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Monitoring the distribution and dynamics of signaling microdomains in living cells with lipid-specific probes

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Abstract. Specialized lipid microdomains in the cell plasma membrane, referred to as 'lipid rafts', are enriched in sphingolipids and cholesterol and have drawn considerable interest as platforms for the recruitment of signaling molecules. Despite numerous biochemical and cellular studies, debate persists on the size, lifetime and even the existence of lipid rafts, emphasizing the need for reliable lipid probes to study *in situ* membrane lipid organization. In this review, we summarize our recent data on living cells using two specific probes of raft components: lysenin, a sphingomyelin-binding protein and the fluorescein ester of poly(ethyleneglycol)cholesteryl ether that labels cholesterol-rich domains. Sphingomyelin-rich domains that are spatially and functionally distinct from the GM1 ganglioside-rich domains were found at the plasma membrane of Jurkat T cells. In addition, the dynamics of cholesterol-rich domains could be monitored at the cell surface as well as inside the cells.

Keywords. Lipid raft, microdomain, sphingomyelin, cholesterol, lysenin.

Introduction

Considerable evidence supports lateral heterogeneity in lipid composition and physical properties of biological membranes [1]. Based on model membrane studies, it is commonly assumed that in the cell plasma membrane, sphingolipids and cholesterol form a liquid-ordered (lo) phase that coexists with the bulk of the phospholipids organized in a liquid-disordered (ld) phase that is more fluid. In fact, the long, saturated acyl chains of sphingolipids allow them to tightly pack together, but with a high degree of lateral mobility due to the presence of cholesterol [2]. However, there is considerable controversy with regard to the size of the lipid rafts, since they are speculated to be too small to be observed by light microscopy (with a diameter as small as 100 nm). These microdomains could play an important role in many cellular processes, including signal transduction, membrane trafficking, cytoskeletal organization, adhesion, migration and pathogen entry. This diversity of function is accompanied by the diversity of lipid as well as protein composition, in addition to the different cellular localizations (e.g., the plasma membrane, Golgi, endosomes). Furthermore, another level of heterogeneity derives from the dynamic association of these microdomains. Due to the difficulty in visualizing these domains in living cells, most of the data regarding their existence and function have been gathered by indirect methods such as by applying non-ionic detergent at low temperature or cholesterol depletion with methyl-β-cyclodextrin (MβCD), and recent reports have pointed out problems with these techniques [3, 4]. The concept of lipid microdomains has thus not yet reached a consensus in terms of their composition, size or even actual functional existence in living cells [5–7]. Thus, reliable lipid probes are urgently needed in order to define the membrane lipid organization *in situ*.

This review will first discuss the functional role of lipid microdomains as signaling platforms in the plasma membrane, taking as an example T cell receptor

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(TCR) activation. Then recent data obtained from living cells using two specific probes will be presented. These probes were developed in the laboratory for the major components of the lipid rafts: lysenin, a sphingomyelin (SM)-binding protein that provides evidence for spatial and functional heterogeneity of SM-rich membrane domains in the cells and the fluorescein ester of poly(ethyleneglycol)cholesteryl ether (fPEG-Chol), which can be used to monitor the dynamics of cholesterol-rich membranes.

Lipid microdomains and signal transduction

Research in cell signaling has led to an understanding of how molecules are organized and sequestered in different compartments within cells. Such compartmentalization has been connected to the presence of specialized lipid domains in the plasma membrane. Whereas the lipid raft membrane concept was originally employed to explain the selective delivery of lipids and lipid-anchored proteins to the apical and basolateral surface of polarized epithelial cells [8], it then evolved into an explanatory model integrating not only lipid-lipid interaction but also protein-lipid interaction, mainly situated at the plasma membrane and in the lipid biosynthetic and endocytic pathways. Several reviews have been published recently that discuss in detail the raft concept and its role in the regulation of cell signaling [9–13].

How do lipid microdomains regulate cell signaling?

Laterally compartmentalized lipid microdomains at the plasma membrane are considered to serve as platforms regulating the induction of signaling pathways, owing to their ability to recruit or exclude specific lipids and proteins [14, 15]. They are often thought to exert a positive effect by maintaining various signaling components (receptors, coupling factors, enzymes and substrates) in a restricted domain, thus inducing a more rapid and efficient coupling. They can provide regulation *via* compartmentalization by limiting the access of nonspecific components or inhibitors. In certain systems such as TCR or some of the tyrosine kinase receptors such as the epidermal growth factor (EGF) receptor, rafts play a more subtle regulatory role due to the fact that different signaling components can be localized in both the raft and non-raft parts of the membrane [16, 17].

The fact that lipid microdomains are dynamic structures in which both lipids and proteins can move with different kinetics is essential for their role in signal transduction, but this dynamism has also made it exceedingly difficult to study their functional presence in living cell membranes. Thus, controversy has arisen concerning their size and even their existence under steady-state conditions (resting cells) versus the activated state [18]. Characterizing the topography of signaling molecules in living cell membrane using lipid-specific probes at the nanometer scale is crucial for mapping the interactions of proteins and lipids during cell signaling and for visualizing the clustering (and co-clustering) of lipid microdomains. However, due to the heterogeneity of the lipid components of such microdomains, it is essential to be able to confirm and characterize the obtained existence by using more than one probe. This emphasizes the need for reliable and specific probes that do not interfere with the lipid domain topology.

Lipid-specific probes to study cell lipid microdomains

Various probes have been used to study lipid microdomain organization in cell membrane. We will mainly focus on those used in the cell imaging, thus allowing a more direct approach to understanding the spatial organization of these domains and their involvement in the compartmentalization of signaling components. All of them have advantages linked to their lipid specificity as well as limitations. In fact, the major problems using probes that partition in the membrane are interference with membrane organization and unexpected secondary cellular effects after sequestration, as is the case with certain cholesterol probes.

Several cholesterol-binding probes are used for cholesterol imaging in the cell membrane. Filipin, a polyene antibiotic, is used to detect free cholesterol in fixed cells, but it is not suitable for living cells due to cytotoxicity. Furthermore, its detection is limited by rapid photobleaching [19, 20]. Recently, perfringolysin θ (θ toxin), a cytolysin produced by *Clostridium perfringens*, has been shown to selectively bind cholesterol-rich membrane domains in living cells [21]. A protease-nicked and biotinylated derivative (BCθ) has been produced that does not cause membrane damage [22]. However, since it does not have intrinsic fluorescence, its detection requires extrinsic labeling with fluorescent avidin. In contrast, dehydroergosterol (DHE) is a naturally occurring fluorescent cholesterol analog that has been used to study free cholesterol trafficking in living cells [23]. However, due to its absorption and emission in the UV and blue regions, its detection requires a special microscope setting in order to attain sufficiently high quantum efficiency. It is not clear whether exogenously added DHE co-localizes with endogenous cholesterol. Recently, fPEG-Chol was used to follow the dynamics of cholesterol-rich domains in the

membrane due to its water solubility and low toxicity [24, 25]. We will develop its properties in greater detail below.

Among the sphingolipid-binding toxins, the cholera toxin subunit B (CTB), which binds as a pentamer to glycosphingolipid GM1 [Galβ1,3GalNAcβ1, 4(Neu-Acα2,3)Galβ1,4Glcβ1-1′-ceramide), is one of the most widely used lipid raft markers. Although GM1 is not ubiquitous, it is reported that GM1 is enriched in caveolae and lipid rafts [26]. CTB has then been proven to be useful not only to detect GM1-enriched domains at the plasma membrane, but also to follow the retrograde lipid trafficking from the plasma membrane to the Golgi and the endoplasmic reticulum [27]. However, although the compartimentalization of GM1 ganglioside to the site of TCR engagement has been clearly demonstrated [28], GM1-rich domains are not the only domain involved in T cell activation [29], emphasizing the need of more than one specific lipid probe to study raft involvement in cell signaling.

Various toxins are reported to bind SM [30–33]. Among them, lysenin was recently demonstrated as a useful tool to study SM-rich membrane domains [29, 34–36] and will be described in more detail.

In general, lipid-binding proteins, despite their high affinity for lipids, have the possible problem of altering the membrane organization due to their molecular weight being higher than that of lipid molecules. Thus lower molecular weight peptides or chemical compounds would likely be more reliable. The fact that probes like antibodies or CTB can also induce lipid raft clustering by cross-linking has to be carefully considered as well, since they can change the size of the lipid domains. This has been pointed out for CTB, which is pentavalent and can have an effect similar to cross-linking, thereby changing the size of the GM1-enriched domains.

Lipid rafts and the modulation of TCR signaling

The role of plasma membrane lipid microdomains in TCR signaling has been intensively investigated and debated and will be discussed here. One of the first pieces of evidence for the functional role of lipid rafts came from studies of the TCR, which is activated at the cell surface of T lymphocytes after antigen binding, then transmits a signal to the cytoplasm and the nucleus by a cascade of protein-mediated signaling events. Such compartmentalization inside lipid microdomains is considered to be vitally important for the sequential recruitment of the signaling molecules necessary for TCR activation [37]. However, recent results have pointed out different roles for lipid-mediated and protein-mediated interactions in early TCR signaling and during the formation of the larger membrane domains that form the immunological synapse [16, 38].

The TCR is composed of α and β chains and is activated after the binding of peptide antigen presented by major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs) [39]. The TCR short cytoplasmic tails cannot mediate signal on their own but are non-covalently associated with the transmembrane CD3 signaling chains γ, δ, ε and ζ, which sense the MHC peptide, then initiate TCR activation signaling. The CD3 signaling chains are devoid of intrinsic kinase activity, and possess a phosphorylation motif called the immunoreceptor tyrosine-based activation motif (ITAM). Upon TCR activation, ITAMs of the CD3 chains become phosphorylated by the Src-family tyrosine kinase Lck allowing the binding of the cytosolic Syk family ZAP-70. Once recruited, ZAP-70 becomes phosphorylated and thus activated, then can in turn phosphorylate many proteins involved in T cell activation: the stimulation of phospholipase Cγ1 (PLCγ1), recruitment of phosphatidylinositol-3-kinase (PI3K) and phosphorylation of the linker for activation of T cells (LAT), which is central to coordinate T cell activation since phosphorylated LAT serves as the docking site for PI3K, PLCγ1 and Grb2 [40, 41]. In addition, other surface receptors participate in T cell activation: the CD4 and CD8 co-receptors increase MHC-peptide recognition by the TCR by directing Lck to the TCR/CD3 complex.

Early studies have suggested that lipid raft partitioning controls interactions among proteins of the TCR signaling machinery and modulates their function. Using the detergent extraction or cholesterol depletion approach, transient raft-facilitated interactions of the proteins involved in TCR signaling, Lck or LAT, have been shown. The tyrosine kinase Lck, which contains a dual acylation, is considered to be constitutively present in lipid rafts [42, 43]. Similarly, the transmembrane adaptor LAT is targeted to rafts through a dual palmitoylation [44]. Once LAT is phosphorylayed during initiation of T cell signaling, other signaling proteins can bind to it and thus may become indirectly associated to lipid rafts [45–47]. For example, the CD8 co-receptor appears to increase TCR activation through the stronger raft partitioning of its CD8αβ heterodimer [48, 49]. Moreover, the ability of various components to translocate in and out of lipid raft domains is important for regulation of TCR activation. For example, hyperphosphorylated and thus inactive Lck in lipid rafts appears to be sequestered from the tyrosine phosphatase CD45, which is absent from rafts [50].

Despite extensive characterization, the true physiological role of lipid rafts in TCR activation remains

controversial. The importance of lipid membrane organization has been confirmed by confocal microscopy observation of the accumulation of the raft marker GM1 at the TCR active sites leading to higher and more stable tyrosine phosphorylation [28, 51]. Recently, condensation of the plasma membrane at the sites of TCR activation was observed in the membrane of T lymphocytes using the fluorescent probe Laurdan, a lipophilic molecule whose fluorescence emission spectrum changes depending on the condensation and order of its membrane environment [52]. Thus, such condensed membrane domains, i.e., liquid-ordered domains, reflect lipid raft accumulation. They were dependent on the activity of the Src family kinase (Lck, Fyn) and the presence of LAT and stabilized by the actin cytoskeleton. This emphasizes the connection between the actin cytoskeleton, large scale-ordered membrane domains and TCR signaling [53].

Fluorescence resonance energy transfer (FRET) analysis revealed the existence of nanometer-size clusters around glycosyl-phosphatidylinositol (GPI)-anchored proteins in the CHO cell membrane [54] as well as, at least in part, cholesterol-dependent clusters of glycosphingolipid GM1 and GPI-anchored proteins in the COS-7 cell membrane [55]. However, several studies using biochemical experiments together with fluorescence microscopy as well as FRET analysis have reported that the accumulation of GM1, cholesterol or GPI-anchored proteins that are considered to be typical raft makers did not occur after TCR activation [45, 56, 57].

The controversial results concerning the role and size of lipid raft microdomains in T cell activation may be due to the complex diversity and dynamics of these membrane domains. A recently proposed view is that there is a dynamic confinement of membrane microdomain components. Thus, elementary lipid rafts are considered to be dynamic and small domains containing a few molecules of proteins important for the formation of transient (a lifetime of several minutes) and early signaling events in the immediate vicinity of activated TCR. These domains will then participate in the organization of a larger-scale rearrangement of cell membrane domains, the 'immunologic synapse', mediated by the actin cytoskeleton, which can be maintained over many hours [58, 59]. In fact, many raft-associated signaling molecules, such as Lck or LAT are indeed concentrated in the immunologic synapse with the TCR and could enable sustained signaling for a full T cell response.

Such small dynamic rafts, subsequently clustered to promote T cell signaling and dependent on the cytoskeleton, have been actively studied by Kusumi and co-workers using single-particle tracking (SPT) [60, 61]. In these studies, the trajectories of small particles (40 nm) tagged to membrane lipids or membrane-associated proteins are followed by high-resolution microscopy with 10–20 nm precision. Reduced lipid diffusion was found within transient confinement areas that were dependent on membrane-associated proteins interacting with the cytoskeleton so as to create 'pickets' and 'fences' within the plasma membrane. Upon stimulation, the small, unstable rafts coalesced to form larger, stabilized membrane domains, indicating that the pickets within the membrane as well as the fence on the cytoplasmic surface could jointly allow a spatial regulation of the signal complexes. Such stabilized raft domains could function as signaling centers and thus resemble the signaling domains generated by TCR cross-linking. Interestingly, upon TCR clustering at a single site in Jurkat cells, Lck and its N-terminal-10 amino acid sequence (N10) fused to the green fluorescent protein (GFP) became concentrated around the TCR cluster in a cholesterol-and actin-dependent manner [62], indicating the critical role of rafts and their association with actin. Such interaction has been shown to be dynamic, both Lck- and N10-GFP exchanging rapidly with those in the surrounding area.

Whereas heterogeneity in the composition, cholesterol-dependence and localization of membrane lipid rafts seem important for the control of TCR activity, such a heterogeneous collection of domains presents a serious challenge to characterization. Despite the recent development of different techniques to visualize native raft domains, a consensus on the size and density of rafts is still lacking [7, 63]. This emphasizes the need for specific probes able to demonstrate and characterize lipid microdomains in living cells.

Lysenin, a novel probe to demonstrate spatial and functional heterogeneity of sphingomyelin-rich membrane domains in living cells

SM, one of the major lipids of the plasma membrane of most mammalian cells, is found concentrated in the outer leaflet and demonstrated to be a component of lipid rafts. However, compared to the GM1 ganglioside, the involvement of which in membrane lipid domains is largely documented thanks to the use of CTB, little is known about SM distribution and dynamics in membranes, mainly due to the lack of reliable probes. Among the toxins that specifically bind SM, lysenin was demonstrated recently in the laboratory as a useful tool for studying SM-rich membrane domains.

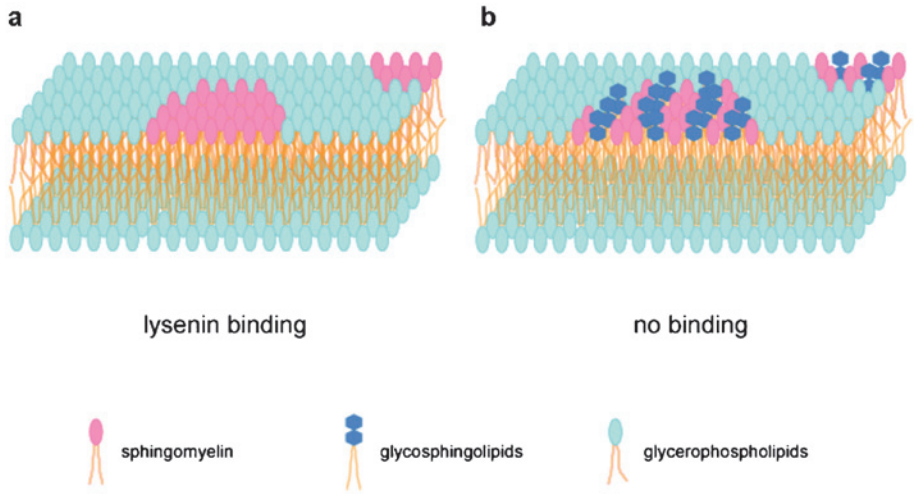

Figure 1. Lysenin recognizes the heterogeneous organization of sphingomyelin. Lysenin binds membranes when SM (sphingomyelin) forms clusters (*a*). The presence of glycosphingolipids decreases the local density of SM in the membrane and thus inhibits the binding of lysenin (*b*). Figure from [93], Oxford University Press.

Lysenin is a sphingomyelin-specific pore-forming toxin

Lysenin was isolated from the coelomic fluid of *Eisenia foetida* [64]. It binds specifically to SM. The phosphocholine as well as the ceramide moieties of SM are required for its recognition by lysenin, thus indicating that lysenin interacts with both the hydrophilic and hydrophobic moieties of SM. Membrane binding of lysenin induces its SDS-resistant oligomerization in an SM-dependent manner, leading to the formation of pores with an approximate diameter of 3 nm. Oligomerization, but not binding, is affected by the fatty acid composition of SM, suggesting the role of membrane fluidity in the oligomerization step, i.e., more fluid membrane accelerates the oligomerization of lysenin. Both tryptophan fluorescence and differential scanning calorimetry indicated that the lysenin oligomer was inserted into the hydrophobic region of the membrane [65].

The importance of aromatic amino acids of lysenin in its activity was demonstrated by systematic tryptophan to alanine mutation. Such mutation did not significantly affect the tertiary structure of the protein as revealed by CD spectra, but did modify the intensity of the intrinsic tryptophan fluorescence. In fact, lysenin contains six tryptophans, of which five are also conserved in two lysenin-related proteins 1 and 2 (LRP-1 and -2). These conserved tryptophans are directly or indirectly involved in the SM recognition and the cytolytic activity of lysenin [66].

Lysenin can detect heterogeneous organization of SM in the membrane

Interestingly, recent data indicate that, in addition to its SM specificity, the binding of lysenin to SM is dependent on the local density of SM, i.e., the binding increases as SM forms clusters [34]. The accessibility of lysenin to SM has been shown to be different between the apical and basolateral membranes of polarized epithelial Madin-Darby canine kidney (MDCK) cells known to display different lipid compositions [8]. The glycosphingolipid enrichment of the apical membrane results in a decreased local density of SM and thus a decrease of lysenin binding and toxicity. This is further supported by the higher binding and toxicity of lysenin towards the glycolipid-deficient mutant melanoma cell line GM-95 compared to its parental cell line MEB-4 [67]. In fact, the organization of SM in the membrane is affected by other lipids. Model membrane experiments have shown that lysenin binds SM only when SM forms clusters (Fig. 1). As with most sphingolipids, SM has a relatively high phase-transition temperature, and thus spontaneously forms clusters in the presence of liquid-crystalline lipids such as diC18:1 PC. In contrast, in the presence of acyl chain-ordered lipids like diC16:0 PC, the local density of SM decreases. By isothermal titration calorimetry (ITC), it has been shown that one molecule of lysenin binds five SM molecules. Thus lysenin is not only an SM-specific protein, but can also be used to study heterogeneous organization of SM. Interestingly, this organization differs both between different cell types and also between different membrane domains within the same cells. The fact that most mammalian cells are susceptible to lysenin suggests that SM exists in the form of tightly packed complexes in many biomembranes.

SM-rich domains are spatially and functionally distinct from GM1-rich membrane domains

While the toxicity of lysenin has been shown to be useful to isolate cell mutants defective in SM synthesis and ceramide transport [68, 69], it became a problem

to study SM distribution in living cells. A truncated lysenin mutant that does not oligomerize and thus is non-toxic was generated [29]. Studies with lysenin deletion mutants indicated that the N-terminal part of lysenin is not required for SM recognition, whereas deletion of the C-terminal end significantly diminished recognition. Note that all deletion mutants tested lost their hemolytic activity. The minimal C-terminal fragment (amino acids 161–297) able to recognize SM, referred as 'non-toxic lysenin' (NT-Lys), displayed the same selectivity as full-length lysenin for SM-containing membranes *in vitro* and recognized cell surface SM in living Jurkat cells. However, its dissociation was 100 times faster than that of the full-length, indicating that the process of oligomerization observed with the full-length lysenin stabilizes its binding to SM-containing membranes. The addition of various tags at the N-terminal of NT-Lys (glutathione-S-transferase GST, GFP-derived fluorescent protein Venus) does not alter the binding specificity of non-toxic lysenin.

It is worth noting that using this non-toxic mutant of lysenin, SM-rich domains spatially distinct from ganglioside GM1-rich membrane domains were detected in the plasma membrane of Jurkat T cells [29]. Pretreatment of Jurkat cells with either non-toxic lysenin or CTB did not affect binding of either toxin, suggesting that GM1 and SM are spatially segregated. Fluorescence microscopy analysis after double labeling with non-toxic lysenin and CTB displayed a uniform staining of the Jurkat cell plasma membrane, indicating that the size of the GM1 and SM microdomains was below the resolution of the light microscope. However, electron microscopy analysis of membrane fragments indicated that both SM and GM1 distributed extensively over the entire membrane and formed spatially distinct domains with a radius of 60–80 nm (Fig. 2).

Furthermore, this spatial heterogeneity could be related to specific SM-dependent signaling pathways different from the pathways linked to TCR activation or GM1 clustering. As previously described, TCR activation through cross-linking of the TCR-CD3 complex by anti-CD3 antibody induces an intracellular calcium increase (from intracellular stores, then influx *via* calcium channels) as well as activation of the serine/threonine tyrosine kinases ERK1/2 (or p44 MAP and p42 MAP kinases) [70]. Similarly, cross-linking of GM1 by CTB directly or with antibody against CTB [71, 72] induces calcium increase and ERK1/2 phosphorylation, which is known to be important for cytokine production. Neither of these events was affected by cell surface SM depletion after sphingomyelinase treatment, indicating that SM is dispensable in the signal transduction induced by TCR activation and GM1 cross-linking.

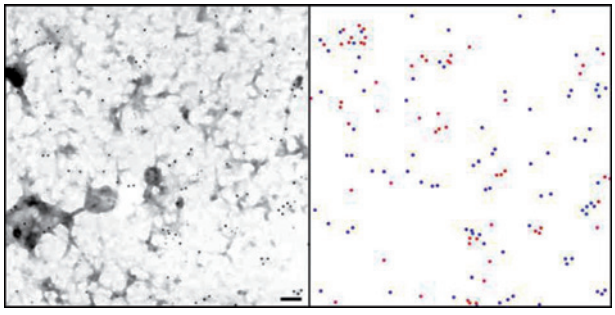

Figure 2. Cell surface distribution of SM-rich domains and GM1-rich domains on two-dimensional sheets of plasma membrane. Jurkat cells were first labeled with HmV-NT-Lys and biotinylated cholera toxin B fragments at 4 °C and then fixed with 4% paraformaldehyde and 0.02% glutaraldehyde for 10 min at 4 °C. The fixed cells were labeled with anti-GFP rabbit polyclonal antibody at 4 °C followed by labeling with goat anti-rabbit Ig(immunoglobulin)G-5-nm gold and goat anti-biotin IgG-10-nm gold at 4 °C. The distribution of gold particles on the plasma membrane was examined under electron microscope after ripping off. Bar, 100 nm. In the right panel, distribution of SM (5-nm gold) is colored in red, whereas the distribution of GM1 (10-nm gold) is in blue. Figure from [29].

Lysophosphatidic acid (LPA), a lipid mediator with a broad range of effects in various cell types, is reported to induce calcium increase and ERK1/2 activation through binding to G-protein-coupled receptors (GPCRs) termed LPA1–3, members of the 'endothelial differentiation gene' EDG family. These receptors are widely expressed and mediate signaling through the G_q, G_i and $G_{12/13}$ families of heterotrimeric G-proteins [73]. LPA treatment of Jurkat cells induces a calcium increase as well as ERK1/2 phosphorylation, as already observed. However, in contrast to TCR activation with anti-CD3 antibody or GM1 cross-linking, these effects were inhibited by SM depletion, indicating that SM is participating in these LPA-dependent signaling pathways. Furthermore, binding of GST-NT-Lys to Jurkat cell membrane (and thus the SM-rich domains) and subsequent cross-linking by anti-GST antibody alone or in combination with a secondary antibody to increase cross-linking, induces a sustained calcium increase, which unlike the case with CD3 activation, is fully dependent on exogenous calcium and can be blocked by the calcium channel blocker nifedipine. In addition, the cross-linking of SM-rich domains also induces ERK phosphorylation, confirming that these domains provide a signaling platform for calcium mobilization and ERK activation (Fig. 3).

Interestingly, key regulators of T cell activation such as LAT and Lck are differentially involved after SM cross-linking in Jurkat cells. Unlike CD3 or GM1 cross-linking, cross-linking of SM does not induce

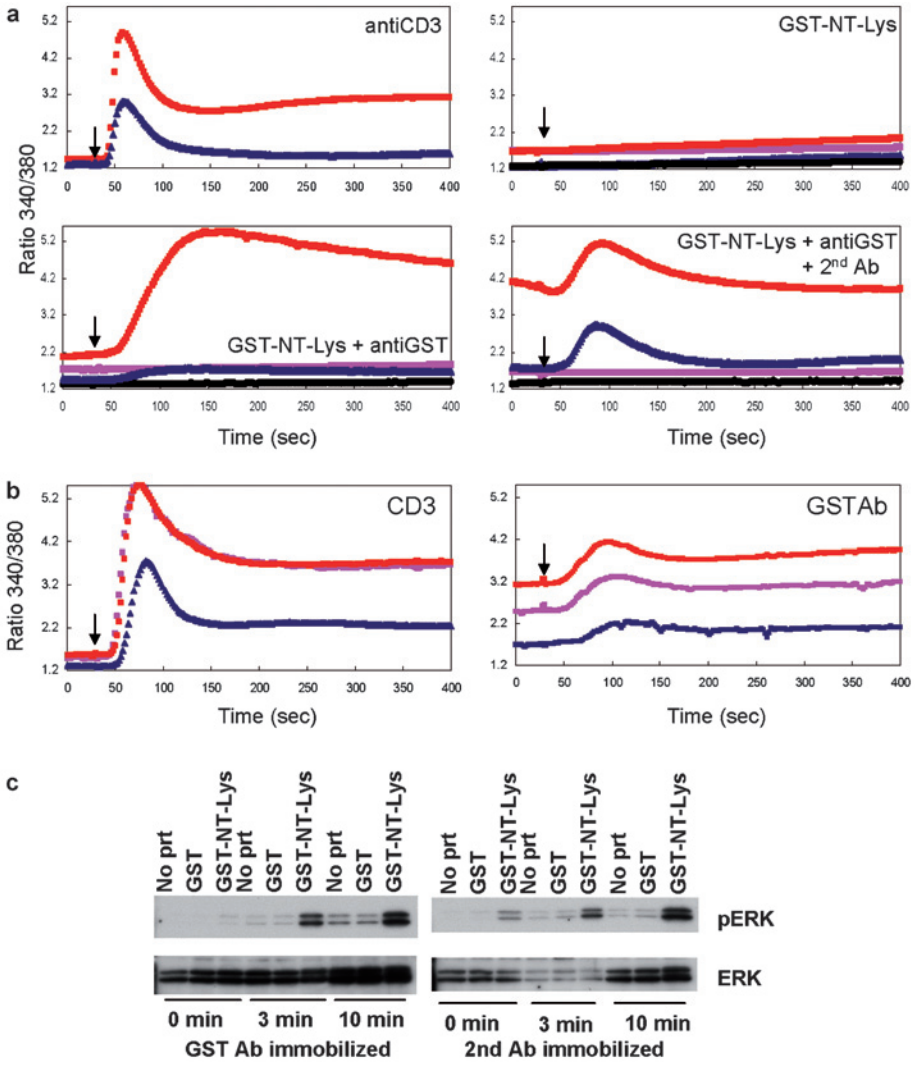

Figure 3. Induction of calcium influx and ERK activation by cross-linking of SM-rich domains. (*a*) Cross-linking of SM-rich domains induces calcium influx. Jurkat cells were loaded with Fura-2AM and the proteins were added at 30 s (indicated by arrows). Upper left, red squares show the result in the presence of 1 mM calcium buffer, and blue triangles show the result in the presence of 1 mM EGTA. 1 μg/ml anti-CD3 antibody was added. In the remaining panels, red squares and blue triangles are the results from cells incubated with GST-NT-Lys and measured in the presence of calcium or EGTA, respectively. Pink squares and black circles are the results from cells incubated with GST and measured in the presence of calcium or EGTA, respectively. Upper right, GST or GST-NT-Lys was added at 30 s after loading Fura-2AM. Lower left, cells were first labeled with GST or GST-NT-Lys on ice for 30 min and then loaded with Fura-2AM, and anti-GST antibody was added as indicated by the arrow. Lower right, cells were labeled with proteins and anti-GST antibody on ice and loaded with Fura-2AM, and anti-mouse Ig antibody was added as indicated by the arrow. Results shown here are representative data from at least two experiments. (*b*) Calcium channel blocker inhibits calcium influx triggered by cross-linking of SM-rich domains. Left, Jurkat cells were loaded with Fura-2AM in the absence of dimethyl sulfoxide (Me_2SO) (red squares), in the presence of Me_2SO (pink squares), and in the presence of 200 μM nifedipine in Me_2SO (blue triangles). 1 μg/ml anti-CD3 antibody was added at 30 s as indicated by the arrow. Right, cells were incubated with GST-NT-Lys on ice for 30 min in the absence of Me_2SO (red squares), in the presence of Me_2SO (pink squares), and in the presence of 200 μM nifedipine in Me_2SO (blue triangles). Cells were then loaded with Fura-2AM and activated according to the protocol in (*a*), lower left. Results shown here are representative data from at least two experiments. (*c*) Cross-linking of SM-rich domain induces ERK (extracellular signal-related kinase) phosphorylation. Jurkat cells were incubated with 50 μg/ml GST or GST-NT-Lys or without any addition (No prt) and replated onto plates coated with anti-GST antibody (left panel), or cells were further incubated with anti-GST antibody and replated onto plates coated with the corresponding secondary antibody (right panel). After the indicated periods, cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. Upper row, blotted against anti-phosphospecific antibody; lower row, anti-ERK antibody. Ab, antibody; pERK, phospho-ERK. Figure from [29].

significant protein tyrosine phosphorylation. Furthermore, whereas anti-CD3 treatment of Jurkat cells induces LAT tyrosine phosphorylation, as already described, there is only a small increase in tyrosine phosphorylation of LAT after SM-rich domain cross-linking. However, the involvement of the transmembrane tyrosine phosphatase CD45, the Src-family tyrosine kinase Lck and the Syk family kinase ZAP-

70, already known to be essential for TCR activation, are also necessary for ERK phosphorylation after SM cross-linking. In addition, the role of PLCγ, which is positively regulated in TCR activation, is limited for SM-dependent ERK phosphorylation. This could be related to the differences in calcium mobilization between the signaling pathways dependent on GM1- and SM-rich domains (cf. Fig. 3). Finally, the use of various kinase inhibitors has demonstrated the involvement of the protein kinase C (PKC) and/or calmodulin kinase II in SM-dependent signaling pathways. The inhibition of ERK phosphorylation by the pertussis toxin PTX, a specific inhibitor of Gαi/o, has confirmed that SM-rich domains are able to provide a platform for GPCR signaling. In addition, a slight inhibition by dominant negative H-Ras suggests that the Ras-mediated pathway is not the main pathway initiated by SM clustering. Interestingly, besides the Ras/ERK pathway, the PI3K pathway is reported to be an important intermediate of the LPA mitogenic effect in non-hematopoietic cells [74]. In addition, it was shown recently that cholesterol depletion by MβCD or cholesterol add-back experiments strongly modulates PI3K activation in response to LPA in Vero cells [75] as well as in HeLa cells [76]. Furthermore p85-dependent PI3Ks as well as the EGFR (epidermal growth receptor) have been already identified in lipid rafts, suggesting that lipid microdomains play a positive role in LPA signaling by bringing together EGFR, PI3K and the PI3K substrate phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P2], thus providing a favorable environment for PI3K activation. It remains to be demonstrated whether, in Jurkat cells, SM-rich domains connected to the LPA receptor are similarly linked to the compartmentalization of the PI3K pathway components on the inner leaflet of the plasma membrane.

The above results make evident that two well-recognized lipid raft markers, SM and GM1, are distributed in different domains detected at the submicron scale on the cell plasma membrane. In addition, such SM-rich and GM1-rich domains afford different signaling platforms: SM-rich domains mediate signaling events dependent on GPCRs such as the LPA receptors, while GM1-rich domains participate in the signal cascade for TCR activation. Although GM1 ganglioside, cholesterol and a set of raft-associated proteins like the GPI-anchored proteins CD59 are often used as raft membrane markers to indicate raft involvement in TCR activation, investigation of other lipid domains, such as the SM-enriched domains described here, provides new and important insights into T lymphocyte activation.

Fluorescein ester of poly(ethyleneglycol)cholesteryl ether (fPEG-Chol) and dynamics of cholesterol-rich membranes

Cholesterol is considered to play a critical role in maintaining raft structure, and as a matter of fact, its depletion with drugs such as MβCD has been widely used to verify raft involvement in signaling pathways [15], despite concerns about side-effects [77]. Instead of manipulating the cholesterol level in lipid rafts, visualizing the dynamics of cholesterol-rich membranes using reliable probes is a powerful tool not only for demonstrating the role of membrane lipid domains in signaling, but also to follow cholesterol trafficking. The cellular content and distribution of cholesterol are tightly regulated by complex mechanisms [78]. Cholesterol is delivered to other organelles by a combination of vesicular and non-vesicular transport processes [79, 80]. The formation, regulation and role of membrane microdomains in cholesterol trafficking are still unclear, due to the lack of reliable probes. Recently, fPEG-Chol was synthesized and proven to be a sensitive probe for elucidating the dynamics of membrane cholesterol-rich domains due to its water-solubility and low toxicity [24, 25].

fPEG-Chol as a probe for cholesterol-rich domains

When fPEG-Chol is added to fixed and permeabilized fibroblasts, the Golgi apparatus and intracellular small vesicles become fluorescent, as already observed with filipin. In contrast to normal fibroblasts, Niemann-Pick type C (NPC) cells, characterized by intracellular accumulation of unesterified cholesterol, display numerous perinuclear compartments previously identified as lysobisphosphatidic acid (LBPA) or bis(monoacylglycero)phosphate (BMP)-enriched late endosomes loaded with cholesterol [81]. Furthermore, an *in vitro* liposome-binding assay showed that fPEG-Chol binding to the membrane increased with increasing cholesterol content. In addition, fPEG-Chol rapidly moves from cholesterol-poor liposomes to cholesterol-rich ones, indicating that fPEG-Chol preferentially distributes to cholesterol-rich domains.

Dynamics of cell surface cholesterol-rich domains

Due to its water solubility and low toxicity, fPEG-Chol is distributed exclusively on the outer leaflet of the plasma membrane. After human skin fibroblasts were briefly incubated with fPEG-Chol followed by fixation, uneven distribution of the fluorescence could be detected by wide-angle video-enhanced fluorescence microscopy. Interestingly, many of the labeled fPEG-Chol positive structures colocalized with the GM1 probe CTB. Furthermore, addition of EGF induced a clustering of the EGFRs as well as a

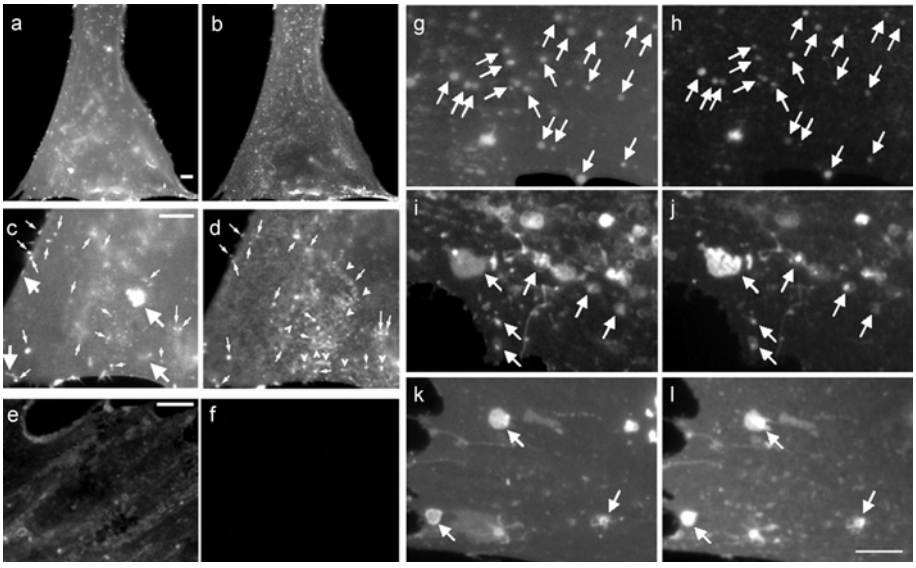

Figure 4. PEG-Chol reveals redistribution of cholesterol-rich plasma membrane domains in fibroblasts. (*a–d*) Normal human skin fibroblasts were incubated with 1 μM fPEG-Chol [fluorescein ester of poly(ethyleneglycol)cholesteryl ether] and 5 μM AlexaFluor 594-labeled cholera toxin for 90 s at room temperature and then fixed with paraformaldehyde for 10 min. (*a* and *c*) fPEG-Chol fluorescence. (*b* and *d*) AlexaFluor 594 fluorescence. Small arrows show the structures that were doubly labeled with fPEG-Chol and cholera toxin. Big arrows show those labeled solely with fPEG-Chol. Arrowheads indicate the spots positive with cholera toxin alone. In (*e*) and (*f*), cells were treated with (*f*) and without (*e*) 10 mM MβCD for 30 min at 37 °C before fixation. Cells were then labeled with 1 μM fPEG-Chol. *Bar*, 4 μm. (*g–l*) Normal skin fibroblasts were labeled with 2 μM fPEG-Chol and incubated with 5 μg/ml biotinylated EGF for 20 min at 4 °C (*g* and *h*) or for 2 min at 37 °C (*i–l*). Cells were then fixed with 3% paraformaldehyde, 8% sucrose in phosphate-buffered saline, quenched, and incubated with TRITC(tetramethyl rhodamine iso-thiocyanate)-labeled streptavidin for 20 min at 4 °C. The specimens were observed under a Nikon TE 300 microscope equipped with a Hamamatsu C-4742-98 cooled CCD camera. (*g* and *i*) fPEG-Chol fluorescence. (*h* and *j*), TRITC-fluorescence. In (*k*) and (*l*), cells were doubly labeled with 1 μM fPEG-Chol- and AlexaFluor 594-labeled cholera toxin B subunit before stimulation by non-labeled EGF. (*K*) fPEG-Chol fluorescence. (*l*) Cholera toxin fluorescence. *Arrows* show the structures that were doubly labeled. *Bar*, 4 μm. Figure from [24].

redistribution of the fPEG-Chol and GM1 enriched in the same clusters (Fig. 4). This is consistent with the fact that the EGFR colocalizes with cholesterol-rich plasma membrane domains. The cell surface cholesterol level is known to modulate the binding of EGF as well as the activity of the EGFR, i.e., cholesterol depletion increases EGF binding and EGFR activation, whereas cholesterol enrichment lowers them [82–84]. Thus it seems that lipid rafts function as negative regulators of EGFR signaling. Recently, the mechanism underlying the effect of cholesterol has been demonstrated using single-molecule fluorescence imaging [85]. Modification of the membrane cholesterol content dramatically alters the diffusion pattern of EGFR. Interestingly, F-actin depolymerization partially restored receptor mobility in cholesterol-depleted membranes, indicating that cholesterol depletion might lead to receptor confinement by acting on F-actin beneath the lipid rafts. This reinforces the concept of an intimate relationship between cholesterol-enriched lipid rafts and the actin cytoskeleton, possibly by the recruitment of PI(4,5)P2, as described previously for the TCR.

fPEG-Chol slowly internalized with lipid raft markers

Incubation with low concentrations of fPEG-Chol does not interfere with endocytosis and allows the fate of cell surface cholesterol-rich membrane domains to be followed [24, 25]. After 5 min labeling at room temperature, internalization of fluorescence was chased at 37 °C in human skin fibroblasts. Most of the fluorescence stayed at the plasma membrane after 10 min of chase. After 60 min, fPEG-Chol fluorescence surrounded the nucleus and then intracellular vesicles. After 180 min, the Golgi apparatus was prominently labeled with fPEG-Chol. Internalized fPEG-Chol colocalized with lipid raft markers: the GPI-anchored protein CD59 and the ganglioside GM1, visualized respectively with anti-CD59 monoclonal antibody and CTB (Fig. 5). Interestingly, endocytosis of these raft components follows a clathrin-independent route [86]. In contrast, fPEG-Chol was not co-localized with internalized transferrin, the endocytosis of which is clathrin-dependent. It thus appears that rafts domains are internalized *via* a clathrin-independent pathway into acidic organelles, supporting the idea that raft-preferring lipids are delivered to late endosomes/lysosomes. After pro-

longed incubation, fPEG-Chol is further transported to the Golgi.

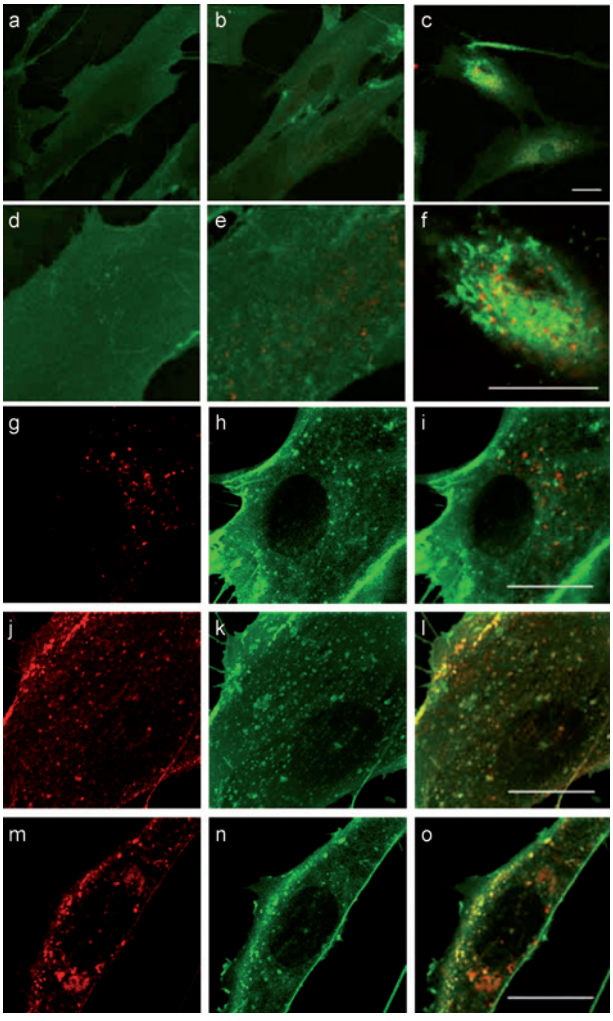

Figure 5. PEG-Chol is internalized together with lipid raft components. (*a–f*) Normal human skin fibroblasts were incubated with 1 μM fPEG-Chol for 5 min at room temperature. Cells were then washed and incubated for 10 (*a* and *d*), 60 (*b* and *e*), and 180 min (*c* and *d*) at 37 °C in the presence of 1 mg/ml rhodamine dextran. (*g–o*) Normal fibroblasts were incubated with 1 μM fPEG-Chol together with Texas Red transferrin (*g–i*) AlexaFluor 594-labeled CTxB (*j–l*, or phycoerythrin-conjugated anti-CD59 monoclonal antibody (*m–o*) for 5 min at room temperature. Cells were then washed and further incubated for 20 min. Cells were treated with ammonium chloride before taking images. Bar, 20 μm. Figure from [24].

Cholesterol-rich domains in intracellular organelle membranes

Little is known about the transbilayer membrane distribution of cholesterol in live cell organelles. Since fPEG-Chol is not permeable across the membrane, it was microinjected in order to monitor the distribution of cholesterol on the cytoplasmic side of intracellular organelles. Interestingly, fPEG-Chol accumulates mainly in the Golgi of normal as well as NPC (Niemann-Pick type C) fibroblasts, indicating that the Golgi apparatus exposes cholesterol-rich domains to the cytosol. Furthermore, since the staining of cholesterol-rich late endosomes of NPC fibroblasts could only be observed after permeabilization, this indicates that NPC cells accumulate cholesterol only on the luminal side of late endosomes/lysosomes where BMP is located, reinforcing the idea of a direct interaction between these lipids [81, 87].

Trafficking of cholesterol in the outer and inner leaflets of the plasma membrane

The transbilayer distribution of cholesterol as well as its intracellular transport remain largely unknown [10]. Transport of cell surface cholesterol was recently studied in CHO cells using DHE (dehydroergosterol). Internalized DHE colocalized extensively with transferrin (Tf), a marker for the endocytic recycling compartment [88]. The results obtained with fPEG-Chol are different, suggesting that the internalization of DHE and fPEG-Chol follows different routes. This might be linked to the different kinetic behavior of the probes between the plasma membrane leaflets. As is the case with cholesterol [89], DHE was shown to undergo rapid transbilayer movement from the outer to the inner leaflet [90], thus favoring its subsequent accumulation in recycling endosomes. In contrast, fPEG-Chol is restricted to the cholesterol-rich domains of the outer leaflet, likely following the slow clathrin-independent traffic to the Golgi. Furthermore, these results might indicate that the trafficking of cholesterol in the outer and inner leaflets of the plasma membrane occurs independently, in relation to the different lipid composition of the leaflets [10, 91, 92].

Conclusions

The use of specific probes such as lysenin and fPEG-Chol for the elucidation of the lipid raft structure in living cells will improve our understanding of the organization and function of such specialized lipid domains, not only at the plasma membrane, but also within the membrane of intracellular organelles. Reliable lipid probes as well as complementary techniques (e.g., high resolution electron microscopy and FRET) are important to map the composition, dynamics and function of lipid microdomains in signal transduction. Such information will be critical for the development of new drugs in a variety of diseases in which the importance of lipid organization has been demonstrated, diseases such as atherosclerosis, lipid storage disorders, viral infections, various neurodegenerative diseases and cancer.

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