Lipid rafts: bringing order to chaos

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Abstract   Lipid rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. They exist as distinct liquid-ordered regions of the membrane that are resistant to extraction with nonionic detergents. Rafts appear to be small in size, but may constitute a relatively large fraction of the plasma membrane. While rafts have a distinctive protein and lipid composition, all rafts do not appear to be identical in terms of either the proteins or the lipids that they contain. A variety of proteins, especially those involved in cell signaling, have been shown to partition into lipid rafts. As a result, lipid rafts are thought to be involved in the regulation of signal transduction. Experimental evidence suggests that there are probably several different mechanisms through which rafts control cell signaling. For example, rafts may contain incomplete signaling pathways that are activated when a receptor or other required molecule is recruited into the raft. Rafts may also be important in limiting signaling, either by physical sequestration of signaling components to block nonspecific interactions, or by suppressing the intrinsic activity of signaling proteins present within rafts. This review provides an overview of the physical characteristics of lipid rafts and summarizes studies that have helped to elucidate the role of lipid rafts in signaling via receptor tyrosine kinases and G protein-coupled receptors.—Pike, L. J. **Lipid rafts: bringing order to chaos.** *J. Lipid Res.* **2003.** 44: 655–667.

Supplementary key words   cholesterol • signal transduction • liquid-ordered domains

For 30 years, the fluid mosaic model of Singer and Nicolson (1) has provided the foundation for our understanding of the structure of cellular membranes. In this model, membrane proteins are viewed as icebergs floating in a sea of lipids. However, work over the last decade has provided evidence that the plasma membrane is not a random ocean of lipids. Rather, there is structure within this sea of lipids that in turn imposes organization on the distribution of proteins in the bilayer. The lipid "structures" within the membrane ocean are called lipid rafts.

Lipid rafts are localized regions of elevated cholesterol and glycosphingolipid content within cell membranes (see **Fig. 1**). The fatty-acid side chains of the phospholipids present in lipid rafts tend to be more highly saturated than those in the surrounding membrane. This allows close packing with the saturated acyl chains of sphingolipids, and probably leads to phase separation. Due to the presence of cholesterol, a liquid-ordered domain is formed that exhibits less fluidity than the surrounding plasma membrane. This tight packing of lipids and phase separation is probably responsible for the signature property of lipid rafts: their insolubility in nonionic detergents (2).

Caveolae are small plasma-membrane invaginations that can be viewed as a subset of lipid rafts. Like lipid rafts, caveolae have a high content of cholesterol and glycosphingolipids; however, caveolae are distinguished from lipid rafts by the presence of the cholesterol-binding protein caveolin-1 (3) that appears to be responsible for stabilizing the invaginated structure of caveolae (4, 5).

The presence within lipid rafts (and caveolae) of a variety of membrane proteins involved in cell signaling (6, 7) has led to the consensus that these lipid domains play an important role in the process of signal transduction. This review will focus on the lipid rafts found in plasma membranes and their role in signal transduction. Except in specific cases, a distinction between flat rafts and invaginated caveolae will not be made, since most studies do not unequivocally distinguish between these raft subtypes. Reviews focusing on the structure and function of invaginated caveolae have been published recently (7–9).

ISOLATION OF LIPID RAFTS

Historically, lipid rafts have been defined functionally by their low density and insolubility in cold 1% Triton X-100 (10). This has given rise to the acronyms DRM (detergent-resistant membrane), TIM (Triton-insoluble membranes), and TIFF (Triton-insoluble floating fraction).

Abbreviations: EGF, epidermal growth factor; GPI, glycosylphosphatidylinositol; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate.

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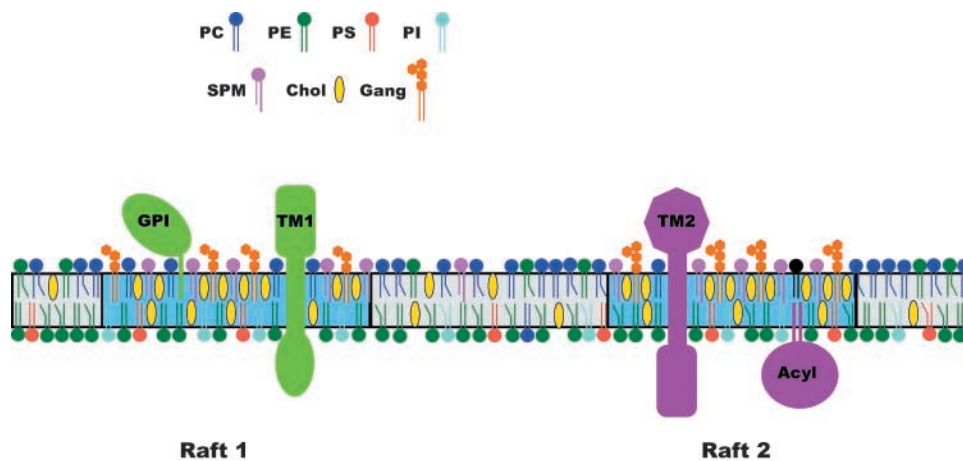   e-mail: pike@biochem.wustl.edu

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Fig. 1. Structure of lipid rafts. Lipid rafts (blue bilayer) are specialized membrane domains containing high concentrations of cholesterol, sphingomyelin, and gangliosides. They are also enriched in phospholipids that contain saturated fatty acyl chains (straight lines in lipid tails). This composition results in lateral phase separation and the generation of a liquid-ordered domain. Bulk plasma membrane (gray) contains less cholesterol, sphingomyelin, and gangliosides, and more phospholipids with unsaturated acyl chains. As a result, it is more fluid than lipid rafts. A variety of proteins partition into lipid rafts: glycosylphosphatidylinositol-anchored proteins; transmembrane proteins (TM); dually acylated proteins (Acyl). As shown in the diagram, not all lipid rafts have the identical protein or lipid composition (Raft 1 vs. Raft 2). Not shown are invaginated caveolae, a subclass of lipid rafts that contains caveolin. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol, SPM, sphingomyelin, Chol, cholesterol; Gang, gangliosides.

The traditional method of preparation of detergent-resistant lipid rafts involves scraping cells into cold buffer containing 1% Triton X-100, and homogenizing the lysate (10). Rafts are isolated by flotation in a 5% to 30% linear sucrose density gradient where they distribute in the top few fractions of the gradient (10). This procedure yields a fairly consistent product that is enriched in cholesterol and raft marker proteins such as flotillin and glycosylphosphatidylinositol (GPI)-linked proteins. Differences can arise, however, if the extent of physical manipulation of the detergent lysates is varied. For example, epidermal growth factor (EGF) receptors are retained in Triton X-100-resistant lipid rafts if the lysate is placed in a tube and simply inverted several times prior to centrifugation and flotation of rafts. By contrast, EGF receptors are lost from DRMs if the original detergent lysate is homogenized prior to centrifugation (unpublished observations). Thus, care must be taken to be consistent in every aspect of the isolation procedure to obtain preparations that are comparable across experiments.

Recently, a wide variety of detergents other than Triton X-100 have been used to isolate low-density detergent-insoluble membrane fractions. These include NP-40, octyl-glucoside, CHAPS, Lubrol, and Brij 98, as well as concentrations of less than 1% Triton X-100 (11–13). While there is substantial overlap in the protein and lipid content of lipid rafts prepared by these various methods, significant differences also exist among them (11–13). This suggests that the various methods for lipid raft preparation do not yield identical membrane fractions. Thus, to a large extent, detergent-insoluble lipid rafts are truly the unique product of the method by which they have been made.

Several detergent-free preparations of lipid rafts have also been reported. One preparation involves the lysis of whole cells in a sodium carbonate buffer (pH 11). This buffer is used because the elevated pH helps in the removal of peripheral membrane proteins. Following sonication of the lysate, rafts are centrifuged on a discontinuous sucrose gradient and band at the 5% and 35% sucrose interface (14). This is a relatively straightforward preparation, but flotation through 35% sucrose is not a particularly stringent test for low-density membranes. In addition, problems can arise because the material that is ultimately sonicated and separated by density-gradient centrifugation contains all of the intracellular membranes. Because rafts are present on intracellular membranes as well as the plasma membrane, there is no guarantee that a protein found in the light fraction at the end of this procedure was actually present in the plasma membrane at the beginning of the preparation.

A more selective procedure for the purification of non-detergent lipid rafts was reported by Smart et al. (15). In this method, cells are lysed in isotonic sucrose buffer, and a postnuclear supernatant is isolated. A purified plasma membrane fraction is prepared by sedimentation of the postnuclear supernatant in a self-forming Percoll gradient. Plasma membranes are readily separated from Golgi, endoplasmic reticulum, and mitochondria by this method. The banding pattern of these various membrane fractions can be modified by altering the pH and ionic composition of the Percoll gradient to obtain optimal separation (16). The purified plasma membranes are sonicated to release lipid rafts (and caveolae), which are isolated by flotation in a continuous gradient of Opti-Prep in isotonic solution.

This method yields a highly purified lipid raft preparation that probably closely reflects the composition of these domains in intact cells.

All of the above preparations isolate all forms of lipid rafts present in the cell, i.e., flat rafts and invaginated caveolae. The caveolin-containing lipid rafts can be separated from noninvaginated rafts by anticaveolin immunoaffinity purification (17). If caveolae are to be isolated from vascular endothelial cells, a procedure is available that physically separates caveolae from lipid rafts (18). Rat lung vasculature is perfused with cationic colloidal silica particles to coat the extracellular side of the plasma membrane. Lipid rafts, being flat, are surface-coated in this procedure. However, because the necks of caveolae are so narrow, the interior of these invaginations is not coated. Endothelial cells are isolated from these perfused vessels and, subsequently, plasma membranes are prepared. The plasma membranes are homogenized to release the caveolae, which float at a lower density in sucrose density gradients than do the silica-coated plasma membranes containing the flat lipid rafts. The plasma membranes, stripped of caveolae, can be washed with a high-salt solution to remove the silica. Extraction of these membranes with Triton X-100 and flotation in a density gradient results in the isolation of a low-density membrane fraction that is devoid of caveolin but enriched in other lipid raft markers, and probably corresponds to purified flat lipid rafts. This preparation, though cumbersome and not generally applicable to all types of cells, generates what are probably the most highly purified preparations of caveolae and lipid rafts.

SIZE OF LIPID RAFTS

Because caveolae represent a morphologically identifiable domain, the size of this subclass of lipid rafts can be readily determined via electron microscopy. Caveolae are, in general, found to be flask-shaped invaginations of ~100 nm diameter (19). However, caveolae are often found in grape-like clusters that have a much larger overall size. In addition, caveolin-3, the muscle-specific form of the caveolar structural protein, caveolin-1, is involved in the development of T-tubules that can be microns in length (20).

The size of flattened lipid rafts cannot be measured directly, because the domains cannot be distinguished from the surrounding membrane. Therefore, relatively indirect methods have been employed to determine the size of these domains. These studies have generated highly variable estimates of raft size.

GPI-linked proteins are known to partition into lipid rafts and are often used as markers for these domains (10, 18). Analysis of the rate of lateral diffusion of GPI-linked proteins as well as gangliosides, a raft lipid marker, suggested that the domains are 200 nm to 300 nm in diameter (21–23). Fluorescence depolarization studies of the GPI-linked folate receptor obtained results consistent with a size of <70 nm for lipid rafts (24). Single particle track-

ing of GPI-anchored proteins yielded a size of ~26 nm (25). Somewhat higher values (0.2–2 μm) were obtained in a study that used single-dye tracing to examine the fluorescence of a fluorescent lipid probe (26). By contrast, several studies using fluorescence resonance energy transfer have failed to find evidence for stable lipid domains of this size, and suggest that such domains may exist only transiently in some membranes (27, 28). Every technique applied to study lipid raft size has its own unique strengths and weaknesses. Based on the summation of currently available data, a conservative interpretation is that lipid rafts are probably structures with an average diameter in the range of 100 nm to 200 nm, well below the resolution of the light microscope.

Another important consideration with respect to raft size is the fraction of the plasma membrane that is actually covered by lipid rafts. If one assumes that lipid rafts represent everything that is left over after a cell has been subjected to extraction with 1% Triton X-100, then lipid rafts appear to represent ~50% of the plasma membrane surface area (29, 30). However, significantly lower estimates for the fraction of plasma membrane present as rafts (13%) have been derived from single-molecule microscopy studies (26, 30). The surface area encompassed by lipid rafts almost certainly varies among cell types, and this could account for the variability in published estimates of raft coverage.

Firmer values for both the size and membrane fraction of lipid rafts await the development of better physical methods. Nonetheless, the currently available information does provide an explanation for the observation that many proteins that can be localized to rafts biochemically often appear to be diffusely distributed on the cell membrane rather than present in a punctate pattern. A raft diameter below the limit of resolution of the light microscope coupled with the rather extensive coverage of the plasma membrane surface by these domains would result in an apparently even distribution of raft-localized proteins, as visualized by immunofluorescence methods.

COMPOSITION OF LIPID RAFTS

Several different lipid raft preparations have been analyzed using various methodologies to determine their lipid composition. In general, these studies have shown that DRMs are enriched in cholesterol and glycosphingolipids, but are often poor in glycerophospholipids. In a study of 1% Triton X-100-insoluble, low-density membranes from MDCK cells, Brown and Rose (10) reported that the vesicles contained 32 mol% cholesterol and 14 mol% sphingomyelin compared with ~12 mol% cholesterol and ~1 mol% sphingomyelin in whole cells. The DRMs were also enriched about 5-fold in glycolipids, such as gangliosides and sulfatides, as compared with intact cells. Similar results were reported by Prinetti et al. using a metabolic labeling approach (31).

A tandem high resolution mass spectrometry analysis of 0.1% Triton X-100-resistant lipid rafts isolated from RBL-

2H3 mast cells provided an extensive analysis of the fatty acid composition of the different phospholipids present in these DRMs (32). These studies demonstrated that 50% of the fatty acid side chains in lipids from plasma membranes contained zero or one double bond, but this increased to 60% in lipids from the DRMs. Thus, DRMs showed a moderate increase in saturated fatty acids as compared with plasma membranes.

Analysis of lipid rafts prepared from KB cells by a detergent-free protocol demonstrated many similarities but also some differences from the above analyses of DRMs (33). Nondetergent lipid rafts were found to be 2-fold enriched in cholesterol and ~30% increased in sphingomyelin content as compared with bulk plasma membrane. Interestingly, the nondetergent rafts were enriched in ethanolamine plasmalogens, particularly those containing arachidonic acid. Given that DRMs are expected to be low in PUFAs, the preference for arachidonic acid-containing lipids was unanticipated. This finding suggests that these arachidonic acid-containing plasmalogens may be important for the function of lipid rafts. In this regard, a recent report that ethanolamine plasmalogens are required for the transport of cholesterol from the plasma membrane to the endoplasmic reticulum is particularly intriguing (34).

Most of the typical raft lipids (e.g., cholesterol, sphingomyelin, and glycosphingolipids) tend to be found primarily in the exofacial leaflet of the membrane. By contrast, ethanolamine-containing glycerophospholipids are preferentially localized to the cytofacial leaflet of the plasma membrane. The finding that rafts contain a distinct subset of these cytofacial lipids (33) suggests that the composition of both the exofacial and cytofacial leaflets of rafts are specific to these domains, and implies that rafts are probably bilayer structures.

Analysis of DRMs prepared from these same KB cells (33) indicates that DRMs are low in glycerophospholipids, as compared with nondetergent rafts (**Fig. 2A**). The DRMs are particularly low in inner leaflet lipids such as anionic phospholipids and phosphatidylethanolamine, and are not enriched in ethanolamine plasmalogens as are the nondetergent rafts. These differences between the lipid composition of DRMs and nondetergent rafts suggest that detergent treatment of membranes may selectively extract the exofacial leaflet of rafts and leave behind the lipids from the inner leaflet.

The degree of saturation-unsaturation of fatty-acid side chains in rafts versus plasma membranes was also assessed in the studies in KB cells (33). The percent of phospholipid species containing only saturated or monounsaturated fatty acid side chains was 40% in plasma membranes as well as in nondetergent lipid rafts. However, in membranes prepared from the same cells by extraction with 1% Triton X-100, 60% of the phospholipid species contained only saturated or monounsaturated fatty acid side chains. Thus, only the rafts prepared by detergent extraction showed an elevated level of saturated fatty acyl side chains.

Analysis of the distribution by class of the unsaturations in
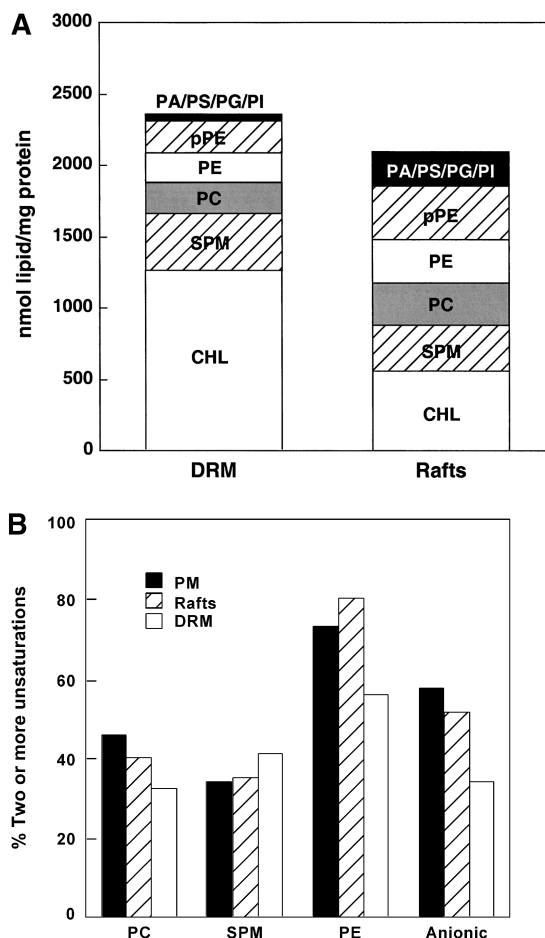
Fig. 2. Comparison of lipid composition and side chain unsaturation in nondetergent lipid rafts and Triton X-100 insoluble lipid rafts. Purified plasma membranes (PM), nondetergent lipid rafts (Rafts), and detergent-resistant membranes (DRMs) were prepared from KB cells (33). Electrospray ionization-mass spectrometry was used to determine the structure of the phospholipids present in each membrane preparation. A: Lipid composition of DRMs and nondetergent rafts. Results are shown as nmol lipid/mg protein. B: Distribution of double bonds in acyl side chains by class of lipids. The percent of two or more double bonds was calculated by determining the fraction of individual phospholipid species that contained a total of two or more double bonds between the two acyl groups present in the lipid.

the fatty acid side chains indicates that the higher level of saturated lipids present in DRMs can be accounted for largely by the exclusion from these preparations of inner leaflet lipids that contain double bonds in their fatty acid side chains (Fig. 2B). The fraction of phosphatidylcholine and sphingomyelin species that contain less than two total double bonds is roughly equivalent in plasma membranes, nondetergent rafts, and DRMs. However, plasma membranes and nondetergent rafts exhibit many more phosphatidylethanolamine and anionic phospholipid species that contain two or more double bonds than do DRMs. As phosphatidylethanolamine and the anionic phospholipids are normally associated with the inner leaflet of the membrane, this observation is consistent with the hypothesis that detergent treatment selectively extracts the outer leaflet of rafts.

PROTEIN COMPOSITION OF LIPID RAFTS

A variety of proteins have been found to be enriched in lipid rafts and/or caveolae. This includes caveolins, flotillins, GPI-linked proteins, low molecular weight and heterotrimeric G proteins, src family kinases, EGF receptors, platelet-derived growth factor (PDGF) receptors, endothelin receptors, the phosphotyrosine phosphatase syp, Grb2, Shc, MAP kinase (MAPK), protein kinase C, and the p85 subunit of PI 3-kinase (7, 10, 14, 35–41).

The mechanisms through which raft association occurs seem to be variable. Caveolin, an intrinsic membrane protein, is a cholesterol binding protein and is probably concentrated in caveolae because of its ability to bind this sterol (3). By contrast, GPI-anchored proteins, src family kinases, and endothelial nitric oxide synthase appear to localize to lipid rafts as a result of lipid modifications (42, 43). For some transmembrane proteins, the membrane-spanning domain appears to mediate the partitioning of the protein into cholesterol-enriched membrane domains (44). Surveys of the behavior of other transmembrane proteins (TM) have suggested that the association of proteins with cholesterol-enriched domains is influenced by the length of their transmembrane domains (45). Extracellular carbohydrate-containing motifs have also been implicated in directing the association of TMs with cholesterol-enriched domains (46, 47). More recently, Yamabhai and Anderson (48) demonstrated that sequences in the most membrane-proximal portion of the extracellular domain of the EGF receptor target it to lipid rafts, independent of any carbohydrate modifications of the sequence. Thus, a variety of mechanisms appear to be employed for localizing proteins to lipid rafts.

SEGREGATION OF COMPONENTS INTO RAFTS OF DISTINCT COMPOSITION

There is increasing evidence that not all lipid rafts are equivalent. A variety of studies have demonstrated that lipid rafts with distinctly different protein and/or lipid components coexist within cells (see Fig. 1). Madore et al. (49) examined the distribution of Thy-1 and prion protein (PrP), two GPI-anchored proteins, in low-density fractions from Brij 96-solubilized rat brain membranes. Immunoaffinity purification of the preparation with anti-Thy-1 antibodies led to the isolation of a fraction that contained Thy-1 plus much of the PrP. However, immunoaffinity purification using anti-PrP resulted in the isolation of membranes that contained essentially all of the PrP but only ∼10% of the Thy-1. Similarly, Drevot et al. (12) demonstrated that depletion of DRMs from T-cells with anti-Thy-1 antibodies quantitatively removed T-cell receptors (TCR) from the rafts. However, predepletion of the rafts with anti-TCR antibody removed little Thy-1. These data are consistent with the interpretation that Thy-1 is present in the bulk of T-cell lipid rafts, while only a subset of those rafts contain expressed PrP (49) or TCR (12). More generally, this implies that not all rafts contain the same subset of proteins.

Immunofluorescence microscopy has also been used to identify distinct classes of lipid rafts. The GPI-anchored protein, placental alkaline phosphatase, and prominin (a pentaspan membrane protein) were shown to be present in a low-density, detergent-resistant fraction. However, by immunofluorescence, these proteins exhibited distinct, punctate cell surface distributions (11). Similar methodology was used by Gomez-Mouton et al. (50) to demonstrate that in migrating T-cells, leading edge rafts contained chemokine receptors and a urokinase plasminogen activator receptor as well as GM_3, but lacked GM_1. Conversely, uropod rafts contained CD44 and GM_1, but lacked GM_3. In yeast, there is a clustering of ergosterol (the yeast equivalent of cholesterol) and mating-specific raft proteins at the tip of the mating projection. Raft proteins that are not involved in mating are not similarly localized (51). These experiments clearly indicate that distinct populations of rafts exist in cells and that they can be mobilized to different regions of the cell following a stimulus.

LIPID RAFTS AND CELL SIGNALING

The existence of different classes of lipid rafts (in addition to invaginated caveolae) has significant implications for the function of these membrane domains in cell signaling. A number of possibilities are summarized below to provide a framework for interpreting the myriad of sometimes conflicting experimental observations relating to rafts and cell signaling. This is followed by a discussion of the studies on the role of lipid rafts in two different signaling systems: receptor tyrosine kinases and G protein-coupled receptor systems.

In the simplest case, rafts can be viewed as signaling platforms that serve to colocalize the requisite components, facilitating their interaction and supporting signaling. In this scenario, receptors, coupling factors, effector enzymes, and substrates would be colocalized in a single raft. The pathway would be activated by hormone binding. Signal transduction would occur rapidly and efficiently because of the spatial proximity of the interacting components. Specificity of signaling could be enhanced by restricting receptor localization to a particular class of rafts that contains a specific subset of signaling components. This restriction would limit access of the receptor to the components of other signaling pathways and prevent nonspecific signaling. Furthermore, the potential for spatial localization of the lipid rafts themselves could support focal activation of the pathway, introducing additional specificity into the response.

In a more complicated model, complementary components of a signaling pathway would be segregated into different lipid rafts under basal conditions. Stimulation of the cell with a hormone or growth factor would lead to the transient fusion of lipid rafts. Alternatively, rafts could contain a nearly complete signaling pathway that would be activated when a receptor or other required molecule that is normally localized in the nonraft portion of the membrane is recruited into the raft. This would colocalize

the various components, promoting interactions and leading to the activation of a signaling pathway. In these scenarios, rafts may provide regulation via compartmentalization; the flux through a given signaling pathway is restricted due to the localization of the interacting components in different physical compartments. In this situation, localization of proteins to rafts is not specifically required for the functioning of the pathway. Rather, the rafts provide a physical separation of proteins that would otherwise interact, leading to unregulated activation of a pathway. This implies that the disruption of lipid rafts could lead to the deregulation of some signaling pathways, a prediction that has been borne out experimentally.

Lipid rafts could also control cell signaling by modulating the intrinsic activities of proteins located within them. This could be due to the specific lipid environment present in rafts or could be the result of the close approximation in rafts of a signaling protein with a regulatory molecule. The observation that some receptor tyrosine kinases behave differently when they are in lipid rafts than when they are in nonraft membranes supports a role for rafts in modulating the activity of raft proteins (see below).

In some systems, rafts may play a more subtle role in signaling. Many raft proteins are not wholly localized to lipid rafts, but instead partition to various degrees between the raft and nonraft portions of the membrane. This is the case for the receptor tyrosine kinases. If these tyrosine kinases function differently in raft and nonraft membranes and have access to different subsets of signaling partners in the different compartments, it is possible that the same receptor could activate different signaling pathways, depending on where it was localized. Thus, changes in the partitioning of molecules between raft and nonraft compartments (due to changes in cholesterol levels?) could alter the ratio of signaling via different pathways.

Rafts may also play a role in signal termination. Rafts are known to be involved in endocytic events (5, 8), and could limit cell signaling by internalizing specific components, preventing them from further participation in a particular pathway.

LIPID RAFTS IN RECEPTOR TYROSINE KINASE SIGNALING

Many receptor tyrosine kinases, including the EGF receptor, the PDGF receptor, and the insulin receptor, have been shown to be localized to lipid rafts (35, 36, 52–54). Unlike some other lipid raft proteins, these transmembrane receptor tyrosine kinases are, in general, not recovered in 1% Triton X-100-resistant fractions but rather are isolated with other lipid raft markers only when nondetergent methods of preparation are used (55–57); however, methods that use lower concentrations of Triton X-100 or milder detergents will sometimes produce material in which these raft-localized receptors are retained in the detergent-resistant fraction (57, 58). Thus, it is clear that the nature of the association of receptor tyrosine kinases with

rafts differs from that of other raft markers, such as GPI-anchored proteins, that are highly detergent-resistant. This has given rise to the hypothesis that lipid shells may assist in targeting such TMs to rafts (59).

Although many receptor tyrosine kinases are localized to lipid rafts, the effect of ligand on this association is highly variable (summarized in **Table 1**). EGF receptors rapidly move out of lipid rafts upon activation by ligand (60), a behavior that is unique among receptor tyrosine kinases. In cells that contain caveolae, insulin receptors are constitutively sequestered in caveolae (58). However, in cells that lack caveolae, insulin receptors are recruited into rafts by the addition of insulin. The localization of PDGF and nerve growth factor (NGF) receptors to rafts appears to be relatively unaffected by ligand (35, 61). The functional implications of such changes in receptor compartmentalization is unclear; however, a rough correlation between the effect of ligand on receptor localization and the effect of cholesterol depletion on receptor-mediated signaling (**Table 2**) suggests that those receptors that remain in, or are recruited to, rafts following ligand binding are much more dependent on raft integrity for function than are receptors that exit rafts upon ligand binding.

Several methods have been used to investigate the role of lipid rafts in cell signaling. One approach has been to stimulate cells with a growth factor and then isolate the raft and nonraft membranes and determine in which compartment a specific signaling event has occurred. Using this approach, it has been shown that autophosphorylation of the PDGF receptor as well as tyrosine phosphorylation of other cellular substrates occurs primarily in lipid rafts and is very weak in nonraft membranes (35, 62).

TABLE 1. Summary of the effects of agonist binding on receptor localization

Receptor	Moves into Rafts	Moves out of Rafts	Unaffected by Agonist	References
Tyrosine kinases				
EGF		X		(60)
ErbB2			X[a]	(60)
Insulin	X[b]		X[b]	(52, 58)
NGF			X	(35, 61)
PDGF			X	
G Protein-coupled				
Adenosine A1		X		(85)
Angiotensin II type 1	X			(90)
β_2-Adrenergic		X		(82)
β_1-Adrenergic			X	(82)
m2 Muscarinic cholinergic	X			(80)
Bradykinin 1	X			(91)
Bradykinin 2	X			(87, 88)
EDG-1	X			(86)
Endothelin			X	(39)
Rhodopsin			X	(81)

EGF, epidermal growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor.

[a] ErbB2 does not directly bind an agonist ligand but is activated by forming heterodimers with ligand-activated EGF receptors.

[b] Insulin receptors reside constitutively in caveolae in cells that express caveolin but move in to lipid rafts upon insulin binding in cells that do not express caveolin.

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TABLE 2. Summary of the effects of cholesterol depletion on signaling via receptor tyrosine kinases

Signaling Pathway	Receptor	Effect of Cholesterol Depletion	Reference(s)
Binding	EGF	+	(74–76)
Dimer formation	EGF	+	(75, 79)
Autophosphorylation	EGF	+	(73–76, 79)
MAPK activation	EGF	+	(73, 74)
PI turnover	EGF	−	(68)
Binding	Insulin	0	(69)
Autophosphorylation	Insulin	0 or −	(58, 69)
IRS-1 phosphorylation	Insulin	−	(58, 69)
ATP citrate lyase phosphorylation	Insulin	−	(52)
Glucose uptake	Insulin	−	(52, 69)
Glucose oxidation	Insulin	−	(70)
Activation of PKB/Akt	Insulin	−	(69)
MAPK	Insulin	0	(69)
Autophosphorylation	PDGF	−	(62)
Tyrosine phosphorylation of endogenous proteins	PDGF	−	(62)
PI 3-kinase activation	PDGF	−	(71)
Autophosphorylation	TrkA (NGF)	−	(72)
MAPK	TrkA (NGF)	−	(72)

MAPK, MAP kinase. + indicates a positive effect. − indicates a negative effect. 0 indicates no change.

Likewise, phosphorylation of the TrkA NGF receptor occurs largely within lipid rafts, and only in this compartment is TrkA found associated with the downstream signaling molecules, Shc and phospholipase Cγ (61). Consistent with its movement out of rafts following ligand binding, autophosphorylated EGF receptor appears both in raft and nonraft compartments (unpublished observations); however, the EGF receptor appears to activate the MAPK signaling pathway in a manner that involves lipid rafts. Stimulation of Rat-1 cells with EGF was found to lead to the recruitment of Raf-1 to lipid rafts within 30 sec (36). Since the recruitment of Raf-1 to membranes is an initiating step in the activation of MEK and subsequently MAPK, this EGF-induced localization of Raf-1 to lipid rafts suggests that MAPK signaling can be initiated within this compartment. Consistent with this interpretation is the finding that the activation of MAPK by PDGF can be recapitulated in vitro using only isolated lipid raft membranes (63).

In addition to MAPK activation, tyrosine kinases appear to activate other signal transduction pathways largely from within rafts. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) is the major substrate for growth factor-stimulated phosphatidylinositol turnover, generating the two intracellular second messengers inositol trisphosphate and diacylglycerol. Studies with MDCK cells, Neuro 2a cells, and A431 cells indicate that as much as half of the cellular PtdIns(4,5)P$_2$ is present in lipid rafts (56, 64, 65). Stimulation of A431 cells with either EGF or bradykinin led to the time-dependent loss of PtdIns(4,5)P$_2$ from the low-density fraction with no change in the level of nonraft PtdIns(4,5)P$_2$, indicating that phosphatidylinositol turnover occurs in lipid rafts rather than in bulk plasma membrane (65). Similarly, stimulation of PC12 cells with NGF induced the hydrolysis of only that sphingomyelin that is present in lipid rafts (54). Thus, a variety of signaling pathways utilizing both protein and lipid components seem to be initiated in lipid rafts.

A second approach for studying the function of lipid rafts involves depleting cells of cholesterol. Lipid rafts are held together to a large extent via interactions between cholesterol and sphingolipids. Thus, the integrity of these domains can be disrupted by the treatment of cells with agents such as filipin or methyl-β-cyclodextrin that sequester or remove cholesterol (66–68). In contrast to studies outlined above in which membrane fractionation was ultimately used to identify raft function, cholesterol depletion permits an analysis of the role of lipid rafts in cell signaling in intact cells.

Consistent with a positive role for lipid rafts in signal transduction, cholesterol depletion generally leads to significant impairment in the ability of receptor tyrosine kinases to signal (summarized in Table 2). Treatment with methyl-β-cyclodextrin diminished insulin-stimulated phosphorylation of its receptor, IRS-1, and ATP citrate lysase, as well as insulin-stimulated glucose uptake and oxidation (52, 58, 69, 70). Insulin-stimulated PI 3-kinase activation, as measured via PKB/Akt activation as well as MAPK activation, was also reduced following cholesterol depletion (69). Depletion of cellular cholesterol with either filipin or lovastatin inhibited PDGF-stimulated PI-3 kinase activation (71) and tyrosine kinase activity (62). Similarly, cholesterol depletion impaired NGF-stimulated MAPK activation and receptor autophosphorylation (72), and inhibited EGF-stimulated PI turnover (68).

The obvious exception to the rule that cholesterol depletion inhibits receptor tyrosine kinase signaling is the EGF receptor (Table 2). For this receptor, ligand binding, receptor dimerization, and autophosphorylation, as well as MAPK activation, are all enhanced following disruption of lipid rafts (73–76). Given that the EGF receptor is also the only receptor that moves out of rafts upon agonist binding, it is tempting to speculate that these two unique behaviors are related.

The observation that the EGF receptor can activate MAPK in the apparent absence of lipid rafts suggests that

rafts may not be absolutely required for activating this pathway. Indeed, experimental evidence suggests that activation of components of the MAPK signaling cascade does not occur exclusively in lipid rafts. For example, the binding of GTP to H-Ras leads to the release of this signaling protein from rafts (77). Furthermore, the addition of targeting signals to Raf-1, the protein immediately downstream of Ras, to either direct it into rafts or prevent it from entering rafts, does not alter the ability of the modified Raf-1 protein to participate in MAPK activation (78). Thus, the later steps of the MAPK pathway may not take place in rafts at all, and only the early steps of MAPK activation may occur in both raft and nonraft fractions, depending on the receptor type.

The observation that some hormone-stimulated signaling events are enhanced following cholesterol depletion reveals a negative regulatory role of rafts in signal transduction. As noted above, cholesterol depletion increases basal MAPK activity and enhances EGF-stimulated MAPK activation (73, 74, 78). The increase in basal MAPK activity appears to be dependent on PI 3-kinase activity (78) and to involve ligand-independent activation of the EGF receptor (79). Apparently, this PI 3-kinase dependent pathway of activation is normally suppressed in the presence of intact rafts. The basis for enhanced EGF-stimulated MAPK activation in cholesterol-depleted cells (73, 74, 78) is not known, but may be due to the activation of a similar nontraditional pathway that is normally suppressed when the signaling components are restricted to lipid rafts. Alternatively, this may be the result of the ability of the specialized environment of lipid rafts to modulate the intrinsic activity of the EGF receptor. Depletion of cholesterol by treatment of cells with methyl-β-cyclodextrin leads to an increase in both EGF binding and intrinsic tyrosine protein kinase activity (74-76). These findings suggest that the function of the EGF receptor may be suppressed by cholesterol or localization to cholesterol-enriched domains. Thus, in addition to serving as platforms for signal initiation, lipid rafts may provide a tonic level of negative regulation of signaling molecules, helping to suppress spurious signaling.

While it is clear that cholesterol-enriched membrane domains are important in receptor tyrosine kinase signal transduction, a general model has yet to be developed for their role in this process. Some pathways, such as PI turnover, may universally require rafts to colocalize the activating receptor with its effector molecules and a source of substrate for the production of second messengers. This pathway may represent the model in which a raft contains some or all of the necessary signaling components, requiring only hormones to recruit the final elements and trigger activation of the pathway. For other pathways such as MAPK activation, the role of lipid rafts appears to be more complex and receptor specific. Receptors that are recruited or restricted to lipid rafts may preferentially initiate MAPK signaling from that compartment, but later events can occur outside that domain. In this situation, rafts may facilitate signaling by localizing components in a restricted space, allowing more rapid and efficient inter-action. For receptors like the EGF receptor that can move out of rafts, the function of lipid rafts is less clear. Some components of the MAPK cascade are recruited to rafts upon hormone binding, but activation of the pathway does not appear to be restricted to that domain, since receptors that are outside of rafts are able to engage the pathway. In this regard, the EGF receptor differs from other receptor tyrosine kinases that do not appear to be able to initiate MAPK signaling in the absence of lipid rafts. What property of the EGF receptor confers this unique capability remains to be determined, but may be related to the two other unique properties of EGF receptor: *i*) its ability to exit rafts following ligand binding, and *ii*) inhibition of intrinsic EGF receptor binding and kinase activities by cholesterol or lipid rafts. These latter observations hint at the possibility that in some systems, rafts are more important for suppressing signaling than for supporting signaling.

LIPID RAFTS IN G PROTEIN-COUPLED RECEPTOR SYSTEMS

A large number of G protein-coupled receptors have been shown to be enriched in lipid rafts or caveolae. This includes β_1- and β_2-adrenergic receptors, adenosine A_1 receptors, angiotensin II type 1 receptors, EDG-1 receptors, endothelin receptors, m_2 muscarinic cholinergic receptors (80), rhodopsin (81), and bradykinin B_1 and B_2 receptors (39, 82–88). Like the receptor tyrosine kinases, the localization of G protein-coupled receptors to lipid rafts is modulated by ligand (Table 1). For the β_2-adrenergic receptor (89, 90) and the adenosine A_1 receptor (85), treatment with agonist causes translocation of the cognate receptor out of lipid rafts or caveolae. By contrast, the angiotensin II type 1 receptor (90), the muscarinic receptor (80), the EDG-1 receptor (86), and the bradykinin B_1 and B_2 receptors (87, 88, 91) are targeted to rafts upon activation by agonist. The localization of the endothelin receptor is apparently unaffected by agonist (39).

The functional significance of these agonist-induced changes in receptor localization has not been unequivocally demonstrated. However, it is possible that ligand-induced movement of receptors into lipid rafts may promote receptor association with the G proteins and effector enzymes that are enriched in this compartment. In addition, many G protein-coupled receptors are desensitized via a mechanism that involves sequestration of the receptors from the cell surface (92). Lipid rafts and caveolae are known to be involved in endocytosis (5, 8). Thus, for receptors that are recruited into lipid rafts-caveolae following addition of agonist, it is possible that the recruitment to rafts not only initiates signaling but also represents the first stage of the desensitization of that signal. Experiments on the muscarinic acetyl choline receptor and the angiotensin II type 1 receptor support this hypothesis (89, 90).

Equally as important as the localization of the G protein-coupled receptors to lipid rafts is the distribution of

the G proteins themselves. A variety of G proteins have been reported to be enriched in lipid rafts and/or caveolae including G_s, G_i, G_o, G_q, and transducin (15, 37, 38, 68, 81, 93–95). Localization of G proteins to lipid rafts appears to be the result of the acylation of the α subunit of these proteins (96). Oh and Schnitzer (97) reported that G_q interacts with caveolin and is therefore concentrated in caveolae, whereas G_i and G_s do not bind caveolin and are therefore targeted to lipid rafts by default. Thus, different G proteins may segregate into different subtypes of lipid rafts depending on the presence of other components in the cell. An interesting possibility is that the differential targeting of G proteins to caveolae or lipid rafts could lead to a parallel agonist-induced segregation of the receptors that interact with those G proteins in the same compartment. Such a mechanism could enhance the selectivity of the receptor for activation of specific signaling pathways localized to a subset of lipid rafts or caveolae.

In addition to the enrichment of receptors and G proteins in lipid rafts or caveolae, several G protein effector enzymes have been reported to be present in or recruited to lipid rafts following receptor activation. The cyclic guanosine monophosphate (cGMP)-phosphodiesterase involved in the visual signal transduction system has been shown to be recruited by DRMs following stimulation of rod outer-segment membranes with light and guanosine triphosphate gamma thio (GTPγS) (81). Several different forms of adenylate cyclase, including types III, IV, V, and VI, have been found to be localized to lipid rafts (82, 84, 95, 98–100). Endogenous adenylate cyclase appears to consistently concentrate in lipid rafts, while overexpressed adenylate cyclase distributes to a variable degree between rafts and bulk plasma membranes, depending on the cell type examined (82, 84, 100).

Approaches similar to those used to study receptor tyrosine kinase systems have been employed to investigate G protein-coupled receptor signaling in lipid rafts. Using membrane fractionation, rhodopsin was shown to be present in lipid rafts, and activation by light induced the translocation of transducin and cGMP-phosphodiesterase into rafts (81). This suggests that the early steps of visual

signal transduction are likely to occur in this membrane compartment.

More extensive work has demonstrated the importance of raft localization in the β-adrenergic receptor signaling system. In neonatal cardiac myocytes, $β_2$-adrenergic receptors are highly enriched in lipid rafts-caveolae. By contrast, $β_1$-adrenergic receptors and adenylate cyclase distribute between rafts and bulk plasma membrane (82, 84). Prostanoid EP2 receptors are excluded from rafts. Overexpression of adenylate cyclase, which becomes enriched in lipid rafts, led to a greater enhancement of $β_1$-adrenergic-stimulated activity than $β_2$-adrenergic-stimulated activity, and failed to increase the response to prostaglandin E_2 (84). Ostrom et al. (84) suggested that the ability of overexpressed adenylate cyclase to enhance β-receptor signaling correlated with the degree to which the $β_1$- and $β_2$-receptors colocalized with adenylate cyclase in lipid rafts. The selective increase in the response to $β_1$- as compared with $β_2$-agonists was thought to derive from the fact that the $β_1$-adrenergic receptor remained in rafts after hormone stimulation, while agonists induced the migration of the $β_2$-adrenergic receptor out of lipid rafts. This would separate the $β_2$-adrenergic receptor from its G protein and adenylate cyclase effector, and limit signaling.

This agonist-promoted exit of the $β_2$-adrenergic receptor from rafts was inhibited by expression of the C-terminal peptide of β-adrenergic receptor kinase (βARK), which blocks activation of endogenous βARK by sequestering Gβγ subunits (101). Concomitant with this inhibition of receptor movement out of lipid rafts, there was an enhancement of $β_2$-adrenergic stimulated cAMP production. Together, these data are consistent with the hypothesis that localization of β-adrenergic receptors in lipid rafts-caveolae promotes their interaction with G proteins and adenylate cyclase, and enhances their response to agonist.

Other G protein-mediated signaling pathways also appear to be activated principally in lipid rafts. In A431 cells, a large portion of the $PtdIns(4,5)P_2$ is concentrated in lipid rafts, and bradykinin-stimulated hydrolysis of PtdIns(4,5)P_2 utilizes only this raft-localized pool of Ptd-

TABLE 3. Summary of the effects of cholesterol depletion on signaling via G protein-coupled receptors

Signaling Pathway	Receptor	Effect of Cholesterol Depletion	Reference(s)
Arterial SMC contraction	$α_1$-adrenergic	0	(105)
Adenylate cyclase activation	$β_2$-adrenergic	+	(82)
Myocyte contraction	$β_2$-adrenergic	+	(83)
Myocyte contraction	$β_1$-adrenergic	0	(83)
Transactivation of the EGF receptor	Angiotensin II	−	(106)
PI turnover	Bradykinin	−	(68)
Phospholipase A2 activation	Bradykinin	0	(91)
Arterial SMC contraction	Endothelin	−	(105)
MAPK activation	Endothelin	−	(104)
Focal adhesion kinase activation	Endothelin	−	(104)
Arterial SMC contraction	5-hydroxytryptamine	−	(105)
Receptor affinity	Oxytocin	−	(108)
Activation	Rhodopsin	+	(107)
PI 3-kinase activation	Thrombin	−	(102)
Arterial SMC contraction	Vasopressin	−	(105)

+ indicates a positive effect. − indicates a negative effect. 0 indicates no change.

Ins(4,5)P$_2$ (65). Similarly, in platelets the thrombin-stimulated production of phosphatidic acid and phosphatidylinositol 3,4,5-trisphosphate is largely concentrated in rafts (102). The production of phosphatidic acid could be a result of the generation of diacylglycerol due to PI turnover, and is therefore consistent with the compartmentalization of phosphatidylinositol turnover in lipid rafts. The focal production of phosphatidylinositol 3,4,5-trisphosphate suggests that, in addition to PI turnover, phosphatidylinositol 3-kinase activation in platelets may also be localized to lipid rafts. Endothelin stimulates the activation of MAPK via a mechanism that involves G$_q$ and phospholipase C-β activation. The ability of endothelin to stimulate MAPK is dependent on palmitoylation of the endothelin receptor, a modification that would target it to lipid rafts (103). This implies that localization of the endothelin receptor to lipid rafts is required for the stimulation of this G$_q$-mediated signaling pathway, and is consistent with the conclusion that PI turnover is initiated in lipid rafts.

In general, the conclusions suggested by membrane fractionation studies are largely confirmed in experiments in which G protein-coupled receptor signaling is probed via cholesterol depletion (**Table 3**). As was true for receptor tyrosine kinase signaling, cholesterol depletion generally impairs G protein-mediated signaling. Bradykinin-stimulated PI turnover was found to be inhibited by cholesterol depletion, which delocalizes the PtdIns(4,5)P$_2$ and other raft components (68). Similarly, thrombin-stimulated phosphatidic acid generation and phosphatidylinositol 3,4,5-trisphosphate production is inhibited by cholesterol depletion (102). Like the fractionation studies, these data suggest that these G protein-mediated signaling events require intact lipid rafts and proceed within this compartment.

Other G protein-coupled receptor-activated signaling pathways are also sensitive to cholesterol depletion. Endothelin-stimulated tyrosine phosphorylation of focal adhesion kinase and MAPK was inhibited by treatment of primary astrocytes with filipin (104). Likewise, treatment of rat tail artery denuded of endothelium with cyclodextrin led to the inhibition of endothelin-, 5-hydroxytryptamine-, and vasopressin-stimulated smooth muscle cell contraction (105). Transactivation of the EGF receptor by angiotensin, a pathway involving Ca^{2+} and *c*-src, was also impaired following cholesterol depletion (106). These data indicate that intact rafts are necessary for the activation of a variety of G protein-coupled receptor signaling pathways.

As was true for receptor tyrosine kinases, there is an exception to the general conclusion that cholesterol depletion inhibits G protein-coupled receptor signaling. Both adenylate cyclase activation and myocyte contraction mediated via the β$_2$-adrenergic receptor are enhanced by cholesterol depletion (82, 83). Interestingly, like the EGF receptor, which is the tyrosine kinase exception to the general "inhibited-by-cholesterol-depletion" rule, the β$_2$-adrenergic receptor migrates out of lipid rafts following agonist binding. It is possible that, like the EGF receptor, the activity of the β$_2$-adrenergic receptor is inhibited by

cholesterol or the lipid raft environment. This inhibition may be relieved upon release of the receptor from rafts, either by ligand binding or cholesterol depletion. Release of all raft components into the bulk plasma membrane by treatment with cyclodextrin may then permit more productive receptor-G protein-cyclase interactions, resulting in elevated cAMP production. Additional studies on the effect of cholesterol on β$_2$-adrenergic receptor function will be needed to clarify these observations. The finding that cholesterol depletion enhances the fraction of rhodopsin that converts to the activated conformation upon photolysis (107) is consistent with the possibility cholesterol and lipid rafts may negatively regulate the function of some receptors.

SUMMARY

Lipid rafts are organized subdomains of the plasma membrane and other intracellular membranes, such as the Golgi. They appear to be small in size, but may constitute a relatively large fraction of the plasma membrane. While rafts have a distinctive protein and lipid composition, studies suggest that not all rafts are identical in terms of either the proteins or the lipids that they contain. Furthermore, rafts of different protein or lipid composition can be spatially segregated in cells to accomplish specific tasks, as in mating yeast or migrating lymphocytes. These compositional and spatial differences are likely to be important for raft function in cell signaling.

The overall picture that emerges from studies on the role of lipid rafts in signal transduction is one in which rafts appear to exert both positive and negative control on signal transduction. In their positive role, rafts containing different signaling proteins may cluster or fuse upon agonist stimulation, leading to the mixing of components and resulting in the activation of signaling pathways. In their negative role, rafts may spatially segregate interacting components to block nonspecific pathway activation, or may directly suppress the activity of signaling proteins present in rafts.

Although extensive research has been done on the role of rafts in signal transduction, many of the studies utilize fairly indirect approaches, such as cholesterol depletion, to implicate rafts in signaling. Truly unequivocal experiments are rare. Thus, while the data are consistent with a role for rafts, no unifying model of exactly how rafts function in signal transduction has yet evolved. Further progress in defining the role of rafts in cell signaling will require the development of new tools to visualize lipid rafts more effectively and to isolate and study distinct populations of these domains.

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