Václav Hořejší

The roles of membrane microdomains (rafts) in T cell activation

Author's address
Václav Hořejší,
Institute of Molecular Genetics, Academy of Sciences of the Czech Republic,
Praha, Czech Republic.

Correspondence to:
Václav Hořejší
Institute of Molecular Genetics
Academy of Sciences of the Czech Republic
Vídeňská 1083
14220 Praha 4
Czech Republic
Tel.: 420 2 41729908
Fax: 420 2 44472282
E-mail: horejsi@biomed.cas.cz

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Summary: Detergent-resistant membrane microdomains enriched in sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins play essential roles in T cell receptor (TCR) signaling. These 'membrane rafts' accumulate several cytoplasmic lipid-modified molecules, including Src-family kinases, coreceptors CD4 and CD8 and transmembrane adapters LAT and PAG/Cbp, essential for either initiation or amplification of the signaling process, while most other abundant transmembrane proteins are excluded from these structures. TCRs in various T cell subpopulations may differ in their use of membrane rafts. Membrane rafts also seem to be involved in many other aspects of T cell biology, such as functioning of cytokine and chemokine receptors, adhesion molecules, antigen presentation, establishing cell polarity or interaction with important pathogens. Although the concept of membrane rafts explains several diverse biological phenomena, many basic issues, such as composition, size and heterogeneity, under native conditions, as well as the dynamics of their interactions with TCRs and other immunoreceptors, remain unclear, partially because of technical problems.

Introduction

Various types of T lymphocytes are stimulated to proliferation, terminal differentiation into effector cells and effector function execution by ligation of their T cell receptors (TCRs) in appropriate context. Despite considerable progress in elucidating the mechanisms of TCR signaling, many basic questions remain unresolved. The traditional view has been that TCRs, and also other similar immunoreceptors (B cell receptors, Fc-receptors), work basically as protein tyrosine kinase (PTK)-associated receptors. Src-family kinases (e.g. Fyn) were thought to be constitutively associated with cytoplasmic tails of the CD3 chains; aggregation of such PTK-associated TCRs by physiologic or surrogate ligands was thought to result in approximation and cross-phosphorylation-based activation of the associated PTKs, followed by tyrosine phosphorylation of further components of the signaling cascades. A basic problem with this simple model was that it was difficult to demonstrate

association of the PTKs with unstimulated TCRs. This problem was apparently elegantly solved by the recently proposed model based on ligation-induced association of TCRs (free of any associated PTKs) with membrane microdomains, rich in Src-family PTKs and other signaling molecules. This paper presents a critical review of this model.

Membrane rafts – general background

More than 20 years ago it was discovered that T cell activation could also be achieved by cross-linking of T cell surface proteins such as Thy-1 (1, 2) or Ly-6 (3). Initially it was speculated that these molecules might be components of the TCR complex, but this association has not been confirmed. Thy-1, Ly-6 and several other activating proteins were later found to be anchored in the membrane through a glycolipid (glycosylphosphatidylinositol, GPI) covalently attached to their C-termini (4, 5). These molecules have no transmembrane and cytoplasmic domains, and thus it was difficult to explain how they might transmit the activating signals into the cell interior. It was soon found that essentially all GPI-anchored proteins and also some glycolipids transmit activating signals upon effective cross-linking. A plausible explanation came with the discovery that GPI-anchored proteins and glycosphingolipids are components of detergent-resistant membrane microdomains also rich in protein kinases (6–12). The existence of distinct lipid-based membrane domains in leukocyte membranes was observed more than 20 years ago by studies employing incorporation of various types of fluorescently labeled lipid probes (13); their possible involvement in TCR signaling was indicated by early studies based on the inhibitory effects of free unsaturated fatty acids incorporation (14).

Composition and properties of membrane rafts

These 'islets', also known as membrane rafts, lipid rafts, glycosphingolipid-enriched microdomains (GEMs) or detergent-resistant microdomains (DRMs), are, in contrast to the bulk membrane, relatively resistant to solubilization with commonly used detergents such as Triton X-100, NP40 or CHAPS (but are easily solubilized, for example, by n-octylglucoside or sodium dodecyl sulfate). These structures are held together mainly by hydrophobic interactions between saturated fatty acid residues of their main lipid constituents, sphingomyelin and glycosphingolipids (such as GM3 in T lymphocytes) and further enhanced by intercalated cholesterol molecules (15–17). It is not clear whether both leaflets of the raft's bilayer have similar (compatible) lipid composition or whether more or less independent rafts exist in each leaflet. Cholesterol redistribution between the membrane leaflets and presumably also between the raft and nonraft areas is actively mediated by the multidrug transporter, P-glycoprotein (18). Important lipid constituents of the raft cytoplasmic leaflet are phosphorylated phosphatidylinositols (PIP_2, PIP_3) (19, 20). Another important lipid stabilizing and aggregating the rafts is ceramide, produced by acidic sphingomyelinase from sphingolipids (21). As stated above, characteristic protein components of lipid rafts are extracellularly oriented GPI-anchored proteins such as CD14, CD16b, CD24, CD48, CD52, CD55, CD59, CD73, CD87, CD90 (Thy-1), CD108, CD157, CD230 (prion protein) or Ly-6. Membrane rafts also exist in cells deficient in expression of GPI-anchored proteins (22) or deficient in glycosphingolipids synthesis (23). However, their integrity is compromised after cholesterol depletion (23, 24) or biosynthetic replacement of saturated fatty acid residues in their sphingolipids by unsaturated ones (25–27). Compared to the bulk membrane, membrane rafts appear to possess a thicker membrane (17) and selectively sequester a set of important cytoplasmic signaling molecules such as Src-family kinases (7–11) and G proteins (28–30). Most transmembrane proteins are excluded, notable exceptions being the coreceptors CD4 and CD8αβ (31–36), pre-TCR (in early thymocytes) (37), adhesion receptor CD44 (38, 39), proteolipid MAL (40), influenza virus hemagglutinin (41, 42), transmembrane adapter proteins LAT (43, 44) and PAG/Cbp (45, 46) or several members of the tumor necrosis factor (TNF)-receptor family (21, 47–51). The cytoplasmic proteins, as well as most of the transmembrane proteins, are targeted to rafts due to their modifications with saturated fatty acid residues (myristoylation and palmitoylation) (52, 53); palmitoylation-deficient mutants of Src-family PTKs (54–56), CD8β (34, 35), pre-TCR (37) or LAT (43) are no longer targeted to membrane rafts and are functionally defective. It is possible to artificially target various proteins to membrane rafts by grafting onto them sequences required for either modification by the GPI moiety or double acylation (as in Src-kinases) (57–64).

Membrane rafts, defined as lipid–protein complexes insoluble in cold solutions of Triton X-100, can be isolated, because of their high lipid content and thus low buoyant density, by density gradient ultracentrifugation (12). Such structures could be demonstrated in essentially all cell types tested. Their existence in T lymphocytes neatly explained the puzzling signaling capacity of GPI-anchored proteins and glycolipids: their cross-linking by antibodies or other multivalent ligands may induce redistribution (clustering) of the

raft-associated Src-kinases, resulting presumably in their cross-phosphorylation and activation, followed by phosphorylation of some of their substrates and thus initiation of the signaling process. This raft aggregation appears to be accompanied also by entrapment and phosphorylation of TCR molecules, or at least the ζchains, which results in mimicking some aspects of TCR signaling (65–67).

A special type of raft microdomains is called caveolae, morphologically characteristic invaginations of plasma membrane observable by electron microscopy in many cell types (but not in lymphocytes) (68, 69). These structures are organized by caveolins, palmitoylated proteins with an affinity for cholesterol. Caveolae are not present in lymphocytes due to their lack of caveolin expression; forced expression of caveolin-1 in these cells results in caveolae formation (70). Membrane rafts and caveolae have been implicated in an ever-increasing number of biologically important phenomena such as signaling through TCRs (discussed later), B cell receptors (BCRs) (71–75), pre-BCRs (76), Fc-receptors (77–84), lipopolysaccharide (LPS) receptors (85, 86), apoptotic receptors (21, 49–51), cytokine and chemokine receptors (87–93), collagen receptors (94, 95), several other receptors (96–99), natural killer (NK) cells (100), bactericidal activity of neutrophils (101), antigen presentation (102–105), cell interaction with pathogens (105–108) and bacterial toxins (109–111), pathogenesis of prion and other neurodegenerative diseases (112, 113), specific forms of endocytosis (114, 115), vesicle trafficking (116, 117), adhesion (93, 118, 119) and establishing of cell polarity (120–122). At present, it is sometimes difficult to escape the impression that nearly all important membrane events occur in membrane rafts. There are numerous recent reviews on various aspects of membrane rafts (15–17, 75, 91, 116, 123–143).

Conceptual and technical problems

A basic problem with noncaveolar membrane rafts is that because of their small size they cannot be easily observed on intact cell surfaces by microscopic techniques. Therefore, most of the data demonstrating their functional roles (and even their very existence) are based on membrane solubilization in suitable detergents and isolation of the lipid-rich detergent-resistant rafts by density gradient ultracentrifugation. This process is of course a source of potential artifacts; it is not clear how closely the composition and size of such biochemically isolated rafts correspond to the presumed native structures present in live cell membranes. Several years ago a skeptical view prevailed that the purified rafts are entirely detergent artifacts formed as a result of selective membrane lipid solubilization by the detergents at low temperature (144, 145). More recently, however, several approaches have demonstrated that raft-like membrane microdomains do exist even under physiological conditions.

First, a detergent (Brij-98) was found that preserves membrane rafts even at 37 °C, thus at least partially excluding the objection that low-temperature solubilization induces lipid phase transitions artificially creating the detergent-resistant protein–lipid complexes (146). Second, formation of clearly separated and observable microdomains corresponding remarkably well to lipid rafts could be demonstrated in model membranes and liposomes composed of well-defined lipid mixtures (147–149). In some cases even a fine structure of these domains could be demonstrated, which consisted of a more detergent-resistant, glycolipid-rich core and a less-resistant peripheral zone (149). These purely lipid-based microdomains are formed spontaneously, primarily due to intrinsic mutual affinity of the (glyco)lipid species with long, saturated hydrocarbon chains. Such lipids, especially if doped with a suitable amount of cholesterol, form a specific 'liquid ordered phase' distinguished from the more disordered and more fluid bulk phase formed by lipids containing more disordered unsaturated fatty acid residues (15, 16). Such a spontaneous physico-chemical process also seems to be primarily responsible for the formation of lipid rafts in membranes of living cells. However, the formation, properties and stability of natural membrane rafts are likely to be markedly affected by incorporation of proteins (discussed later) and also by the fact that in contrast to the artificial lipid membranes, biological membranes are highly dynamic, nonequilibrium systems (132). Third, methods such as fluorescence energy transfer (150), chemical cross-linking (151), single particle tracking (152), laser trap (153) or single dye tracking (154) produced results compatible with the idea that the lipid and protein components examined by these methods were confined within cholesterol-dependent membrane microdomains physically more or less separated from the bulk membrane. The dimensions of the microdomains determined by these methods vary from 40 nm to more than 1 μm depending on the cell type and the method used. This fits fairly well with the early estimates of size of the detergent-resistant complexes (50–200 nm) (8, 12) and also with recent data obtained by transmission electron microscopy (155). Unfortunately, light microscopic methods cannot be used for direct microscopic visualization of membrane rafts, as they are too small, comparable with or smaller than the wavelength of visible light. However, novel sophisticated

optical techniques may soon enable direct observation of objects as small as 50 nm (156); the field would obviously greatly benefit from availability of such methods enabling direct observation of rafts.

Distribution of GPI-anchored proteins or glycolipids in fine dots apparently corresponding to membrane rafts can be often observed when these molecules are visualized by means of fluorescently labeled antibodies or other ligands (e.g. cholera toxin that has five binding sites for the GM1 glycolipid). However, this seems to be largely due to aggregation induced by the multivalent probes. Such artifacts should be prevented by suitable fixation of the cells before immunostaining; however, GPI-anchored proteins and glycolipids are reportedly difficult to immobilize by fixation methods compatible with the immunostaining. So far, distribution of raft markers in discrete dots has not been reported when rigorously purified monovalent, fluorescently labeled Fab fragments are used for detection. Similarly, when GPI-anchored fluorescent proteins are expressed in cells, they are distributed essentially homogeneously (157), which is compatible with the concept of very small rafts under the discrimination limit of optical microscopy. On the other hand, when purified, fluorescently labeled GPI-anchored glycoprotein CD59 was incorporated into leukocyte membrane, distinct dotted distribution was observed, the appearance of which correlated with acquiring signaling capacity of the incorporated protein (158). It is not clear whether in this case the host cells possessed unusually large intrinsic rafts and the method visualized genuine rafts or whether the newly incorporated (presumably at least partially aggregated) GPI-protein associated with residual detergent induced their fusion into larger patches.

It is not clear what percentage of the cell surface is covered by the rafts. It certainly depends very much on the cell type; in polarized epithelial cells, most of the apical surface appears to be composed of the rafts. Earlier estimates for other cell types, such as leukocytes, were in the range 5–10%, but more recently higher values of around 50% appear to be more realistic (a somewhat paradoxical situation when there may be more islets than ocean) (132). However, in the absence of direct visualization, these estimates remain speculative, as they are inevitably based on indirect indications and the stringency of the biochemical definition of rafts used (discussed later).

Another related question concerns heterogeneity of rafts in a given cell type; there could be essentially one type of raft containing various types of raft-resident glycolipids and proteins or rather several, or even many types, each enriched in specific molecules only (e.g. one Lck-rich, another LAT-rich, etc.). Recent data indicate that the latter case is closer to reality. For example, two major glycolipids, GM1 and GM3, were demonstrated to reside in two clearly distinct types of rafts in motile T cells, one of them originating from the leading edge and the other from the uropod (159). In another study, cholesterol extraction was found to destabilize microdomains of T cell lymphoblasts containing Lck, while the rafts containing LAT remained intact under the same conditions (155). Cholesterol-independent, temperature-resistant raft-like microdomains were described containing several tetraspanin proteins and phosphatidylinositol 4-kinase (160).

The standard methods of raft isolation based on density gradient ultracentrifugation of detergent-solubilized membranes produce a mixture of different rafts that are difficult to separate. However, the use of shallow-density gradients or methods combining separation according to density and size do yield fractions differing remarkably in protein composition (161, 162).

Perhaps the most important technical point is that the present operational definition of membrane rafts is based on their resistance to detergents such as 1% Triton X-100 at low temperature and their low buoyant density (approximately 1.05–1.09 g/cm^3), causing them to float under the conditions of density gradient ultracentrifugation. It is very important to note that very different rafts can be obtained if other detergents are used or if much lower concentrations of the standard Triton X-100 are used. The use of milder detergents, such as Brij-98, Brij-58 or Lubrol, produces higher yields of buoyant rafts containing close to 100% of total cellular GPI-anchored proteins, transmembrane adapters LAT or PAG/Cbp, substantial fractions of Src family kinases but also small amounts of several 'atypical' transmembrane proteins such as CD45 or major histocompatibility complex (MHC) molecules (unpublished data). In contrast, when more stringent 1% Triton X-100 or NP40 is used, the low-density gradient fractions may contain less than 50% of the typical raft molecules (GPI-proteins, transmembrane adapters), less than 10% of total Src kinases and only traces of any CD45 or MHC proteins. As mentioned earlier, some 'mild' detergents such as *n*-octylglucoside or *n*-dodecylmaltoside (also called laurylmaltoside) effectively dissolve rafts even at low temperature, although they preserve most protein–protein interactions. It is even possible to disintegrate membranes by homogenization in the absence of any detergent and separate the mixture obtained by density gradient ultracentrifugation. The results are then relatively similar to those obtained after solubilization with very mild detergents such as Brij-58 (163, 164). It is only a matter of convention how the results obtained by various detergents should be interpreted. One possibility is that the

very mild detergents preserve best the native structures and that the atypical components (CD45, etc.) are indeed attached weakly to native rafts; the native rafts may consist of a more resistant core that survives even more stringent detergents, and a sensitive peripheral region that is dissolved by Triton X-100 but withstands Brij-58. Another possible interpretation is that the rafts obtained by means of Triton X-100 are closer to the native ones, while the milder detergents produce a contamination of the 'true' rafts by remnants of poorly solubilized nonraft membrane. In our experience, the results are also markedly affected by technical details such as whether the detergent is present in the whole gradient or just in the solubilized sample applied at the bottom of the gradient. In the latter variant (used by most studies), there is a much higher tendency to a 'nonspecific' sticking of various transmembrane proteins to the buoyant rafts (unpublished observations).

Another important, often neglected, technical detail is how the detergent-solubilized cells are treated before separation by density gradient ultracentrifugation. In our experience it is very important to use the whole suspension of the detergent-treated cells as the sample for ultracentrifugation; insoluble nuclei and cytoskeleton should not be first removed by low-speed centrifugation, because this step usually leads to great losses of membrane rafts associated with cytoskeleton. Also, it is important to analyze the insoluble nuclear/cytoskeletal pellet after gradient ultracentrifugation to get an idea of which part of the raft molecules might have been lost in this way. It may be even better to isolate and solubilize plasma membrane first and isolate membrane rafts from this better-defined material.

An additional possible source of ambiguity could stem from the fact that only the buoyant rafts have been studied. It is, however, possible that 'heavy rafts' with a higher protein/lipid ratio exist and are hidden in the bottom fraction of the gradient mixed with the completely solubilized proteins. A simple approach to discover and study such hypothetical atypical membrane rafts might be based on the use of optimally engineered gradients and/or on separation of the heavy rafts from the complex mixture in the dense fractions of the gradient by sizing methods.

A widely used approach to demonstrate involvement of lipid rafts in cellular functions has been based on extraction of cholesterol using methyl-β-cyclodextrin (MbCD) (24, 41, 165, 166). Detergent solubilization of cells after such a treatment does not produce the typical low-density complexes of glycolipids, GPI-anchored proteins and cytoplasmic signaling molecules. Detergent-resistant rafts are affected by other agents (filipin, nystatin) that do not extract cholesterol but form complexes with it *in situ* (165, 167, 168). Thus, if some cellular function is affected in such treatments, it is taken as evidence for raft involvement. One disturbing but largely neglected fact has been that MbCD is evidently not specific for cholesterol present in rafts but extracts this membrane lipid from the bulk nonraft membrane (and at higher concentrations it may even extract other lipids). Furthermore, these treatments are relatively harsh, and the cells change their shape markedly and often die. It is therefore difficult to determine which effects unrelated to rafts are in fact caused by MbCD or similar treatments (169).

Other raft-disrupting treatments exist. One of them is based on cholesterol oxidase, which disrupts rafts due to a chemical modification of cholesterol, and this treatment does affect TCR signaling (146). Another such treatment is based on biosynthetic replacement of a fraction of saturated fatty acids in the membrane lipids by polyunsaturated ones (25–27). Such a treatment was accompanied by displacement of Src-family kinases and LAT from the detergent-resistant rafts, while GPI-anchored proteins and ganglioside GM1, both residing in the outer membrane leaflet, remained in the buoyant rafts, indicating that this particular treatment probably selectively modifies the cytoplasmic layer of membrane rafts. All such treatments should be carefully examined as to what extent the observed inhibitory effects are perhaps again due to some 'nonspecific' effects other than disruption of the normal structure of membrane rafts.

It would be ideal to have some mutant cell lines devoid of any rafts, similar, for example, to mutants devoid of caveolar microdomains due to lack of caveolin expression (170, 171). There are at least two problems with such hypothetical raftless cells. First, as stated earlier, the rafts are not directly observable as morphologic entities, so it would be difficult to define unequivocally such mutants unless the microscopic techniques are refined to achieve much higher resolution. Second, both components, critically important for the raft integrity, cholesterol and (glyco)sphingolipids possessing long saturated fatty acid chains, seem to be vitally important for any cell, at least in some minimal amounts. As stated earlier, lipid rafts (defined as detergent-resistant buoyant complexes) still exist in mutant cells possessing no glycosphingolipids (but containing sphingomyelin) (23) or in cells lacking any GPI-anchored proteins (22). Cells from mice deficient in acidic sphingomyelinase were reported to have marked disturbances in the composition of their membrane lipids (marked loss of cholesterol) and to be devoid of conventionally defined Triton X-100-resistant lipid rafts (172); thus, these cells might be close to the desired nonraft mutants.

Although there is at present no definitive consensus, it seems likely that 'elementary rafts' are quite small (diameter <10 nm) and contain very few (perhaps even single and some none at all) protein molecules surrounded by a 'shell' of about 100 of the specific lipid molecules (141). Lipid composition of these lipid shells around various membrane proteins is probably dictated by properties of the transmembrane domains, other membrane-interacting areas of the proteins and/or their lipid modifications. Such elementary microdomains may coalesce with some specificity into larger patches (125), either after engagement of their protein or glycolipid components with their natural ligands or antibodies or after exposure to some types of mild detergents.

Functional interactions of membrane rafts with TCR

A simple model of the TCR–raft interaction

A real boom of immunologists' interest in membrane rafts came when it was found that these structures may play essential roles in signaling initiated through TCRs (165, 173), BCRs (71–75) and several Fc-receptors (77–84). According to the simplest, currently popular model, these receptors are in their resting state devoid of any associated protein tyrosine kinases. Upon cross-linking by their natural or surrogate ligands (antibodies), their aggregates merge with membrane rafts and immune receptor-based tyrosine activation motifs (ITAMs) present in cytoplasmic tails of their signaling chains (CD3, CD79, ζ-family proteins) become exposed to the Src-kinases present in the rafts (126, 128). Several other components of the earliest phases of immunoreceptor signaling (LAT, PIP_2) also reside constitutively in membrane rafts. Very recently it was demonstrated that TCRs may be preassociated with membrane rafts (146, 174) and their cross-linking may reorganize this assembly to allow for optimal exposure of the CD3 and ζchains to the Src-family kinases. This attractively simple model is supported by the following observations:

- A fraction of TCRs and also several cytoplasmic signaling molecules become rapidly associated with buoyant rafts following TCR cross-linking (165, 173) or are preassociated with them (146, 175).
- Constitutive association of the pre-TCR with lipid rafts is sufficient for eliciting signals needed for prethymocyte survival (37).
- Partial dispersion of rafts by cholesterol depletion or by modifications in fatty acid composition of their lipid and protein components inhibits early TCR-triggered events such as tyrosine phosphorylation of the ζchain and elevation of cytoplasmic calcium (26, 165) (but paradoxically, cholesterol depletion induced tyrosine phosphorylation of multiple T cell signaling proteins in another study; 174).
- Wild-type Lck and LAT molecules capable of targeting to rafts, but not their membrane-associated raft-nontargeted mutants, are able to reconstitute TCR signaling defects in appropriate T cell mutant cell lines (43, 54–56). In addition, artificial targeting of other cytoplasmic molecules, such as SHP-1 (58), CD45 (64) or PLCγ (52), to membrane rafts (by grafting onto them motifs capable of double acylation similar to that naturally occurring in most Src-family kinases) results in marked functional effects on TCR-induced signaling.
- Co-cross-linking of TCR/CD3 with some T cell surface GPI-anchored proteins has, depending on the conditions used, markedly synergistic costimulatory or inhibitory effects (176–178). Signaling induced by Thy-1 aggregation is dependent on TCR coexpression (65).
- T cells from acid sphingomyelinase knockout mice, which are deficient in membrane rafts due to drastically decreased cholesterol content, have defects in TCR-induced tyrosine phosphorylation and T cell proliferation (172).
- Membrane rafts are also partially disrupted (due to an unknown mechanism) in T cells exposed to corticosteroids, and this disruption correlates with defective TCR signaling (179). TCR signaling is also inhibited following treatment with cholesterol oxidase (146).
- T cells from GPI-deficient or Thy-1-deficient cells have TCR signaling defects (180, 181).
- Mutation of the TCR connecting peptide that interferes with increased ζchain raft association in response to partial agonist antigen affects Erk activation and thymocyte positive selection (182). Similar signaling defects are observed in T cells of CD3δ knockouts, the TCR/CD3 complex of which is also deficient in membrane raft association (183).

Do membrane rafts participate in the earliest phases of the TCR signaling?

Several recent studies indicate that especially under more natural conditions of T cell stimulation, that is with antigen-pulsed antigen-presenting cells (APCs) rater than with anti-CD3 antibodies, initial phases of TCR signaling, namely phosphorylation of the ζchains, may be largely independent of lipid rafts. Rather, the TCR–raft interactions may be important in later phases of the signaling and/or in the process of costimulation

by receptors such as CD28. This later role is also indicated by the fact that rapid ζchain phosphorylation is essentially normal in T cells expressing defective TCR complexes unable to associate with rafts, while these cells have severe defects in distal parts of the TCR signaling pathways (182, 183). This view is also supported by the recent observation by Viola and collaborators that, in contrast to earlier claims, there is essentially no inhibition of ζchain phosphorylation and cytoplasmic Ca^{2+} elevation by MbCD treatment, which disperses the rafts (169). According to these authors, the previously reported MbCD-caused inhibition of TCR-induced cytoplasmic calcium elevation is mainly due to a nonspecific depletion of intercellular Ca^{2+} stores and plasma membrane depolarization inhibiting capacitative calcium channels function. When these nonspecific effects were properly accounted for, little specific effects on the TCR-mediated signaling could be demonstrated. Interestingly, the elevation of cytoplasmic Ca^{2+} level elicited by cross-linking of a GPI-anchored protein CD59 was completely inhibited in a specific manner, even after correction for the nonspecific effects (169). Thus, while signaling through GPI-anchored proteins indeed requires raft integrity, the TCR-induced signaling, at least its initial phases, may occur independently of membrane compartmentalization. If so, it remains unclear what is the mechanism of the earliest steps of TCR signaling, and especially what is the source of the Src-kinase(s) performing the initial phosphorylation of ITAM motifs in the CD3 and ζchains. However, as discussed earlier, currently there are serious problems with the basic definition of membrane rafts and thus the fact that a treatment (such as MbCD) results in loss of Triton-X100-resistant rafts does not have to mean that the rafts *in situ* are necessarily completely destroyed and totally nonfunctional. It can be speculated that the MbCD treatment disturbs the native structure of rafts only partially in such a way that they become more susceptible to detergents and abolishes the intermolecular interactions responsible for the signaling potential of CD59 molecules, but the 'disturbed rafts' may still be structurally and functionally 'intact enough' to support TCR signaling.

An important point is that the membrane rafts attracted to the clustered TCR appear to be mostly of intracellular origin (184–186). Activation-induced redistribution of intracellular Lck-rich rafts from a so far poorly characterized intracellular compartment to the plasma membrane may be an important feature distinguishing naïve from effector and memory T cells. A higher concentration of surface rafts on the latter may be responsible for their well-known higher sensitivity to antigen stimulation and lack of need for costimulation (184–186).

It is tempting to speculate that two types of rafts are involved in TCR signaling: first, those present on the T cell surface and preassociated with the TCR (146) may be the source of the kinases needed for initiation of the signaling process; another type, stored intracellularly, translocates to the aggregated TCR and is involved in costimulation and signal amplification (184–186). A similar two-step model has been proposed by Miceli and colleagues based on elegant experiments with Lck SH3 mutants (133, 187).

The TCR–membrane raft interactions are also relevant with respect to formation of the immunological synapse between the T cell and the APC (188). According to the simplest model, membrane rafts should accumulate in the central region of the mature synapse rich in TCR and costimulatory molecules. Somewhat surprisingly, in a recent study the only raft marker accumulating in this area was the transmembrane adapter protein LAT and not other typical raft markers (189). A similar observation was also made in mast cells stimulated via FcεRI (190). This might indicate that only one type of raft (LAT-enriched) is specifically recruited to the synapse. In contrast, a recent electron microscopy study demonstrated that Lck and LAT, present in separate microdomains in unstimulated T cells, converged into common patches upon TCR cross-linking (155). The role of immunological synapse in initiation of TCR signaling was recently questioned, as it has been suggested that the early phases of the signaling take place before synapse formation and that the synapse may play some role unrelated to signaling (cell polarization, internalization of the signaling complexes) (191).

Raft components important for TCR signaling

Irrespective of the kinetic details, the importance of membrane rafts in at least some aspects of TCR signaling has been firmly established. Several raft-associated molecules are relevant in these interactions. First, major subsets of the CD4 and CD8 coreceptors associated with Lck are located in membrane rafts (31–33, 36). Co-engagement of TCRs and the coreceptors with the MHC–peptide complex on the APC surface is probably a major factor bringing cytoplasmic domains of the CD3 and ζchains to the proximity of the coreceptor-associated, but presumably also additional coreceptor-free, raft-associated Lck. Another Src-family PTK, Fyn, is also associated with membrane rafts and is involved in T cell activation (192, 193). An unclear issue is whether sequestration into membrane rafts helps to keep Src-family kinases in an active or an inactive state. A major positive regulator of these enzymes is membrane phosphatase CD45, which appears to be excluded from membrane rafts (194). On the contrary, a fraction of the negative regulator of Src-family kinases activity, the PTK Csk, is a raft component (discussed later). Therefore, Src-family

kinases present in rafts should be maintained in an inactive state; this conclusion has been reached in some studies (195) but disputed in others (196). However, CD45 may also have negative regulatory effects on Src-family kinases (197, 198), so the effect of CD45 exclusion from membrane rafts may be ambiguous and dependent on other factors. Furthermore, it can be argued that a small fraction of CD45 might be loosely associated with the peripheral parts of membrane rafts and have access to the Src-family kinases located there. Interestingly, forced targeting of the CD45 cytoplasmic domain to T cell rafts results in inhibition of T cell activation (64).

As mentioned in the introduction, important signaling components of membrane rafts are heterotrimeric G proteins (28, 30, 199, 200) and also small G proteins of the Ras (29, 201) or Rho families (29, 201–203), but the role of their microdomain compartmentalization in T cell activation has not yet been examined.

Among the functionally most important T cell raft proteins are the transmembrane adapters LAT (43) and PAG/Cbp (45, 46). As these molecules are dealt with in detail in other contributions in this issue of *Immunological Reviews*, I will only briefly mention that LAT serves as a key scaffold molecule around which a complex of several other signaling cytoplasmic proteins is organized following TCR ligation. The critical tyrosine residues of LAT are phosphorylated by phospho-ζ-associated ZAP-70 or Syk and then serve as docking sites for SH2 domains of PLCγ1, PI3-K and several cytoplasmic adapters (204–206). LAT knockout mice have severe defects in thymocyte development (207). Importantly, functionality of LAT is dependent on its palmitoylation and incorporation into lipid rafts (43). Most of LAT can be removed from T cell rafts by feeding the cells with polyunsaturated fatty acids (PUFAs) that partially replace saturated fatty acid residues in sphingolipids necessary for raft integrity (26). Such a treatment results in inhibition of TCR signaling and it is probably behind the well-known immunosuppressive effects of PUFAs *in vivo*.

A LAT-like molecule, identified recently in our laboratory and named NTAL, is present in the membrane rafts of non-T cells and plays a role in signaling through BCR, FcεRI and FcγRI (208). In addition, yet another transmembrane adapter protein, pp35, present in rafts of resting T cells and apparently playing a role in regulation of TCR signaling, was recently discovered and is currently under study in our laboratory (unpublished observations).

Tyrosine-phosphorylated transmembrane adapter PAG/Cbp (45, 46) binds and thereby activates (209) the cytoplasmic PTK Csk, a critical negative regulator of Src-family kinases. Thus, the presence of the PAG–Csk complex in membrane rafts serves to dampen the activity of the raft-associated Src kinases, and in this way it may contribute to setting a threshold for TCR activation. Cross-linking of TCR on resting αβT cells results in rapid transient dephosphorylation of PAG/Cbp and release of Csk (45, 210, 211). This presumably contributes to increased Src PTK activity, which is needed for the signaling. This regulatory system is further linked to PKA type I, which is also directed to membrane rafts of activated T cells (presumably due to its association with a so far unidentified raft-resident scaffolding protein) (212, 213). PKA phosphorylates and thereby activates the raft-associated Csk, which in turn contributes to inhibition of Src-family PTKs (212). Interestingly, tyrosine phosphorylation of PAG/Cbp and its Csk association increases following FcεRI cross-linking; this results in Lyn PTK inhibition, and negative regulation of mast cell degranulation (214). PAG/Cbp also binds the PTK Fyn, but this interaction is independent of tyrosine phosphorylation and its biological importance is not yet clear (45). Moreover, the C-terminal motif of PAG/Cbp binds to the PDZ domain of another cytoplasmic adapter protein, EBP50 (also known as NHERF) and thereby can be linked to the ezrin/radixin/moesin proteins and through them to fibrillar actin (211, 215). The PAG/Cbp–EBP50 complex appears to dissociate following TCR engagement (211). Thus, PAG/Cbp might serve to anchor membrane rafts reversibly to actin cytoskeleton (discussed later). Interestingly, in contrast to LAT, PAG/Cbp is not removed from membrane rafts following PUFA treatment (26); it seems likely that the PAG 16aa extracellular peptide plays a role in the raft targeting.

Several other molecules relevant for TCR signaling are associated with membrane rafts, and following TCR cross-linking, they associate with the clusters of TCR. One of them is Itk, a member of the Tec PTK family, which associates with membrane rafts due to interaction of its pleckstrin homology domain with phosphoinositides; this interaction is further enhanced by associations with LAT, SLP-76 and Grb-2, all present in rafts of activated T cells (216). Another such raft-associated molecule is protein kinase C θ (PKCθ), which after tyrosine phosphorylation physically associates with Lck and translocates to lipid rafts associated with immunological synapse (135, 217). Recently, CARMA1, a lymphocyte-specific member of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins, was found to be constitutively associated with lipid rafts and serve as a critical regulator of TCR-induced NF-κB activation and CD28 costimulation-dependent JNK activation (218). A major negative

regulator of the TCR–raft interaction appears to be the cytoplasmic adapter protein Cbl-b (219). The loss of this molecule results in enhanced TCR clustering and TCR-induced raft aggregation accompanied by sustained tyrosine phosphorylation even in the absence of CD28 costimulation. This results in hyperproliferation of the T cells and spontaneous autoimmunity in knockout mice (219).

Thus, membrane rafts clearly contain molecules needed for TCR signaling initiation as well as those inhibiting it. Activation-induced association of TCRs with membrane rafts seems to be dependent on an extracellular proteoglycan agrin, previously known to aggregate receptors at the neuromuscular junction. A T cell specific form of agrin is reported to aggregate lipid rafts and enhance T cell activation induced either by anti-CD3 monoclonal antibody or by peptide antigen (220). It is possible that agrin functions like antibodies to GPI-anchored proteins present in the rafts, although at present it is not clear what its ligand is. In contrast, the association of ligated TCR with membrane rafts can be blocked by soluble lectin galectin-1, which results in partial signals allowing for interferon-(IFNγ) production or apoptosis but preventing full proliferative response involving interleukin (IL)-2 production (221, 222). Employing or mimicking this galectin-1 activity might present an attractive opportunity for therapeutic immunosuppression (223, 224).

The role of membrane rafts in T cell costimulation and cytoskeletal rearrangements

As mentioned earlier, membrane rafts play also important roles in the process of T cell costimulation. The fact that antibody-mediated co-cross-linking of GPI-anchored proteins (typical extracellular raft components) with TCR/CD3 has potent costimulatory effects suggests that a similar process might also operate *in vivo*. Although the major costimulatory receptor, CD28, is not constitutively present in membrane rafts, the CD28-mediated costimulation is accompanied by a major redistribution of rafts, mainly from intracellular reservoirs to the cell surface (184–186). This appears to be due to (or correlated with) a so far poorly characterized cytoskeleton rearrangement. An important mechanism contributing to the costimulation dependent on CD28 and membrane rafts may be based on the interaction of a cytoplasmic CD28 motif with the SH3 domain of the raft-associated Lck, which markedly increases its kinase activity (225). Efficient costimulation can also be achieved by co-cross-linking of TCRs with several other T cell surface molecules such as CD2, CD5, CD9 (226), CD47 (227–229), CD54 (230) or leukocyte function-associated antigen-1 (231, 232). Subsets of some of these molecules are associated with rafts, either constitutively or following cross-linking (226, 227, 229, 233). It is easy to imagine that even a very minor, poorly detectable, raft-associated subset of such abundant transmembrane proteins, usually considered as nonraft molecules, can be responsible for the costimulatory effects observed. Interestingly, the major negative regulator of T cell activation and a counter-player of the CD28 costimulatory receptor, CTLA-4 (CD152), is associated with membrane rafts on the activated T cell surface and coclusters with TCRs and lipid raft ganglioside GM1 within the immunological synapse (234). Another study demonstrated that CTLA-4, unlike CD28, inhibits redistribution of intracellular rafts to the surface of activated T cells (186), possibly being a major mechanism of its negative costimulatory function.

The TCR interactions with membrane rafts have been repeatedly correlated with actin cytoskeleton rearrangements (235). Lipid raft polarization to the immunological synapse depends on an intracellular pathway that involves Vav-1, Rac, cdc42, WASP and actin reorganization (236). Another molecular complex playing a role in these raft-related cytoskeletal rearrangements is composed of SLP-76, Nck, Fyn and PAK (237); PKCθ is reported to be essential in regulation of these events by phosphorylation of the linker protein moesin (238). Lipid rafts do not translocate to the T cell–APC contact site in Vav-1-deficient T cells (239). The activated TCR complex can be linked to actin cytoskeleton through the tyrosine-phosphorylated ζ chain, and this interaction is enhanced through an unclear mechanism by association of aggregated TCRs with lipid rafts (240–242). Association of lipid rafts with actin cytoskeleton may be mediated through some of the proteins residing in rafts, such as the above-mentioned PAG/Cbp (connected to F-actin through EPB50 and ezrin/radixin/moesin) (210, 215) or the adhesion protein CD44 (associating directly with ezrin) (243, 244). The phospholipid PIP_2 is also important in recruitment of the WASP protein to aggregated membrane rafts where it functions in actin nucleation through the associated Arp2/3 complex (245). Accumulation of F-actin under the patches of aggregated rafts correlates with the presence of tyrosine-phosphorylated proteins (246). It is not known whether reorganization of actin cytoskeleton is needed for raft clustering or, conversely, whether raft clustering promotes rearrangements of actin cytoskeleton. Some of the above-mentioned results seem to be compatible with the idea that rafts are associated in the resting state with F-actin and this tethering inhibits the actin cytoskeleton reorganization. Following TCR activation, this inhibitory interaction may be relieved, which allows for the cytoskeletal rearrangements needed for proper TCR signaling.

T cell subsets differ in the use of membrane rafts in TCR signaling

The interactions between TCRs and lipid rafts appear to differ in various T cell subsets, and these differences probably contribute to the markedly different outcomes of TCR signaling in various T cell subsets. Also, the type of T cell response to various types of TCR ligands (agonist, partial agonist, antagonist) may be due to differences in the TCR–raft communication dictated by the primary characteristics of the TCR–antigen interaction. Thus, signaling through the TCRs of immature ($CD4^+CD8^+$) thymocytes and anergic T cells apparently does not involve lipid rafts and its final result corresponds to an 'incomplete' type of signal, resulting in apoptosis (247, 248). A similar response (apoptosis or anergy) occurs in mature T cells if their TCRs are prevented (e.g. by galectin-1) from effective association with membrane rafts (221, 222). As mentioned earlier, effector and memory T cells have more surface rafts compared to naïve T cells, and this occurrence may be the reason for much easier, costimulation-independent triggering of their activation via TCRs (184–186). Activated Th1 and Th2 cells markedly differ in the role played by membrane rafts in TCR signaling: while in Th1 cells the TCR (and also CD45 PTPase) becomes associated with the rafts (this association being largely dependent on CD4), stimulation of Th2 cells does not result in such associations (249). The causes of these remarkable differences are presently unknown but may be due to the different composition and quantity of lipid rafts in the Th cell subsets.

Roles of membrane rafts in other aspects of T cell biology

As mentioned in the introduction, membrane rafts are important in many diverse biological phenomena. More specifically, in T cells they are involved in functioning of IL-2 and IFNγ receptors (87–90). Importantly, both types of human immunodeficiency virus (HIV) receptors (CD4 and the chemokine receptors) reside in membrane rafts, and the integrity of these structures is necessary for HIV infectivity (105–107) as well as for virus particle budding (250). One of the critical pathogenic components of HIV, the protein Nef, is its lipid modification targeted to lipid rafts, and this feature seems to play an essential role in its ability to prime T cells for activation (251). Finally, it should be mentioned that the complexes of antigenic peptides with MHC on the APC surface are also embedded in a type of membrane raft (102–104). This specific lipid environment helps to maintain the clusters of these antigen-presenting molecules in an arrangement optimal for recognition by the TCR. Thus, there is a remarkable symmetry between functional lipid rafts on the apposed surfaces of the APC and the T cell.

Conclusions

During the past approximately 12 years, the field of 'raftology' went through a remarkably rapid, sometimes controversial and slightly confusing development, from the initial skeptical period to the present explosion of interest in almost all areas of membrane biology. There are many branches of molecular immunology in which the raft concept was fruitful and brought fresh explanations of immunologically relevant phenomena. It is clear that membrane rafts play important roles in the TCR (and other immunoreceptor) signaling pathways. Manipulation of the TCR interactions with membrane rafts may present interesting potential targets for manipulation of immune responses for therapeutic purposes. However, as is possibly obvious from this review, the field is still full of open questions and uncertainties, some of them concerning the very essentials of membrane rafts. It will be very important to define in the future which other types of lipid-based microdomains exist on the cell surface in addition to those very few presently known and relatively easily biochemically accessible. At present it seems that too many things are happening in the rafts and too much of the cell surface is covered by them. Perhaps, soon, it will be more interesting to study the minor, nonraft fraction of the membrane and the reactions happening there?

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