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REVIEW

Cytoskeleton–membrane interactions in membrane raft structure

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Abstract Cell membranes are structurally heterogeneous, composed of discrete domains with unique physical and biological properties. Membrane domains can form through a number of mechanisms involving lipid–lipid and protein–lipid interactions. One type of membrane domain is the cholesterol-dependent membrane raft. How rafts form remains a current topic in membrane biology. We review here evidence of structuring of rafts by the cortical actin cytoskeleton. This includes evidence that the actin cytoskeleton associates with rafts, and that many of the structural and functional properties of rafts require an intact actin cytoskeleton. We discuss the mechanisms of the actin-dependent raft organization, and the properties of the actin cytoskeleton in regulating raft-associated signaling events. We end with a discussion of membrane rafts and the actin cytoskeleton in T cell activation, which function synergistically to initiate the adaptive immune response.

Keywords Membrane rafts · Actin cytoskeleton · Phosphatidylinositol 4 · 5 bisphosphate · T cell signaling · Src family kinases

Introduction

Eukaryotic cell membranes consist of a heterogeneous but regulated environment that serves as a dynamic platform for cell functions. Membrane heterogeneity includes discrete membrane domains that form through a combination of protein and lipid interactions [1, 2]. Structurally, membrane domains range from nanoscale structures to molecular assemblies that are microns in size [3–6]. Research shows that establishing and maintaining this membrane heterogeneity is important for cell viability [7–10]. Similarly, some pathologies are associated with changes in the structure and composition of membrane domains [11–13], and some pathogens utilize membrane domains to gain entry or exit from the target cell [14–17]. The functional properties of membrane domains in cell viability and pathology underscore the importance in understanding membrane structure and the mechanisms by which it is established.

One important example of structural and functional membrane domains is the cholesterol-dependent rafts (Fig. 1) [6, 18, 19]. Many of the properties of rafts have been inferred from detergent-resistant membranes (DRMs) that occur in nonionic detergent lysates of animal cells [2, 20, 21]. In the membrane raft model, the DRMs represent poorly solubilized rafts [20], and the composition of the DRMs has served as a guide to the structural and functional properties of rafts. For example, DRMs are enriched with cholesterol and sphingolipids, and it is posited that these lipids provide the structural framework by which the rafts form. Experiments with model membranes show these lipids interact to form a discrete liquid-ordered (Lo) phase in the bilayer [22–24]. Furthermore, Lo phase lipids produced in liposomes exhibit many of the physical properties evidenced for rafts in cell membranes, including resistance to solubilization by nonionic detergents and enrichment with proteins and lipids that occur in DRMs [2, 25]. DRM studies have also identified the signals that target proteins to rafts, and these include palmitoylation of a membrane-proximal cysteine, and addition of a glycophosphatidylinositol (GPI) anchor [20, 26].

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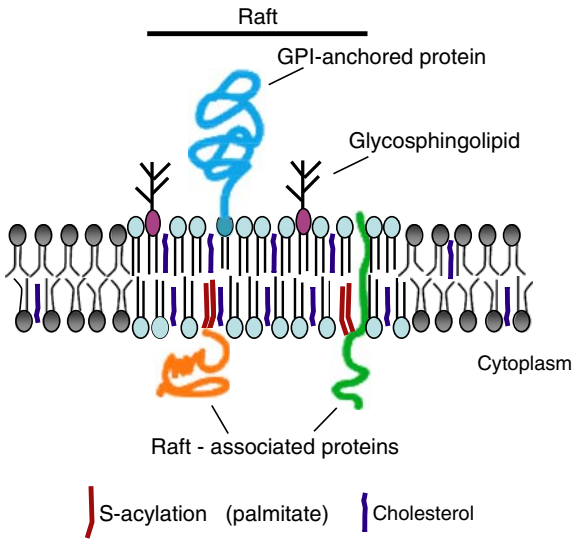

Fig. 1 Membrane raft model. Cholesterol associations with other membrane lipids, such as sphingolipids, generate a discrete lipid compartment or domain with unique physical and biological properties. The cholesterol confers an ordering on the lipids that imparts changes in the physical properties of the bilayer, including an increase in bilayer width. Through a poorly understood mechanism, the rafts are coupled across the bilayer. Proteins that prefer an ordered lipid environment associate with the domains, often through a discrete targeting signal. A frequent raft-targeting signal for proteins is S-acylation, represented by palmitoylation of a membrane-proximal cysteine. Also enriched in rafts are GPI-anchored proteins. The *blue* and *gray circles* represent raft and nonraft lipids, respectively

One shortcoming of the DRM studies is that the detergents are disruptive to membrane structure, and their effects on cell membranes are too complex to draw substantive conclusions regarding the organization of lipids and proteins in the native membrane. For example, detergents used to generate DRMs have been shown to create domains, and to cause mixing and retention of proteins and lipids irrespective of their intrinsic affinity for ordered lipids [27]. Despite the argument that DRMs are detergent artifacts produced during cell lysis [28], in situ imaging studies have provided compelling evidence of nonrandom and clustered distributions for molecules that occur in DRMs [1].

Imaging studies of DRM-associated proteins and lipids show a hierarchical nature to membrane raft structure [1, 3, 4, 29, 30]. The smallest are raft nanoclusters that are approximately 10 nm in diameter, and these are enriched in larger domains that can be several hundred nanometers in size. Even larger are micron-size raft macrodomains that occur in activated cells. The raft macrodomains distinguish themselves as large-scale raft structures by their specific enrichment of proteins and lipids that occur in membrane rafts, and by their resistance to solubilization by nonionic detergents [30, 31]. Examples of raft macrodomains are: the immunological synapse (IS), which forms in activated lymphocytes where they bind to cells bearing antigen [32]; the leading edge and uropod of motile cells [33–35]; and cytoskeleton-rich adhesion complexes [36, 37]. These include both cell-to-cell and cell-to-matrix complexes, such as the adherent junctions and focal adhesions [38, 39] [40–42].

Structuring of membrane rafts by the actin cytoskeleton

Early descriptions of the membrane raft model evoked the notion that interactions between cholesterol and the sphingolipids generate a lipid platform with which specific proteins associate [2, 21] (Fig. 1). Findings that are more recent, however, suggest that the rafts form in part through capture and stabilization of raft lipids by proteins. As one example, measurements of protein distributions in the plasma membrane showed that clustering of raft-associated proteins was not saturable: the fraction of protein that clustered remained constant with increasing protein expression [43, 44]. This finding suggests that the rafts come about through an ordering of lipids by membrane proteins rather than the rafts occurring as pre-formed lipid complexes with which specific proteins associate. Consistent with this interpretation, protein-dependent ordering of lipids to form Lo phase lipids has been demonstrated in at least two separate studies: cholera toxin B subunit (CTB) binding to liposomes that contain its ligand the ganglioside GM1 [45, 46], and GAP-43 and MARCKS binding to membranes that contain phosphatidylinositol 4, 5 bisphosphate (PIP_2) [47].

Refinements of the lipid raft model now ascribe an important role for membrane-associated proteins in forming rafts [43, 48]. One example is the cortical actin cytoskeleton, which is composed of a lattice network of filaments that underlie and attach to the plasma membrane. Proteomic studies show that DRMs are particularly enriched with cytoskeletal proteins, indicative of interactions between the actin cytoskeleton and membrane rafts that could be important in forming and maintaining the rafts. Figure 2 illustrates examples of cytoskeletal proteins that are enriched in rafts, and these include actin, tubulin, myosin, actinin, and supervillin [49–54].

In a recent study from our laboratory, we compared the role of cholesterol and F-actin in the clustering of membrane-targeted fluorescent proteins by imaging their fluorescence resonance energy transfer (FRET) [29]. First, we observed a co-clustering that was specific to where both the donor and acceptor were associated with rafts. The co-clustering of raft proteins occurred for probes that contained entirely different membrane-targeting signals, thus showing that it was not restricted to one type of raft-targeting signal. As predicted from the membrane raft model,

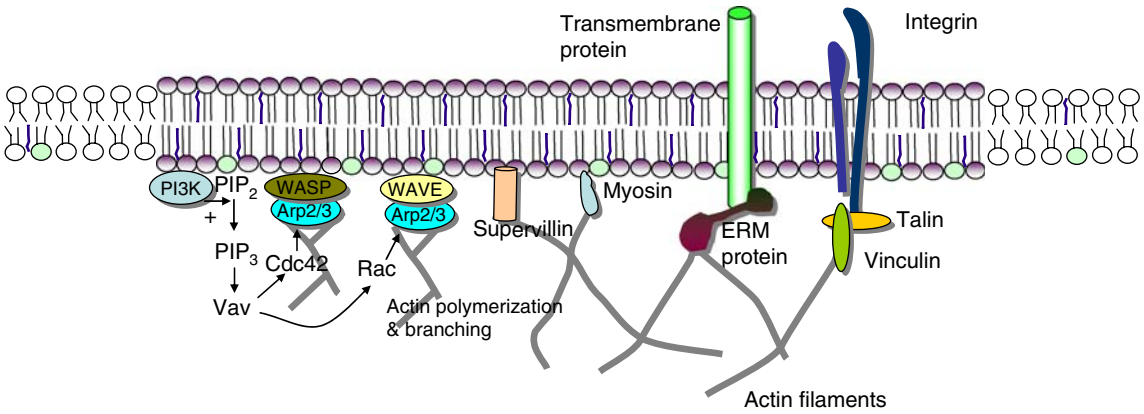

Fig. 2 Mechanisms of raft association with the actin cytoskeleton. Measurements of DRMs show rafts are enriched with protein and lipid effectors that function in tethering actin filaments to the plasma membrane. One important example is the lipid cofactor PIP_2, which occurs in the cytoplasmic leaflet of the plasma membrane. Another important lipid effector for raft-actin interactions is the phosphoinositide 3-kinase (PI3K)–product PIP_3. The PIP_3 is necessary for activation of the Rho family GEF Vav. The Rho GTPases activate actin polymerization via Wiskott–Aldrich syndrome family protein (WASP) and WASP family Verprolin-homologous protein (WAVE). These in turn activate actin polymerization and branching through the Arp2/3 complex. Other raft-associated proteins that bind actin filaments are supervillin, myosin-IIA, and myosin IG. Ezrin–radixin–moesin (ERM) proteins link transmembrane proteins, such as adhesion receptors, to the actin cytoskeleton. ERM proteins are regulated by PIP_2, which binds the FERM domain of the ERM proteins. Activated integrins associate with membrane rafts. Talin links integrins to the actin cytoskeleton either directly or indirectly by interacting with another cytoskeletal protein vinculin. PIP_2 regulates talin interactions with integrins, actin, and vinculin

treating the cells with the cholesterol-binding agent filipin specifically inhibited the co-clustering of probes that associate with DRMs. Interestingly, disrupting the actin cytoskeleton with latrunculin B (Lat B) was in some instances more effective than filipin in disrupting co-clustering of raft-associated probes. Finally, co-treating cells with Lat B and filipin resulted in an entirely random distribution of the donor and acceptor probes. Thus, membrane cholesterol and the actin cytoskeleton were sufficient to account for all co-clustering of the raft markers.

Experiments with model membranes show that actin filaments impart an ordering effect on bilayer lipids [55], a property that may underlie structuring of membrane rafts by the actin cytoskeleton. Similarly, environment sensitive lipophilic fluorescent dyes show that the lipids in actin-rich macrodomains of the plasma membrane are condensed in relation to remaining regions of the plasma membrane [56]. This is consistent with the lipids occurring in a relatively ordered state. In Fig. 3 are data that show a decondensation of the plasma membrane in cells treated with Lat B, indicating that an actin-dependent ordering of lipids is a global property of the plasma membrane.

The model in Fig. 4 relates the nano- and micrometer scale clustering of rafts to the structure of the actin cytoskeleton. The nanoclusters are dispersed as complexes associated with filamentous actin. Activation signals bring about an enrichment of actin filaments in the cortical cytoskeleton, which drives the large-scale clustering of rafts to form macrodomains. Also illustrated in Fig. 4 is a network of compartments or corrals established by the actin filaments. This produces a caging effect that can transiently hinder the diffusion of proteins in the plasma membrane. However, the caging is largely nonspecific in nature [57–59], and this contrasts with the specific clustering of raft proteins that actin filaments can generate. The contribution of the caging or corralling in establishing rafts is not known, but current evidence suggests that it represents a separate event that occurs on a time scale that is much shorter than the lifetime of the rafts [58–60].

The notion of an actin-dependent structuring of membrane rafts is an attractive model in that it merges the dynamic properties of the actin cytoskeleton with the important roles that rafts have in membrane functions. Accordingly, signals that cause a localized enrichment and membrane-attachment of actin filaments are predicted to cause a co-enrichment of membrane rafts. This property is exemplified by the raft macrodomains, which form as a result of stimulatory signals that activate actin polymerization and attachment of actin filaments to the plasma membranes [32, 33, 61–64]. The macrodomains in turn have specific functions in cell signaling, adhesion, and motility, and the interactions of rafts with the cytoskeleton localize and maintain these functions to discrete regions of the cell surface.

Compartmentalization of PIP_2 signaling to membrane rafts

Structuring of rafts by the actin cytoskeleton suggests that proteins and lipids that tether actin filaments to cell

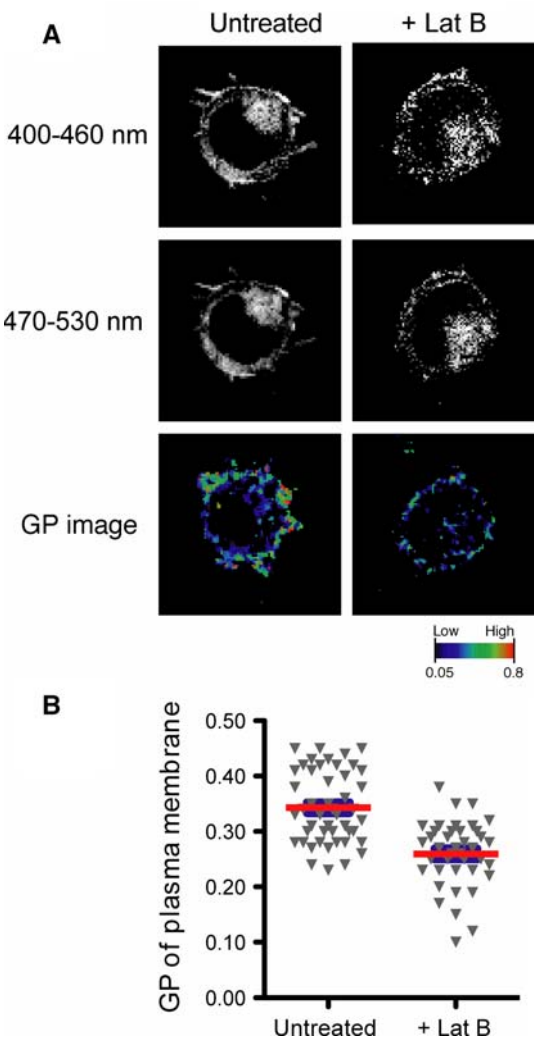

Fig. 3 Global condensation of the plasma membrane by the actin cytoskeleton. The lipophilic dye Laurdan is an environment sensitive fluororophore that serves as a reporter of water penetration into membrane bilayers [126]. Increased lipid packing due to lipid ordering results in a blue shift in the Laurdan emission spectrum, from centered at approximately 500 nm in fluid bilayers to 445 nm in ordered membranes [127]. The normalized ratio of emission channels centered on these wavelengths has been used as a measure of relative membrane ordering [128]. T cells were treated with Laurdan before disrupting the actin cytoskeleton by treating with latrunculin B (Lat B). The samples were imaged in two separate channels, represented by emission wavelengths 400–460 and 470–530 nm using a Leica SP2 multi-photon confocal microscope. General polarization (GP), which reflects the relative lipid condensation [128], is therefore an indicator of lipid ordering. GP was calculated for each pixel in the plasma membrane using the equation $GP = I_{(400–460)} - I_{(470–530)} / (I_{(400–460)} + I_{(470–530)})$ [56]. GP values range between −1.0 and +1.0, and they are directly proportional to the relative membrane condensation. **a** Images in the indicated channels of untreated control and Lat B-treated T cells. The *bottom row* shows the calculated GP image for each sample. **b** Average GP values of the plasma membrane measured in approximately 50 separate untreated or Lat B-treated T cells

membranes are also important for forming rafts. An important regulator of membrane–cytoskeleton interactions is the phosphoinositide PIP_2, which serves as a co-factor for many of the proteins that anchor actin filaments to the plasma membrane [65, 66]. Protein binding to PIP_2 often occurs through a PIP_2-specific recognition sequence, in many cases represented by a PIP_2-specific pleckstrin homology (PH) domain [67–69]. PIP_2-regulated proteins include the ezrin–radixin–moesin (ERM) proteins and talin (Fig. 2) [70–73] [74, 75]. The ERM proteins anchor F-actin to membrane proteins that contain a FERM-binding sequence. Talin tethers actin filaments to integrins. Related to PIP_2 is the PI3K product PIP_3, which also regulates interactions between the cytoskeleton and plasma membrane [76, 77]. For example, PIP_3 activates the guanine nucleotide exchange factor (GEF) Vav [78]. Downstream of Vav are the Rho GTPases Rho, Cdc42, and Rac. Cdc42 and Rac activate Wiskott–Aldrich syndrome family protein (WASP) and WASP family Verprolin-homologous protein (WAVE), respectively [79–81]. These in turn activate the Arp2/3 complex, which binds F-actin and brings about further actin polymerization and branching of actin filaments [82, 83].

Although PIP_2 is enriched in rafts [80, 84–86], the notion of raft compartmentalization of its functions to rafts has been controversial. For example, evidence of PIP_2 compartmentalization has often been surmised based on an inhibition of its functions by cholesterol-binding agents such as methyl-β-cyclodextrin (MβCD). Drug treatment such as this can produce nonspecific changes in membrane structure that indiscriminately affect membrane functions. Similarly, Jalink and co-workers failed to detect a cholesterol-dependent clustering of PIP_2 using FRET to measure the proximity of labeled PIP_2-specific PH domains [87].

One recent study from our group detected compartmentalization of PIP_2 functions in intact cells by expressing membrane-targeted forms of the PIP_2-specific phosphatase Inp54p [88]. Inp54p was targeted to either the raft or nonraft membrane fractions using minimal membrane-anchoring signals, and this selectively increased or decreased the raft pools of PIP_2 without changing the global PIP_2 content of the cell. Reducing raft PIP_2 levels produced cells that had a smooth morphology that was void of the membrane ruffles and filopodia that occurred in normal control cells. Another property was an increase in the number of blebs that formed from the plasma membrane, indicating disassociation of the plasma membrane from the underlying actin cytoskeleton [89]. In contrast, increasing raft PIP_2 levels resulted in cells with a striking morphology that included numerous filopodia and

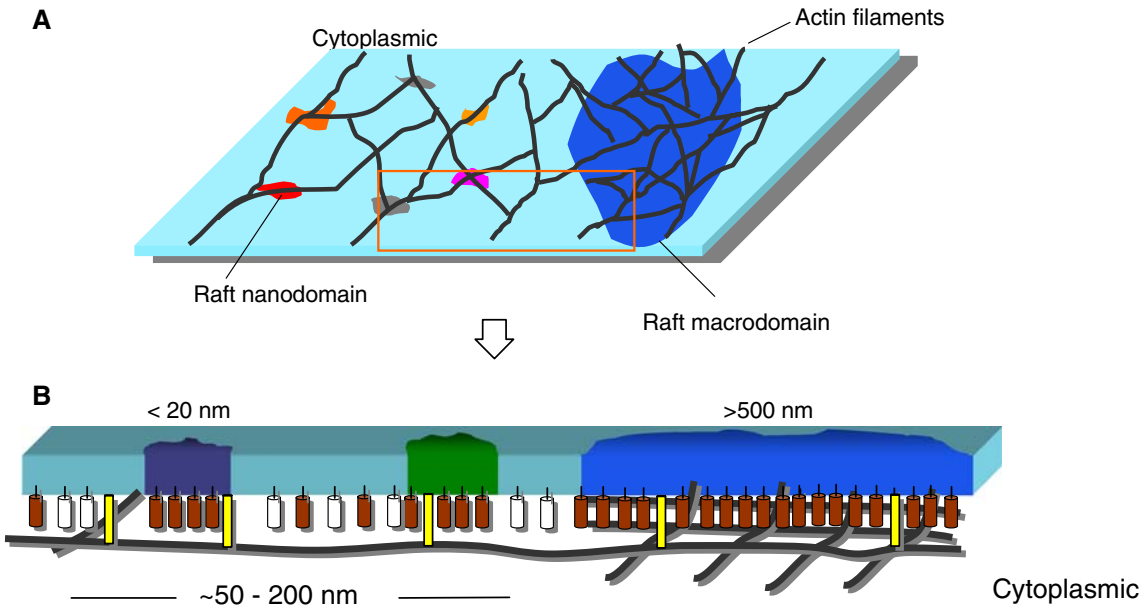

Fig. 4 Actin cytoskeleton-dependent raft organization. **a** Topography of raft nanoclusters and macrodomains. Rafts occur as nanoclusters that are less than 20 nm in diameter, but can cluster to larger structures to include macrodomains that are microns in size. Both the nanoclusters and larger assembles form in an actin-dependent manner [29, 31, 129]. The macrodomains form as a result of signals that activate actin polymerization and attachment of actin filaments to cell membranes. Examples of raft macrodomains include immunological synapses that form in activated lymphocytes, cell–cell adhesion complexes, leading edge and uropod of migrating cells. **b** The cortical actin cytoskeleton is tethered to the plasma membrane through protein linkers, and these interactions likely structure rafts in the membrane. The meshwork of cortical actin filaments also compartmentalizes the membrane into areas approximately 50–200 nm in size. The compartments represent areas of transient and nonspecific membrane protein confinement by the underlying actin filaments

extensive membrane ruffling. Enrichment of raft PIP_2 also increased cell spreading on poly-L-lysine coated glass surface. Both the cell morphology and cell spreading were sensitive to the PI3K inhibitor wortmannin, suggesting that the phenotype produced by increasing raft PIP_2 was due to compartmentalization of either PIP_3 or production PIP_3-dependent signaling. Interestingly, expression of dominant-negative and constitutively active forms of the Rac generates cell morphologies similar to that which we reported for the raft- and non-raft-targeted Inp54p molecules, respectively [90]. Accordingly, the morphology that was evidenced by increasing raft PIP_2 may reflect activation of signals in the Vav–Rac pathway (Fig. 2). This pathway may also give rise to rafts by activating interactions between the cytoskeleton and plasma membrane.

Protein regulation by raft-actin interactions

Membrane rafts in most cell types are enriched with signaling molecules, and a wealth of biochemical and genetic data have provided credence to the notion that rafts function as a specialized signaling platform in cell membranes [26, 91–96]. Furthermore, data show that the actin cytoskeleton participates in regulating and activating raft-associated signaling events [97–99]. For example, separate studies have shown where protein activity and regulation are tied directly to an intact actin cytoskeleton, and its association with membrane rafts. Examples of this property include G-protein coupled receptors (GPCRs) [100], ERK [101], and Src family kinases (SFKs)[29].

The SFKs illustrate an actin-dependent compartmentalization of protein regulation through sequestering from an important activator. As exemplified by the T cell specific SFK $p56^{lck}$ (Lck), the raft-associated pool of SFK is down-regulated relative to that in the nonraft membrane fraction due to sequestering from its activator CD45 [102]. Additional regulation of SFKs by membrane rafts is achieved through phosphorylation by Csk, which associates with rafts by binding to Csk binding protein (Cbp) or phosphoprotein associated with GEMs (glycolipid-enriched membrane) (PAG) [103]. Experiments from our group showed that treating T cells with Lat B resulted in deregulation of Lck. Furthermore, the changes in Lck regulation were CD45-dependent since the Lat B treatment had no affect on Lck in CD45-deficient T cells [29]. These data together with that regarding actin-dependent clustering of raft proteins suggest a model where membrane-associated actin filaments establish membrane rafts, which then sequester Lck and other raft-associated SFKs from CD45 (Fig. 5). Accordingly, treating T cells with Lat B disrupts the rafts and the sequestering of SFKs from CD45, leading

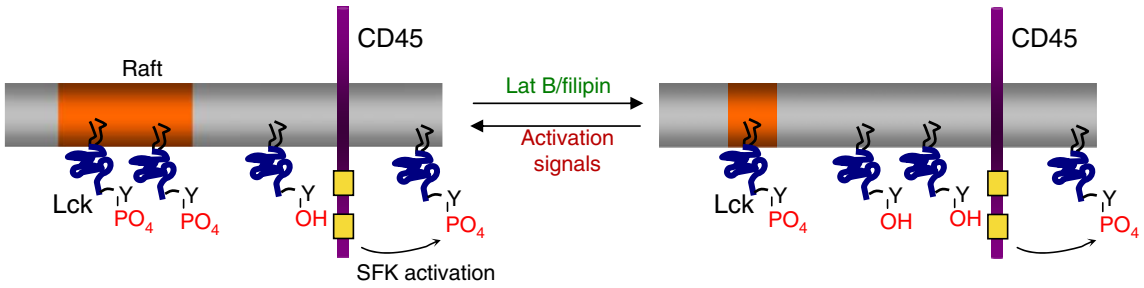

Fig. 5 Model for raft protein regulation by cholesterol and the actin cytoskeleton. The Src family kinase (SFK) Lck is activated by the membrane phosphatase CD45 through dephosphorylation of its regulatory C-terminal tyrosine Tyr^{505}. A significant fraction of Lck associates with rafts, yet CD45 is restricted to the nonraft membrane fraction. Raft association of Lck therefore sequesters it from CD45, leading to reduced activity for the raft-associated pool of molecules. Drug treatments that disrupt rafts, such as filipin and Lat B, eliminate this sequestration, resulting in activation of Lck [29]. Conversely, signals that activate actin polymerization and assembly of rafts, such as that from the TCR in T cells, are predicted to enhance the sequestration and down-regulation of raft-associated Lck

to deregulation of the SFKs. Consistent with this model, filipin treatment also deregulated Lck. The greatest Lck deregulation occurred by co-treating the cells with Lat B and filipin. FRET measurements showed these conditions abolished all co-clustering of raft-associated probes.

Converse to the changes in raft structure and regulation by the drug treatments, activation signals lead to clustering of small raft structures to form large complexes such as the IS [1]. This is predicted to lead to enhance sequestering of CD45 from raft-associated complexes. In the case of T cells, this sequestration is necessary to maintain activation signals since the phosphatase activity of CD45 will quench the TCR-dependent phosphotyrosine signals [104]. Accordingly, T cell activation consists of a choreography where CD45 is first localized at the cell interface with the APC, and then excluded as the IS matures. These properties may underlie interactions between CD45 and SFKs that are first necessary for protein activation, and then removed as the rafts accumulate to form an IS and maintain the activation signals.

Membrane rafts, actin cytoskeleton, and T cell activation

T cell receptor (TCR) signaling exemplifies the interplay between membrane rafts, the actin cytoskeleton, and cell signaling. T cells are activated by antigenic peptide that is presented to the TCR in the context of major histocompatibility complex (MHC) by antigen presenting cells (APCs). TCR signaling is compartmentalized to rafts, and one of the earliest outcomes of these signals is actin polymerization and an actin-driven clustering of rafts [95, 105]. Both the rafts and the signal-driven actin polymerization are necessary for T cell activation; disrupting either the rafts or the actin cytoskeleton inhibits activation [105, 106]. The raft clustering following TCR engagement culminates with formation of an IS where the T cell contacts the APC [107–110]. The IS is necessary to sustain the TCR signals [111–113]. Disrupting the IS inhibits activation-dependent cell proliferation and cytokine secretion [32].

The co-stimulatory receptor CD28 [114], the integrin LFA-1 [115], and the adhesion receptor CD2 [116] can each provide signals that activate actin polymerization and raft clustering independent of the TCR. In some cases, these signals are sufficient to form an IS independently of the TCR [117]. Furthermore, signals from the TCR alone are often not sufficient to cluster rafts and form an IS. This is particularly the case with naïve T cells, which require a co-stimulatory second signal for their raft clustering [94]. The requirement of TCR and co-stimulatory signals for raft clustering coincides with the two-signal requirement for T cell activation [94, 118]. CD28 is the principal co-stimulatory receptor in naïve T cells, engagement of which is necessary to stimulate IL-2 production [119]. Biochemically, CD28 has no intrinsic activity, but rather functions as a linker protein that recruits specific effectors, including PI3K, Lck, Itk, and filamin A [109, 110, 120, 121]. These effectors also activate actin polymerization or actin-binding to the plasma membrane. However, the hierarchy regarding those most important in providing signals for the actin-dependent raft clustering is largely undefined.

Although the CD28-dependent properties of raft clustering and co-stimulatory signals suggests these events are related, the kinetics by which they occur suggest otherwise. For example, CD28 co-stimulatory signals coincide temporally with those from the TCR [122], which are facile and occur within seconds of its engagement with peptide-bound MHC (pMHC) [123]. However, the actin-driven clustering of rafts takes tens of seconds or minutes [124], indicating that it is too slow to participate proximal to the TCR signaling. Nevertheless, T cell rafts undergo an Ag-independent clustering during initial interactions with the APC as they survey its surface for Ag. We term this early clustering event raft clustering during T cell surveying (RaCS). The RaCS occurs before evidence of TCR-

dependent signals, such as TCR stop signals and Ca^{2+} flux, and it concentrates the CD4 co-receptor and TCR at the APC [125]. Optimization of the T cell membrane environment for TCR signaling by RaCS is predicted to lower the threshold of Ag that is necessary to active the T cell, which is also an important property of co-stimulation. However, it is not known if the early raft clustering event reflects co-stimulatory functions.

Conclusions and perspectives

Recent findings show a synergistic interaction between the actin cytoskeleton and membrane rafts. Actin filaments closely associate with the plasma membrane in many cellular processes. Membrane rafts are enriched with the modulators of cortical actin cytoskeleton and with factors that anchor actin filaments to the plasma membrane. Furthermore, the actin cytoskeleton regulates the clustering of membrane raft proteins in a specific manner and at nanoscale level of membrane structure. The actin cytoskeleton is a highly dynamic structure, and its role in establishing membrane rafts provides one avenue for tuning the membrane microenvironment in a manner that favors or inhibits discrete membrane functions. However, further studies are necessary to better resolve the mechanism for the actin-dependent structuring of membrane rafts.

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