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Review

Membrane microdomains in immunoreceptor signaling

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ABSTRACT

Membrane microdomains denoted commonly as lipid rafts (or membrane rafts) have been implicated in T-cell receptor (TCR), and more generally immunoreceptor, signaling for over 25 years. However, this area of research has been complicated by doubts about the real nature (and even existence) of these membrane entities, especially because of methodological problems connected with possible detergent artefacts. Recent progress in biophysical approaches and functional studies of raft resident proteins apparently clarified many controversial aspects in this area. At present, the prevailing view is that these membrane microdomains are indeed involved in many aspects of cell biology, including immunoreceptor signaling. Moreover, several other types of raft-like microdomains (perhaps better termed nanodomains) have been described, which apparently also play important biological roles.

1. Introduction

The concept of membrane microdomains has been formulated already in 1982 [1] and reflected the experimental evidence for membrane lateral heterogeneity observed by various biophysical techniques in model membrane systems as well as in native cell membranes. It was suggested that membrane lipids can undergo phase separations, interact more or less selectively with membrane proteins and with submembrane cytoskeletal elements. Later, a strong biochemical indication of heterogeneity of biological membranes was based on selective resistance of certain membrane proteins to solubilization by some detergents, e.g. Triton X100, Brij-series, NP-40 or CHAPS.

The detergent-resistant membrane microdomains (DRMs) started to be called lipid rafts [2] and for some time these terms were considered as more or less synonymous. These entities became especially interesting for immunologists when it was found that they contain several important signaling molecules involved in immunoreceptor signaling [3]. For years, lipid rafts (more correctly membrane rafts, as they are not composed solely of lipids) of immunocytes and other cell types were defined (mostly based on the results of biochemical experiments involving detergent-resistance) as membrane microdomains enriched in glycosphingolipids and glycerolipids containing mainly saturated fatty acid residues, cholesterol and lipid-modified proteins, including especially the glycosylphosphatidylinositol (GPI)-anchored ones.

Studies on artificial membrane systems indicated that these membrane microdomains are held together mainly by hydrophobic interactions between saturated fatty acid residues of their main lipid constituents and further stabilized by cholesterol molecules, which are in biological membranes intercalated between bulky glycolipids [4]. This particular lipid mixture may form a specific "ordered liquid phase", the physical properties of which are different from the rest of the plasma membrane. Treatments of membranes with cholesterol-depleting agents [5], cholesterol-modifying enzymes or biosynthetic replacement of saturated fatty acid residues in their sphingolipids by unsaturated ones [6] were found to destabilize the rafts so they lost their detergent resistance.

Later it became obvious that the use of detergents may produce more or less significant artefacts – the composition and properties of the DRMs were clearly dependent on the chemical nature and concentration of the detergent, temperature and duration of the solubilization (see below). Thus, DRMs generally should not be equated with native rafts; some authors even doubted about the very existence of raft microdomains in native membranes. Many studies aimed to demonstrate the existence and properties of the raft microdomains in more or less native biological membranes. An obvious approach has been based on the use of microscopic methods. These are however of limited use because the size of these microdomains appears to be in most cases under the resolution limit of conventional optical microscopy. Nevertheless, the use of lipid and protein probes preferentially incorporating into membrane areas enriched in certain types of lipids confirmed the lateral heterogeneity of not only artificial, but also native biological membranes [7–9].

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A specific type of raft-like microdomains are caveoli, morphologically distinct plasma membrane invaginations stabilized by cholesterol-binding protein caveolin and therefore readily observable by electron microscopy [10].

A major advance in the studies of raft microdomains in relatively native cell membranes was the introduction of the techniques producing plasma membrane vesicles. In this system, phase separation of distinct lipid-based membrane domains can be observed and conditions affecting this process and its biological implications can be studied under relatively natural conditions. The microscopically observable membrane domains observed in these studies apparently arise due to spontaneous coalescence of dynamic "elementary membrane rafts" present under physiological conditions [11]. Importantly, in agreement with previous biochemical, detergent-based experiments, different membrane proteins are selectively segregated into such relatively native raft membrane domains, often based on their palmitoylation status.

Another powerful approach to study the role of raft microdomains in physiological functions of proteins residing there is the use of mutants targeted outside of rafts. This has been achieved mostly by modifications altering the palmitoylation status of such proteins (see below). A very telling example of importance of targeting of biologically active molecules into raft microdomains was demonstrated by Simons and colleagues [12]; raft-targeted inhibitors of a raft-associated enzyme were markedly more active than those targeted outside rafts.

The currently widely accepted idea about membrane rafts is that they are tiny, very dynamic "islets" (only tens of nanometers in size), containing a few hundreds of lipid molecules and mostly single protein molecules, spontaneously formed as a result of phase transitions in complex mixtures of membrane lipids and proteins, that cannot be easily observed on intact cell surface by existing microscopic techniques. These "elementary rafts" can be often stabilized to form larger, readily observable membrane domains following various physical or chemical perturbations affecting the protein-protein or protein-lipid interactions [13]. The properties of raft microdomains in native cell membranes may be strongly influenced by interactions with submembrane skeleton and cytoskeleton. One so far unresolved problem is to what extent there is a coupling of the raft microdomains of both membrane leaflets (i.e. the external and cytoplasmic leaflet in the case of plasma membrane). It can be speculated that such coupling may be mediated by specific "raftophilic" transmembrane proteins, such as the palmitoylated transmembrane adaptor proteins mentioned below.

2. Involvement of lipid rafts in immunoreceptor signaling

The existence of a possible relationship between T cell activation and membrane rafts became apparent when it was realized that T cells can be activated by antibody-mediated cross-linking of surface glycoproteins such as Thy-1 or Ly-6. This was somewhat mysterious, as these molecules apparently cannot directly communicate with cytoplasmic signaling molecules, because they are entirely extracellularly oriented and anchored in the external plasma membrane leaflet by means of a covalently attached glycolipid moiety, glycosylphosphatidylinositol (GPI). Cross-linking of numerous GPI-anchored proteins (and also glycolipids) results in cellular responses strikingly similar to those elicited by immunoreceptors, such as T-cell receptor (TCR), B-cell receptor (BCR) or some Fc-receptors (FcR). These observations could be rationalized by the fact that GPI-anchored proteins are components of membrane rafts, which contain several key signaling components involved also in immunoreceptor signaling (Src-family kinases (SFKs), transmembrane adaptor proteins, phosphatidylinositol bis-phosphate (PIP2), G-proteins). Importantly, T-cell activation via cross-linking of GPI-anchored proteins was found to be dependent on expression of TCR ζ chain. Therefore a plausible model was that cross-linking of GPI-anchored proteins (or raft glycolipids) results in partial co-cross-linking of TCR and possible mimicking of early steps in physiological TCR activation (reviewed in [14]). However, a question remained whether this is just an experimental artefact, or if this is an "informative artefact" and membrane rafts are actually involved in physiological activation of signaling cascades initiated by immunoreceptors. Indeed, biochemical studies in several types of immunocytes revealed that experimental cross-linking of the respective immunoreceptors (TCR, BCR, FcR) is accompanied by association of the receptors with DRMs, i.e. presumably membrane rafts. Thus, also cross-linking by their natural ligands may induce their functionally relevant merging with membrane rafts. As a result, the tyrosine based-activation motifs (ITAM) in cytoplasmic tails of immunoreceptor complexes (CD3, ζ-chain, Ig-α,β FcR γ-chain) become exposed to SFKs present in the rafts. Phosphorylated ITAMs of these signaling chains then serve as docking sites for Syk family kinases (ZAP70 or Syk). Activated ZAP70 in T cells phosphorylates another membrane raft component – the transmembrane adaptor protein LAT ("linker for activation of T cells"), resulting in its association with several other cytoplasmic signaling proteins, including phospholipase Cγ1 (PL Cγ1). This promotes further steps in the TCR-induced signaling cascades. Importantly, also TCR co-receptors, CD4 and CD8, are palmitoylated proteins associated with membrane rafts. Therefore, their association with TCR after contact of the T cell with antigen presenting cells (APC) may contribute to co-aggregation of the receptor complex with membrane rafts. Alternatively, TCR (and other immunoreceptors) may be pre-associated with membrane rafts [15] and its ligation just reorganizes somehow this assembly to allow for optimal exposure of the CD3 and ζ chains to the SFKs.

The importance of membrane rafts immunoreceptor signaling is supported by findings that palmitoylation-deficient mutants of several of the raft resident proteins such as SFKs, CD8β, pre-TCR or LAT [16–20] are excluded from the rafts which results in functional defects. A fraction of a negative regulator of Src-family kinases activity, the protein tyrosine kinase (PTK) Csk, is also found in membrane rafts, due to its association with the phosphorylated transmembrane adaptor protein PAG ("phosphoprotein associated with GEMs") also called Cbp ("Csk binding protein") [21,22], a palmitoylated membrane raft resident molecule. Cross-linking of TCR on resting $\alpha\beta$T cells causes rapidly a transient dephosphorylation of PAG accompanied by Csk dissociation. This in turn contributes to increased SFK (Lck, Fyn) activity needed for TCR signaling, because the negative regulator of these SFKs is now removed from their vicinity. On the other hand, protein kinase A type I, which also associates with membrane rafts of activated T cells, activates by phosphorylation the raft-associated Csk and thereby contributes to inhibition of SFKs [23]. Another raft-associated transmembrane adaptor, LIME ("Lck interacting molecule"), becomes tyrosine phosphorylated and Csk-associated after cross-linking of the CD4 or CD8 co-receptors [24]; however, the biological importance of this effect is not clear because the LIME gene knock-out apparently does not have any defects in TCR signaling.

The major costimulatory receptor of T cells, CD28, is present in the non-raft part of the resting T cell membrane, but after activation-induced cross-linking it may relocate to rafts [25]. The major negative regulator of T cell activation and CD28 competitor, CTLA-4 (CD152), was reported to be constitutively associated with membrane rafts of activated T cells and may interfere with relocation rafts activated T cell plasma membrane [26].

Among other important raft-associated signaling molecule of activated T cell are the scaffolding protein CARMA1 and protein kinase Cθ. The former molecule is the critical regulator of TCR-induced NF-κB activation [27], while the latter cytoplasmic

enzyme associates with Lck and translocates to membrane rafts associated with immunological synapse [28].

In addition to the signaling molecules involved in productive immunoreceptor activation, apoptotic signaling is also initiated from receptor complexes localized to membrane rafts. Stimulation of activated human $CD4^+$ T cells may result in translocation of the major apoptotic receptor Fas (CD95) into a sort of membrane raft microdomains, which apparently sensitizes these cells sensitive to apoptosis after ligation to Fas ligand (FasL) [29]. Somewhat paradoxically, disruption of membrane rafts by cholesterol depletion results in spontaneous activation of Fas and apoptosis [30], indicating a diversity of apoptosis-inducing mechanisms.

Yet another membrane raft associated transmembrane adaptor protein is NTAL ("non-T cell activation linker"), also called LAB ("linker for activation of B cells") [31,32]. This protein is involved in regulation of immunoreceptor signaling in mast cells, macrophages, B-lymphocytes, but also activated T-cells [33,34]. Recent results indicate its importance (together with LAT) in fine-tuning the mastocyte response to ligands of different affinity for high-affinity IgE receptor [35].

3. Is LAT association with membrane rafts really important? a novel type of membrane rafts

Early studies demonstrated that palmitoylation-deficient LAT was unable to support TCR signaling. However, a later study described a mutant LAT construct, which did not contain any palmitoylation motif, was not associated with buoyant DRMs but was fully functional in TCR signaling [36]. This chimeric construct was composed of LAT cytoplasmic domain and extracellular and transmembrane domains of another transmembrane adaptor protein, LAX ("linker for activation of X"; not present in conventionally defined membrane rafts). Moreover, it turned out that the previously studied palmitoylation-deficient cysteine mutant of LAT is actually not effectively incorporated into plasma membrane and remains stuck in endoplasmic reticulum [37]. Thus, the conclusion was that membrane rafts may not be, after all, important in the TCR signaling process. However, in our subsequent study we demonstrated that the results of Zhang and colleagues can be explained by existence of a novel type of membrane microdomains (to be called "type 2 rafts" here) producing upon detergent solubilization "heavy DRMs" [38]. These were similar in some respects to "classical" DRMs – resistant to solubilization by polyoxyethylene type detergents, such as Brij-98, large, as determined by gel chromatography, sensitive to laurylmaltoside and to cholesterol extraction, but in contrast to conventional raft-derived DRMs they did not flotate in density gradients (apparently due to a higher ratio of proteins vs. lipids). Their integrity is also more dependent on protein–protein interactions, as evidenced by their sensitivity to the treatment with the chaotropic agent 0.6 M potassium iodide. These "heavy DRMs" (and thereby the presumed original membrane microdomains) contained a number of protein molecules, including the adaptor proteins LAX and TRIM, H-Ras, CD45, CD28, CD5, HLA class I, or CD71. As expected, the transmembrane domain of the adaptor protein LAX was found to be critical for targeting the LAX–LAT chimeric construct into the type 2 rafts [39].

Therefore, our conclusion was that at moderate level of expression, LAT targeted to the standard rafts supported more efficiently TCR signaling than constructs targeted to the newly described type 2 rafts or to non-raft membrane; the difference can be however compensated by increased level of expression in the suboptimal types of membrane microdomains. Thus, the lipid environment corresponding to classical rafts may be optimal for signaling functions of LAT (and perhaps also some other molecules). Nevertheless, the type 2 rafts (detected as heavy DRMs) are in principle also able to communicate with TCR complex and participate at the signaling processes, although less efficiently. Obviously, the lipid environment of the type 2 rafts may be favorable for other membrane molecules with respect to their signaling or other functions.

Among the most important molecules involved in initiation of immunoreceptor signaling are SFKs (Lck and Fyn in the case of T cells). As stated above, their access to the signaling chains of the immunoreceptors may be regulated by membrane compartmentalization. It is not quite clear what is the relative importance of non-raft and raft-associated SFKs in this respect. To answer this question we made use of the fact that the SFK activity is negatively regulated by cytoplasmic tyrosine kinase Csk which phosphorylates critical tyrosine residues near C-termini of SFK molecules. We made a set of constructs composed of constitutively active Csk mutants targeted to various types of T cell membrane microdomains. Interestingly, only Csk targeted through different N-terminal motifs into standard rafts (the sites of SKKs residence) but not to type 2 rafts or non-raft membrane effectively inhibited TCR signaling, demonstrating the critical role of membrane raft-associated SFKs in this process [40]. These results are complementary to those obtained by a previous study based on Lck mutant transfectants [41].

An interesting model of a functional interplay between classical rafts and type 2 rafts has been recently proposed to explain the collaboration of two SFKs, Fyn and Lck, in the early phases of TCR signaling [42,43].

4. Tetraspanin microdomains

Tetraspanins are small (25–50 kDa) proteins with four transmembrane segments and two extracellular loops. Apparently all cell types possess several tetraspanin family members, usually expressed at high level. Tetraspanins typically oligomerize and associate with other membrane proteins, including integrins, growth factor receptors and cytoplasmic signaling proteins [44], resulting in "tetraspanin microdomains" (TEM) [13,45]. In addition to proteins, TEMs contain also lipid components including cholesterol and gangliosides, thus resembling partially those characteristic for membrane rafts. Similar to the membrane raft resident proteins, tetraspanins are multiply palmitoylated; this modification seems to be crucial for their oligomerization [46] and TEM assembly [47]. TEMs are also partially detergent-resistant and produce a specific type of DRMs the composition of which is dependent on the detergent used for solubilization of the membrane. Microscopic observations revealed that plasma membrane contains hundreds of spatially separated TEMs (of average size 200 nm), each composed of two or more different tetraspanins [48]. Some properties of TEMs indicate that they may be related to the above mentioned type 2 rafts, in which also protein–protein interactions seem to play important roles in maintaining their integrity. Just as classical rafts, several palmitoylated transmembrane adaptor proteins are present in tetraspanin microdomains, such as LST1/A [49] and SCIMP [50]. The former one may be involved in signaling through a so far unidentified myeloid cell receptor, while the latter one probably plays a role in signaling through MHC class II glycoproteins or other receptors of antigen presenting cells.

5. Membrane microdomain heterogeneity

At the moment it is not clear how heterogeneous different DRMs (and the corresponding native microdomains) are – i.e. whether one raft type is e.g. rich in LAT only, another in Lck, etc., or whether various "mixed" DRMs (and microdomains) exist, containing various combinations of these molecules. There are indications that the former model of simple elementary rafts containing

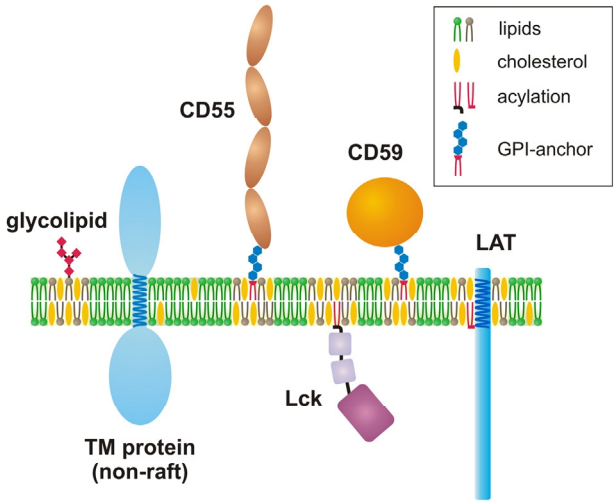

Fig. 1. Schematic representation of membrane raft microdomains in plasma membrane. The "elementary rafts" are relatively small dynamic assemblies of relatively specific lipids (predominantly saturated fatty acid chains of sphingolipids and glycerolipids and cholesterol) containing in most cases just single protein molecules, such as GPI-anchored proteins (here CD55 and CD59), cytoplasmic double acylated proteins (Lck) or some palmitoylated proteins (LAT). Some, or perhaps most of these raft micro/nano-domains contain specific glycolipids. Most of transmembrane proteins are present in the non-raft part of the membrane glycolipids.

mostly only single protein molecules is more correct (Fig. 1); for example, two clearly distinct types of rafts were demonstrated in motile T cells, one of them originating from the leading edge and containing GM1 as the major glycolipid the and the other, containing GM3 from the uropod [51]. The membranes of activated T cells contain separate microdomains differentially sensitive to cholesterol depletion, enriched in Lck or LAT, respectively [52]. However, so far little has been done in terms of development of effective separation methods to tackle this problem. In contrast to membrane rafts, tetraspanin microdomains probably do contain several species of membrane proteins (tetraspanins and others).

6. The detergent problem

The results based on DRMs are of course potentially problematic because it is not clear to what extent the composition and properties of native rafts in the unperturbed membrane correspond to the DRMs derived from them by detergent extraction. Some components present in the lipid rafts in vivo may be lost by detergent extraction, while the exposure of the membrane to a detergent, especially at low temperature, may induce artificial associations and even lipid phase transitions. The composition of the isolated DRMs is dependent on the nature of the detergent used and in most cases also on its concentration, temperature and time of exposure. Some "milder" detergents such as Brij-98, Brij-58 or Lubrol produce higher yields of buoyant SFKs. The DRMs prepared by means of these detergents are also much more temperature stable and much less affected by increased detergent concentrations. More stringent 1% detergents such as Triton X-100 or NP40 typically produce even at low temperature much less buoyant fractions containing e.g., only less than 50% of the typical raft molecules (GPI-proteins, transmembrane adaptors, SFKs, CD4). Moreover, increased temperature and detergent concentrations, as well as prolonged exposure to these detergents, result in gradual dissolution of the DRMs. The simplest explanation is that the very mild detergents preserve well the native structures (Fig. 2); it is conceivable that the native raft microdomains may consist of a core resistant even to the more stringent detergents and a more detergent-sensitive periphery. Various other interpretations are possible as excellently discussed by Pike [53,54]. It should be emphasized that some other "mild" detergents, especially those of the alkyl-glycosidic type (octylglucoside, laurylmaltoside) preserve apparently very well the multisubunit complexes of membrane-embedded receptors, but effectively dissolve membrane rafts and tetraspanin microdomains.

Because of these potential detergent problems, the very existence of lipid rafts in vivo has been repeatedly put in doubt. However, results derived from microscopic approaches, as well as the above mentioned striking physiological effects of targeting membrane molecules to raft vs. non-raft microdomains are in basic agreement with the biochemical results based on detergent solubilization. Also, it should be remembered that the results of the experiments with artificial membrane systems are in a very good agreement with the idea of membrane raft microdomains Another strong argument in support of the raft concept is the above mentioned technique employing membrane vesicles derived from natural plasma membrane [11], in which spontaneous formation of well observable raft-like areas is demonstrated. Furthermore, it should be noted that exactly the same detergent-based methods are routinely used in the studies on membrane receptor complexes. If we dismiss the detergent-based studies on membrane microdomains, we would have to do the same in the case of the membrane receptors – this would be certainly rather absurd. However, there is no doubt that the detergent-based biochemical methods must be properly complemented by other independent methods.

A further relevant point for discussions on the nature of membrane rafts in vivo is what percentage of the cell surface is actually covered by the raft micro- (or rather nano-) domains. While most of the apical surface of polarized epithelial cells appears to be

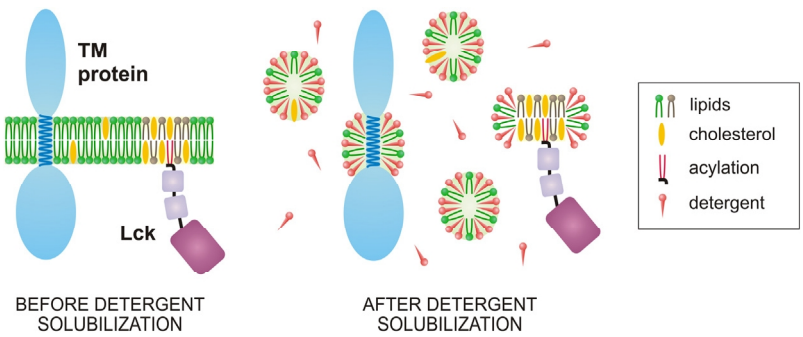

Fig. 2. Detergent resistance of membrane rafts. Plasma membrane upon exposure to detergents such as Brij-98 is mostly fully solubilized (formation of mixed lipid-detergent micelles, solubilized transmembrane proteins) while raft microdomains remain more or less intact as a specific form of micelles. The detergent resistance is mainly due to specific nature of the raft lipids (combination of predominantly saturated fatty acid chains of sphingolipids and glycerolipids and cholesterol).

composed of the rafts, in other cell types, such as leukocytes, the estimates vary; the present consensus values are probably around 50%. The leukocyte cell surface may be actually a complex and dynamic mosaic of various types of microdomains (heterogeneous classical rafts, type 2 rafts, tetraspanin microdomains, probably other so far undiscovered types). It is reasonable to expect that combination of advanced microscopic, biophysical and biochemical approaches will elucidate these important issues of membrane biology.

7. Concluding remarks

This brief review concentrated only on a very small part of the raft-related topics. Although for many years this area has been controversial and many important relevant issues remain partially unresolved, the concept of membrane rafts (and generally membrane microdomains) has been certainly fruitful in many areas of cell biology, including molecular immunology. It has brought plausible, yet debatable explanations of central aspects of receptor signaling. Rational targeting of membrane rafts or similar types of microdomains and manipulation of their interactions with signaling components of membrane receptors may soon become promising even for therapeutic purposes. Furthermore, it may be important to develop techniques suitable for studies of other, so far poorly known types of membrane microdomains which may play important physiological roles.

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