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Review

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Recent controversy surrounding lipid rafts

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Summary

Lipid rafts (LRs) are highly enriched in glycosphingolipids, sphingomyelins, and cholesterol membrane microdomains, existing in a liquid-ordered phase of the plasma membrane. In the literature, LRs are also known as detergent-insoluble membranes, detergent-resistant membranes, glycosphingolipids-enriched membranes, detergent-insoluble glycolipid-rich membranes, and Triton-insoluble floating fractions. These properties enabled their separation from the rest of the phospholipid bilayer, providing new insight into the structure of the plasma membrane, which until then was believed to represent a two-dimensional liquid structure with proteins uniformly solubilized in the lipid solvent. Although there have been many articles concerning LRs, there is still controversy about their existence in the natural state, their size, definition, and function. Different techniques have been developed to visualize LRs in living cells, but the results are contradictory. In this minireview, some recent papers concerning LRs, their existence *in vivo*, their dynamics, size, methods of isolation, and their association with different proteins are discussed.

Key words: **lipid rafts**

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Lipid rafts (LRs) were initially defined as plasma membrane microdomains insoluble in the non-ionic detergent Triton X-100 at 4°C and enriched in glycosphingolipids, sphingomyelins, and cholesterol[13]. Further investigation of LRs from different cells revealed that they contain glycosylphosphatidylinositol (GPI)--anchored proteins (e.g. CD90, CD59, ephrinA5), transmembrane proteins (e.g. EGF, FcεRI), Src-family kinases (e.g. Lck, Fyn, c-Yes), adaptors (e.g. LAT), guanine exchange factors (e.g. Ras), and intracellular Ca^{2+}-dependent lipid-binding proteins (e.g. annexin II, annexin XIIIb)[2, 6, 13, 23, 29, 30]. These findings suggested that LRs may be involved in inter- and intra-cellular signaling. Ikonen and Simons[13] proposed that LRs function as platforms for signal transduction and play a role during the initiation of cell activation.

It is possible, however, that more important role of LRs is in signal maintenance and amplification[12]. Membrane proteins are thought to be constitutively or inducibly associated with LRs, to which they can be recruited or from which they can be excluded, depending on the role in cellular signaling**.** It was suggested that double acylation of Src-family kinases by saturated lipid chains determines the preferential partitioning into rafts[13]. For GPI-anchored proteins, the lipid anchor, with its two (usually) saturated fatty acyl chains, determines raft association[3, 13]. The concentration or separation of different membrane proteins on the plane of the membrane is the fundamental principle by which LRs are thought to exert their function. There are data indicating a crucial role of LRs in organizing early events in T cell receptor (TCR) signaling both prior to and following receptor engagement[4]. The importance of LRs has also been recognized in a number of other cellular processes, including membrane and protein sorting, transporting the newly synthesized products to the cell membrane, clathrin-independent endocytosis, and the pathogeneses of several human diseases[11, 13].

It is thus clear that the studies on LRs have had a strong impact on the field of plasma membrane topology and function and have contributed to a better understanding of signaling cascades in the cell. Recently, however, a number of reports have questioned the validity of the original definition of LRs, stirring up a controversy which raises doubts about the physiological significance of some of the earlier conclusions[17, 19]. The main questions concern the existence of LRs *in vivo*, the methods of isolation and visualization, estimation of their size, their definition, and their association with different proteins.

Considering the existence of LRs in a natural state, it has to be said that they have never been isolated in native form[17]. In early studies, LRs were obtained by ultracentrifugation of detergent-lysed cells in sucrose density gradient. However, this method of isolation itself may cause many perturbations in the cell structure, creating the "phenomenon" of rafts, and the use of detergent may induce artifacts[17] and promote artificial co-localization of some proteins[2]. The most commonly used detergent for LR isolation is Triton X-100. It has, however, been demonstrated that treating cells with Triton X-100 causes the aggregation of detergent-insoluble microdomains, increasing the size of GPI-anchored protein complexes[7]. In addition, it turned out that the amount of CD90 complexes associated with LRs often depends on the use of different detergent and fractionation methods[27]. Recently, a wider spectrum of weaker detergents has been used to extract LRs, e.g. Triton X-114, CHAPS, Lubrol WX, Brij96, and Brij98[1]. On the basis of their differential solubility in different reagents, LRs have been subclassified into distinct types. It was found that a number of proteins that were established to be raft associated were outside rafts when another detergent was used, and that proteins in the same microdomain can display different levels of detergent solubility[1]. Furthermore, not only the choice of detergent matters, but also the temperature is very important. There is a large body of work in which cold detergent TX-100 for preparing LRs was utilized. Drevot et al.[4] proposed a new method for isolating rafts and suggested that chilling and the use of TX-100 may strongly modify the behavior of membranes. Instead, the new procedure with Brij98 for LR extraction at 37°C was used and allowed more accurate analysis of LR structure[4], though some authors argue that none of the detergents can be used to isolate a transient protein-lipid aggregate in its native form[1]. Membrane fractions with similar biochemical characteristics to those of LRs were also isolated by detergent-free methods[30], but it is doubtful whether any existing technology can provide the adequate molecular resolution to isolate LRs precisely from their native enviroment[1].

A lot of information about LRs comes from biophysical studies of artificial lipid layers and observations of LRs after isolation, i.e. from indirect studies. However, the information from those sources is also contradictory. For example, no consensus has been reached with regard to the size of LRs. Studies of an artificial lipid reconstitution system have shown that *in vitro* such structures yield large sheets of over 1 μm^2. Some authors calculated that GPI-anchored receptors should be clustered in domains and be of about 70 nm in diameter[16]. To confirm this, studies of LRs after isolation have demonstrated that they were visible as small vesicles floating on the sucrose gradi-

ent and measured about 70 nm across[4]. However, there have been other data indicating that LRs are much smaller, as little as 25 nm in diameter[14].

One obvious prediction following from the *in vivo* existence of LRs is that proteins associated with them should be clustered. Friedrichson and Kurzchalia[7] used chemical crosslinking to demonstrate that LRs consisting of GPI-anchored proteins exist at the surface of living cells. After chemical crosslinking of cells they observed the formation of oligomers of GPI-anchored proteins, indicating that these molecules are close together on the surface in steady state[7]. With this approach it was estimated that LRs consist of at least 15 protein molecules. Nevertheless it was still impossible to define precisely the number of molecules in a cluster[7]. In addition, the clustering has never been observed using microscopic techniques[11]. Previous use of conventional immunofluorescence techniques also revealed no clusters of molecules responsible for signal transduction on the cell surface after activation[3].

Different techniques have been recently developed to visualize LRs in living cells and to try to estimate their size, e.g. fluorescence resonance energy transfer (FRET)[28], single particle tracking[21, 26], and 2-photon microscopy[8]. Using 2-photon microscopy, Gaus et al.[8] directly visualized the membrane lipid structure of living cells. The existence of a phase separation *in vivo* was demonstrated with the use of an environmentally sensitive fluorescent probe. It showed that membrane filopodia of macrophages, adhesion points, and cell-cell contact regions are particularly enriched in LRs. However, the surface area covered by LRs was difficult to determine, and the number of individual LRs per cell could not be readily counted. The suggested size varied 30–50 nm to several micrometers, but it could not be estimated exactly because of the limitations of 2-photon fluorescence microscopy[8].

It seemed that FRET microscopy, which increases the resolution of immunofluorescence microscopy to the molecular scale [15], could better detect the close proximity of molecules on a scale of 1–10 nm. Using so-called homo-FRET, which monitors the extent of depolarization of fluorescence emission, Varma and Mayor[28] measured the distances between GPI--anchored proteins (folate receptors), determining their non-random organization. It was shown that GPI-anchored proteins are organized into small submicron domains at the cell surface. The size of LRs in living cells was estimated as smaller than 70 nm, containing fewer than 50 molecules of GPI-anchored proteins[28]. At the same time, another paper, analyzing the distribution of GPI-anchored proteins (GPI-anchored protein 5' nucleotidase) in the cell membrane by the hetero-FRET imaging, was published. These studies, based on detecting fluorescence resonance energy transfer between different fluorophores, reported the absence of detectable clustering of GPI-anchored proteins across the apical surface of the cells under steady-state conditions. It was shown that proteins and lipids that share a common property of detergent insolubility are not necessarily associated in intact cell membranes[15]. The question of the size and nature of LRs before detergent extraction remained unanswered.

The failure to observe GPI-anchored protein clustering in LRs was explained by their mobility on the plane of the membrane. To establish the size and dynamics of LRs in living cells, the local diffusion of single membrane proteins was measured by high-resolution single particle tracking. A microsphere was attached to the studied protein and the motion was confined by a laser trap. The sphere's thermal position fluctuation inside the trapping potential was tracked with subnanometer and microsecond resolution. The measurement demonstrated that LRs are cholesterol-stabilized complexes of 26±13 nm in diameter. However, it was shown that proteins associated with LRs are quite stable and diffuse together as one entity within minutes (up to 10 min). This means that the diffusion coefficient measured is dominated by the motion of the entire LR[21].

Recently, a combination of hetero-FRET and homo--FRET microscopy in conjunction with a theoretical modeling of FRET efficiencies has been used. The studies revealed an unexpected organization of GPI--anchored proteins on the cell surface, suggesting that they were present as monomers, organized in extremely high-density, cholesterol-sensitive clusters measuring less than 5 nm across. These clusters were composed of at most four molecules and gathered diverse GPI-anchored protein species[24]. This suggested that the lipid-dependent clusters in the membranes of living cells are unexpectedly small. This might explain why also in recent studies by Glebov and Nichols[10], where the analysis of the T cell lines with the use of hetero-FRET was conducted, no clusters of GPI-anchored proteins were observed. It seems possible that resting T cells contain LRs that elude detection by conventional FRET technologies. However, in activated T cells the clustering of GPI-anchored proteins is already expected. Indeed, after TCR activation the fluorescence of two different GPI-anchored proteins was increased, but it was concluded that this could simply reflect changes in the membrane shape. Thus, in this study no clustering

was ascertained[10]. The data gave convincing evidence that the immunological synapse did not contain a concentration of LRs enriched by GPI-linked proteins, and they weakened conclusions about the role of LRs as signaling platforms[20].

Clearly, although different techniques have been developed to visualize LRs in living cells, consensus on their size and existence has still not been reached. Moreover, there are many contradictory data as to whether certain proteins are associated with or are excluded from LRs. Thus the constitutive presence of CD45 in the raft or non-raft environment is still unclear. Some data suggest that phosphatase CD45, which regulates the activity of Lck kinase, is excluded from LRs in T lymphocytes[9, 14, 18, 22], while another report shows that CD45 is continuously present in the LRs in T cells and only its amount is reduced upon stimulation[5]. It seems that although LRs in certain cells have a distinctive protein and lipid composition, it is hard to define them in terms of the proteins and lipids they contain.

At this point, it is worth mentioning that the definition of LRs has not yet been definitely established. It is not clear whether structures extracted in a way different from that presented in the original definition can be called LRs, and whether proteins found as present in those structures are associated with LRs or result from a number of nonphysiological rearrangements of the bilayer during detergent solubilization[16]. It has been shown that LRs in different cells and different cell regions behave differently[21]. According to these findings it seems that the definition of LRs should be redefined and updated. One criterion for proteins that might exist in LRs is the abrogation of association with LRs after cholesterol depletion[8, 21, 25, 28]. However, cholesterol depletion can cause the alteration of cell morphology, exocytosis and trafficking, and disruption of the actin cytoskeleton, and could thus have a number of pleiotropic effects[17], including the dissociation of various proteins from LRs. There are also data suggesting that cholesterol extraction by commonly used methyl β-cyclodextrin increases the fluidity and disrupted lipid raft organization[19].

In summary, it is certain that the cell membrane is not a homogenous mixture of lipids and proteins, and this might indicate the presence of LRs in the plasma membrane. However, the large amount of data claiming that the phenomenon of LRs is created by extraction procedures raises controversy. Still, there has been no conclusion considering the size of LRs, their definition, and the proteins found either to be associated with rafts or excluded from them. According to one hypothesis of membrane structure, proposed by Viola and Pizzo[29], raft borders are not well defined, so that regions where the concentration of glycosphingolipid is very high may be surrounded by regions where the rafts gradually merge with the bulk membrane phospholipids. Thus, after detergent extraction some proteins may not be present in rafts[29]. This LR model may explain the incoherence of results obtained by extraction methods. However, the data gained using FRET imaging of living cells is at present also inconsistent. It seems that further investigations using more refined methods, such as FRET and single particle tracking, will be needed to resolve the controversy surrounding LRs.

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