties to the in vitro lipid domains. Firstly, trapping in zones was also observed with unsaturated lipids, which were excluded from the cholesterol-rich domains in vitro. Secondly, the size of the zones seen in vivo was unaffected by temperature changes over a large range (4–37°C), while the size of the lipid domains seen in vitro was highly temperature dependent, with the domains apparently disappearing at physiological temperatures. This suggests that the mechanism underlying confinement of the putative raft probes in vivo may be distinct from the lipid domain based segregation seen in vitro. The confinement zones might instead be explained by the older model of "fences" that form by interactions between the cytoskeleton and membrane proteins and which then transiently impede lateral diffusion (Jacobson et al., 1981; Tsuji et al., 1988). Indeed, it has recently been reported that such zones of transient confinement are dependent on the actin cytoskeleton, but are not affected by cholesterol depletion (Fujiwara et al., 2002). In addition, single particle tracking studies have found the dynamics of the GPI-anchored protein CD59 to be almost identical to those of a fluorescent nonraft phospholipid (Subczynski and Kusumi, 2003). Therefore, the confinement zones seen in vivo could be accounted for by compartmentalization of the plasma membrane by the underlying cytoskeleton and the transmembrane proteins attached to it and need not reflect the existence of cholesterol-rich raft domains.

An alternative approach to obtaining visual evidence of rafts has been to examine those signal transduction events that involve a clustering of signaling components on the surface of cells. In particular, T lymphocytes form extensive contacts or "synapses" with their target cells during activation of the T cell receptor (TCR). As with other signaling systems, evidence for raft involvement has come from changes in Triton resistance of signaling components, the effects of cholesterol depletion, and the clustering of putative raft lipids in the synapse along with the TCR and associated signaling proteins (Bromley et al., 2001; Pizzo and Viola, 2003). The initial evidence for clustering of raft lipids during TCR activation came from studies using the nontoxic B subunit of cholera toxin (CT-B). This pentamer binds the glycosphingolipid GM1, and it has been widely used as a putative reporter for the distribution of lipid rafts (Harder et al., 1998; Janes et al., 1999). Binding of the CT-B pentamer brings together 5 GM1 molecules and masks their negative charge (Merritt et al., 1994). In addition, although GM1 is the major surface receptor for CT-B, the toxin can also bind to other sugar structures with terminal galactose residues. Although this is with a greatly reduced affinity, it is sufficient for CT-B to be purified on immobilized galactose (Uesaka et al., 1994), raising the possibility that some of the surface bound CT-B is attached to glycoproteins. Finally, it has recently been found that CT-B can induce signaling in B cells through a region distinct from its GM1 binding site, suggesting it may bind an additional ligand, at least on B cells (Aman et al., 2001). As such, CT-B does not seem an ideal reporter

to examine the lateral distribution of lipids in the plasma membrane.

A couple of recent studies have used alternative means to examine the distribution of putative raft constituents during TCR signaling. One examined a different reporter, a fluorescent protein attached to a GPI anchor, in living cells, and found that it did not cluster during TCR activation, even though many signaling proteins previously proposed to be in rafts did cluster with the TCR (Bunnell et al., 2002). A similar conclusion came from a different approach in which anti-TCR-coated microbeads were attached to T cells and ripped away with native membrane attached (Harder and Kuhn, 2000). Although these beads were found to recruit TCR and associated signaling molecules, there was no increased recruitment of GM1 or cholesterol. The authors also examined a fluorescent protein fusion to LAT (linker for activation of T cells), a transmembrane protein that is one of the central scaffold molecules for TCR signaling and is found in detergent-resistant membranes (Zhang et al., 1998). Mutation of the tyrosine phosphorylation sites in the cytoplasmic tail of LAT did not reduce association with detergent-resistant membranes, but blocked association with signaling molecules and recruitment into the T cell synapse following activation (Hartgroves et al., 2003). These results suggest that raft lipids, and detergent-resistant proteins are not clustered into the T cell synapse during T cell signaling, and that instead protein-protein interactions could account for the formation of signaling assemblies.

## Rafts and the Function of GPI-Anchored Proteins

It is worth considering some of the other consequences for cells if there were raft domains in the outer leaflet of the plasma membrane. A major class of proteins proposed to reside in rafts is GPI-anchored proteins (Brown and Rose, 1992; Foster et al., 2003). However, for many GPI-anchored proteins it is unclear why they should need to be clustered, as they are enzymes such as alkaline phosphatase and acetylcholine esterase that degrade small molecules. Other GPI-anchored proteins are coreceptors for extracellular ligands that need to be presented to transmembrane proteins to mediate signal transduction, and it is argued that this would bring the transmembrane protein into contact with cytosolic-signaling molecules associated with the raft (Brown, 1993; Simons and Toomre, 2000). However, if the GPI anchor did not confer clustering, but rather rapid lateral diffusion, it would allow the coreceptor to capture ligands and present them to less mobile signaling receptors, and hence increase the sensitivity of ligand recognition (Chan et al., 1991; Ferguson, 1999). Indeed for some GPI-anchored proteins, it would seem disadvantageous for them to be clustered. One example is the class of GPI-anchored proteins that inhibit complement activation on the cell surface. Complement in blood is constitutively activated at a low level that can be rapidly amplified to provide an immunoglobulin-independent "alternative" pathway of activation. Cells are protected from the activated complement by the proteins decay accelerating factor, CD59 and homologous restriction factor. All of these proteins are GPI anchored, and their importance is illustrated by the genetic disease paroxysmal nocturnal hemoglobinuria in which patients lack all GPI-anchored proteins in their blood cells, and are anemic due to extensive red cell lysis (Luzzatto et al., 1997). These proteins act by binding directly to membrane bound components of the complement cascade, and then either displacing them from the membrane, or preventing them recruiting the further components that mediate lysis. It seems hard to reconcile a function that requires a GPI-anchored protein to search the entire plasma membrane for attached complement with that protein being restricted to microdomains that would comprise only part of the plasma membrane.

## Lipid Domains Other Than Plasma Membrane Rafts

It is not the intention of this review to argue that there cannot be any lateral heterogeneity of lipids in eukaryotic membranes. It is clear that there is extensive heterogeneity of proteins in the plasma membrane, and indeed putative raft components such as GPI-anchored proteins and lipid-anchored signaling proteins are amongst the few plasma membrane proteins that show a uniform distribution at the level of light microscopy. For instance, plasma membrane structures such as clathrin-coated pits, caveolae, cilia, focal adhesions, microvilli, and synapses all contain specific sets of proteins, and their organization is dependent on a subset of these, usually recruited from the cytoplasm. It is quite possible that a unique protein content, or physical shape distinct from the surrounding bilayer, could enrich or exclude particular lipid species. This could have some purpose, such as inducing partitioning of other proteins in the bilayer (Nichols, 2003), or it could simply reflect the adaptation of the bilayer to accommodate an unusual structure. In addition, localized vesicle delivery to the plasma membrane could cause transient local concentration of particular proteins and lipids (Gheber and Edidin, 1999; Valdez-Taubas and Pelham, 2003).

In addition to the plasma membrane, there is also heterogeneity in the lateral distribution of some proteins in internal membranes, and it is quite conceivable that some of this could be lipid-mediated. Certainly, there must be mechanisms to sort lipids themselves to ensure that the plasma membrane has a lipid composition distinct from that of internal membranes, and that in polarized cells, the apical and basolateral domains of the plasma membrane have differing lipid compositions. At present it is uncertain if this lipid sorting reflects the lipids themselves partitioning into microdomains, or the selection of particular lipids by the protein content and physical shape of transport vesicles and tubes (Mukherjee et al., 1999; Sprong et al., 2001; Sharma et al., 2003). Nonetheless, a number of intracellular sorting processes have been proposed to be mediated by membrane proteins being either included or excluded by the lipid content of the bilayer of transport carriers and vesicles leaving organelles, including the retention of Golgi resident proteins (Bretscher and Munro, 1993; Levine et al., 2000), and the selective targeting to the apical surface of polarized cells (Simons and van Meer, 1988; Weimbs et al., 1997). Of course, the different functional requirements and lipid content of the different organelles of the cell may mean that there are constraints

on which organelles can contain lipid domains. More importantly, it should be noted that as with lipid rafts in the plasma membrane, these models have yet to be proven.

## In Conclusion

The model of lipid rafts in the plasma membrane has attracted much recent attention, and rafts have been proposed to be involved in an astonishing diversity of cellular processes. However, a number of studies are beginning to highlight problems with this model. Firstly, the behavior of cholesterol and sphingolipids in model systems, and their high levels in the outer leaflet of the plasma membrane, indicate that if they were to form a $l_o$ phase in cells it would cover much, if not all of the plasma membrane. Secondly, there is as yet no clear evidence for a mechanism for the formation of lipid domains in the inner leaflet of the plasma membrane. Thirdly, detergent resistance as an assay for rafts may not unequivocally report the distribution of proteins in the unperturbed cell. Fourthly, the evidence for the existence of rafts in the plasma membrane of living cells is not yet compelling.

A role for cholesterol and sphingolipids in reducing bilayer permeability could provide an alternative explanation for at least some phenomena attributed to rafts. If there are no rafts in the plasma membrane it could provide a rationale for why this membrane nonetheless contains such high levels of these lipids. Moreover, the unusually asymmetric distribution of lipids between the leaflets of the plasma membrane would mean that it is principally the outer leaflet that has the properties of a permeability barrier. As such it would be refractory to penetration by small molecules, but also resistant to perturbation by detergents, and in both cases these properties would be perturbed by depletion of cholesterol. Alteration of bilayer physical properties and permeability would be expected to affect many cellular functions.

This is not to say that the notion of lipid rafts in the plasma membrane is demonstrably wrong. However, it would seem that the existence of lipid rafts cannot be taken as the established fact implied by some of the recent literature, but rather as a speculative, and as yet unproven, model. It is undeniable that this model has had the very beneficial effect of stimulating renewed interest in the biology and physics of lipids. A more detailed understanding of lipid phase behavior, particularly at physiological temperatures and compositions, detailed examination of the contribution of cholesterol and sphingolipids to bilayer properties in vivo, and the continued application of new methods such as single particle tracking to examine the localization of proteins in living cells should resolve many of these issues. However, until then it would seem prudent to proceed with caution and an open mind to alternative mechanisms for phenomena currently attributed to rafts.

### Acknowledgments

I am indebted to many colleagues for helpful discussions, and to Mark Bretscher, Matthew Freeman, Dagmar Iber, Michael Neuberger, Ben Nichols, Katja Röper, and Katja Schmidt for valuable comments on the manuscript.

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