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# Signaling through sphingolipid microdomains of the plasma membrane: The concept of signaling platform

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**Transmembrane signaling requires modular interactions between signaling proteins, phosphorylation or dephosphorylation of the interacting protein partners [1] and temporary elaboration of supramolecular structures [2], to convey the molecular information from the cell surface to the nucleus. Such signaling complexes at the plasma membrane are instrumental in translating the extracellular cues into intracellular signals for gene activation. In the most straightforward case, ligand binding promotes homodimerization of the transmembrane receptor which facilitates modular interactions between the receptor's cytoplasmic domains and intracellular signaling and adaptor proteins [3]. For example, most growth factor receptors contain a cytoplasmic protein tyrosine kinase (PTK) domain and ligand-mediated receptor dimerization leads to cross phosphorylation of tyrosines in the receptor's cytoplasmic domains, an event that initiates the signaling cascade [4]. In other signaling pathways where the receptors have no intrinsic kinase activity, intracellular non-receptor PTKs (i.e. Src family PTKs, JAKs) are recruited to the cytoplasmic domain of the engaged receptor. Execution of these initial phosphorylations and their translation into efficient cellular stimulation requires concomitant activation of diverse signaling pathways. Availability of stable, preassembled matrices at the plasma membrane would facilitate scaffolding of a large array of receptors, coreceptors, tyrosine kinases and other signaling and adapter proteins, as it is the case in signaling via the T cell antigen receptor [5]. The concept of the *signaling platform* [6] has gained usage to characterize the membrane structure where many different membrane-bound components need to be assembled in a coordinated manner to carry out signaling. The structural basis of the signaling platform lies in preferential assembly of certain classes of lipids into distinct physical and functional compartments within the plasma membrane [7,8]. These membrane microdomains or rafts (Figure 1) serve as privileged sites where receptors and proximal signaling molecules optimally interact [9]. In this review, we shall discuss first how signaling platforms are assembled and how receptors and their signaling machinery could be functionally linked in such structures. The second part of our review will deal with selected examples of raft-based signaling pathways in T lymphocytes and NK cells to illustrate the ways in which rafts may facilitate signaling.**

***Keywords:*** **rafts, transmembrane signaling, sphingolipids, protein tyrosine kinase**

## Rafts are distinct plasma membrane compartments

The fluid mosaic model of Singer and Nicolson [10] has been actively revised in recent years, especially to accommodate the heterogeneity in lateral diffusion of membrane proteins, which are by no means freely diffusing in the 'sea of lipids' of the plasma membrane [11]. Application of novel biophysical methods such as single-particle tracking to address the question of protein diffusion in membranes [12], has led to the view that stable assemblies of outer leaflet sphingolipids constitute confinement zones wherein glycosylphosphatidylinositol (GPI)-anchored proteins in particular, are likely to be retained [13]. The physical nature of such confinement zones was hinted at by studies showing that GPI-anchored proteins are resistant to solubilization in cold non-ionic detergents and are usually recovered as large molecular complexes by molecular sizing columns [14,15]. Brown and Rose observed that these complexes are enriched in sphingolipids and cholesterol, have low buoyant density and can be separated from the bulk of the solubilized membrane lipids and proteins by equilibrium gradient centrifugation [16]. These findings led to the concept that GPI-anchored proteins and sphingolipids are organized in specialized membrane microdomains or rafts.

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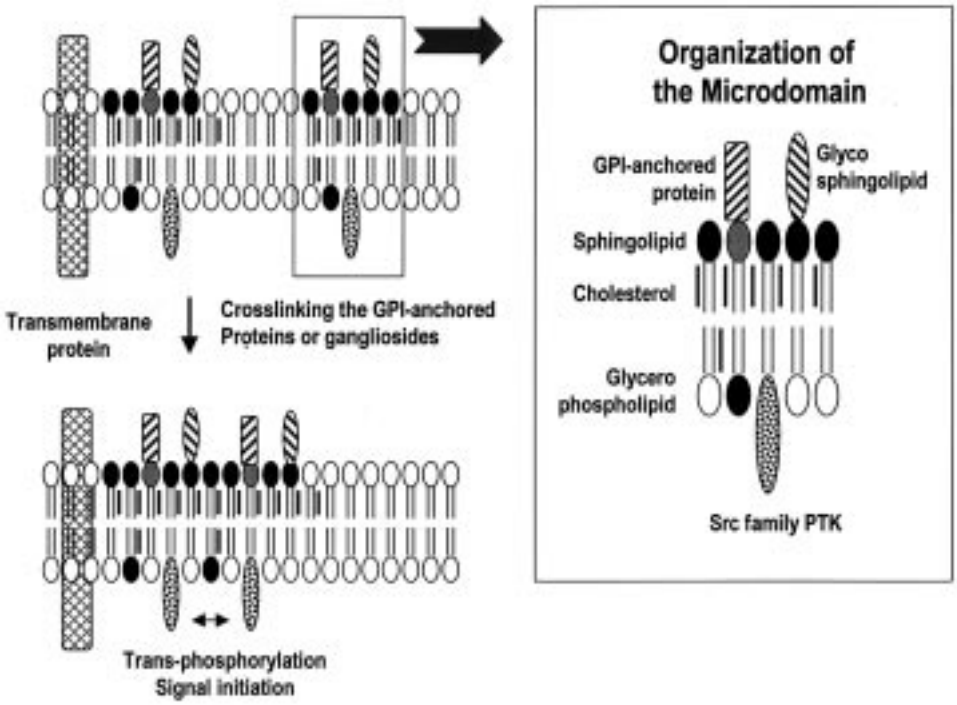


**Figure 1.** Schematic representation of the organization of the microdomains. Sphingolipids and cholesterol segregate laterally within the plane of the plasma membrane to form microdomains or rafts. Transmembrane proteins are normally excluded from the microdomains. GPI-anchored proteins and glycosphingolipids preferentially associate with the microdomains on the exoplasmic leaflet. Doubly acylated Src family PTKs are associated on the cytoplasmic leaflet of the microdomains. Cell surface crosslinking of the GPI-anchored proteins or gangliosides leads to coalescence of the microdomains bringing the Src family PTKs on the cytoplasmic side in close proximity to each other facilitating transphosphorylation and signal initiation.

Measurements on the proximity of the GPI-anchored molecules on intact cells by fluorescence resonance energy transfer [17] and chemical crosslinking [18] estimated that GPI-anchored proteins are confined to relatively small membrane microdomains of 50–70 nm, each containing about 15 GPI-anchored molecules.

It has been argued for some time that the isolated detergent-resistant membranes could be artefactual complexes of membrane lipids and GPI-anchored proteins induced by detergents. Much towards establishing that detergent-resistant membranes are genuine membrane subdomains has been achieved by showing that GPI-anchored proteins do not transfer at random from one type of vesicle to another [15] and most decisively, by reconstituting detergent-resistant complexes with sphingolipids and cholesterol [19]. A rigorous in vivo definition seems to be emerging rapidly from biophysical measurements of the confinement zones of GPI-anchored proteins [20] and saturated acyl chain lipids [21], as well as the proximity analysis of GPI-anchored molecules [17,18] at single cell level [17,21].

Lipid reconstitution experiments suggest that the plasma membrane microdomains or rafts arise from the phase behavior of the plasma membrane lipids [22]. Interactions among the long, saturated acyl chains of sphingolipids promotes their clustering into a liquid ordered phase (lo) in the presence of cholesterol, clusters that coexist in the same membrane with the glycerophospholipids in liquid crystalline (lc) phase [19]. The saturated acyl chains of glycosyl-phosphatidyl-inositol (GPI)-anchored proteins are stable within clusters of liquid-ordered lipids [23,24] and GPI-linked surface glycoproteins can be considered to be reliable markers for rafts. Saturated acyl chains of other, non ceramide-based lipids may actually substitute for glycosphingolipids to form microdomains, as rafts also occur in mutant cells devoid of gangliosides [25]. The presence of cholesterol is necessary to stabilize saturated acyl chain lipids in a liquid-ordered state [7,26]. Although the need for cholesterol in promoting lateral assembly of gangliosides has been questioned [27], removal of cholesterol from plasma membranes profoundly perturbs the physical state of rafts and microdomains [28] and compromises their function [29].

Caveolae, the plasma membrane pits of 50–70 nm, also constitute detergent-resistant microdomains wherein membrane lipids may be organized by the cholesterol-binding protein caveolin [30]. Rafts and caveolae may overlap in some situations, but several studies strongly support the notion that rafts and caveolae are distinct entities. For the sake of concision, we shall concentrate on rafts in this review and refer the interested reader to specialized reviews on caveolae for further information [30,31].

## Signaling via GPI-anchored proteins, the Src family PTKs and the inner leaflet of the microdomains

Despite being restricted to the outer leaflet of the plasma membrane most GPI-anchored proteins transduce cellular activation signals [32]. A detailed listing of the signaling functions of GPI-anchored proteins can be found elsewhere [33].

A few GPI-anchored proteins such as glial cell derived neurotropic factor receptor (GDNFR) and ciliary neurotropic factor receptor (CNTFR) owe their signaling function to direct interactions with transmembrane proteins containing intrinsic PTK activity (GDNFR-Ret) or associated with non-receptor protein tyrosine kinases (CNTFR-gp130) [34–36]. The signaling mechanism of most other GPI-anchored proteins remained a puzzle until it was shown that Src family PTKs coisolate with GPI-anchored receptors in different cell types [37], suggesting a functional link between GPI-anchored proteins and Src kinases. However, the localization of Src kinases to the inner leaflet of the plasma membrane still required a transmembrane connecting mechanism to couple the GPI-anchored protein with the Src kinases [38]. This long-sought transmembrane link has turned out to be contained in the raft structure itself, and how the functional linkage actually operates is beginning to be appreciated.

Most Src kinases are modified by co-translational addition of myristate to Gly2 and by post-translational addition of one or more palmitates to Cys residues at the amino terminal end [39], a modification that makes their association with rafts possible. Prevention of fatty acyl modifications by mutation of the Cys and/or Gly residues abolished the coprecipitation of Src kinases with GPI-anchored proteins and compromised their signaling functions [40, 41]. The importance of raft targeting of the T cell specific Lck and Fyn kinases is illustrated later in more detail.

Crosslinking the GPI-anchored proteins or gangliosides on the surface has been shown to result in the aggregation of the Src kinases and accumulation of Tyr phosphorylated protein substrates on the cytoplasmic face of the aggregated rafts [42, 43]. This suggests that the two leaflets corresponding to the microdomains communicate with each other, but how exactly this occurs remains unclear. Organization of the raft structure is initiated at the outer leaflet of the membrane bilayer, but the long and saturated acyl chains of raft sphingolipids are thought to interdigitate with inner leaflet lipids, and secondarily organize the corresponding inner leaflet [44]. However, the composition and physical nature of raft inner leaflets has remained difficult to investigate. In particular, it is not known whether the inner raft leaflet contains glycerophospholipids in a physical state reflecting the liquid-ordered state of the outer leaflet. Disruption of the microdomain association of Lck by polyunsaturated fatty acids (PUFA) was interpreted to reflect the composition of the microdomain inner leaflet as a structure containing predominantly saturated lipids [45], but recent findings show that PUFA acts similar to 2-bromopalmitate (a palmitate analog) to suppress palmitoylation of Lck and Fyn thereby preventing their targeting to the microdomains [46].

## Microdomains in T cell antigen receptor signaling

Both helper and cytotoxic T lymphocytes are stimulated via the $\alpha\beta$ heterodimeric T cell receptor for antigen (TCR), which forms a multiprotein transmembrane receptor complex with the CD3 $\gamma, \delta, \varepsilon, \zeta$ and $\eta$ chains (Figure 2). The TCR $\alpha\beta$ heterodimers recognize the antigen in conjunction with the cognate MHC molecules, and the CD3 proteins transduce the signals across the plasma membrane. The biochemical pathways of T cell activation have been extensively reviewed [47]. The CD3 proteins owe their signaling capacity to the presence of specific signaling sequences called 'immunoreceptor activation motifs' (ITAMs) in their cytoplasmic portions. Neither the TCR nor the CD3 chains have any kinase activity, but they form close contacts with the membrane associated Src family PTKs Lck and Fyn in a number of ways. Lck is bound to both CD4 and CD8 co-receptors and is brought in close proximity to the TCR engaged with the peptide-MHC complex presented on the surface of an antigen-presenting cell. The initial signaling events that follow TCR engagement include activation of the cytoplasmic PTKs of the Src family and phosphorylation of Tyr residues of ITAMs. Subsequent recruitment to the phosphorylated ITAMs and activation of the Syk family PTK ZAP70 is followed by Tyr phosphorylation of a number of intracellular signaling molecules such as LAT, PLC$\gamma$ and Vav. Ensuing mobilization of the intracellular calcium and activation of the mitogen activated protein kinase (MAPK) cascade lead to nuclear translocation of transcription factors, cell proliferation and effector functions.

The most recent advance in T cell signaling has been the realization that plasma membrane microdomains represent structural and functional organizers of the 'immune synapse' (see [5] for review). A potential role for the microdomains in T cell signaling was inferred from observations that crosslinking of GPI-anchored molecules which reside in microdomains of the T-cell surface can modulate TCR signaling [48–54]. For several signaling molecules participating in T-cell activation, association with rafts was shown to be necessary for effective signaling in well-defined situations: (a) an engineered form of Lck, which is targeted to the plasma membrane but not to the microdomains, failed to phosphorylate the CD3$\zeta$ chain upon activation [55], (b) the phosphorylated CD3$\zeta$ was found exclusively in the plasma membrane microdomains [56] and (c) phosphorylation of CD3$\zeta$ only occurred when Fyn was properly targeted to the microdomains [57]. Further support for raft involvement was provided by disruption of the microdomain architecture with methyl-$\beta$-cyclodextrin (a specific cholesterol-binding agent) that abolished Tyr phosphorylation of PLC$\gamma$ and the intracellular calcium flux during T cell stimulation [58].

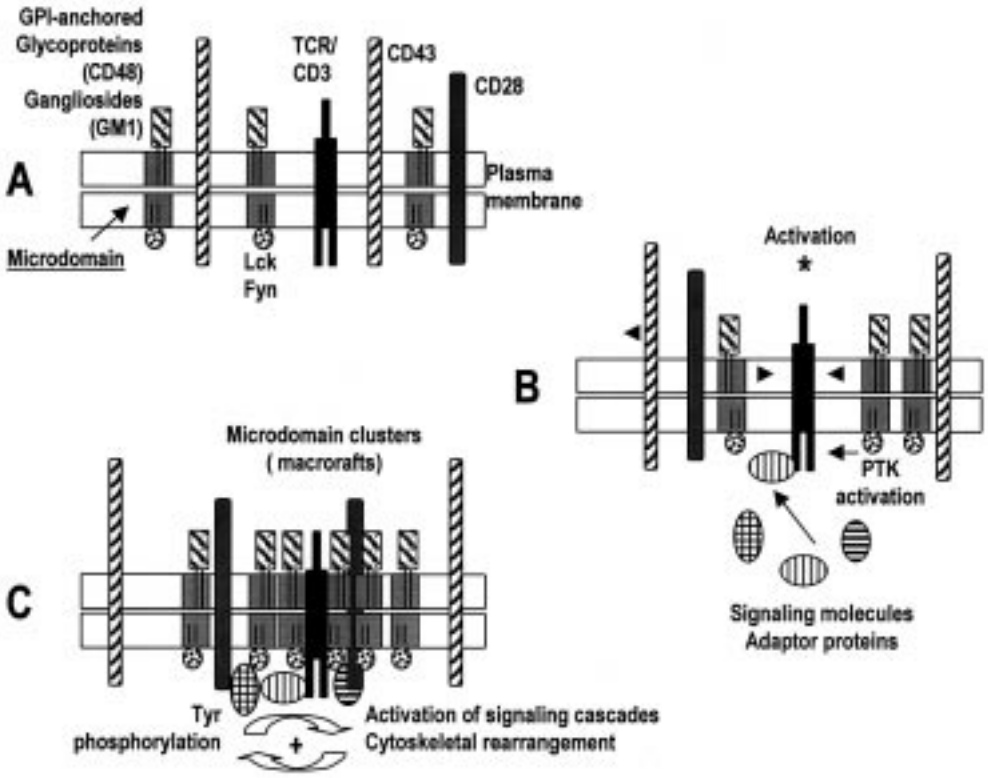


**Figure 2.** Current view of the TCR signal integration in T lymphocyte microdomains. (A) In the resting T cells, microdomains are dispersed within the plasma membrane. The microdomain-associated GPI-anchored proteins and gangliosides are restricted in their movement within the microdomains. The CD3/TCR complex, the costimulatory molecule CD28 and the large surface glycoproteins such as CD43, CD44 and CD45 are evenly distributed on the cell surface. The TCR is not associated with microdomains in the resting T cell. (B) Upon activation, the microdomain associated Lck and Fyn kinases phosphorylate the ITAM motifs of the TCR/CD3 complex. This is accompanied by movement of the microdomains towards the TCR (arrowheads pointing to the TCR). During the interaction between the T cell and the antigen presenting cell, the large CD43 molecules move away from the TCR (arrowhead). CD28 is presumed to be interacting with the microdomain constituents. Modular protein interactions recruit cytosolic signaling molecules and adaptor proteins to build a signaling scaffold. (C) Initiation of the signaling cascades and cytoskeletal rearrangement generates a positive feedback signaling loop within the 'supramolecular activation cluster' leading to the aggregation of the microdomains into 'macrorafts' that can be visualized.

A major step towards unraveling the mechanisms of signaling through rafts was the identification and characterization of LAT (linker for T cell activation). LAT is a transmembrane protein, doubly acylated in the juxtamembrane segment, which is Tyr phosphorylated by the ZAP70 non-receptor tyrosine kinase, and targeted to the microdomains upon T cell activation [59]. The phosphotyrosine residue of LAT then recruits the PLC$\gamma$ enzyme close to the microdomain-resident Lck and Lck in turn activates PLC$\gamma$ to produce $IP_3$ and mobilize Ca++. Via the adaptor protein Grb2, phospho-LAT can also recruit the guanine nucleotide exchange factor Vav to the microdomains and possibly influences the actin reorganization via the Rho family GTPases at the signaling pole of the cell.

Of special interest to understand what is happening in the 'immune synapse' is the work of Kupffer and colleagues who mapped the movement of molecules taking place at the T cell-antigen-presenting cell (APC) interface using deconvolution confocal microscopy [60]. The 'supramolecular activation clusters' at the immune synapse contain the TCR/CD3 complex and the PKC$\theta$, Lck and Fyn kinases at the center while LFA1 (an adhesion molecule) and talin (a membrane-cytoskeleton linker) accumulate at the periphery. The interacting molecules at the T cell-APC interface namely, TCR, CD3, CD4/CD8 on the T cell and the peptide-MHC complex on the APC are rather short (15 nm) whereas the highly glycosylated, more abundant CD43, CD44 and CD45 molecules span more than twice that distance. It has been postulated [61] and actually demonstrated [62] that large, glycosylated CD43 molecules will move peripherally to the point where the immune synapse is established. This extensive remodeling of the T-cell surface in preparation for the immune synapse (Figure 2), implies a regulated movement of molecules in and out of the synapse and strongly suggests the participation of rafts in coordinating this molecular traffic [5]. For instance, raft aggregation occurs when T-cell stimulation is mimicked by double stimulation with antibodies to CD3 and CD28 (a major co-stimulatory receptor) [63]. CD28 is important in this context because it activates the Ras-

MAPK, PI3K and PLC$\gamma$ signaling pathways, which overlap with the TCR-mediated signaling cascades, and thus strongly reinforce TCR signaling pathways. Simultaneous engagement of CD3 and CD28 results in activation of more Lck and prolonged Tyr phosphorylation of intracellular substrates [63]. CD28 has been shown to activate Lck directly by allosteric mechanisms [64]. The physical and functional relationship of CD28-stimulated signaling pathways with TCR/microdomain signaling events remain to be explored.

## Microdomains in NK cell function

Recent studies on natural killer (NK) cells take our understanding of the signaling events occurring in microdomains one important step further. NK cells recognize viral or tumor antigens on sensitive target cells and are equipped to kill that target cell upon contact. No killing occurs if the NK cell 'killer inhibitory receptor' (KIR) recognize MHC class I ligands on a normal target cell. Recently, Lou et al. have demonstrated that rafts aggregate at the NK cell-target cell junction only when NK cells recognize the target in the absence of KIR engagement and signaling, and proceed to kill their target [65]. This process of raft aggregation depends upon the PTK activity stimulated by signaling, but the elevated PTK activity is also reinforced by raft aggregation, suggesting the existence of a positive feedback loop between raft aggregation and signal propagation. Moreover, the signaling pathways triggered by engagement of KIR receptors activate cytoplasmic protein tyrosine phosphatases (PTPases) and result in the dispersion of aggregated rafts. These results suggest that raft aggregation is tightly regulated by tyrosine phosphorylation/dephosphorylation. In the T cell context, PTK inhibitors also prevented the actin reorganization following surface cross-linking of the GPI-anchored glycoproteins, but aggregation of the GM1 patches was not inhibited [43].

## What makes rafts favorable environments for signaling?

Studies from our laboratory suggest that the raft/microdomain membrane environment maintains the Src kinases Lck and Fyn in a higher state of activation [66]. Moreover, as mentioned earlier, the Fyn kinase phosphorylates its CD3$\zeta$ substrate only when correctly acylated and targeted to rafts [57] and a similar observation holds true for Lck [55]. It therefore appears that the raft membrane environment provides optimal conditions for the catalytic activity of kinases and their modular interactions.

The influence of specific membrane microenvironment in modulating the membrane associated enzyme activities is a well recognized phenomenon. For example, lipid binding of protein kinase C modulate the enzyme activity as well as its susceptibility to proteases [67]. Src family PTKs are regulated negatively by an intramolecular interaction between the Src homology domain 2 (SH2) and the C-terminal regulatory phosphotyrosine (P-Y) that locks the kinase into an inactive conformation [68]. However, the locked conformation is believed to be dynamic, opening and closing intermittently (breathing) to allow transient activation. The inactive Src kinases can be activated allosterically, without dephosphorylating the regulatory P-Y, by peptides that bind to the SH2 or SH3 domains [69]. In the 'open' kinase, autophosphorylation of the Tyr in the activation loop positively regulates the catalytic activity. In T lymphocytes, Lck is regulated by reversible phosphorylation and dephosphorylation of the regulatory Tyr (Y505) by Csk and CD45, respectively. Since the CD45 protein tyrosine phosphatase is excluded from the microdomains, it is speculated that the specialized lipid microenvironment of the microdomains itself may allosterically activate the Src-family PTKs [66]. In this context, it is also intriguing that CD28, which is required for microdomain aggregation during T cell stimulation via the TCR, can allosterically activate Lck. Further work is needed to determine whether it is the lipid environment and/or the modular protein interactions within the microdomains that are important in regulating microdomain-associated Src family PTK activities.

## Transmembrane receptors in the microdomains

Most transmembrane proteins are excluded from the microdomains. However, the number of transmembrane receptors and proteins reported to be associated with the microdomains is increasing. For instance, phosphorylated Fc$\varepsilon$RI $\beta$ and $\gamma$ chains [70] and CD3$\zeta$ chain [56] are recovered from the microdomains following stimulation via Fc$\varepsilon$RI and TCR, respectively. The mechanism underlying the partial redistribution of these transmembrane signaling components remains however unclear. Deletion and domain swapping experiments suggest a role for the transmembrane segment of Fc$\varepsilon$RI subunit. Recent observations indicate that phosphorylation is a prerequisite for microdomain targeting of the CD3$\zeta$ chain [71], however the fact that other phosphorylated CD3 chains are not found in the microdomains suggest that phosphorylation alone is not sufficient for microdomain localization. It is not unlikely that ligand binding might impose structural changes within the transmembrane segments of the Fc$\varepsilon$RI $\beta$ and $\gamma$ chains and the CD3$\zeta$ chain that prefer the microdomain lipid environment. Ensuing intracellular phosphorylation events and modular protein-protein interactions may stabilize their association with the microdomains.

Variable amounts of other transmembrane proteins such as CD44 and CD26 in T cells also co-isolate with microdomains [24,72]. CD44 is a type I transmembrane protein whereas CD26 is a type II membrane protein. Mutation analysis has demonstrated a role for the transmembrane segment of CD44 in microdomain targeting [73]. A number of multispanning membrane proteins have also been shown to associate with rafts and could possibly represent stabilizing components of the raft structure. The MAL proteolipid is a particularly

interesting tetraspan, non-glycosylated protein that resides in rafts and promotes apical transport in polarized cells [74,75], and may link surface GPI-anchored receptors with Src kinases in lymphocytes [76]. Two other multispanning membrane proteins expressed at the lymphocyte surface have also been reported to associate with rafts: (a) the CD20 tetraspan protein of B lymphocytes which relocalizes selectively to rafts following surface cross-linking with one layer of anti-CD20 antibodies [77], and (b) the hexaspan LMP1 protein (encoded by the Epstein-Barr virus) which resides partially in rafts [78]. Generally speaking, multispanning proteins may contribute to the ordering of lipids in rafts. For instance, the MAL proteolipid incorporates into membranes irrespective of the type of lipid [79] and confers raft-like properties to those membranes.

## Conclusions and perspectives

The concept of rafts/microdomains highlights the need to understand how the organization of the plasma membrane conditions the cellular responses to extracellular stimuli. The biochemical problem of transmembrane signaling is chiefly one of defining how lipids and proteins interact within the distinct phases of the plasma membrane, forming complexes that are transient and dynamic. The rafts themselves represent one modality of membrane organization which is not readily demonstrable by microscopy and this difficulty has often been a reason for misunderstanding, in particular regarding the question of the size of the rafts. However, the availability of well-defined biological systems (i.e. the T-cell and NK cell synapses, for instance) offers very interesting possibilities to refine our functional analyses of rafts with the help of the latest advances in confocal microscopy and image processing. Finally, the sustained interest of biophysicists in membrane microdomain studies is certainly going to help defining the notion of 'raft' more rigorously and the availability of micromethods for the analysis of membrane lipids should make it easier to sort out the various molecules involved.

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