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Lipid Raft Distribution of CD4 Depends on its Palmitoylation and Association with Lck, and Evidence for CD4-Induced Lipid Raft Aggregation as an Additional Mechanism to Enhance CD3 Signaling

Roben Fragoso,* Dejian Ren,† Xiaoping Zhang,‡ Michael Wei-Chih Su,* Steven J. Burakoff,* and Yong-Jiu Jin‡

By mutagenesis, we demonstrated that the palmitoylation of the membrane-proximal Cys<sup>396</sup> and Cys<sup>399</sup> of CD4, and the association of CD4 with Lck contribute to the enrichment of CD4 in lipid rafts. Ab cross-linking of CD4 induces an extensive membrane patching on the T cell surface, which is related to lipid raft aggregation. The lipid raft localization of CD4 is critical for CD4 to induce the aggregation of lipid rafts. The localization of CD4 in lipid rafts also correlates to the ability of CD4 to enhance receptor tyrosine phosphorylation. Thus, our data suggest that CD4-induced aggregation of lipid rafts may play an additional role in CD4 signaling besides its adhesion to MHC molecules and association with Lck. *The Journal of Immunology, 2003, 170: 913–921.

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D4, a 55-kDa integral plasma membrane glycoprotein, is expressed by T lymphocytes that recognize Ag in association with MHC class II molecules (1–4). It functions as a T cell coreceptor to augment T cell activation. So far, it has been known that CD4 does so in two ways. Its extracellular domain interacts with nonpolymorphic regions of the MHC class II molecules, stabilizing the interaction between TCR and its ligand (5). CD4 also enhances signal transduction by the interaction between its cytoplasmic tail and Lck, an Src family tyrosine kinase (6–8).

CD4 is enriched in the lipid rafts of the plasma membrane (9–11). Lipid rafts are the nonionic detergent-insoluble lipid microdomains composed primarily of sphingolipids and cholesterol. Lipid rafts are enriched with GPI-linked proteins, surface receptors and tyrosine kinases, suggesting that lipid rafts may serve as the functional modules for signal transduction and membrane trafficking (12–16). In the T cell surface membrane, lipid rafts are enriched with molecules involved in T cell activation, such as CD3ξ (17), coreceptors CD4 and CD8 (9), Src family kinase Lck and Fyn (18, 19), transmembrane adaptor linker for activation of T cells (LAT) (20) and the Src homology domain-containing leukocyte protein of 76 kDa (21). In contrast, protein tyrosine phosphatases (PTPs), including CD45 and membrane-associated fractions of Src homology protein-1 and Src homology protein-2 are basically not present in lipid rafts (22–24). When T cells are stimulated through TCR engagement or through CD3/CD28 costimulation, lipid rafts cluster at the site of engagement (25–27). Ag-induced translocation of PKC-θ to lipid rafts at the synapse is required for T cell activation (16). Thus, the aggregation of lipid rafts at the contact area may lead to the concentration of protein tyrosine kinases in the contact area while excluding PTPs from the area, a mechanism that may drive the signaling cascades of tyrosine phosphorylation for T cell activation. Recently, it has been reported (25) that the costimulatory role of CD28 is associated with the induction of membrane capping. Viola et al. (25) showed that lipid rafts form caps in the contact areas between T cells and the beads coated with CD3 plus CD28, but not the beads coated with CD3 alone. Others have shown that the costimulatory mechanism of adhesion molecules, including CD2, CD48, CD44, CD5, and CD9, also involves lipid raft reorganization (28–31). It is possible that CD4 also plays its costimulatory role by inducing the reorganization of lipid rafts.

Previously, it was shown that the membrane-proximal cysteine residues of CD4 are palmitoylated when expressed in HeLa cells (32). The palmitoylation of membrane-proximal cysteines is known to be essential for the lipid raft localization of several transmembrane proteins, including LAT and CD8β (20, 33). Src family kinases Lck, Fyn, Hck, and Lyn are palmitoylated at the amino-terminal cysteines, which are critical for both their membrane association and lipid raft targeting (34–40). Significantly, these palmitoylated cysteines are all present in a conserved motif consisting of cysteines and hydrophobic residues (CVRC in LAT, GCVC in Lck and Fyn, and CVRR in CD8β). CD4 also contains a CVRC motif at the membrane-proximal region that may be critical for CD4 targeting into lipid rafts.

In this report, we demonstrated that the enrichment of CD4 in lipid rafts relies on the palmitoylation at membrane-proximal cysteines and CD4’s association with Lck. Our results suggest that CD4-induced aggregation of lipid rafts may play some roles in CD4 coreceptor signaling.

Materials and Methods

Cells and Abs

Jurkat T cell line J77, a variant of clone E6-1 (American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium, supplemented with 10% FCS at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.
JCAM.1.1 T cells were obtained from American Type Culture Collection. Anti-CD3 mAb (OKT3), anti-CD4 mAb (OKT4), and anti-CD8 mAb (OKT8) were prepared from the hybridomas obtained from American Type Culture Collection. Anti-CD4 and anti-CD8 polyclonal Abs and anti-CD3 and anti-CD45 mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-Tyr (4G10) mAb and anti-Lck and anti-LAT polyclonal Abs were purchased from Upstate Biotechnology (Lake Placid, NY). PE-conjugated anti-human CD4 (Leu-3A-PE), CyChrome-conjugated anti-human CD8, and rhodamine-conjugated anti-mouse Ig were purchased from BD Pharmingen (San Diego, CA). Anti-p-Tyr (RC20) was purchased from BD Transduction Laboratories (Lexington, KY). Anti-streptavidin-HRP, anti-mouse Ig-HRP, and anti-rabbit Ig-HRP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). FITC-conjugated cholera toxin (CTx) and HRP-conjugated CTx were purchased from Sigma-Aldrich (St. Louis, MO).

Construction and stable expression of plasmids of CD4 mutants in Jurkat T cells

To construct CD4 mutant plasmids, PCR was performed with a human CD4 template using 5′-end PCR primer 5′-GCTGACGGGCAGTGGC 5′-GAGCTGTGGGCAAGGAAGATGCCTAGCCC, 5′-CGCTCTGCTTGGCGCCTTCGGTGCCGGCACCTGACTCAGA and 5′-CGCTCTGCTTGGCGCCTTCGGTGCCGGCACCTGACTCAGA for the 2C CD4 mutant, 2Cm CD4 mutant, and tailless CD4 mutant, respectively. The changed nucleotides are underlined. The PCR products were digested by BstEII/BamHI and used to replace the same restriction fragment of wild-type (wt) CD4 subcloned in pBlueScript. The 4C CD4 mutant was generated by using the same approach that was used to generate the 2C CD4 mutant, but with the 2Cm CD4 as the template. The CD4 tailless mutant was generated by introduction of a stop codon in place of Cys396. CD4 mutants were subcloned into the mammalian expression vector pEFI-Myc-His (Invitrogen, Carlsbad, CA) using the EcoRI and Xhol sites. The chimeric CD4aCD4 was generated by PCR using human CD8a and CD4 in pEBB as the templates. The complementary primer for sense CD8aCD4 chimeric junctions is 5′-GGCTGGACACTTGCGCGCCCTCGTTGTCGGCGCCGGCCGGCCCTCTCCTG (the underlined is from CD4 transmembrane region), which was used to generate 5′ to 3′ PCR fragments by using additional primers internal to pEBB. The fragments were then isolated and mixed and then PCR extended. The resulting product was digested with BamHI and subcloned into pcDNA3. Plasmids (5 μg) were transfected into Jurkat T cells (1 × 10⁶/500 μl) by electroporation with the settings of 800 μF, low ohm, and 250 V. The clones with mutant CD4 and CD8α stable expression were selected in medium containing neomycin (G418) at 2.5 mg/ml.

Metabolic labeling of CD4 and CD8 with [3H]palmitate and biotin

For [3H]palmitate labeling, Jurkat T cells (2 × 10⁷) were cultured with 1 mCi [3H]palmitate (NEN, Boston, MA) for 4 h in 2 ml RPMI 1640 medium containing 5% diazoy FCS. Cells were then washed three times with RPMI 1640 medium and then lysed for immunoprecipitation. For biotin labeling, cells were washed three times with cold PBS (Ca²⁺, Mg²⁺) (20 mM sodium phosphate (pH 7.4), 0.15 M NaCl, 1 mM MgCl₂, and 0.1 mM CaCl₂), and then incubated with sulfo-LC-NHS-biotin (Pierce, Rockford, IL) at a concentration of 0.5 mg/ml in PBS (Ca²⁺, Mg²⁺) at 4°C for 30 min with gentle agitation. The reaction was stopped by cell pelleting and washed two times with 50 mM glycine in PBS (Ca²⁺, Mg²⁺) at 4°C for a total of 10 min.

Sucrose density gradient fractionation of crude T cell membrane

Crude T cell membrane was prepared as described previously (24). The crude membrane preparation was lysed at 4°C for 1 h in 1 ml of 1% Triton X-100/0.1 M EDTA, which was used for immunoprecipitation with Lck, whereas the CD4 mutants lacking Cys422 and Cys424, including two in the membrane-proximal CVRC motif and two at the Lck binding site, were localized outside lipid rafts. To define the amino acid residues responsible for the localization of CD4 in lipid rafts, we generated a panel of mutant CD4 constructs, including 2C CD4, 2Cm CD4, VR CD4, 4C CD4, and tailless CD4 (Fig. 1A). In the 2C CD4 mutant, Cys422 and Cys424, which are essential for Lck binding, were changed to alanines. In the 2Cm CD4 mutant, membrane-proximal CVRC motifs were changed to threonine and glutamic acid. All of these mutants were stably expressed in Jurkat T cells. The levels of surface expression of the CD4 mutants were shown by FACS analysis (Fig. 1B). The immunoprecipitation with anti-Cd showed that the wt CD4, 2Cm CD4, and VR CD4 were coimmunoprecipitated with Lck, whereas the CD4 mutants lacking Cys422 and Cys424, including 2C, 4C, and tailless CD4, were not coimmunoprecipitated with Lck (Fig. 1C). The levels of expression of Lck in these CD4-transfected Jurkat T cells are similar to one another, as determined by anti-Lck immunoblotting (data not shown).

To determine the distribution of mutant CD4 in lipid rafts, the crude membrane preparations were lysed in 1% Triton X-100 solution and segregated into nine fractions using sucrose gradient sedimentation. Fraction 3 was the most enriched with lipid rafts.
(insoluble in Triton X-100), and fractions 8 and 9 were derived from outside the lipid rafts (soluble in Triton X-100) (Fig. 2A). The immunoblotting with anti-CD4 showed that the wt CD4 was enriched in lipid rafts, whereas tailless CD4 was eliminated from lipid rafts. This indicates that the localization of CD4 in lipid rafts is dependent on the cytoplasmic tail of CD4. The 2C CD4 mutant, lacking two cysteines for Lck binding, was less enriched in lipid rafts. 2Cm CD4, with two cysteines mutated in the CVRC motif, was also less enriched in lipid rafts. 4C CD4, with all four cysteines changed to alanines, was essentially outside lipid rafts. As the control, anti-LAT immunoblotting showed that the lipid raft distribution of LAT in these cell lines was not changed. The results indicate that the cysteine residues at the potential palmitoylation site and at the Lck binding site are responsible for the localization of CD4 in lipid rafts. VR mutation of CD4 did not affect the lipid raft localization of CD4, which indicates that the conserved hydrophobic residues in the CVRC motif are not critical for lipid raft targeting. We obtained similar results when surface biotin-labeled CD4 mutants were analyzed by sucrose density gradient centrifugation (data not shown).

The role of these four cysteines in lipid raft localization of CD4 was further examined in JCAM.1 T cells. JCAM.1 is a variant of Jurkat lacking the expression of functional Lck (42). Although a
low level of truncated ~49-kDa CD4 (<10%) can be detected in JCAM.1. It is poorly associated with CD4 (data not shown). Therefore, it allowed us to assess the lipid raft localization of CD4 in the absence of Lck association. As shown in Fig. 2B, ~20% of the wt CD4 was distributed in lipid rafts of JCAM.1 T cells, which is about half of the wt CD4 distributed in lipid rafts of Jurkat T cells. With the Cys306 and Cys399 of the CVRC motif changed to alanines, 2Cm CD4 in JCAM.1 T cells was predominantly outside lipid rafts, although Cys422 and Cys424 are intact. The lipid raft distribution of LAT in these JCAM cell lines was not changed. The results indicate that the membrane-proximal Cys306 and Cys399 are lipid raft-targeting signal for CD4 and that Cys422 and Cys424 function through their association with Lck.

The percentage of CD4 distributed in lipid rafts was analyzed by densitometry (Fig. 2C). In Jurkat T cells, ~60–65% of wt CD4 and VR CD4 and ~20% of 2C CD4 and 2Cm CD4 are distributed in lipid rafts. Tailless and 4C CD4 are essentially not present in lipid rafts. In JCAM.1 T cells, ~20% of wt CD4 is in lipid rafts, whereas only 4% of 2Cm CD4 is in lipid rafts. The presence of a small amount of 2Cm CD4 in lipid rafts may be due to its association with the truncated Lck.

Palmitoylation of Cys306 and Cys399 in CVRC motif is critical for lipid raft targeting

Previously, Cys306 and Cys399 of CD4 were shown to be palmitoylated in HeLa cells (32). We examined whether Cys306 and Cys399 of CD4 are specifically palmitoylated in T cells. CD4 mutants were metabolically labeled with [3H]palmitate. After immunoprecipitation, the CD4 complex was analyzed on an 8% nonreducing SDS-PAGE, which separated CD4 from Lck. [3H]Palmitate was found by fluorography to be incorporated into the wt CD4, 2C CD4, and VR CD4, but not 2Cm CD4, indicating that Cys306 and Cys399 of CD4 are the specific palmitoylation sites (Fig. 3A). A 56-kDa protein that coimmunoprecipitated with wt CD4, 2Cm CD4, and VR CD4 was also [3H]palmitoylated. This protein was identified to be Lck by specific immunoblotting against Lck. The [3H]palmitoylated Lck was coimmunoprecipitated with wt CD4, 2Cm CD4, and VR CD4, but not 2C CD4. The VR CD4 mutant was [3H]palmitoylated, indicating that a mutation in the valine and the arginine did not affect the palmitoylation of Cys306 and Cys399 in the CVRC motif.

To determine whether the palmitoylation of CD4 is required for its lipid raft localization, Jurkat T cells expressing the wt CD4 and

FIGURE 3. Palmitoylation of CD4 is critical for its lipid raft localization. A, [3H]palmitoylation of CD4 in Jurkat T cells. Following [3H]palmitate labeling, CD4 was immunoprecipitated with OKT4. The samples were resolved by 8% nonreducing SDS-PAGE, and then subjected to [3H]autofluorography for 3 wk (left panel) or immunoblotted with specific Abs (right panel). B, 2-bromopalmitate blocked the distribution of CD4 in lipid rafts. Jurkat T cells were treated with 2-bromopalmitate followed by sucrose gradient segregation. Twenty microliters of each fraction from wt CD4 cells was immunoblotted with anti-CD4, anti-CD3ζ, and anti-CD45. Fractions from 2C CD4 were immunoblotted with anti-CD4.

FIGURE 4. Lipid raft distribution and [3H]palmitoylation of CD8α in Jurkat T cells. A, Schematic map of the intracellular region of CD8α wt and chimeric CD8αCD4. B, Flow cytometry of CD8α expressed in Jurkat T cells. CD8α was stained with CyChrome-conjugated anti-human CD8. C, Sucrose gradient analysis of CD8α in lipid rafts. T cells were surface biotin labeled. A volume of 0.5 ml of each fraction was added to 1 ml of 1.5% NP-40 lysis buffer and 5 μg/ml anti-CD8α (OKT8) for immunoprecipitation. The samples were immunoblotted with anti-streptavidin-HRP. D, [3H]Palmitoylation of CD8α. T cells were labeled with [3H]palmitate. CD8α was immunoprecipitated with OKT8. After SDS-PAGE, it was subjected to [3H]autofluorography for 3 wk (left panel) or immunoblotted with polyclonal anti-CD8α (right panel).
2C CD4 were incubated with 2-bromopalmitate, which inhibits protein palmitoylation (43). After incubation, no palmitoylation was detected in these CD4 molecules (data not shown). In sucrose gradient, the wt CD4 distributed in lipid rafts was greatly reduced while 2C CD4 was detected mostly outside lipid rafts (Fig. 3B). In contrast, the distribution of CD3/H9256 and CD45 were shown to be largely unaffected by 2-bromopalmitate treatment. These results indicate that the palmitoylation of CD4 at Cys396 and Cys399 is the signal for its lipid raft targeting.

Introduction of CD4 tail into CD8/H9251 enhances its lipid raft localization

We have shown that the palmitoylation of cysteines in the CVRC motif of CD4 and its association with Lck are responsible for targeting CD4 into lipid rafts. This mechanism was further investigated by introducing the CD4 tail into the CD8/H9251 molecule (Fig. 4). CD8/H9251 contains a single membrane-proximal cysteine but lacks the second cysteine. Previously, CD8/H9251 homodimers have been shown to be predominantly outside lipid rafts (33). The chimeric CD8α/CD4 was constructed by using the CD8α extracellular domain fused to the transmembrane and the intracellular domains of CD4 (Fig. 4A). CD8α/CD4 mutant was stably expressed in Jurkat T cells. The cell surface expressions of CD8α wt and CD8α/CD4 were shown by cytometric analysis (Fig. 4B). After surface biotin labeling, the distribution of CD8α was analyzed by sucrose gradient sedimentation (Fig. 4C). The wt CD8α was shown to be predominantly distributed outside lipid rafts. The chimeric CD8α/CD4, like the wt CD4 molecule, was greatly enriched in lipid rafts. The results indicated that the CD4 tail contains the signals for lipid raft targeting. We also performed the experiments by immunoblotting with anti-CD8α and obtained similar results, although the signal is relatively weaker (data not shown).

CD8α contains a membrane-proximal cysteine. The distribution of CD8α outside the lipid rafts suggests that CD8α may not be palmitoylated. However, by [3H]palmitate labeling, we found that wt CD8α is palmitoylated (Fig. 4D). The results suggest that palmitoylation at the single membrane-proximal cysteine may not be sufficient to confer strong lipid raft localization.

The lipid raft localization of CD4 correlates to its ability to enhance tyrosine phosphorylation

To determine whether the distribution of CD4 in lipid rafts is relevant to its biological function, we examined the receptor tyrosine phosphorylation by costimulation of CD4 and CD3 in Jurkat T cells (Fig. 5). With the low concentration of plate-bound OKT3, an anti-CD3ε mAb, CD3 stimulation alone induced a relatively low level of tyrosine phosphorylation, which was enhanced by plate-bound OKT4 costimulation. The anti-Ptyr immunoprecipitation and the anti-ζ immunoprecipitation showed that the overall tyrosine phosphorylation and the specific CD3ζ phosphorylation were significantly enhanced by costimulation with wt CD4, VR CD4, 2Cm CD4, and 2C CD4, but less enhanced by 4C CD4 and tailless CD4. The anti-Ptyr immunoblots (bottom) were stripped and then reimmunoblotted with anti-CD3ζ, which showed that

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**FIGURE 5.** Distribution of CD4 in lipid rafts is critical for CD4 to augment CD3-induced tyrosine phosphorylation. Tyrosine phosphorylation was induced by plate-bound OKT3 and OKT4 in Jurkat T cells expressing wt CD4, VR CD4, 2Cm CD4, 2C CD4, 4C CD4, and tailless CD4. After stimulation, cells were lysed in 1% NP-40 lysis buffer and immunoprecipitated with anti-pTyr (4G10) (top panels) or with anti-CD3ζ (bottom panels). It was immunoblotted with an anti-pTyr Ab (RC20).

**FIGURE 6.** Effects of CD4 cross-linking on lipid raft distribution of CD4 and other membrane proteins in Jurkat T cells. A, Cells expressing wt CD4 were cross-linked in solution with OKT4, OKT3, or OKT4 plus OKT3. Sucrose gradient fraction 3 (lipid raft fraction) and fraction 9 (non-lipid raft) were immunoblotted with anti-CD4. B, Cells expressing wt CD4 were cross-linked with OKT4. Fractions 1–9 were immunoblotted with specific Abs for CD4, Lck, LAT, CD3ζ, and CD45 and with HRP-CTx for GM1. C, Cells expressing wt CD4 (JA20) and different CD4 mutants were cross-linked with OKT4. Fractions 1–9 were immunoblotted with anti-CD4.
similar amounts of CD3ζ were immunoprecipitated in these cell lines with each stimulation as indicated (data not shown). The tyrosine phosphorylated 70-kDa, 56-kDa, and 38-kDa proteins in the immunoprecipitation of anti-Pyr are most likely ζ-associated protein 70, Lck, and LAT, respectively. Of note, 2C CD4 is capable of enhancing the tyrosine phosphorylation, although it is not associated with Lck (Fig. 1). The wt CD4 and VR CD4 appear to be more effective than 2C CD4 and 2Cm CD4 in the enhancement of CD3-induced tyrosine phosphorylation. These results indicate that the lipid raft localization of CD4 correlates to its ability to enhance CD3-induced tyrosine phosphorylation.

Cross-linking of CD4 with Ab induced the strong lipid raft aggregation on the T cell plasma membrane

The correlation between the lipid raft localization of CD4 and its ability to enhance tyrosine phosphorylation suggests that CD4 may function through lipid rafts. Such a mechanism of amplifying receptor signaling has been proposed for the CD28 costimulatory molecule, which is involved in the redistribution of lipid rafts and the enhancement of substrate tyrosine phosphorylation (25). The aggregation of lipid rafts was also proposed for the costimulatory mechanism of adhesion molecules, including CD2, CD48, and, recently, CD44 molecules (28–30).

The reorganization of lipid rafts upon Ab cross-linking of CD4 was evidenced by sucrose gradient analysis (Fig. 6). We found that the amount of CD4 in fraction 3 (lipid rafts) was markedly increased upon cross-linking of CD4 while decreased in fraction 9 (nonlipid) (Fig. 6A). Such increase was not observed with cross-linking of CD3 alone. Co-cross-linking of CD4 with CD3 had an effect similar to that of the cross-linking of CD4 alone. To understand the mechanism underlying the CD4 redistribution, we analyzed the distribution of other membrane proteins after CD4 cross-linking (Fig. 6B). The results showed that the lipid raft-localized Lck, LAT, and CD3ζ were similarly increased in lipid raft fraction as CD4, whereas the non-lipid raft protein, such as CD45, was not affected. GM1, a marker of lipid rafts, was also increased upon cross-linking of CD4. Because no evidence showed a direct association of CD4 with LAT, CD3ζ, and GM1, CD4 cross-linking appeared to increase the amount of lipid rafts in fraction 3. It is known that clusters of lipid rafts are more resistant to nonionic detergent and therefore can keep more lipid raft components in Triton X-100-resistant fractions (44). We hypothesize that the redistribution induced by cross-linking of CD4 is most likely due to the aggregation of lipid rafts.

Next, we analyzed the effects of Ab cross-linking on the redistribution of CD4 mutants (Fig. 6C). The results showed that wt CD4 from JA20 T cell, a cell line expressing CD4 derived from Jurkat T cells by cell sorting and cloning (obtained from Dr. O. Acuto (Institute Pasteur, Paris, France)), and CD4 mutants VR, 2Cm, and 2C were increased in lipid raft fraction, whereas 4C and tailless CD4 were not. These results indicate that the aggregation of lipid rafts is induced only by CD4 presented in lipid rafts.

Furthermore, CD4-induced aggregation of lipid rafts was examined on plasma membrane by inspection of membrane patching using confocal microscopy (Fig. 7). We focused our studies on 2C CD4 and tailless CD4, because both mutants are not associated with Lck but show the difference in inducing lipid raft aggregation and tyrosine phosphorylation (Figs. 5 and 6). As shown in Fig. 7A, CD4 was evenly stained over the surface membrane before cross-linking (a). After cross-linking, CD4 molecules, including wt

**FIGURE 7.** Confocal microscopy shows the patching and topological redistribution of CD4 and GM1 upon cross-linking of CD4. A. Patching of CD4 mutants in Jurkat T cells. The cells in a were fixed and then stained with Leu 3A-PE; the cells in b, c, and d were cross-linked with OKT4 and rhodamine-conjugated anti-mouse Ig and then fixed. B. GM1 staining (green) after cross-linking of mutant CD4 in Jurkat. wt CD4 T cells (a) were not cross-linked; cells in b, c, and d were cross-linked with OKT4 and rhodamine-conjugated anti-mouse Ig. The top panels show patching of CD4; the bottom panels show staining of GM1. C. Patching of CD4 and staining of GM1 in JCAM T cells. wt CD4 (a) and 2C CD4 (b) were cross-linked as described above and then stained with FITC-CTx. The top panels show patching of CD4; the bottom panels show the GM1 staining. All results are the representative areas from three experiments, and >1000 cells were examined.
CD4, 2C CD4, and tailless CD4, were found clustered in almost all of the cells examined (b, c, and d). In each case, we have examined several separate areas (50–100 cells) several times and obtained similar results. The clustering or patching of tailless CD4 was less apparent than that of wt CD4 and 2C CD4 based on the average size of the clusters. To find whether the patching of these CD4 mutants is related to the aggregation of lipid rafts, we stained the glycosphingolipid GM1 with FITC-CTx, a marker for lipid rafts (45). As shown in Fig. 7B, the patching of wt CD4 and 2C CD4 matched well with the GM1 staining, similar to the observation reported recently by Popik et al. (46). In contrast, although tailless CD4 induced some clustering on the T cell membrane, the GM1 largely remained evenly distributed.

The results showed that 2C CD4 without Lck association induced the lipid raft aggregation. To find whether CD4 may induce lipid raft aggregation in the absence of Lck, we investigated the lipid raft aggregation in JCAM T cells. As shown in Fig. 7C, after cross-linking, both wt CD4 and 2C CD4 were patched. However, there was no apparent lipid raft aggregation shown by GM1 staining. The results indicate that the kinase activity of Lck is required for CD4-induced lipid raft aggregation, although the direct association between CD4 and Lck is not essential.

Discussion

Although CD4 is known to be enriched in lipid rafts, the requirement for its lipid raft localization remained ill-defined. In this study, we demonstrated that the palmitoylation of Cys394 and Cys397 in the CVRC motif of CD4 and CD4’s association with Lck are essential for keeping CD4 highly concentrated in lipid rafts (Figs. 2 and 3). We also found that both CD4 and Lck were palmitoylated in the complex formed by CD4 and Lck, which may enhance or stabilize their localization in lipid rafts (Fig. 3). However, the hydrophobic residues in the CVRC motif conserved in many palmitoylated proteins did not play an important role in the palmitoylation and localization of CD4. In contrast, we found that dual cysteine palmitoylation of the motif was important. CD4 was more enriched than the CD8α homodimer in lipid rafts (Figs. 2–4). The scant presence of CD8α in lipid rafts is likely due to the lack of a second cysteine. Previously, the molecules LAT and growth-associated protein 43 kDa were shown to be no longer enriched in lipid rafts when any one of the cysteines in the dual cysteine motif was mutated (20, 47). Therefore, our results support the notion that the dual cysteine motif near the plasma membrane is the optimal structure to localize transmembrane proteins into lipid rafts.

Ab cross-linking of CD4 on the T cell surface induced an extensive membrane capping, which is shown to be related to the aggregation of lipid rafts (Figs. 6 and 7). The ability of CD4 to mediate the aggregation of lipid rafts relies on its allocation in lipid rafts. In our experiments, cross-linking of CD4 induced more clustering than that of CD3 (data not shown). This may be attributed to the more enriched or more stable lipid raft localization of CD4 than that of CD3. Among the CD3 subunits, only CD3ζ can be clearly detected in detergent insoluble lipid rafts (Figs. 3 and 6) (17, 23, 24, 48). These results suggested that TCR/CD3 stimulation may require the coordinated action of CD4 to promote the aggregation of lipid rafts, which may facilitate the formation of the immunological synapse in the contact area between T cells and the APCs.

The lipid raft aggregation induced by cross-linking of CD4 may explain some observations of CD4 function that could not be adequately explained before. First, chimeric CD4/Lck, composed of the CD4 extracellular and transmembrane domain ligated to the catalytic domain-depleted form of Lck, is capable of enhancing the tyrosine phosphorylation in Jurkat T cells and enhancing IL-2 production in a CD4-dependent T cell hybridoma (49, 50). In our experiment, the cross-linking of 2C CD4 mutant that lacks the binding site for Lck enhanced the receptor tyrosine phosphorylation (Fig. 5). The cross-linking of CD4 alone was capable of stimulating a small increase in phosphatidylinositol and Ca2+ or inhibiting the CD3-induced calcium mobilization (51–53). Second, for costimulation of CD4, the direct co-cross-linking of CD3 with CD4 appears not to be necessary. It has been well demonstrated, as well as shown in Fig. 5, that anti-CD3 and anti-CD4 Abs immobilized to the same solid support can augment TCR/CD3-induced T cell response (54–56, 51). CD4 can enhance the T cell response regardless of whether it binds to the same or different MHC molecules as the TCR (2). The engagement of CD4 with ligand or anti-CD4 in solution can positively or negatively affect the outcome of TCR/CD3 stimulation, which appears to be dependent on the concentration of anti-CD4 Abs and the conditions used for costimulation of CD4 (57–62). The inhibition of CD3/TCR signaling was also observed by cross-linking of CD4 incapable of Lck binding (equivalent to our 2C CD4) (62). This suggests that the desensitization of TCR/CD3 stimulation upon cross-linking of CD4 may also be related to the aggregation of lipid rafts when imposed nonsynchronously with TCR/CD3 stimulation. It may be hypothesized that the effective TCR/CD3 stimulation should be accompanied by capping. CD4 treatment is not capable of inducing the effective T cell activation but instead results in lipid raft aggregation or capping, which inhibits the following “restimulation” with CD3. It is also possible that the structure and the constituents of a formed cap may be changed gradually, such as by the recruitment of PTPs, which leads to the down-regulation of tyrosine phosphorylation. Third, with the aid of three-dimensional video microscopy, it was recently demonstrated that, after the onset of TCR activation, CD3 was found clustered in the central contact area, whereas CD4 molecules moved to the periphery (27). This apparent disparity between CD3 and CD4 in the locality of the immunological synapse again suggests that CD4 may function without direct association with the TCR/CD3 complex. Indeed, the direct binding between CD4 and CD3 complex has not been demonstrated so far. In contrast, some evidence has already shown that CD4 engagement may affect the structure of the cytoskeleton, a cellular component closely linked to lipid rafts (63). Ab cross-linking of CD4 also induced an actin cytoskeleton polymerization-dependent attenuation of IL-2 receptor signaling (64). These structural reorganizations may involve protein tyrosine phosphorylation, which depends on Lck. This could explain why cross-linking of CD4 in JCAM T cells failed to induce lipid raft aggregation (Fig. 7C).

Our results show that the cross-linking of the non-lipid raft-localized tailless CD4 induced the membrane patching, which probably resulted from the clustering of non-lipid membrane microdomain (44) (Fig. 7B). The patching induced by tailless CD4 failed in enhancing the receptor tyrosine phosphorylation (Fig. 5). These different patterns of membrane patching induced by CD4 may ultimately influence the potency of TCR/CD3 signaling. Thus, while a potent agonist ligand often triggers the formation of large caps on the surface of T cells, weak agonist and antagonist ligands, which do not bind or bind weakly to CD4, can induce only small, unstable patterns of raft clustering (65–68). We speculate that the quality and extent of CD4-mediated redistribution of lipid rafts may help to discriminate between the quality of ligands and may affect the overall potency of TCR stimulation, as suggested by others (57–62, 69).

Our finding that CD4 mediates the redistribution and clustering of intracellular kinase-rich raft microdomains furthers our understanding of the coreceptor function of CD4. Such a mechanism of amplifying receptor signaling has been proposed for the CD28...
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