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Review

Rafts: Scale-Dependent, Active Lipid Organization at the Cell Surface

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Rafts have been conceptualized as lateral heterogeneities in the organization of cholesterol and sphingolipids, endowed with sorting and signaling functions. In this review we critically examine evidence for the main tenet of the 'raft hypothesis', namely lipid-dependent segregation of specific membrane components in the plasma membrane. We suggest that conventional approaches to studying raft organization wherein membranes are treated as passive, thermally equilibrated systems are unlikely to provide an adequate framework to understand the mechanisms of raft-organization *in vivo*. An emerging view of raft organization is that it is spatio-temporally regulated at different scales by the cell. This argues that rafts must be defined by simultaneous observation of components involved in particular functions. Recent evidence from the study of glycosylphosphatidyl inositol-anchored proteins, a common raft-marker, supports this picture in which larger scale, more stable rafts are induced from preexisting small-scale lipid-dependent structures actively maintained by cellular processes.

Key words: active, cholesterol, glycosylphosphatidyl inositol-anchored proteins, membrane structure, rafts, scale dependence, signaling, sorting

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The surface of eukaryotic cells is a complex assembly of a variety of molecular components which actively partitions the interior of the cell from the external environment. Over several decades investigators have focused their attention on the structural and functional organization of this multicomponent milieu in an attempt to understand how the cell engages with the outside and how it controls the exchange of chemicals and information across this barrier.

The cortical layer of the cell, viewed across a transverse section 700 nm wide, is organized in heterogeneous multilayers. This begins with the extracellular matrix, followed by a semipermeable lipid bilayer, the plasma membrane consisting of lipids and embedded proteins and finally ends in the complex cytoskeletal meshwork loosely attached to the plasma membrane via anchoring proteins. These layers are structurally and dynamically coupled to one another at different spatio-temporal scales in ways that we are just beginning to understand.

More recently, however, it is the *lateral* organization of the cell surface and its interactions with the above components that have been the subjects of intense scrutiny (1,2). In this article, we critically review our understanding of *membrane rafts*, lateral heterogeneities composed of specific cell surface lipids and proteins. We will discuss attempts at their identification *in vivo* and contrast them with their 'realization' in model artificial membranes. We then provide a synthesis of the available information with the aim of developing a new conceptual framework to understand lipid-dependent organization in the surface of living cells.

Composition of Eukaryotic Membranes

Recent methodologies of biomolecular structure determination based on ultra-sensitive mass spectrometry (3) have led to a detailed characterization of the chemical composition of cellular membranes (4). The lipid composition of the plasma membrane and other endomembranes of living eukaryotic cells is extremely complex, consisting of up to 500 different lipid species, classified according to head-group and backbone structure. These include neutral glycerolipids, glycerophospholipids, ceramides, glycosphingolipids and sphingomyelins (1,5). A major lipid component of the plasma membranes is cholesterol or its closely related analog ergosterol (6).

The eukaryotic cell is functionally compartmentalized via membrane-limited organelles that continually exchange biomolecules, including lipids, by a variety of membrane trafficking mechanisms (7,8). In the face of this dynamic exchange, heterogeneity in the lipid composition of the membranes of different organelles appears to be maintained. For instance, the plasma membrane of most eukaryotic cells is highly enriched in cholesterol and glycosphingolipids, while the endoplasmic reticulum (ER)

is poor in these components (6,9). In addition, there is a transbilayer lipid compositional asymmetry within the same membrane (5). Compositional heterogeneity has important functional consequences; a dramatic demonstration occurs during *apoptosis*, when the predominantly inner-leaflet lipid species, phosphatidylserine, fails to be actively 'flipped' and accumulates at the outer leaflet, thus serving as a signal for clearance of 'cell corpses' by a macrophage cell with scavenger functions (10,11). Although precise details of how compositional heterogeneity is achieved are not available, it is abundantly clear that this is done by a complex mechanism involving lipid synthesis, turnover and *active* transport. Curiously, recent studies on the lateral organization of chemical heterogeneity on the plasma membrane have not taken this into account.

The Fluid-Mosaic Model of the Plasma Membrane

The first attempt at portraying the lateral organization of lipids and proteins on the plasma membrane was the *fluid-mosaic* model (12) following observations of translational diffusion of lipids and proteins based on lipid mixing experiments in fusing cell membranes (13). While the mobility of cell surface lipids and proteins was consistent with simple Brownian diffusion, the measured translational diffusion coefficients were different from those measured in artificial membranes, often by an order of magnitude. This suggested that whereas the lipid environment was similar to artificial fluid membranes, its interaction with the embedded proteins resulted in increased drag experienced by these molecules. The embedded proteins in turn were influenced by the underlying cytoskeleton (14). Within the fluid-mosaic model, the multicomponent lipid nature of the plasma membrane facilitates the 'solvation' of a variety of membrane proteins via specific interactions such as hydrophobic shielding, electrostatics, hydrogen bonding and van der Waals. In this multicomponent chemical milieu, it is not unreasonable to expect some level of physical heterogeneity as a result of macroscopic *phase segregation*, or transient, short-scale, heterogeneities induced by thermal fluctuations in the mixed state (especially when close to a *phase boundary*). The fluid-mosaic model, however, does not endow such lipid-based heterogeneities with any functional significance, and it is difficult to imagine how such equilibrium heterogeneities, created by thermal fluctuations, can be utilized by the cell in a regulated and precise way. To summarize, the fluid-mosaic view of the plasma membrane is that of a passive, equilibrium, multicomponent lipid bilayer with functionally active proteins embedded in it. It is largely this view that has promoted studies on artificial multicomponent membranes as good model systems to describe the physical properties of the cell surface.

The Original 'Raft' Hypothesis

Since the fluid-mosaic proposal, numerous studies revisiting the architecture of the plasma membrane at different length and time scales have provided a much more complex picture of cell surface organization (reviewed in (15)), in particular the recent proposal of *membrane rafts* (1,14). Unlike the fluid-mosaic model, the membrane raft hypothesis addresses the possibility of functionally relevant lateral compartmentalization of specific lipids. In its original form (Figure 1A), the hypothesis postulated that lipids of specific chemistry, namely cholesterol and sphingolipids, spontaneously associate with each other to form platforms for the segregation of proteins such as GPI-anchored proteins (16). These segregated domains were presumed to have a role in membrane protein sorting and the construction of signaling complexes (17).

The predominantly circumstantial evidence for this lateral functional organization (see Table 1) was given an operational basis by the discovery that a specific set of membrane components were insoluble in a solvent containing nonionic detergents (chiefly Triton X-100) at low temperatures (4 °C), resulting in detergent-resistant membranes (DRMs). Resistance to detergent extraction has since become a 'definition' of membrane rafts. Compositional analyses of DRMs showed a high proportion of cholesterol, sphingolipids and a variety of phosphatidylcholines (PCs), together with GPI-anchored proteins (18). Many specific membrane components are selectively associated with these membranes in a cholesterol-sensitive fashion (19). Based on this definition, rafts have been correlated with a variety of signaling and sorting properties of membrane components. In parallel, lipid depletion (specifically cholesterol and sphingolipid) has also been shown to perturb sorting and signaling properties of many membrane proteins (see Table 1). These characteristics have been found to be congruent with a raft-based, lipid-dependent functional organization. However, as we shall discuss below, neither DRM association (see Box 1) nor lipid depletion protocols provide unambiguous evidence for preexisting lipid-dependent assemblies in living cell membranes. In this context it should be noted that alteration in cholesterol and sphingolipid levels may perturb several different aspects of cell physiology. For instance, cholesterol depletion via extraction with a cholesterol complexing agent, methyl-B-cyclodextrin in addition affects the lateral mobility of lipids, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin cytoskeleton (20); sphingolipid depletion may alter levels of sphingolipid metabolites that are important lipid second messengers involved in a variety of signaling pathways (21,22).

Looking for 'Rafts'

Even if we take DRMs as a useful operational definition for membrane rafts (see Box 1), it is clear that such a *biochemical* criterion cannot provide information on the

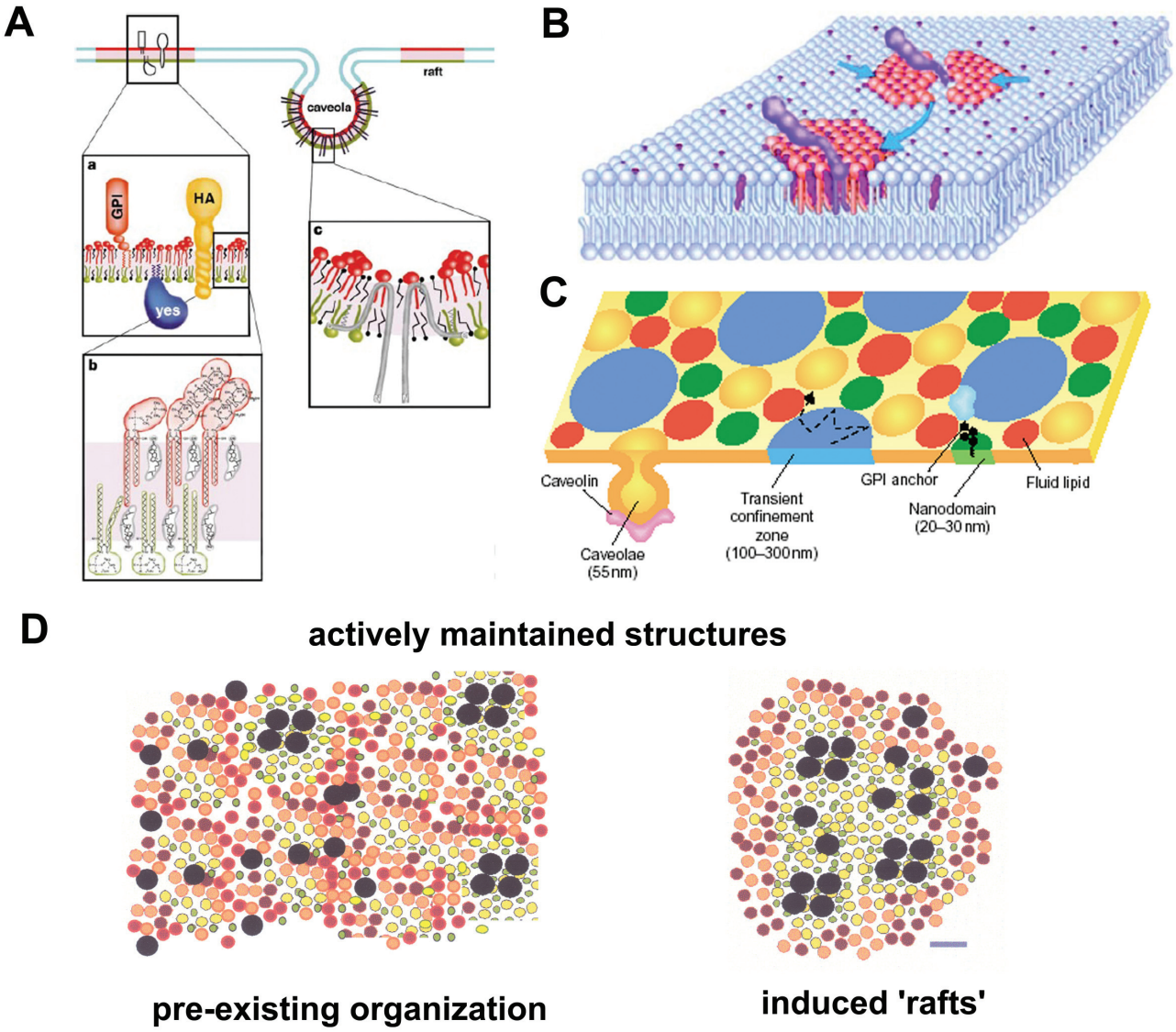

Figure 1: A) The most commonly cited hypothesis for membrane rafts proposed by K. Simons (Dresden, Germany) (16) depicts rafts that are relatively large structures (~50 nm) (83), enriched with cholesterol and sphingolipid (SL), with which proteins are likely to associate. B) Anderson & Jacobson (84) visualize rafts as lipid shells that are small, dynamic molecular-scale assemblies in which 'raft' proteins preferentially associate with certain types of lipids. The recruitment of these 'shells' into functional structures could be a dynamic and regulated process. C) Another point of view is that a large fraction of the cell membrane is raft-like and exists as a 'mosaic of domains'; cells regulate the amount of the different types of domains via a cholesterol-based mechanism (45). D) Actively generated spatial and temporal organization of raft components. A different picture, which is consistent with data from GPI-anchored protein studies in living cells (52,60), suggests that preexisting lipid assemblies are small and dynamic, and coexist with monomers. They are actively induced to form large-scale stable 'rafts'. Black circles, GPI-anchored proteins; red and pink circles, nonraft associated lipids; yellow circles, raft-associated lipids; green, cholesterol. Scale bar ~5 nm.

physical organization of its components. These concerns have led to a variety of physical methods of varying sophistication designed to study the nature of lateral organization of specific lipids and proteins at different scales both on the cell surface and in artificial membrane systems.

'Rafts' in artificial membranes

If we were to view *functional* heterogeneities on the cell surface as an extension of the ideas portrayed in the fluid-mosaic model, then we would continue to treat artificial multicomponent membranes as model systems describing the physical properties of the plasma membrane. Thus, a lot of work has concentrated on establishing the existence of domains in artificial membranes composed of specific lipids, for example a 1 : 1 : 1 proportion of DPPC : Sph : Chol, resembling those obtained from DRMs (reviewed in (2,23,24)). In this point of view, 'rafts' are *preexisting* structures on the cell surface which are

Table 1: Cellular functions that implicate 'rafts'

Cellular function/Observations	Inference	References
Apical membrane is richer in specific lipids compared to basolateral membranes of polarized epithelia	Polarized traffic of lipids in epithelial cells	(62)
GPI-anchored proteins are selectively delivered to the apical surface of polarized epithelia	GPI-anchoring acts as an apical sorting signal	(63,64)
DRM association of GPI-anchored proteins during biosynthetic transport to the apical surface of polarized epithelia	Apically transported proteins are located in DRM domains	(18)
Cholesterol- and sphingolipid-dependent apical sorting of GPI-anchored proteins	Cholesterol- and sphingolipid-sensitive structures mediate apical protein sorting	(65–67)
Cholesterol and sphingolipid-sensitive endocytic sorting of GPI-anchored proteins at the cell surface and in endosomes.	Cholesterol and sphingolipid-sensitive structures mediate endocytic protein sorting	(68,69)
ER-Golgi traffic of GPI-anchored proteins in distinct carrier vesicles.	a) Role of 'rafts' in membrane traffic in yeast	(70–71)
Polarized delivery of DRM components in yeast Smoo formation	b) Role of 'rafts' in signal-dependent cell polarity in yeast	(72)
Cholesterol-sensitive transbilayer signaling via GPI-anchored proteins mediated by src-family nonreceptor protein tyrosine kinases (NRPTKs)	Cholesterol-sensitive DRM association of NRPTKs suggests the role of rafts in transbilayer signaling	(73–75)
Reversible DRM association of signaling receptors and down stream components: T- and B-cell signaling	T- and B-cell receptors modulate their signaling via rafts	(76,77)
Distinct lipid requirements for Ras isoform signaling and the detection of distinct domains for different mechanisms of membrane anchorage of H-Ras and K-Ras	Modulation of signaling via inner leaflet proteins takes place by differential association with distinct rafts	(57,78)

spontaneously formed by *equilibrium phase segregation* in a multicomponent system or *equilibrium thermal fluctuations* resulting in transient small-scale domains even in the homogeneous mixed phase. These possibilities have been examined in numerous artificial membrane bilayer systems (25–29), and even in Monte Carlo/molecular dynamics simulations using simple model lipid potentials in two dimensions (reviewed in (30)).

Using the aforementioned lipid composition, freely suspended monolayers at the air–water interface, suspended lipid bilayers, lamellar stacks of lipid bilayers and artificial giant unilamellar vesicles (GUVs) have been prepared and subjected to a variety of techniques suited for assessing heterogeneities at different scales:

- *thermodynamic measurements* such as differential scanning calorimetry (DSC), and surface pressure-area isotherms coupled with preferential partitioning of lipid probes;
- *diffusion measurements* via intervesicular transfer rates of various lipids, fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and single particle tracking (SPT);
- *spectroscopic measurements* such as fluorescence quenching and fluorescence energy transfer (FRET);
- *direct visualization and imaging* by confocal microscopy, scanning atomic force microscopy (AFM) and near-field scanning optical microscopy (NSOM).

Many of these techniques and the results obtained pertaining to micron and submicron scale structures on artificial membranes have been reviewed recently (31–33) and will not be gone into in detail here.

These studies have shown that while the binary lipid system of Sph : PC shows a liquid–gel coexistence at temperatures below the main transition of sphingolipids ($T_m = 40\,°C$), the ternary mixture of Sph : PC : Chol shows a liquid–liquid coexistence within a range of compositions and temperatures (26). A range of domain sizes have been reported ranging from the nanometer to the micron scale (25,29,34,35). Using fluorescent probes attached to glycolipids such as GM1 and GPI-anchored molecules, several researchers have demonstrated preferential partitioning of these molecules into liquid domains enriched in Sph/Chol with differing diffusion properties (36,37).

Liquid–liquid coexistence in the ternary system has been interpreted as being a coexistence between the high temperature liquid disordered (l_d) phase with a cholesterol-poor composition and a liquid-ordered (l_o) phase enriched

Box 1

'Rafts' and their relationship to DRMs

The chemical composition used in many studies on rafts was suggested predominantly by the ability of components to associate with DRMs (38,79). While this has been a popular method to implicate rafts in functional terms (see Table 1), DRM association has recently been subject to the most intense critical scrutiny. Despite the correlation of DRMs with l_o phases in artificial systems (79), the mechanism of detergent solubilization has only been recently investigated. Using pulsed DSC, Heerklotz and coworkers show that titrated addition of Triton X-100 to a multicomponent lipid bilayer in the l_d phase *induces* domains with l_o characteristics; the size of these domains are as yet unknown (80). The detergent also severely perturbs preexisting l_o-domains (81). In parallel, Prieto et al. constructed a ternary phase diagram of a Chol/Sph/PC lipid mixture based on differential partitioning of lipid probes and examined the effect of Triton X-100 at a particular composition (26). They observed that extraction with Triton X-100 (TX100) of 1 : 1 : 1 Chol : SM : PC liposomes at 4 °C leaves an insoluble lipid membrane residue (DRM) whose composition coincides with the composition of the 'l_o' domain observed at 37 °C in the ternary phase diagram, but not with the composition of the l_o phase at 4 °C. This indicates that detergent extraction dramatically alters the lipid composition of preexisting domains. These studies suggest that if rafts are indeed formed by the spontaneous de-mixing of cholesterol and sphingolipids from a complex milieu of phospholipids, they are likely to be very sensitive to perturbations, especially those that involve incorporation of detergent molecules into the bilayer.

Analyses of the protein composition of DRMs has provided a list of potential raft-associated molecules (19). However, the use of different detergents, detergent to protein ratios, temperatures and cell types appears to give rise to a different composition of DRM-associated molecules (82). Even the ratio of the lipid constituents vary dramatically between the different protocols followed. This challenges the credibility of such a technique to define 'rafts' in an absolute sense. In a complex environment as a cell membrane, DRM association may at best serve to define a circumstantial biochemical characteristic. It cannot provide information regarding the preexisting organization of membrane components on the multicomponent cell surface.

To summarize, with the understanding of the physical process of detergent-mediated lipid insolubility, the relationship of DRMs with any preexisting lipid-dependent organization has been seriously challenged. Furthermore, simply correlation of function with the lipid status may not be a sufficient criterion for understanding raft-based organization and function.

in sphingolipids and cholesterol. The difference between l_d and l_o phase is that the latter is characterized by a sharp reduction in the area per lipid as a result of stiffening of the acyl chains (24,38,39). Direct measurement of acyl chain stiffening in l_o regions may be made by small angle X-ray scattering from oriented lamellar samples, or by measuring the torsional flexibility of labeled acyl chains using nuclear magnetic resonance or electron spin resonance. However, data from X-ray diffraction studies are not conclusive, presumably due to lack of registry of the components in different layers (40).

The interpretation of the 'l_o' nature of this phase comes primarily from observations of reduced area per lipid obtained from surface pressure-area isotherms, and preferential partitioning of saturated long-chain fatty acids. A recent alternate proposal (reviewed in (23)) is that this new phase represents a liquid rich in *condensed complexes*; a chemical complex of cholesterol and sphingolipids formed in the reversible reaction $p\ C + q\ S \rightleftharpoons (CS)$. Even in the absence of macroscopic phase segregation, equilibrium thermal fluctuations in the mixed phase of a multicomponent system may give rise to transient, small scale l_o domains or, more significantly, condensed complexes whose lifetime could be enhanced by proximity to a phase boundary. This interpretation however, has not been as clearly validated for bilayer vesicles. To test this interesting proposal, one might need additional spectroscopic evidence to measure molecular complexation (for example see (29)).

What are the intermolecular forces responsible for the phase segregation that brings sphingolipids and cholesterol (raft components) together? This is a difficult question to address experimentally since in addition to two-body forces such as hydrogen bonding between the OH group of cholesterol and the amide group of sphingolipids (or even ceramides), weak dipolar interactions between sphingolipids, and van der Waals interactions between saturated acyl chain and cholesterol, there are many body interactions such as hydrophobic shielding or the 'umbrella effect' (wherein cholesterol may segregate into regions of the membrane with strongly hydrated phospholipid head groups due to steric considerations) (personal communication, P. Kinunnen, Helsinki, Finland). Any observed clustering on artificial membranes is most likely due to a combination of all these physical forces.

A closely related point of view is that the constituents of the cell membrane are in a mixed, equilibrated phase, poised close to a *phase* boundary. In this view, any slight

perturbation drives the system across the phase boundary, inducing large scale segregation of specific lipid components, as observed in experiments involving the depletion of cholesterol in living cells, which gave rise to large scale segregation of probes preferring the l_d phase (41).

At the very least, ignoring all active processes and the multitude of components present in the cellular context, any comparison of lipid organization in artificial membrane systems with cellular membranes can be made only when the composition and external thermodynamic parameters such as temperature and surface pressure are maintained the same. But there is a more fundamental criticism – thermodynamically predicated or thermally induced structures (phase segregated domains or transient fluctuations) cannot be effectively regulated and utilized for specific cellular function. The basic problem is that this route of investigation is firmly grounded in the fluid-mosaic picture. Actively maintained lateral compositional heterogeneity and transbilayer lipid and protein asymmetry contribute to holding the cell membrane in a state far from equilibrium. This immediately questions whether lessons obtained from the study of lateral lipid segregation under equilibrium conditions are likely to be relevant to understanding the structure of rafts or functional lipid assemblies present in living cell membranes.

'Rafts' on the cell surface

It appears that the only way to address the question, 'what is the physical nature of 'rafts' in the cell membrane?', is to directly observe raft-assemblies in living cells (31). Fluorescence microscopy in living cells has consistently failed to reveal large-scale laterally segregated structures enriched in a major raft-component, GPI-anchored proteins (42,43). This suggests that any preexisting cellular rafts must be much smaller than those recently characterized in artificial systems and hence undetectable by the limited resolution of the fluorescence microscope (>300 nm), and/or extremely dynamic (31,32). Their detection is also likely to be beyond the scope of conventional electron microscopy (42–44). Conventional optical microscopy fails to reveal any large-scale heterogeneities (32). At this scale the membrane is consistent with the fluid-mosaic picture. To face this challenge, a number of new methodologies for detecting membrane heterogeneity in cell membranes have emerged.

Probe partitioning methods

Recent studies examining the distribution of lipid probes capable of differential partitioning into l_o or l_d domains in living cell membranes have been *interpreted* in terms of a preexisting 'mosaic of domains' of varying size, composition, timescale and physical properties (45). This interpretation should be viewed with some caution, since studies on the molecular origins of differential partitioning in artificial membranes suggest a complex of interactions involving both the head (steric and dipolar) and the long-saturated acyl chains (free volume, van der Waals (46,47)). In light of this, and the ability of exogenously added detergents to significantly alter preexisting domains (see Box 1), one needs to carefully check that the lipid probes faithfully report on preexisting structures and not on structures induced by them. The absolute concentration of lipid probes is an important parameter in this regard, since at the probe levels high enough to be visualized (e.g. 1000 molecules/μm^2 of a probe results in at least 0.1% probe to membrane lipid fraction), the probes may themselves need to be treated as a separate component. For a similar reason one should take care that the fluorescent markers used to tag specific lipids and proteins do not induce aggregation of the tagged molecules. However, multiphoton imaging with appropriate lipid probes such as Laurdan, capable of differing fluorescence properties (generalized polarization (GP)) in l_o and l_d domains (48) has recently revealed regions of the living cell membrane with fluorescence characteristics consistent with 'l_o' domains (49). It remains to be determined whether this 'l_o' characteristic is due to preexisting lipidic structures or protein interactions, since crosslinking of a 'non-raft or DRM-associated protein', the transferrin receptor, increases the extent of these domains.

Methods of detecting proximity

In native cell membranes, methods designed to detect proximity between molecules have observed inhomogeneous distributions of many molecular components of rafts, including GPI-anchored proteins.

Chemical crosslinking with short (1.1 nm) crosslinkers (50) suggest that cholesterol-sensitive complexes of GPI-anchored proteins exist at the cell surface containing anywhere from two to 14 molecules. These experiments were conducted using nonspecific cell-impermeable crosslinkers at low temperatures for an extended period of time. While this procedure facilitates detection of relatively long-lived preexisting structures, it is difficult to quantify the actual size or abundance of preexisting clusters in the membrane with this methodology.

Fluorescence resonance energy transfer (FRET) methods are designed to detect proximity between fluorophores at 1–10 nm scale (51). Earlier work from our laboratory monitoring FRET between identical fluorophores (homo-FRET (52)) had suggested that GPI-anchored proteins occur in cholesterol-sensitive, submicron-sized 'domains' at the surface of living cells. Recently, data from our laboratories have shown that a small but significant fraction (20–40%) of GPI-anchored proteins form extremely high density clusters of nanometer size ($\sim$4–5 nm), each consisting of a few ($\leqslant 4$) molecules and different GPI-anchored protein-species (60). The high local density of GPI-anchored protein molecules was directly derived from the FRET-related

fast anisotropy decay rates observed in time-resolved anisotropy measurements in experiments conducted on three different proteins, the human folate receptor (FR-GPI) labeled via a monovalent fluorescent folic acid analog, N-α-pteroyl-N-ε-(4′- fluorescein -thiocarbamoyl)-L-lysine (PLF), GPI-anchored Enhanced Green Fluorescent Protein (GFP-GPI) and variants of GFP, mCFP- and mYFP-GPI, in a variety of cell types. Using fluorescence photobleaching experiments and theoretical modeling of the resultant changes in anisotropy, in conjunction with a knowledge of the interprotein distances, we have been able to show that that 20–40% of GPI-anchored protein species are present in clusters on the scale of the Forster's radius R_0 (i.e. < 4.65 nm). Interestingly, these results resolve the apparent discrepancy between the lack of detectable hetero-FRET from clustered GPI-anchored proteins (53,54) and the detection of robust homo-FRET (52) and significant chemical-crosslinking of diverse GPI-anchored proteins with a nanometer-sized spacer (50). These nanoscale structures are sensitive to cholesterol levels in living cells. On the other hand, sphingolipid depletion does not directly alter the structure of this organization, it instead makes these nanoscale structures more susceptible to cholesterol depletion. A particularly intriguing feature of this organization is that it exhibits a constant fraction of clusters and monomers over a large range (10–20-fold) of GPI-anchored protein expression levels. We believe that this methodology is most suited for the elucidation of nanoscale organization in living cell membranes in other contexts as well.

Single particle tracking (SPT)

Numerous SPT studies have been conducted to examine the diffusion characteristics of membrane components (14). Observations made at video rate (33 frames/s) of particles attached to potential raft-molecules have not provided any conclusive evidence of regions of the membrane that exhibit characteristics expected for l_o domains as observed in artificial membrane experiments. Observations at this time-resolution from a variety of groups suggest 'sizes' ranging from zero to 26–500 nm, likely to be due to intrinsic differences in the protocol for making single particles and cell type variation (55). In a *tour-de-force* of precision experimentation, A. Kusumi and colleagues have collected SPT data at an extremely high time resolution (40 000 frames/ s) to measure the diffusion characteristics of GPI-anchored proteins and fluorescent lipids in living cell membranes at different spatial and temporal scales (33,55). These studies suggest that the membrane of living cells is predominantly compartmentalized via membrane skeleton fences at a cell type-dependent scale ranging from 30 to 230 nanometers, restricting the free diffusion of proteins and lipids; membrane constituents' display confined diffusion at short time scales and hop diffusion at longer (14). Their results also suggest that the raft-constituents attached to single antibody-bead conjugates diffuse as extremely small species consistent with monomers or small preexisting assemblies, but inconsistent with any large scale organization (> 100 nm) of stable rafts. An important note of caution emerging from the studies of Kusumi and coworkers is that even mildly crosslinked GPI-anchored protein species exhibit diffusion characteristics that are distinct from monomers in the membranes of living cells (55). Thus, probes with potential for crosslinking GPI-anchored proteins are likely to report anomalous diffusion characteristics for these molecules; the use of single fluorophore reporters would fix this experimental bottle neck. Data from single fluorophore tracking studies conducted on a GPI-anchored isoform of class II MHC molecules (56), albeit at much lower time resolution, are consistent with the SPT studies of Kusumi and coworkers. These studies report that most GPI-anchored proteins appear to exhibit fast diffusion consistent with the monomer species identified by Kusumi, whereas only a small fraction (between 6 and 20%) of the labeled species are likely to have a significantly slower diffusion coefficient consistent with larger oligomers or rafts. However, these studies were unable to characterize the size or origin of the slowly diffusing species.

'Rafts' in the inner surface of cells

Any functional organization at the outer leaflet of the plasma membrane is likely to be reflected in an organization at the inner leaflet so as to provide a connection between the two leaflets of the bilayer. A large number of inner leaflet molecules such as the Ras family of small molecule GTPases and non receptor tyrosine kinases are lipid anchored with modifications ranging from acylation to poly isoprenylation. Recent data on the size and structure of rafts at the inner leaflet of the plasma membrane, utilizing statistical analysis of the spatial distribution of H-ras and K-ras fusion proteins, detected via EM on fixed cells (57), supports the existence of 40-nm-sized structures covering 20–30% of the cell surface of separately clustered distributions of farnesylated H-Ras and K-Ras (tethered to the inner leaflet via polybasic-amino-acid stretches). Though the H-ras clusters are not correlated to non crosslinked GPI-anchored protein on the external leaf of the plasma membrane, they are disrupted by removal of cholesterol. Moreover, they are stabilized/ expanded by crosslinking an intracellular lectin called Galectin. On the other hand, K-Ras clusters appear fundamentally different and are formed independent of cholesterol. In a separate study, using FRET microscopy, Tsien and coworkers have shown that multiply acylated proteins can co-cluster at the inner leaflet of the plasma membrane, providing evidence for a potentially different type of lipid organization at the inner leaflet (58). At this juncture it is important to obtain quantitative data about the size and composition of these inner leaflet structures and their relation to outer leaflet rafts in living cells at different spatio-temporal scales. Particularly important will be the combined study of structure of these inner leaflet proteins and their modulation by different signaling stimuli.

Functional, Active Lipid Organization: Towards a New Picture

While the picture of rafts or lipid assemblies present in live cell membranes is far from settled, a range of hypotheses have been proposed over the years (see Figure 1). Recent experiments on artificial membranes and cells, using more sophisticated experimental methodologies, provide an emerging picture of rafts which may be summarized as follows (59):

- Considering the complexity of the system and the perturbing nature of DRM formation, it is unlikely that DRMs reflect some preexisting structure/organization in the membrane (see Box 1).
- The ability to partition with the DRM could reflect an important membrane-related biochemical property of the specific component in question, especially under conditions where this property is subject to modulation.
- In living cells, functional lipid organization on the cell surface is unlikely to be a result of equilibrium phase separation, further complicating the relationship between DRMs, l_o phases and rafts.
- In living cells, lipid assemblies in their *preexisting* state are likely to be small and dynamic, implying an intrinsic diversity of composition.
- Functional rafts (i.e. larger, more stable platforms) are then induced upon requirement and in specific cellular contexts of sorting or signaling.

If this is indeed a correct picture of rafts, obtaining an understanding of the cellular mechanisms that govern the generation and utilization of these lipidic structures is going to occupy center stage in the raft field.

In this context the simultaneous study of functional lipid-dependent lateral segregation of GPI-anchored proteins provides a new picture of lipid-dependent assemblies in live cell membranes. Data recently obtained from our laboratories suggest that the formation of the GPI-anchored protein clusters must be maintained actively in the cell (60). This is because any mechanism for the formation of GPI-anchored protein clusters must be consistent with the following observed features: (i) the capacity of the clusters to undergo dynamic exchange, and (ii) the concentration independence of the fraction of monomers and clusters over a large range of expression levels, implying a fixed proportion of monomers and clusters over this concentration range. These features are inconsistent with any kind of equilibrium mixing of the clusters with monomers, and may be resolved only if the clusters are actively maintained in 'larger domains' that do not allow for ready mixing, leading to chemical equilibration. The ability of cholesterol levels to modulate the fraction of clusters and monomers suggests that cholesterol homeostasis may in turn regulate this activity.

The small, dense preexisting clusters and the possibility of inducing larger clusters by crosslinking also has important implications for signaling. The combination of monomers and small clusters provide an optimal solution for the need for high *binding efficiency* and large *dynamic range* (61). The ability of the small clusters to be organized into larger structures may give rise to *thresholding*. Reorganization of the smaller structure by crosslinking could provide a mechanism to *reset* the system.

This type of active organization has fundamental implications for membrane organization across the bilayer. In accord with the notion of actively generated rafts, Kusumi's group reported that stable rafts are formed only after crosslinking unstable raft precursors (33). This process generates a long-lived confinement of the crosslinked species, which depends on actin polymerization and cholesterol levels. Next, signaling machinery including the nonreceptor src family protein kinase, lck, and the small G protein, G_i, involved in generating the Ca^{++} signaling response of GPI-anchored proteins, are recruited, resulting in a stable signaling platform (33,59).

Conclusions

Whatever the eventual picture of the plasma membrane, it is apparent that the old notion of cell surface lipids as a passive, equilibrated, two-dimensional solvent implied by the fluid-mosaic model will have to be replaced by a radically different model, in which certain cell surface lipids are transposed as active players. Rafts then may be considered as preexisting, scale-dependent active structures, poised to be induced to form larger and more stable structures which may be utilized for specific cellular purposes. A primary question is what are the common organizing principles governing the structural and functional architecture of rafts and thereby the dynamic nature of lipid assemblies at the surface of living cells in different functional contexts.

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