Lipids as Targeting Signals: Lipid Rafts and Intracellular Trafficking

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Our view of biological membranes has evolved dramatically over the last few decades. In the bilayer model from Singer & Nicholson (Science 1972;175:720–731), both proteins and lipids freely diffuse within the plane of the membrane. Currently, however, membranes are viewed as a mosaic of different compartments or domains maintained by an active cytoskeleton network (Ritchie et al. Mol Membr Biol 2003; 20:13–18). Due to interactions between membrane components, several types of subdomains can form with different characteristics and functions. Lipids are likely to play an important role in the formation of so-called lipid-enriched microdomains or lipid rafts, adding another order of complexity to the membrane model. Rafts represent a type of domain wherein lipids of specific chemistry may dynamically associate with each other, to form platforms important for membrane protein sorting and construction of signaling complexes (Simons & Toomre. Nat Rev Mol Cell Biol 2000;1:31–39). Currently, there are several hypotheses concerning the nature of rafts (reviewed in (Edidin. Annu Rev Biophys Biomol Struct 2003;32: 257–283; Zurzolo et al. EMBO Rep 2003;4:1117–1121)). The most commonly cited one, proposed by Kai Simons (Simons & Ikonen. Nature 1997;387:569–572; Pralle et al. J Cell Biol 2000;148:997–1008), suggests that rafts are relatively small structures (~ 50 nm) enriched in cholesterol and sphingolipids within which associated proteins are likely to be concentrated. Another proposal (Anderson & Jacobson. Science 2002;296:1821–1825) suggests that rafts are constructed of lipid shells. These are small dynamic assemblies wherein ‘raft’ proteins are preferentially associated with certain types of lipids. These ‘shells’ are thermodynamically stable mobile entities in the plane of the membrane that are able to target the protein they encase to preexisting rafts/caveolae domains. In this review we summarize the data suggesting a specific role for lipid domains in intracellular trafficking and sorting and present a modification of the raft model that may help explain the observed phenomena.

Key words: caveolae, detergent resistant microdomains (DRMs), GPI-anchored proteins, lipid shells, rafts, sorting mechanisms

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The Raft Concept

Our view on the molecular interactions of lipids that result in formation of lipid rafts is based on in vitro model systems (1,2). From these studies it was concluded that lipids could be present in three different phases: the gel phase, the liquid ordered phase (lo), and the liquid crystalline or fluid phase (li). In the gel phase, lipids are immobile, with their fatty acid chains fully extended. Above their melting point, lipids are in a fluid phase and form loosely packed structures with high lateral mobility and disordered fatty acid structures. The liquid ordered phase is an intermediate between the gel phase and the li phase: the lipids have a high lateral mobility but also an ordered structure with extended fatty acids. Interestingly, different phases can coexist in membranes. This coexistence is temperature- and composition-dependent and is expected to manifest itself as a phase-separation in membranes at physiological temperatures. It is classically depicted by a phase diagram for a three-component ‘raft-lipid mixture’, consisting of equal amounts of Chol (cholesterol), SM (sphingomyelin) and PC (phosphatidylcholine) at various temperatures (reviewed in (1)).

The presence of domains in biological membranes has been difficult to assess. One of the first tools has been the use of nonionic detergents which, when added to intact cells, gives rise to a fraction of Detergent-Resistant Membranes (DRMs). Remarkably, extraction with Triton X-100 (TX-100) of Chol:SM:PC (1:1:1) liposomes at 0 °C leaves a detergent-insoluble lipid fraction whose composition is similar to that of the ‘lo’ domain expected at 37 °C. However, because native membranes are more complex than artificial ones, and because TX-100 extraction can lead to domain formation in single-phase membranes (3,4), DRM association can only define a biochemical characteristic of the associated molecule and so it is not possible to compare DRMs with native raft structures occurring in living cells. Other techniques that measure lateral mobility of lipids and proteins in living cell membranes have been recently developed (reviewed in (5) and in this issue (6,7)) but there is still no unifying view on the basis for raft formation, their size and stability in living membranes. A key question that remains is whether in biological membranes conditions exist for spontaneous segregation of lipids as a result of lipid–lipid interactions. Due to the tremendous...
variety of lipid classes and fatty acid composition, this may be unlikely to occur. Indeed, the existence of microdomains in lipid mixtures that truly reflect biological membranes has not yet been reported. Studies in giant unilamellar vesicles (GUVs) formed from lipid mixtures derived from rafts do, however, support the existence of ordered detergent-resistant lipid domains that could be reversibly formed and disrupted upon cooling and heating (8).

An alternative model for the generation of lipid rafts is the shell hypothesis (9), which is based on protein–lipid interactions. Via interactions with lipids, putative raft proteins such as GPI-anchored proteins and selected transmembrane and peripheral proteins are capable of shell formation. It remains questionable, however, whether all raft-associated proteins are capable of shell formation. For example, Ga-subunits of heterotrimeric G proteins that associate with rafts by dual acylation, lack any obvious signals in the protein moiety for lipid shell formation and the two acyl groups are not sufficient for shell formation. The same is true for some GPI-anchored proteins. Additional interactions of the protein with the lipids must be required to create potential lipid shells or rafts.

It is therefore possible that only a selected group of proteins have the intrinsic capacity to form lipid shells around themselves. Typical examples could be caveolins (10), the proteolipid MAL (11,12), flotillins (13), stomatins (14), and some transmembrane proteins (15), which penetrate the lipid bilayer and allow intimate contact with membrane lipids due to the presence of high affinity binding sites for specific lipids. (Figure 1, shell proteins). Based on protein–protein interactions, these shell proteins can coalesce into larger complexes, termed rafts (Figure 1, step A). It is possible that as a consequence of the interaction between these high affinity raft proteins, selected lipids are brought into close proximity, creating and stabilizing liquid ordered phases in biological membranes. It remains to be determined whether shells can already represent a functional unit or whether larger (raft) structures are required to generate functional domains. Other putative raft proteins including dually acylated proteins such as GPI-anchored proteins and Ga-subunits of heterotrimeric G proteins have a low affinity for lipid rafts and may associate with existing rafts by additional protein–protein interactions (Figure 1, step B). Alternatively, by oligomerization of low affinity raft proteins, enough low affinity lipid-interacting moieties may be combined to stabilize a functional raft domain (Figure 1, step C). Under these conditions, the oligomerization process creates (and stabilizes) raft domains. Artificial oligomerization of low-affinity raft proteins by antibodies or toxins may have the same effect. As a consequence, raft association of low affinity raft proteins can be regulated by modifications that regulate protein–protein interactions such as phosphorylation and nucleotide binding. Such a model predicts that lipid rafts are highly dynamic structures, also in terms of their existence, and predicts differences in resistance to detergent-extraction for different types of raft proteins or for different association states. This might be reflected in the known differences in detergent solubility characteristics of different proteins in different detergents (16).

Lipid Rafts in Intracellular Trafficking

Lipid rafts have been proposed to be involved in protein sorting at various places in the cell. We will discuss the involvement of these rafts in membrane transport processes.

Figure 1: Lipid rafts. Proteins with a high affinity for selected lipids are suggested to form lipid shells (9). It remains to be determined whether shells can already represent a functional unit or whether larger (raft) structures are required to generate functional domains. Protein–protein interactions between shell proteins can create larger functional units called lipid rafts (A). Dual-acylated proteins (including GPI-anchored proteins) can associate with pre-existing lipid rafts based on their low affinity for raft lipids of the acyl moiety. This affinity can be enhanced by lipid-protein and/or protein–protein interactions (B). By oligomerization of low-affinity raft proteins, enough low affinity lipid-interacting moieties may be combined to stabilize a functional raft domain (C). Under these conditions the oligomerization process may create and stabilize raft domains.
**Endoplasmic reticulum**

In contrast to what has been shown in mammalian cells, GPI-anchored proteins in yeast associate with DRMs in the endoplasmic reticulum (ER) (17), where their sorting occurs. According to the two-vesicles hypothesis, GPI-anchored proteins exit the yeast ER in vesicles distinct from the ones carrying other secretory and plasma membrane proteins (18). In addition, it has been shown that their transport from ER to the Golgi compartment requires ceramide synthesis (19,20).

One possible explanation of the role of rafts in ER sorting is that rafts stabilize the association of GPI-proteins with the ER membrane. However, whether ceramide is part of rafts within yeast ER membranes remains to be investigated. Alternative explanations could envisage a more direct role for ceramide in stabilizing the protein within the membrane, either by establishing hydrophobic interactions with the GPI-lipid moiety or by furnishing the substrate for remodeling the GPI-lipid moiety (21).

First indications for the existence of ER-localized rafts come from studies of the prion protein PrPc, a GPI-anchored protein responsible (in a misfolded conformation) for neurodegenerative diseases known as Prion diseases or TSE (22). We have observed that DRM association of PrPc occurs in the ER in mammalian cells. Furthermore, perturbation of ER microdomains affects the folding of the immature protein and increases misfolding of some ER-localized mutants, suggesting that ER microdomains are directly involved in PrPc conformation (Sarnataro, Campana and Zurzolo, submitted).

**Golgi apparatus**

The first organelle in which rafts were suggested to have a role in sorting was the Golgi apparatus (23). A role for rafts in protein sorting was first described for apical sorting of GPI-anchored proteins in polarized epithelial cells (24–26). Using innovative experiments of pulse chase followed by TX-100 extraction it was shown that GPI-anchored proteins associate with DRMs during their passage through the Golgi apparatus. Perturbation of this association by cholesterol or sphingolipid depletion resulted in impaired trafficking to the PM or altered polarity of the GPI-anchored proteins (27,28). This not only appears to be a property of epithelial cells but is also common to yeast (as above) and other cell types.

The simplest explanation to explain missorting of GPI-proteins in cholesterol-depleted cells was to link this effect to disruption of DRM association, leading to the conclusion that GPI-anchor-mediated raft association was essential for apical sorting (24). However, the proposal that either the lipid anchor or the subsequent association with DRMs is solely responsible for apical sorting has subsequently been questioned (27,29).

It is now clear that apical sorting can rely on more than one single mechanism and signal: apical proteins can be sorted by both a raft-dependent (reported for GPI-anchored proteins and a few transmembrane proteins (15)) and a raft-independent mechanism (27,30). Furthermore, in some cases the addition of the GPI-anchor to a nonpolarized secretory protein is sufficient to determine apical sorting (31,32), whereas in other cases additional features such as oligosaccharide decoration are required (29,33,34), suggesting the existence of a hierarchical order in apical signals.

Recent findings from our laboratory indicate that basolaterally targeted GPI-anchored proteins can also be associated with DRMs, thus questioning the postulated role for DRMs in GPI-anchored protein apical sorting. It appears that both apically and basolaterally sorted GPI-proteins are present in DRMs in the Golgi, although only apically sorted proteins form high molecular weight complexes. Oligomerization may result in a stabilization of proteins into raft domains (35,36) (see Figure 1). It might also cause coalescence of small rafts into a sorting domain (see Figure 1). This indicates that association with rafts in the Golgi apparatus is not sufficient for apical sorting of GPI-anchored proteins and that an additional step that concentrates the proteins into large complexes is required, as suggested by the fact that this behavior is not observed for basolateral GPI-proteins (Paladino et al., in preparation).

Because oligomerization of apically sorted proteins seems to be connected to their association with DRMs it is possible that differences in the GPI-anchor might determine a different affinity for rafts (or stabilize a different type of raft, see Figure 1), thus affecting the residence time in the raft (or altering the stability of the raft) and therefore the ability of the protein to pack into tight clusters. Alternatively, the recognition of an apical signal in the protein ectodomain by a putative apical receptor (lectin type?) could also be envisaged. The machinery that recognizes the signals and carries out the targeting process is not known, but one protein, VIP17/MAL, which has been found to interact with GPI-anchored proteins and which can be isolated in DRMs, is required for apical protein sorting (11,12).

Further studies are required to assess the composition of the apically sorted oligomers, the differences in the GPI-anchors among differentially sorted proteins, and the molecular and biophysical characterization of raft domains in the Golgi apparatus.

**Identity of lipid rafts in the Golgi complex**

Several GPI-anchored proteins become detergent-insoluble during passage through the Golgi apparatus, i.e. after becoming Endo H resistant (25,37). This finding was in agreement with a concentration gradient of cholesterol along the secretory pathway (38). Therefore, it came as a surprise that upon isolation of detergent-insoluble fractions from isolated Golgi membranes (GICs), several GIC proteins, including flotillin-1, caveolin-1 and two newly
identified proteins, showed a brefeldin A (BFA)-sensitive Golgi localization (39,40; Li and Helms, unpublished data). These data suggest that the major fraction of lipid rafts in the Golgi is at the early Golgi cisternae and not in the TGN. In addition, GICs can be isolated from a fused Golgi-ER organelle (40), indicating that the raft-scaffold is determined by protein–protein interactions rather than lipid–lipid interactions, in agreement with the postulated raft model in Figure 1. Despite the enrichment in early Golgi compartments, lipid raft components are segregated from COPI-vesicles which contain a fourfold reduction in sphingomyelin concentration as compared to donor Golgi membranes (41). In agreement with this, several GIC proteins were found excluded from COPI-vesicles (39,40). These data indicate that GICs may be involved in the sorting of proteins and lipids at early stages of the secretory pathway. In addition, since many GPI-anchored proteins are transported in lipid rafts at the Golgi and are efficiently transported to the plasma membrane, rafts containing GPI-anchored proteins must be efficiently segregated from GIC rafts that remain in early Golgi cisternae. Because both types of rafts are present in the same membrane environment, protein–protein interactions must be involved to define at least two different types of rafts in Golgi cisternae.

How raft proteins other than GIC proteins are transported in an anterograde direction is not known. By affecting the oligomerization of, for example, GPI-anchored proteins, proteins could be transported as nonraft proteins in COPI-coated vesicles (Figure 1). Alternatively, cisternal maturation could be involved in the transport of large oligomeric (raft) structures.

Golgi-resident lipid rafts in early Golgi cisternae may be involved in maintenance of Golgi structure and function. Changes in the cholesterol balance of cells have a dramatic effect on Golgi morphology and on intra Golgi protein transport (42–44; Paladino and Zurzolo, unpublished). It remains to be determined whether this is due to an effect on microdomain integrity, but in agreement with this possibility, subtle changes in Golgi membrane cholesterol content (<10%) affect raft partitioning of a specific subset of proteins (G protein subunits) (44).

**Plasma membrane, endosomes and lysosomes**

The internalization of lipid rafts has been studied by following either the lipids or the proteins associated with DRMs. At the plasma membrane several pathways exist for entry of proteins and lipids into the cells (Figure 2). Association with DRMs is not sufficient to specify a particular pathway of endocytosis occurring at the plasma membrane. However, specific lipid or lipid-anchored protein organization may be important for dictating pathways used by different membrane components. Raft lipid internalization has been studied mainly using fluorescent lipid analogues exogenously added to the cells. Although these analogues might show some differences with respect to their natural counterparts, these experiments show that glycosphingolipids (GSLs) are internalized from the plasma membrane by a clathrin-independent, caveolar-related mechanism and are subsequently transported to the Golgi apparatus via a passage through the endosomal compartment. Indeed, as soon as 5 min after internalization the glycosphingolipid-containing vesicles fuse with the early endosomal system, where they are sorted into two major pools, one that is transported to the Golgi via late endosomes and another that is recycled to the plasma membrane via the recycling compartment (45,46). Cholesterol appears to play a major role in modulating the targeting of glycosphingolipids both in normal cells and in cells derived from patients with sphingolipid (SL) storage diseases (SLSD), leading to the working hypothesis that SL accumulation in SLSD cells induces cholesterol redistribution, leading to alteration in the intracellular sorting of GSLs and to their accumulation in the late endosomal compartment (46).

The best studied case of raft-dependent protein internalization is, again, GPI-anchored proteins. These can be internalized via different mechanisms depending on their interactions with other surface components. The first proposed model was that GPI-anchored proteins are internalized via caveolin-coated structures called caveolae (47). However, unless cross-linked, GPI-proteins are not constitutively enriched in caveolae (48–51). Therefore internalization via caveolae seems to require protein clustering at the cell surface, which could be mediated by protein–protein interactions. Similarly, association of GPI-anchored proteins with other plasma membrane proteins that possess a clathrin-pit localization signal promotes their internalization via coated pits, as in the case of PrPC, several co-receptors, and CD14 (52–54). In the absence of lateral associations or cross-linking, GPI-anchored proteins appear to be selectively internalized via a dynamin-independent pathway. Recent results have indeed shown that when dynamin activity is inhibited, GPI-anchored proteins continue to be internalized and delivered to Rab5-negative early endosomes that are devoid of markers of the clathrin- or the caveolin-mediated pathway (55). This pathway is distinct from that followed by other DRM components (e.g. exogenously introduced sphingolipids and interleukin (IL)-2R), which are internalized preferentially via a dynamin-dependent route (56,57), but it appears to be dependent on raft integrity because it is perturbed by cholesterol depletion (55).

A major fraction of GPI-anchored proteins are delivered to early endosomes and subsequently to recycling endosomes (REC) and to the cell surface (58). Their recycling rates from REC appears to be three- to fourfold slower than other recycling membrane components like TfRs and fluorescently labeled lipids (58) and results in their being sorted away from endocytosed membrane cargo and their accumulation in the REC. In CHO fibroblasts, cholesterol or sphingolipid depletion leads to a faster recycling rate for
GPI-anchored proteins, suggesting a role for rafts in this event.

In addition to recycling, proteins can be sorted to late endosomes. By using a heptameric toxin, aerolysin, which binds GPI-proteins, it has been proposed that endosomal redirectioning of GPI-anchored proteins (from REC to late endosomes) would occur because of a stabilized association with microdomains caused by oligomerization of GPI-anchored proteins with the heptameric toxin (35). This model has many similarities with the one we postulate for apical sorting of GPI-anchored proteins in the exocytic pathway (see above) and implies a role for protein-protein interactions in functional rafts. Another example for the role of oligomerization in raft association and sorting is given by the anthrax toxin, which binds to a cell surface receptor (ATR) and induces its oligomerization and DRM association. However, subsequent internalization of ATR occurs via clathrin-coated vesicles in a dynamin- and EPS15-dependent process (59).

A third sorting pathway occurring in endosomes is towards the Golgi apparatus either via late endosomes, as for the mannose 6-phosphate receptor, or via the REC as for the TGN38 and for Shiga toxin (60,61). Shiga toxin, a GD1-binding toxin, is DRM-associated and is internalized into transferrin receptor-containing endosomes via a dynamin- and clathrin-independent process that is perturbed by cholesterol depletion. Interestingly, Shiga is delivered to the Golgi in a clathrin-dependent mechanism.

All the above data suggest that the same protein can shift into different membrane environments and that this can lead to different sorting. This shift appears to be determined by the interactions that the proteins establish within themselves (in oligomers) or with different partners. Conversely, it is possible that due to a particular membrane composition of a distinct organelle (in terms of lipid and or of lipids and proteins) a protein can shift from a raft to a nonraft environment.

As described above, lipid rafts play an important role in the endosomal recycling pathway. Recycling endosomes of
MDCK cells are enriched in both sphingomyelin and cholesterol (62). In contrast, lipid rafts are segregated from the degradative pathway that leads to lysosomal degradation (63). In lysosomal storage diseases it has been postulated that lipid rafts accumulate in late endosomes and lysosomes (64). Support for this hypothesis comes from overexpression of proteolipid protein which is routed to late endosomes and lysosomes. Concomitantly, cholesterol accumulates in these compartments as well, accompanied by mistrafficking of raft components (46).

In late endosomes, large amounts of internal vesicles termed multivesicular bodies (MVBs) can accumulate. The internal membranes of MVB contain cholesterol-rich membranes (65). In addition, DRMs have been isolated from late endosomes (35). These data may suggest that lipid raft components in the endosomal system are sorted to the internal membranes of MVB. In support of this hypothesis, exosomes were found to contain lipid raft components. In proteomic and biochemical analyses of exosomes, an enrichment of several raft proteins and lipids was observed (66). These observations are particularly intriguing in light of the possible involvement of MVB in the immune system. Indeed, evidence has been obtained that HIV budding in primary macrophages occurs through the exosome release pathway (67). These findings are in agreement with results that indicate the involvement of rafts in the interaction of HIV with host cells. By budding through lipid rafts in T cells, HIV selectively incorporates raft markers and excludes nonraft proteins.

Caveolae
Caveolae are small flask-shaped invaginations of the plasma membrane. Because caveolae contain several specific lipids and proteins typical of DRM components, they can be considered a functional specialized raft. Caveolae arise from the coalescence of small rafts driven by the oligomerization of caveolin-1 that forms the caveolar specialized coat (68,69) as shown by the transfection of the cav 1 gene in cells that do not express caveolins (70,71). The function of these structures remains to be established. From the characterization of these structures and the identification of the GTPase dynamin at the neck of caveolae, several functions have been described, e.g. an involvement in endocytosis, transcytosis and regulation of signaling cascades (10,47). Caveolin-1-null mice, which do not have morphologically recognizable caveolae, are viable, suggesting that at least the structure may not be important for its function (72,73). The molecular mechanisms of the involvement of caveolae in the various processes have yet to be elucidated. For example, caveolae have been observed as interconnected vesicles, possibly defining a continuous transport route. Caveolae can, however, also bud off from plasma membranes in a GTP-dependent manner (reviewed in (10)). These two scenarios are not necessarily exclusive. Using caveolin-1-GFP it was shown that caveolae are largely immobile static structures with little exchange between the plasma membrane pool and intracellular pools of caveolin (74). The addition of Simian Virus 40 (SV40) to cells resulted in enhanced dynamics of caveolin-1-GFP, indicating that endocytosis was stimulated by the virus (75). Real-time video microscopy revealed the presence of a novel endocytic compartment, a caveosome, that lacks classical endocytic markers such as EEA1 and transferrin (76). After uptake of SV40 by endothelial cells, caveolin-1-GFP and SV40 colocalize to caveosomes. Sorting occurs in the caveosomes, resulting in the transport of the virus to the ER and recycling of caveolin-1-GFP to the plasma membrane via the Golgi complex. As described above, endocytosis of lipid rafts is mediated by various mechanisms. Endocytosis of Cholera toxin is mediated by GM1 ganglioside, the toxin receptor that is present in lipid rafts. This raft protein enters the cell by a clathrin-mediated pathway as well as by a caveola-mediated pathway (77,78). When the clathrin-mediated pathway is blocked and the protein is internalized via caveolae, it localizes exclusively to caveosomes. In contrast to SV40, however, Cholera toxin is transported to the ER via the Golgi complex. The mechanisms that underlie sorting in caveosomes and that result in transport to the Golgi complex (caveolin-1 and Cholera toxin) or ER (SV40) are not known. In addition, the transport mechanisms to and from caveosomes remain to be determined. Caveolae that bud from the plasma membranes contain the machinery for vesicular transport including SNARE proteins (79). Transport from caveosomes to the ER may involve ARF and coatamer proteins, components of the retrograde transport machinery between Golgi membranes and ER (80). Thus, retrograde transport from several organelles to the ER may involve the same or similar machinery. It is surprising, however, that COPI vesicles are implicated for the retrograde transport of raft components to the ER because several raft proteins and lipids are segregated from COPI vesicles (40,41). These data may suggest that raft proteins destined for transport to the ER leave the raft scaffold before entering COPI vesicles and being transported to the ER.

Conclusions
In this review we have suggested a modification of existing lipid raft hypotheses that includes the presence of core proteins and low-affinity raft proteins. Core proteins have a high affinity for selected lipids and further research should define the protein–lipid interactions. Low affinity raft proteins need additional interactions for raft localization. We also propose that, depending on the oligomerization status of proteins, rafts can be created or disrupted. This property may be important for the sorting mechanisms and localization of raft components in cells.

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