Lipid Rafts, Detergent-Resistant Membranes, and Raft Targeting Signals

Lipid rafts are liquid-ordered ($l_o$) phase microdomains proposed to exist in biological membranes. Rafts have been widely studied by isolating $l_o$-phase detergent-resistant membranes (DRMs) from cells. Recent findings have shown that DRMs are not the same as preexisting rafts, prompting a major revision of the raft model. Nevertheless, raft-targeting signals identified by DRM analysis are often required for protein function, implicating rafts in a variety of cell processes.

The liquid-ordered ($l_o$) phase is a sterol-dependent state in which lipid acyl chains are tightly packed and highly extended but in which individual lipid molecules have a high degree of lateral diffusion in the bilayer (109). In model membranes, $l_o$-phase domains can coexist with both liquid-disordered ($l_d$)-phase domains, in which lipids are much less condensed, and with gel-phase domains, in which lipids are highly condensed and essentially immobile (44). $l_o$-$l_d$ phase separation in model membranes is not controversial: micron-sized $l_o$-phase domains can be seen by microscopy in giant unilamellar vesicles (GUVs) (109).

The raft hypothesis proposes that $l_o$-phase domains also form in sterol-rich cell membranes, especially the plasma membrane, where they coexist with disordered membrane domains (42, 61, 82).

**Detergent Insolubility and Lipid Rafts**

An important early indication that rafts may exist in cells came from the observation that cell membranes are not fully solubilized by non-ionic detergents such as Triton X-100 (TX100) or CHAPS at low temperatures. Instead, $l_o$-phase DRMs remain and can be isolated on sucrose gradients (15, 95).

**Detergent insolubility in model membranes**

Model membrane studies explained this finding, showing that close packing of lipids in the $l_o$ or gel phases prevents detergent incorporation into the bilayer (115). Two studies showed that detergent insolubility is a useful assay of phase state in model membranes. In one, the phase state of a series of sterol-rich mixtures containing varying amounts of order- and disorder-prefering lipids was first determined by a fluorescence-quenching assay (1). This pinpointed the amount of order-prefering lipid required for phase separation. The same mixtures were then subjected to TX100 extraction. DRMs could only be detected in mixtures that contained $l_o$-phase domains before the addition of TX100 (1). A second approach used two-phase supported lipid bilayers that contained separate $l_d$ and $l_o$ domains visible by microscopy (28). TX100 selectively solubilized $l_d$-phase domains, leaving the $l_o$-phase domains relatively unchanged as DRMs. Both of these approaches showed that TX100 did not substantially change the relative amounts of membrane in the two phases. By contrast, using calorimetry, Heerklotz and colleagues reported that TX100 can induce phase separation in previously uniform mixtures (45, 46). However, results of the other two studies described above suggest that any such effect may be relatively minor.

**Development of the raft model in cells**

These model membrane results initially suggested that DRMs isolated from cells were also derived from preexisting raft domains. This model was attractive because plasma membranes contain significant amounts of order-prefering sphingolipids and sterols, together with disorder-prefering phospholipids. Model membrane studies showed that $l_o$-$l_d$ phase separation could occur at 37°C in lipid mixtures with physiological levels of each of these lipid classes, making phase separation in cell membrane plausible (1, 101). Furthermore, DRMs isolated from cells are enriched in a subset of proteins, mostly from the plasma membrane. DRM enrichment initially suggested that these proteins were concentrated in rafts in cells and that raft targeting might be important in function.

This idea was supported by the use of agents that deplete or sequester membrane cholesterol. β-Cyclodextrins can rapidly and reversibly extract most sterol from cell membranes (25). Polynye antibiotics such as filipin and nystatin partition into membranes and bind sterols, sequestering them from other interactions. For convenience, I will refer to both classes of compound together as cholesterol modifiers. These agents reduce or abolish DRM association of most proteins, suggesting that they disrupt rafts in cells. This idea is reasonable, since phase separation is often enhanced by sterols (1, 102) and could plausibly be abolished by sterol removal. (It is worth noting, however, that $l_o$-phase disruption by cholesterol modifiers has not been demonstrated directly using detergent-free assays in model membrane systems.)
**DRMs are not rafts**

Despite the encouraging precedent provided by model membranes, DRMs isolated from cells do not correspond precisely to preexisting rafts in living cells. This is clear from the fact that isolation of TX100-insoluble DRMs from mammalian cells requires extraction at low temperatures (69). Phase behavior is very temperature dependent. If rafts exist in cell membranes, chilling will certainly expand them, as additional proteins and lipids are recruited to rafts. If rafts do not exist in living cells, chilling could cause them to form by inducing phase separation.

DRMs can be isolated from cells at higher temperatures following extraction with other non-ionic detergents, such as Lubrol WX and detergents of the Brij series (73, 91). This has been taken to suggest that different classes of rafts are isolated by different detergents. However, there is no direct evidence to support this idea. Of concern, these detergents are less efficient than TX100 and may fail to fully solubilize moderately ordered $l_\alpha$-phase membranes. Lipids in the plasma membrane, with its high sterol and sphingolipid content, are expected to be more ordered than those in intracellular membranes, even if phase separation and raft formation do not occur. Thus weak detergents might not fully solubilize the plasma membrane even if no rafts were present (96).

Detergent concentration, and the ratio of detergent to cellular proteins and lipids, can also affect the degree to which proteins associate with DRMs (7, 78). That is, even proteins with low inherent affinity for ordered membranes can be detected in DRMs prepared with low amounts of TX100 (7). It is important in DRM studies to include non-raft-loving proteins (such as the transferrin receptor) as negative controls.

Studies of glycosyl phosphatidylinositol-anchored proteins (GPI-APs) also show that DRMs are not direct descendents of rafts in living cells. GPI-APs have saturated, order-prefering acyl chains and are highly enriched in large (up to 1-$\mu$m diameter) DRMs (15). Nevertheless, analysis by fluorescence resonance energy transfer (FRET) shows that most GPI-APs are uniformly distributed on the cell surface or are present in at most very small (nanoscale) clusters, containing only a few proteins (37, 56, 97). Fluorescence recovery after photobleaching (FRAP) also shows that GPI-APs, as well as other DRM-enriched proteins and lipids, do not diffuse as components of large, stable rafts (55). By extension, DRM-association of proteins other than GPI-APs cannot be taken to show that they are present in rafts in living cells.

Likewise, the ability of cholesterol modulators to affect a process does not prove that rafts are involved. Agents that modulate membrane cholesterol are likely to affect cell function in ways that are unrelated to raft formation. Sterols have profound effects on bilayer structure, even in single-phase membranes (51, 66), and their removal should greatly affect even membranes that do not contain rafts. Thus inhibition of a process by cholesterol modulators suggests that it requires cholesterol but does not prove that it requires rafts. However, a few groups have strengthened the case for raft involvement by showing that functions inhibited by cholesterol depletion can be rescued by repletion of cells with sterols or sterol analogs that promote $l_\alpha$-phase formation in model membranes, but not by compounds that lack this property (16, 77, 90).

In summary, finding that cholesterol modulators affect a given process in cells is consistent with, but does not demonstrate, a role for rafts. Nevertheless, an intriguing report suggests that at least one pathogen may use a cholesterol modulator to avoid host defenses. Pathogenic *Brucella* bacteria, which are phagocytosed by host cells, secrete a cyclic $\beta$-1,2-glucan that weakly extracts cholesterol from membranes. This compound was reported to inhibit phagosome-lysosome fusion, allowing replication of the pathogen in the host cell (6).

**A Current Picture of Raft Structure and Function in Cells**

Much of the evidence for raft function in cells comes from studies pairing DRM-association of key proteins with the effect of cholesterol modulators in disrupting function. Such studies have suggested several important roles for rafts, especially in signal transduction (30, 49, 82) and membrane trafficking (57). Even before their structure was understood, rafts were proposed to act in sorting in the trans-Golgi network of polarized epithelial cells (103). Further work supported roles for rafts in this process (54) and in sorting in endosomes (74). However, intracellular rafts have proven difficult to study because they cannot be directly accessed from the cell surface. In recent years, much attention has shifted to the plasma membrane. Because of the caveats outlined above, the areas in which the evidence for raft function is most compelling are those in which DRM analysis and cholesterol depletion have been combined with microscopy. Thus the cell-surface distribution of proteins and lipids suspected (usually from DRM analysis) of being in rafts is examined microscopically, with the aim of visualizing the putative rafts directly. This approach has been most fruitful in studies of signaling in hematopoietic cells, especially lymphocytes and mast cells. The success of these studies may reflect the fact that cell-surface signaling molecules can assemble into particularly large complexes in these cells, facilitating detection by microscopy. Thus DRM association of the T- and B-cell receptors (21, 73) and of FcεRI (the IgE-binding signaling receptor on mast cells) (33) increases during signaling, and signaling in T cells (114) and mast cells (98) is sensitive to cholesterol
Ironically, some of the strongest evidence for raft function in mammalian cells comes from the behavior of pathogens. A number of pathogenic bacteria (60, 63) and viruses (18, 80) bind order-preferring proteins and lipids on the surface of mammalian cells and appear to co-opt host cell rafts during infection. As an example of how rafts have been implicated in pathogenesis, Wolf and colleagues (113) compared two bacterial toxins that bind different gangliosides on the surface of cultured human intestinal cells. Cholera toxin, which binds the DRM-enriched ganglioside GM1, intoxicated the cells much more efficiently than the related *Escherichia coli* heat-labile type II enterotoxin LTIIb, which does not associate with DRMs (113). Most putative raft-targeting pathogenic agents seem to use rafts for internalization into mammalian cells, suggesting a role for rafts in normal endocytic pathways (22, 57, 80).

Any general model for raft structure and function must incorporate the finding that GPI-anchored proteins, highly enriched in DRMs, appear to be uniformly distributed on the surface of resting cells. An emerging paradigm in the field is that raft formation and/or stabilization may be highly regulated and occur only in response to stimulation, such as receptor clustering. According to this model, rafts in unstimulated cells are small (nanoscale) and unstable, if they exist at all. However, clustering of molecules with high inherent affinity for ordered lipids [helpfully termed raftophilicity by Kusumi and colleagues (59)] can greatly increase raft size and stability. Thus clustering of cell surface receptors, as often occurs during signaling, may increase the affinity of the clustered receptors for rafts and may also stabilize rafts or induce their formation. Proof of principle comes from an elegant recent study (41): clustering of gangliosides in single-phase model membranes close to a phase-separation boundary using cholera toxin induced dramatic large-scale phase separation (shown in **FIGURE 1**). This model suggests that the plasma membrane lipid composition is also maintained close to that required for phase separation, so that small changes in protein or lipid aggregation can have large effects on microdomain organization. Recruitment of raftophilic proteins to rafts may enhance their interactions with each other, for instance during signaling, while reducing their interactions with non-raftophilic proteins.

An example of how such a model could work in practice, from the elegant work of Baird, Holowka, and colleagues on FcγRI signaling in mast cells (49, 119), is shown in **FIGURE 2**. Ligand-induced FcγRI clustering is proposed to stabilize rafts containing both the receptor and the raftophilic kinase Lyn. Lyn itself requires phosphorylation for activation, and raft clustering and stabilization activate Lyn by sequestering it from non-raftophilic phosphatases.
DRM Association is Useful in Demonstrating Raftophilicity and Identifying Raft Targeting Signals

In this context, what do detergent insolubility studies tell us? Enrichment of a protein in DRMs shows that it is raftophilic and is likely to associate with rafts when they form. DRM analysis can also help identify raftophilicity-conferring motifs in proteins, as detailed in the rest of this review. For convenience, I will refer to these as raft targeting signals, although it is important to note that, in most cases, only DRM targeting has been shown. The striking number of cases in which DRM-targeting correlates with function provides some of the strongest available evidence of a physiological role for rafts.

Discussing raft-targeting signals in proteins tacitly assumes that rafts are formed from lipids and that proteins partition into them based on their affinity for an ordered membrane environment. Indeed, $I_w$-phase...
domains form spontaneously in model membranes of appropriate lipid composition, and lipid interactions are undoubtedly also crucial for raft formation in vivo. However, raft formation in cells is very likely to be regulated by proteins. In addition to the transient protein-clustering effects outlined above, proteins such as caveolins (79) and flotillins (88) probably organize rafts into specialized membrane domains. Furthermore, important protein-mediated links of rafts to the actin cytoskeleton are starting to be elucidated (20, 49, 68, 84).

**Raft targeting of peripheral proteins**

Raft targeting of proteins that lack transmembrane spans is best understood. GPI anchors and tandem or closely spaced acyl chains on these proteins are well-known raft-targeting signals (14). Unexpectedly, fluorescence quenching analysis showed that a tandem NH$_2$-terminal myristate/palmitate motif targeted a peripheral peptide to rafts in model membranes more strongly than tandem palmitoylation on two internal sites (112). The myristate/palmitate motif, present in Src-family kinases and some G protein α subunits, appears to be an especially efficient raft-targeting signal. Microscopy analysis in cells supports this idea. A tandem myristoyl/palmitoyl motif can target GFP to clusters on the inner leaflet of the plasma membrane that coincide with rafts (visualized by experimental clustering of outer-leaflet raftophilic proteins) in the extracellular leaflet (40, 87, 120). It is notable that Gαs is modified with an unusual dual-palmitoyl motif, in which the first palmitate is linked to an NH$_2$-terminal Gly instead of the usual internal Cys (58). By analogy with the myristate/palmitate motif, the dual-palmitate motif of Gαs might also confer high raftophilicity. However, this idea has not been tested, and it has been difficult to identify a role for raft targeting in Gαs function (71).

A recent study shows that the matrix domain (MA) of HIV Gag, which is myristoylated, uses a novel variation of this motif (93). As illustrated in **FIGURE 3**, MA binds membrane PI(4,5)P$_2$ in such a way that the polyunsaturated 2’-arachidonate chain is sequestered in a cleft in the protein, whereas the saturated 1’-stearate chain is exposed. PI(4,5)P$_2$ binding also exposes the myristate chain of MA. As a result, MA may be targeted to rafts by membrane insertion of two saturated acyl chains: the covalently linked myristate and the stearate of the bound PI(4,5)P$_2$. Use of a similar strategy by cellular proteins could explain the surprising enrichment of PI(4,5)P$_2$ in DRMs (50), despite the presence of its order-disfavoring arachidonate chain.

Prenyl groups, which are branched, should not fit well into rafts. Model membrane studies verified the expected low affinity of prenylated peptides for rafts (112), and DRMs isolated from cells contain few prenylated proteins (70). Some proteins are modified both tandem palmitoylation (expected to increase raft affinity) and prenylation (expected to reduce raft affinity), raising the possibility that overall raft affinity may be regulated. For heterotrimeric G proteins, this could occur by dissociation of acylated Gα subunits from prenylated Gβγ subunits during signaling. As expected, monomeric Gαi associated better than the Gαiβγ trimeric complex with DRMs isolated from model membranes containing the purified proteins (72). H-Ras, modified by closely spaced dual palmitoylation and farnesylation, is also targeted to rafts, although it is solubilized by standard TX100 extraction (83). The prenyl group may bind reversibly to other proteins to regulate raft-disfavoring insertion of the moiety into the bilayer. Alternatively, other raft-favoring interactions may overcome raft-disfavoring tendency of the prenyl group.

**The vexing question of transmembrane protein targeting to rafts**

As schematized in **FIGURE 4**, transmembrane peptides have rough surfaces and should not fit well into rafts. Detergent-free model membrane studies, examining raft targeting by fluorescence quenching, have confirmed this expectation, showing that such peptides are excluded from rafts (32, 108). As expected, most transmembrane proteins associate poorly with...
DRMs. However, a few such proteins are found in DRMs, and raft targeting appears to be important in their function. It is not known how the strongly raft-disfavoring transmembrane domains are arranged with respect to rafts in the bilayer. Palmitoylated proteins might be expected to prefer domain boundaries, positioning their transmembrane domains in \( l_d \) domains and their palmitate chains in adjacent \( l_o \) domains. However, a dually palmitoylated transmembrane domain peptide corresponding to the T-cell scaffold protein LAT was mostly excluded from rafts in two-phase giant unilamellar vesicles, and showed no enrichment at domain boundaries (99).

Because increases in lipid order increase bilayer thickness, rafts are generally expected to be thicker than coexisting non-raft domains. This has suggested that correspondingly longer transmembrane spans might target some proteins to rafts to avoid hydrophobic mismatch, an idea that has achieved textbook status (2). However, model membrane/peptide studies have shown that the raft-disfavoring difficulty of packing transmembrane spans into an ordered lipid environment outweighs any raft-favoring effect of improved hydrophobic match (67, 108, 110). Transmembrane span length is not known to affect raft affinity of any protein.

**Palmitoylation and raft targeting**

The best-studied raft targeting signal for transmembrane proteins is palmitoylation: usually linkage of two or more palmitoyl chains to the cytoplasmic domain, close to the membrane interface. Palmitoylation is also required for function of some of these proteins, suggesting a physiological role for raft targeting. Most reported cases of this behavior are in hematopoietic cells and (to a lesser extent) neurons. Thus palmitoylation of the tetraspanin CD81 is required to recruit the associated B-cell receptor to rafts in B cells, prolonging its signaling (23). Palmitoylation is also required for raft targeting and efficient function of the T-cell co-receptors CD4 (9, 34) and CD8 (4, 5). Palmitoylation of the T-cell scaffolding protein LAT is required for raft targeting (121), for signaling (65), and for recruitment of the protein to T-cell receptor-enriched immunosolates on activation (43). [However, interpretation of these results might be complicated by another group’s findings that LAT palmitoylation was required for cell-surface trafficking, membrane insertion, and stability (106).]

Palmitoylation of a LAT transmembrane domain peptide slightly increased DRM-association in model membranes (99). However, the intact protein was more highly enriched in DRMs than the acylated peptide, showing that the protein contained additional raft targeting information (99). Palmitoylation of the platelet glycoprotein GP Ib-IX-V complex is required for raft targeting, and the amount of DRM-associated GP Ib-IX-V increases dramatically upon activation (100). Palmitoylation of Fc\(\gamma R I I a\), an IgG receptor expressed on most hematopoietic cells, is required for raft targeting and signaling (10). In neurons, palmitoylation of NCAM is required for raft targeting and for signaling during neurite outgrowth (76). Similarly, the netrin receptor DCC requires palmitoylation for raft association and for function in axon guidance in developing neurons (47). Palmitoylation of the \( \alpha 6\beta 4 \) integrin in epithelial cells is required for raft targeting and for coupling to Src-family kinases and downstream signaling pathways on ligation (35). However, palmitoylation of the luteinizing hormone receptor is needed for raft targeting but not for function (62).

The glycoproteins of many enveloped viruses are palmitoylated, and some require this modification for raft targeting. Nevertheless, a role for acylation in viral life cycles has often been difficult to define. Thus, although palmitoylation of the murine leukemia virus envelope protein is required for raft targeting and enhances cell-surface transport, it does not affect fusion activity (64). Palmitoylation of the HIV-1 envelope glycoprotein (gp160; later cleaved to generate the transmembrane gp41 and the peripheral protein gp120) was initially reported to be required for raft targeting and infectivity (92). However, loss of gp41 palmitoylation was later reported to affect infection only partially (11) or not at all (17). This issue may be complicated by the fact that HIV-1 Gag protein can recruit gp41 to rafts (12). Palmitoylation of influenza hemagglutinin is required for raft targeting (19, 70), but the role of the modification in protein function is controversial, as well summarized earlier (19), and may be subtype specific.

![Transmembrane proteins and lipid rafts](https://physiologyonline.physiology.org/content/physiology/21/6/435/Fig4.png)
Not all palmitoylated transmembrane proteins associate with DRMs, and it is not known what distinguishes the two groups. Increasing the number of acyl chains may enhance DRM association, although protein-specific factors are also clearly important since different numbers of acyl chains are required for DRM association of different proteins. Thus LAT requires two chains (121), whereas influenza HA requires three (70) for DRM association. Many singly palmitoylated proteins, such as VSV G, are excluded from DRMs (15). Transferrin receptor, a widely used non-DRM marker protein, was originally reported to be either singly or doubly palmitoylated, depending on cell type (53), and this issue has not been investigated further.

Transmembrane domain raft targeting signals

Very little is known about how transmembrane domain amino acid sequence affects raft targeting. Several residues of influenza hemagglutinin that are predicted to contact the extracellular half of the bilayer are essential for raft targeting (94, 105). Gosse et al. examined hybrid proteins containing the extracellular domain of the IgE receptor FceRI, the intracellular domain of the \( \zeta \) chain of the T-cell receptor, and variable transmembrane domains (39). On receptor clustering, the hybrid containing the TCR \( \zeta \) chain transmembrane domain associated with DRMs, whereas hybrids containing transmembrane domains from two putative non-raft proteins (CD45 and PTPs) did not. In all cases examined, the ability of the hybrids to signal after clustering correlated with DRM association. Truncation and domain-swapping experiments have implicated transmembrane domains of CD44 (75, 81) and CD40 (13) in raft targeting. Signaling through the Epstein Barr virus latent membrane protein (LMP-1), which is similar to CD40, is essential for EBV-mediated B-cell immortalization (26). Transmembrane domains 1 and 2 of this polytopic protein (26), and in particular a motif (FWLY) in transmembrane domain 1 (118) are essential for raft targeting. The FWLY motif is also required for LMP1-mediated signaling (118). The COOH-terminal transmembrane domain of herpes virus saimiri tip (which induces downregulation of the T-cell receptor and CD4 in T cells) is also required for raft targeting (24).

Extracellular and cytoplasmic domain raft targeting signals

A few raft targeting signals have been identified in extracellular or cytoplasmic domains, although mechanisms have not been defined. Such motifs could act by partially inserting in the bilayer (as proposed below for caveolins), by inducing conformational changes that affect raftphilicity, or indirectly through binding to other raft-targeted proteins. Conformational changes in the extracellular domain affect raft targeting of the T-cell adhesion protein CD2 (117), and a 60-residue membrane-proximal sequence was reported to target the EGF receptor to rafts (116). Both studies showed that glycosylation played no role in raft targeting. The pentapeptide LWYIK, present in the juxtamembrane region of the HIV fusion protein gp41, was shown to bind cholesterol and induce formation of cholesterol-rich domains in vitro (31).

The cytoplasmic domain sequence LIRW aids in DRM association of the proteolipid MAL, although this effect may be secondary to altered targeting of the mutant protein from the TGN to an uncharacterized BFA-insensitive compartment (86). The RING finger domain of tumor necrosis factor receptor-associated factor (TRAF) 2 is required for DRM association of the protein on engagement of TNF receptors and for activation of downstream signaling (8). Replacement of the RING finger with another raft targeting signal can partially rescue signaling. PKA-mediated phosphorylation of the neurotrophin receptor p75NTR is required for both DRM association and signaling (48). A membrane-proximal signal (RHRRR) in the cytoplasmic domain of the T-cell co-receptor CD4 is required for DRM association (85). This effect is independent of palmitoylation, since mutation of the signal abolishes DRM association but not palmitoylation. Analysis of hybrids of the DRM-associated, Ca\(^{2+}\)-sensitive adenylyl cyclase AC5 and the non-DRM-associated, Ca\(^{2+}\)-insensitive adenylyl cyclase AC7 showed that DRM-targeting signals were present in cytoplasmic loops and not in transmembrane domains (27). An intriguing model membrane study suggests that a membrane-proximal peptide may target caveolins to DRMs through insertion of aromatic residue side-chains into the bilayer, favoring recruitment of cholesterol and presumably other small-head group lipids through steric effects (3). However, attempts to localize raft targeting signals by mutagenesis of intact caveolin-1 have been confused by the tendency of mutations throughout the protein to induce misfolding and loss of DRM association (89).

Conclusions

Two simple and widely used methods, DRM analysis and cholesterol depletion, have implicated rafts in a variety of cell functions. Furthermore, DRM association has been used to identify raftphilicity-conferring signals in many proteins. However, raft dynamics in cells remain poorly understood. An important challenge for the future will be to learn how and when proteins associate with rafts and precisely how rafts exist and function in cells. Development of improved methods for studying rafts (61) will be essential for further progress toward these goals.

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References


