The inner side of T cell lipid rafts

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Abstract

A key question in understanding the functional role of lipid rafts is whether lipid microdomains at the plasma membrane outer leaflet are coupled to lipid microdomains at the inner leaflet. By using a cyan-fluorescent protein (CFP) targeted to inner plasma membrane rafts of Jurkat T cells, we found that raft domains at the outer and inner leaflets are physically coupled and that this coupling requires cholesterol. Interestingly, TCR/CD3 cross-linking induces co-capping of the raft bilayer independently of cholesterol or signaling events, indicating that cholesterol-extracting drugs are unable to destroy TCR–lipid raft interaction.

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1. Introduction

The existence of plasma membrane microdomains termed “lipid rafts” is an hypothesis that may not only drastically change our view of the structural organization of the phospholipid bilayer, but also influence our understanding of the mechanisms through which receptor triggering is linked to the activation of an intracellular signaling cascade. The raft hypothesis predicts that attractive forces between sphingolipids and cholesterol mediate the formation of lipid clusters in an unsaturated glycerolphospholipid environment [1,2]. The biochemical techniques used for raft analysis (isolation following cell membrane solubilization by mild detergents) showed that many lipid-modified signaling proteins, such as Src family tyrosine kinases, glycosylphosphatidylinositol (GPI)-linked proteins, and adaptor proteins are concentrated in raft domains [3]. This selective confinement of signaling molecules in membrane subdomains suggested that lipid rafts could function as platforms for the formation of multi-component transduction complexes.

An important question in understanding the functional role of rafts in T lymphocytes is whether and how lipid microdomains present at the inner leaflet of the plasma membrane are coupled to the ones at the outer leaflet. Data obtained using giant unilamellar vesicles (GUV) show exact superposition of similar phase domains in apposing monolayers [4]. In intact cells, outer rafts can be visualized by staining with labeled cholera toxin B (CT-B) subunit, which binds and cross-links the raft-associated glycosphin-golipid GM1. This approach also allows tracking raft cluster redistribution at the cell surface and verification of raft association with certain proteins by co-staining with specific antibodies. Using this approach in T lymphocytes, it was shown that CT-B patches co-localize with Lck and LAT proteins [5,6], which are associated to the inner cytoplasmic surface of the membrane. This finding has been taken as evidence that inner and outer lipid rafts are physically associated. It can nonetheless be argued that the co-localization of these lipid-anchored proteins with outer rafts is due to their binding to raft-associated transmembrane proteins. Given that the T cell receptor (TCR) co-patches with GM1, the redistribution of Lck and LAT could be explained by their functional association with the TCR. In basophils [7] and in B lymphocytes [8], outer lipid raft components co-redistributed with a green fluorescent protein analog of the kinase Lyn, again suggesting coupling of outer and inner domains.
To visualize lipid rafts directly at the cytoplasmic leaflet of the T cells plasma membrane and to study their coupling with outer rafts and with the TCR, we expressed a cyan-fluorescent protein (CFP) carrying consensus sequences for myristoylation plus palmitoylation (MyrPalm-mCFP), in the Jurkat T cell line. This lipid anchor is necessary and sufficient to target the recombinant protein to a membrane fraction with the biochemical characteristics of classical lipid rafts [9]. In addition, CFP is totally irrelevant for signaling and is thus not expected to interact with endogenous transmembrane proteins to any significant extent. Finally, the weak dimerizing tendency of CFP was eliminated by selected point mutations, thus totally irrelevant for signaling and is thus not expected to target the recombinant protein into lipid rafts [9].

2. Materials and methods

2.1. Cell culture, fluorescent protein constructs and transfection

E6.1 Jurkat T cell lines were cultured in RPMI 1640 medium (Euroclone) supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. MyrPalm-mCFP in pcDNA plasmid, coding for lipid-modified fluorescent protein [9], was the kind gift of Dr. P. Keller (Max Planck Inst., Dresden, Germany). The pGFP-GPI plasmid was the gift of Dr. R. Tsien (Howard Hughes Medical Institute, Univ California, San Diego, CA). The pGFP-GPI plasmid was the gift of Dr. P. Keller (Max Planck Inst., Dresden, Germany). The cDNA constructs were transfected by electroporation using 50 μg DNA (20 μg of MyrPalm-mCFP plus 30 μg of pcDNA3) and 10^7 Jurkat cells suspended in 400 μl RPMI supplemented with 20% FCS. Electroporation was done in 0.45 cm electroporation cuvettes (Gene Pulser, Bio-Rad) at 960 μF and 250 V. After 24 h, transfected cells were diluted and selected in 2 mg/ml geneticin-containing medium (G418, Gibco).

2.2. Reagents and treatments

Mouse IgM anti-human CD59 (MEM-125) and CD3 (MEM-92) antibodies were a gift of Dr. V. Horejsi (Inst. Molecular Genetics, Academy of Sciences of the Czech Republic, Prague). Anti-human transferrin receptor (sTfR, CD71) was purchased from BD PharMingen. The monoclonal anti-GM3 antibody (V4) was a gift of Dr. E. Montero (Center of Molecular Immunology, Havana, Cuba). CT-B and rabbit polyclonal anti-CT-B antibodies were from Sigma–Aldrich, and TXRD- or FITC-conjugated secondary antibodies were from Southern Biotechnology. Anti-human transferrin receptor (hTfR, Molecular Genetics, Academy of Sciences of the Czech Republic, Prague). Anti-human transferrin receptor (hTfR, Molecular Genetics, Academy of Sciences of the Czech Republic, Prague).

Stably transfected Jurkat cells were washed twice in serum-free RPMI and suspended at 2 × 10^7 cells/ml. Cells were incubated (37 °C, 30 min) with 12.5 nM 5-methyl-β-cyclodextrin (MβCD, Sigma), or 10 μM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, Calbiochem). Treated cells were washed twice to eliminate dead cells, counted and used immediately.

2.3. Patching and immunofluorescence confocal microscopy

Stable transfected Jurkat cells (2 × 10^6) were resuspended in 150 μl HEPES-RPMI and incubated with 3 μg CT-B (on ice, 20 min). Cells were washed twice, 3 μl of rabbit polyclonal anti-CT-B were added and the cells incubated (on ice, 10 min). Cells were washed twice and incubated (37 °C, 15 min) with 1.5 μg anti-rabbit TXRD. Patching of GM3, CD59 and CD71 was carried out with basically the same protocol described for CT-B, using 1:100 primary antibodies (4 °C, 20 min) followed by 1.5 μg anti-mouse TXRD-conjugated antibody (37 °C, 15 min). Patching of TCR was performed by using 1:100 anti-CD3 (37 °C, 5 min) followed by 1.5 μg anti-mouse TXRD-conjugated antibody. Immediately after patching, cells were fixed with 2 or 4% paraformaldehyde (Sigma) in PBS for 20 min at room temperature, then adhered for 10 min to microscope slides coated with 0.5 mg/ml poly-l-lysine (Sigma), washed and mounted in 2.5% 1,4-diazobicyclo[2.2.2]octane (DABCO, Fluka), 90% glycerol, 10% PBS. Confocal microscopy was performed with a Biorad confocal microscope (BioRad) with 60 and 100× objective lenses (Nikon), using laser excitation at 457, 488 and 543 nm. The widths for CFP, fluorescein and Texas red emission channels were set such that cross-channel bleedthrough was negligible. Images were analyzed using the Adobe Photoshop 7.0 program. Surface plot analysis was performed with the program Image J.

2.4. Fluorescence quantification

To quantify MyrPalm-mCFP recruitment in antibody-induced patches, boxes of equal area were drawn around the patch area, non-patched cell regions, and a background area outside the cell. The relative recruitment index was calculated as indicated: [mean fluorescence intensity (MFI) at patches − background]/MFI at non-patched regions. Quantitative analysis of MFI was performed with the Image J program. A minimum of 20 cells was examined quantitatively for each experiment. Statistical analysis was performed using Student’s t-test (Microsoft Office).

3. Results

3.1. Localization of inner and outer rafts

To analyze whether MyrPalm-mCFP co-distribute with GM1, we performed patching experiments with anti-CT- B antibodies in Jurkat T cells expressing MyrPalm-mCFP. After antibody-induced cross-linking, MyrPalm-mCFP under-
Fig. 1. Co-localization of outer and inner membrane raft markers. Jurkat T cells expressing MyrPalm-mCFP were (A) left untreated or incubated with: (B) CT-B followed by anti-CT-B antibodies to induce GM1 patching or (C) anti-GM3, (D) anti-CD3, or (E) anti-CD71 antibodies. Anti-Ig antibodies conjugated with Texas red (TXRD) were used to visualize patches. Confocal images were taken with identical settings. Data are representative of at least 20 cells in one out of three independent experiments. In each experiment more than 90% of the capped cells showed the indicated phenotypes. In this and the following images mCFP was color-coded green and anti-Ig-TXRD red; in the merged image yellow represents the superimposition of the two colors. Bar, 10 μm.

went a major redistribution and co-patched with CT-B (Fig. 1B). In contrast, a CFP carrying prenylation consensus sequences (GerGer-mCFP), previously shown not to partition into rafts [9], did not redistribute after GM1 cross-linking (not shown). This latter construct, however, appeared largely contained in intracellular membranes in Jurkat cells, making the interpretation of the result ambiguous.

T cell raft domains are suggested to be heterogeneous in composition, and evidence has been provided that different raft-prefering molecules partition into different membrane domains upon cross-linking [10,11]. In particular, the two gangliosides GM1 and GM3 segregate in distinct domains after cross-linking as well as during T cell polarization [10]. To verify whether GM3 cross-linking causes inner raft recruitment into the patches, we performed patching experiments with anti-GM3 antibodies in Jurkat cells stably expressing MyrPalm-mCFP. Under these conditions, MyrPalm-mCFP co-redistributed with GM3 (Fig. 1C), suggesting that cross-linking of both GM1 and GM3 induces recruitment of inner rafts into large/stable raft domains.

TCR association with raft domains is still a matter of discussion, although most biochemical studies indicate that under resting conditions, the TCR is excluded from or only weakly associated with rafts, and is recruited into rafts after receptor cross-linking [12,13]. In specific experimental conditions, however, constitutive association of the TCR–CD3 complex with lipid rafts was demonstrated in murine T cells [14]. Microscopy experiments showing co-patching of cross-linked TCR and GM1 suggest that the TCR might be associated with outer rafts after antibody cross-linking [6]. Here we found that anti-CD3 patching, induced by treatment of cells with a specific primary anti-CD3 antibody, caused MyrPalm-mCFP redistribution in patches that co-localized with the TCR (Fig. 1D). In contrast, cross-linking of the transferrin receptor CD71 induced patches of the latter antigen but not of MyrPalm-mCFP (Fig. 1E).

3.2. Role of cholesterol in coupling inner and outer rafts

Several reports indicate that lipid raft integrity is cholesterol-dependent (reviewed in [15]). Treatment of T lymphocytes with the cholesterol-extracting drug methyl-β-cyclodextrin (MβCD) thus results in loss of all raft markers from detergent-resistant membranes (DRM), as well as inhibition of CD59-mediated signaling [16]. We thus analyzed whether the observed co-redistribution of MyrPalm-mCFP with outer raft markers is a membrane cholesterol-dependent process. Jurkat T cells expressing MyrPalm-mCFP were incubated with 12.5 mM MβCD for 20 min, washed and immediately treated with specific ligands to induce GM1, CD59 or CD3 patch formation. Following this treatment, GM1, CD59 and MyrPalm-mCFP became soluble in 1% Triton-X-100 (not shown). It was still possible to induce GM1 or CD59 patching in MβCD-treated cells, but MyrPalm-mCFP remained evenly distributed at the cell membrane in either case (Fig. 2B and D).

It has been reported that MβCD treatment does not inhibit co-clustering of patched GM1 with CD59 and Lck in Jurkat cells [6]. These data might suggest that reduction of plasma membrane cholesterol levels does not inhibit the formation of large, stable rafts induced by GM1 clustering. Alternatively, GM1, CD59 and Lck might co-cluster due to interactions not involving lipid rafts, but rather protein–protein or lipid–protein associations, explaining why in our experiments MyrPalm-mCFP did not co-cluster with GM1. To test this latter possibility, we transfected Jurkat T cells with a GFP-GPI, a raft-prefering molecule that should not interact with endogenous T cell molecules [17]. As predicted, in untreated cells, GM1 clustering induced co-redistribution of GFP-GPI (Fig. 2D). After cholesterol removal by MβCD, however, GFP-GPI molecules did not co-redistribute with GM1 patches (Fig. 2F), suggesting that indeed the treatment
Fig. 2. Cholesterol extraction affects rafts bilayer formation. Jurkat T cells expressing MyrPalm-mCFP were (A, C and E) left untreated or (B, D and F) treated with 12.5 mM MβCD, and then incubated with (A, B, E and F) CT-B followed by anti-CT-B antibodies or (C and D) anti-CD59 antibodies to induce patching. (E and F) Jurkat T cells expressing GFP-GPI were (E) left untreated or (F) treated with 12.5 mM MβCD and incubated with CT-B followed by anti-CT-B antibodies to induce patching. TXRD-conjugated anti-Ig antibodies were used to visualize anti-CT-B or CD59 patches. Confocal images were taken with identical settings. Data are representative of at least 20 cells of one of two independent experiments. In each experiment more than 90% of the capped cells showed the indicated phenotypes. Bar, 10 μM.

prevents the generation of clustered rafts and validating our data obtained with MyrPalm-mCFP.

3.3. TCR/CD3 association with inner rafts does no depend on cholesterol

To test whether the TCR–lipid rafts association is disrupted by MβCD treatment, treated cells were incubated with anti-CD3 antibodies. In this case, we observed not only co-localization of TCR patches with GM1 but also with MyrPalm-mCFP aggregates (Fig. 3A), indicating that TCR interacts with the raft bilayer in a cholesterol-independent manner. The TCR/CD3 association with inner membrane rafts might be a consequence of signaling events induced by the cross-linking antibody. To verify this possibility, cells were pre-treated with the Src kinase inhibitor PP2, followed by anti-CD3 antibodies. Under these conditions, no increase in tyrosine phosphorylation or [Ca2+] (not shown). In PP2-treated cells, the patches of CD3 appeared much smaller than in control cells. Nonetheless, we still observed co-localization of TCR/CD3 and MyrPalm-mCFP patches, indicating that TCR signaling is not required to associate inner raft domains and the TCR (Fig. 3B). This observation is consistent with data demonstrating that recruitment of cross-linked TCR in DRM is independent of signaling [13]. Quantitative analysis of these results was performed as indicated in Section 2 and is summarized in Fig. 4.

4. Discussion

We used a fluorescent protein carrying consensus sequences for myristoylation plus palmitoylation (MyrPalm-mCFP) to label lipid rafts at the plasma membrane cytoplasmic leaflet of T cells and to study TCR–raft association. In co-patching experiments, we showed that MyrPalm-mCFP, a marker of lipid rafts at the plasma membrane cytoplasmic leaflet, co-localizes with GM1, GM3 and CD59, but not with CD71 patches, indicating that outer and inner lipid microdomains are coupled in T cells. We also found that MyrPalm-mCFP co-localizes with CD3 patches, confirming and extending previous results showing CD3 co-localization with GM1 [6]. In contrast to GM1 or CD59, however, CD3 cross-linking induced MyrPalm-mCFP redistribution in
Fig. 3. The association of CD3 and inner rafts is insensitive to cholesterol extraction and does not require signaling events. (A) Jurkat T cells expressing MyrPalm-mCFP were treated with 12.5 mM M\textsubscript{ij/CD}, and then incubated with anti-CD3 antibodies to induce patching and stained with CT-B-FITC to visualize GM1 expression. (B) Jurkat T cells expressing MyrPalm-mCFP were treated with 10\mu M PP2, then incubated immediately with anti-CD3 antibodies to induce patching. TXRD-conjugated anti-Ig antibodies were used to visualize CD3 patches. Confocal images were taken with identical settings. Data are representative of at least 20 cells of one of two independent experiments and in each experiment more than 90% of the capped cells showed the indicated phenotypes. Bar, 10\mu M. In this experiment mCFP was color-coded green, anti-Ig-TXRD red, and CT-B-FITC blue; thus yellow represents the superimposition of green and red and white that of green-red and blue.

CD3 patches by a process that is not affected by membrane cholesterol depletion. We do not exclude that, by further reducing the membrane cholesterol level, it would be possible to uncouple cross-linked CD3 and the inner raft marker, but a stronger M\textsubscript{ij/CD} treatment dramatically reduce T cell viability [16] and thus the interpretation of the results. Schuck et al. [18] showed that in Madin-Darby canine kidney cells, M\textsubscript{ij/CD} treatment does not destroy lipid rafts, even when cholesterol was depleted by more than 70%. Using the same cells transfected with MyrPalm-mCFP and MyrPalm-mYFP, however, Tsien and co-workers [9] showed the existence of basal, concentration-independent, fluorescence resonance energy transfer (FRET) between the chromophores, which is lost after M\textsubscript{ij/CD} treatment. This was interpreted as evidence for raft disruption, but it may alternatively be speculated that the reduction in FRET efficiency is due to altered mobility of the acylated proteins within lipid rafts. Distinction among the different possibilities is beyond the aim of this manuscript and will require specific studies.

Our results suggest that the generation of large, stable rafts induced by GM1 or CD59 cross-linking is more dependent on plasma membrane cholesterol levels than is the generation of large, stable rafts induced by TCR/CD3 cross-linking. These findings may have important implications for the interpretation of signaling experiments. We previously showed that in T cells M\textsubscript{ij/CD} treatment inhibits signaling through the GPI-anchored protein CD59, but not through the TCR/CD3, indicating that CD59, but not CD3, requires membrane cholesterol for signal transduction [16]. The present data allow a better understanding of those results. We found that cholesterol is required to couple CD59 to inner lipid rafts following anti-CD59 cross-linking; it is thus not surprising that no signal is generated in M\textsubscript{ij/CD}-treated cells after CD59 ligation by specific antibodies. Once CD59 and rafts are dissociated, in fact, there is no possibility of communication between the GPI-anchored protein and the internal signaling molecules. In contrast, cholesterol extraction does not dissociate cross-linked CD3 from inner rafts, consistent with anti-CD3 signaling resistance to M\textsubscript{ij/CD} treatment. In this scenario, therefore, it can be concluded that the effects of M\textsubscript{ij/CD} treatment cannot be considered an argument in favor of, or against, the importance of lipid rafts in TCR signaling, and that alternative approaches must be devised to address this question.
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