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Protein Interactions between CD2 and Lck Are Required for the Lipid Raft Distribution of CD2

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In T lymphocytes, lipid rafts are preferred sites for signal transduction initiation and amplification. Many cell membrane receptors, such as the TCR, coreceptors, and accessory molecules associate within these microdomains upon cell activation. However, it is still unclear in most cases whether these receptors interact with rafts through lipid-based amino acid modifications or whether raft insertion is driven by protein-protein interactions. In murine T cells, a significant fraction of CD2 associates with membrane lipid rafts. We have addressed the mechanisms that control the localization of rat CD2 at the plasma membrane, and its redistribution within lipid rafts induced upon activation. Following incubation of rat CD2-expressing cells with radioactive-labeled palmitic acid, or using CD2 mutants with Cys226 and Cys228 replaced by alanine residues, we found no evidence that rat CD2 was subjected to lipid modifications that could favor the translocation to lipid rafts, discarding palmitoylation as the principal mechanism for raft addressing. In contrast, using Jurkat cells expressing different CD2 and Lck mutants, we show that the association of CD2 with the rafts fully correlates with CD2 capacity to bind to lipid rafts, as CD2 physically interacts with both Lck and Fyn, preferentially inside lipid rafts, and reflecting the increase of CD2 in lipid rafts following activation, CD2 can mediate the interaction between the two kinases and the consequent boost in kinase activity in lipid rafts. The Journal of Immunology, 2008, 180: 988–997.

Cell surface molecule CD2 is a 45- to 58-kDa type I integral protein expressed on virtually all T lineage and NK cells (1). The extracellular domain of CD2 is composed by two Ig superfamily domains (2), of which the membrane-distal V-like domain is involved in the binding to the ligand (3). CD2 has different ligands in rodents (CD48) and humans (CD58), and the mechanisms of ligand binding are substantially different, as assessed by thermodynamic analysis (4, 5). Nevertheless, engagement of CD2 to CD58 in humans, or to CD48 in rodents, facilitates adhesion between T cells and APC and is proposed to promote the formation of an optimal intercellular membrane spacing (∼140 Å) suitable for TCR recognition of a peptide Ag bound to MHC (6).

Concomitantly with its function as an adhesion molecule, CD2 has a role in the process of signal transduction. It is known that stimulation of CD2 with combinations of mAb or with the physiological ligand induces T cell proliferation in rats and human (7, 8). Moreover, ligation of CD2 with CD58 augments the IL-12 responsiveness of activated T cells (9), and can also reverse T cell anergy induced by B7 blockade (10). In contrast, CD2 interacts with the inhibitory receptor CD5 (11, 12), and through this association is able to amplify modulatory signals at the T cell surface (11, 13).

The cytoplasmic domain is required for CD2-mediated activation (14–17), but as it has no intrinsic enzymatic activity, it depends on physical interactions with other signaling molecules to propagate the stimulus received upon ligand binding. CD2 associates with the Src family tyrosine kinases Lck and Fyn and through these kinases couples to downstream signal transduction pathways (18–20). Proline-rich sequences of the cytoplasmic domain of CD2 have been shown to be involved in the interaction with the Src homology 3 (SH3)4 domains of these kinases (20–22), also with the SH3 domain of the adapter protein CD2AP (23), connecting CD2 to the cytoskeleton, and additionally with the SH3 domain of CD2BP1 (24), resulting in the down-regulation of activation-dependent CD2 adhesion. CD2 binds to the GYF domain of CD2BP2 as well, an interaction resulting in cytokine production (25).

The T cell plasma membrane contains regions of distinct lipid composition, enriched mainly in sphingolipids and cholesterol immersed in a phospholipid-rich environment, and termed lipid rafts (26). In resting cells, key signaling proteins such as the adaptors linker for activation of T cells (LAT) (27) or phosphoprotein associated with glycosphingolipid-enriched microdomain (PAG) (28), or the kinase Fyn (29), are resident in rafts, whereas most integral proteins are found outside these platforms. Localization and targeting of signaling molecules to lipid rafts is largely dependent on posttranslational acylation modification of proteins, one of the most important being membrane-proximal cysteine.
palmitoylation. These cysteine residues are usually present within a conserved motif consisting of cysteines and hydrophobic residues, CVRC in LAT and GCVC in Lck and Fyn. The composition of raft-associated proteins may, nevertheless, change upon cell stimulation. Membrane compartmentalization and partitioning of essential T cell-activating components in lipid rafts were shown to be involved in the initial stages of T cell activation (30). Several costimulatory molecules on the T cell surface, such as CD2 (31), CD5, CD9 (32), and CD28 (33) can up-regulate TCR signals by enhancing the association of the TCR with lipid rafts.

Human CD2 has been shown to translocate to lipid rafts upon CD2 Ab cross-linking or following binding to CD8 during conjugate formation (34), and the shift between phases may result in the replacement of CD2BP2 by raft-resident Fyn (22), which competes for the same proline-rich sequence of the cytoplasmic tail of CD2. In contrast to human CD2, which is reported to be entirely non-raft-resident in resting cells (34), mouse CD2 contains a CIC motif at the membrane-proximal region that can putatively address CD2 to lipid rafts (32). We have investigated the membrane localization of rat CD2 and its transition between different phases at the T cell surface, and show that a significant fraction of CD2 is constitutively present in lipid rafts, and that this proportion increases upon CD2 stimulation. However, membrane-proximal cysteine palmitoylation is not the decisive tag addressing rat CD2 to lipid rafts, but rather the interaction with the Src family kinase Lck.

Materials and Methods

Antibodies

Monoclonal Ab recognizing rat CD2 were OX-54, OX-55 (8), and OX-34 (35). None of these Abs cross-react with human CD2 as confirmed by FACS (data not shown). The anti-phosphotyrosine mAb 4G10 HRP-conjugated was from Upstate Biotechnology. Polyclonal Abs and conjugates were: rabbit anti-LAT, from Upstate Biotechnology; rabbit anti-Lck, raised against a peptide of aa 39–64 of murine Lck, a gift from J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands); BL90, polyclonal anti-Fyn, a gift from J. Bolen and M. Tomlinson (DNAX Research Institute, Palo Alto, CA); goat anti-mouse peroxidase conjugate, purchased from Zymed Laboratories.

Cells, cDNA cloning, plasmids, and transfections

Splenocytes were obtained by maceration of spleens from 3-mo old Wistar male rats in ice-cold PBS. The rat thymoma cell line W/FU(C58NT)D (36), and its transition between different phases at the T cell surface, and show that a significant fraction of CD2 is constitutively present in lipid rafts, and that this proportion increases upon CD2 stimulation. However, membrane-proximal cysteine palmitoylation is not the decisive tag addressing rat CD2 to lipid rafts, but rather the interaction with the Src family kinase Lck.

Cells were washed and resuspended in PBS containing 0.2% BSA and 0.1% NaN3 (PBS/BSA/NaN3), at a concentration of 1–107 cells/ml. Staining was performed by incubation of 5 × 106 cells/well with MBS buffer (25 mM MES, 150 mM NaCl (pH 6.5), containing 1% Triton X-100, 1 mM PMSF) and cocktail protease inhibitors (1 mM AEBBS, 0.8 µM aprotinin, 50 µM bestatin, 15 µM E-64, 20 µM leupeptin and 10 µM pepstatin A; Calbiochem). The lysates were homogenized by brief sonication for 10 pulses on ice, using a Heat Systems Ultrasonic sonicator (model W-375) equipped with a microtip and set to 50% duty cycle, output 3. To obtain the rafts fraction, cell lysates were mixed with an equal volume of 85% sucrose in MBS buffer and transferred

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to the bottom of Sorvall ultracentrifuge tubes. The samples were then overlaid with 6 ml of 35% sucrose followed by 2 ml of 5% sucrose. After centrifugation at 200,000 × g for 17 h at 4°C in a TST41.14 swing-out rotor (Sorvall), 10 fractions of 1 ml were collected from the bottom of the tube and analyzed by Western blotting. The fractions are labeled from the top of the gradient.

**Metabolic labeling of cellular proteins with 3H-palmitate**

Aliquots of 1.5 × 10⁷ cells were collected from culture, resuspended in 1.5 ml of RPMI 1640 complete medium supplemented with 300 μCi/ml 3H-palmitic acid, and incubated for 16 h at 37°C. Cells were harvested and lysed in lysis buffer (10 mM Tris Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1% (v/v) Triton X-100). The nuclear pellet was removed by centrifugation at 12,000 × g for 10 min at 4°C, and the lysate run on SDS-PAGE under nonreducing conditions. Immunoprecipitations were performed by incubating the lysate with Abs (5 μg of IgG or 2 μl of antiserum) and 100 μl of 10% protein A-Sepharose CL-4B beads (Amersham Biosciences), for 90 min at 4°C by end-over-end rotation. The beads containing the immune complexes were washed three times in 1 ml of lysis buffer, denatured, and loaded on an SDS-PAGE gel. The gel was soaked for 30 min in fixing solution (25/65/10 isopropanol/H₂O/acetic acid) and additionally treated for 30 min with “Amplify” (Amersham Biosciences), for 90 min at 4°C by end-over-end rotation. The beads were cross-linked with rabbit anti-mouse at 20°C at a 1/100 dilution of ascitic fluid. After 5 min of incubation on ice, cells were washed and resuspended in 1 ml of RPMI 1640 complete medium supplemented with 300 μCi/ml of RPMI 1640 complete medium supplemented with 300 μCi/ml of 3H-palmitate, and fractionated by sucrose gradient centrifugation as described.

**Western blotting**

Proteins were separated by SDS-PAGE under reducing or nonreducing conditions and then transferred to Hybond C-extra membranes by electroblotting. Membranes were blocked in 0.1% Tween 20, containing 5% (w/v) nonfat dried milk, probed with unconjugated primary Ab for 1 h and revealed with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (1/20,000 dilution). For phosphotyrosine detection, SDS-PAGE was performed under reducing conditions, and detection was done with incubation with 4G10 HRP-conjugated. Immunoblots were developed using ECL or ECL-plus (Amersham Biosciences) and exposed to CL-XPosure films (Pierce).

**Densitometry**

Densitometric analyses were performed on autoradiographs of the SDS-PAGE gels, using a GS-800 Densimeter (Bio-Rad) and Quantity One software. All densitometry values obtained were calculated from nonsaturated signals.

**Cell activation**

For lipid rafts analysis, ~1.7 × 10⁶ cells were used per sample. Cells were washed and resuspended in 1 ml of RPMI 1640 medium containing 3H-palmitate and incubated with 1 mM PMSF, and 1% (v/v) Triton X-100. After 5 min of incubation on ice, cells were cross-linked with rabbit anti-mouse at 20 μg/ml and maintained at 37°C for 15 min. Cells were then washed twice with ice-cold PBS, lysed, and fractionated by sucrose gradient centrifugation as described.

For phosphotyrosine detection, the procedure was as described (42), using a combination of OX-54 plus OX-55 at 10 μg/ml each. Cell activation for kinase assays was performed as previously described (11), using OX-54 plus OX-55 at 10 μg/ml each.

**Immunoprecipitations and in vitro kinase assays**

Immunoprecipitations, in vitro kinase assays, and reprecipitation of phosphorylated substrates were performed as previously described (19).

**Results**

A significant proportion of CD2 associates with plasma membrane lipid rafts in resting rat T cells and in a rat thymoma cell line

Previous work has shown that in human resting T lymphocytes the transmembrane glycoprotein CD2 is virtually absent from membrane lipid rafts, as defined based on a Triton X-100 insolubility criterion (34). However, in mouse cells a considerable fraction of CD2 molecules is raft-resident (32). We have investigated the membrane localization of the rat homolog of CD2 in resting primary cells as well as in the rat thymoma cell line C58. Nonactin-treated splenocytes or C58 cells were solubilized in a lysis buffer containing 1% Triton X-100, and the lysates were subjected to sucrose gradient centrifugation. The localization of the molecules within or outside lipid rafts was evaluated by immunoblotting with the mAb OX-55, using as marker for the rafts fractions immunoblotting of the resident palmitoylated molecule LAT (27). As measured by densitometry of immunoblot in different experiments, between 7 and 15% of rat CD2 is constitutively confined to the rafts in unstimulated splenocytes, and the majority of CD2 molecules are recovered from the “soluble” phase (Fig. 1). Using nonstimulated C58 cells, we could observe that CD2 is over-represented in the rafts (~75%), compared with the non-rafts fractions. The increased amount of rat CD2 in the rafts of the thymoma cell line, compared with the resting physiological ex vivo cells, probably reflects the level of permanent activation of the cycling cells.

**CD2 targeting to lipid rafts is not fully determined by palmitoylation of Cys²²⁶ and Cys²²⁸**

The difference in raft localization between human and mouse CD2 has been attributed to two membrane-juxtaposed cysteine residues present in the mouse amino acid sequence and absent from the human molecule, which could be palmitoylated and thus address the molecule to lipid rafts. We examined whether the membrane-juxtaposed cysteine residues Cys²²⁶ and Cys²²⁸, also present in rat CD2, might have a role in targeting the molecule to the rafts, using a mutant for which these residues were replaced by alanines. The mutant CD2(CIC/AIA) was stably expressed in E6.1 Jurkat cells, and surface expression levels were confirmed by flow cytometry (data not shown). E6.1-CD2(CIC/AIA) cells, as well as E6.1 cells expressing full-length rat CD2, E6.1-CD2, were lysed in 1% Triton X-100-based lysis buffer, lysates were subjected to sucrose gradient
separation and analyzed by immunoblotting. As can be seen in Fig. 2, wild-type rat CD2 is largely concentrated in lipid rafts (74% as measured by densitometry); however, rat CD2(CIC/AIA) is still able to localize very significantly to the rafts (49%), indicating that the Cys226 and Cys228 are not fully accountable for the lipid raft-targeting of rat CD2.

We next examined whether rat CD2 could in fact be palmitoylated in the cysteine residues. In these studies we used E6.1-CD2 cells, and cells expressing a cytoplasmic deletion mutant, E6.1-CD2(CY 6), containing only the first six amino acids of the cytoplasmic domain, but still retaining the two cysteine residues. Cells were incubated in medium containing [3H]palmitate, for 16 h at 37°C. Following cell lysis, rat CD2 was immunoprecipitated from E6.1-CD2 and E6.1-CD2(CY 6) cells, and samples from the lysates as well as from the immunoprecipitates were run on SDS-PAGE under nonreducing conditions because the presence of 2-ME is recognized to interfere with S-ester and hydroxyester linkages, as can be the case for the covalent linkage of palmitate with the cysteine residues (43). As a positive control for the experiment, we immunoprecipitated Lck, previously shown to incorporate [3H]palmitate (44).

Fluorography analysis of radiolabeled products in SDS-PAGE showed that a number of cellular proteins did incorporate [3H]palmitate, as can be seen in the lysate lanes (Fig. 3A). Lck was easily detected in the respective lane. However, we were not able to confirm that rat CD2 could be subjected to lipid modification, as no labeled protein bands could be perceived in the lanes of rat CD2. The failure to detect palmitoylated rat CD2 could not be attributable to a low cellular expression of the molecule or to a low affinity of the Abs. As seen in Fig. 3B, rat CD2 immunoprecipitated from E6.1-CD2 and E6.1-CD2(CY 6) cells is clearly evident by immunoblotting. Moreover, rat CD2 was well expressed at the cell surface as confirmed by FACS analysis (Fig. 3C).

FIGURE 3. Analysis of palmitoylation of rat CD2. E6.1-CD2 and E6.1-CD2(CY 6) Jurkat cells were labeled with [3H]palmitate for 16 h, lysed in Triton X-100, and immunoprecipitated with OX-34 (anti-rat CD2) or with Lck sera. Cell lysates and immunoprecipitates were separated on 10% SDS-PAGE under nonreducing conditions and analyzed by fluorography (A) or Western blotting with OX-34 (B). No palmitoylation of rat CD2 or mutant CD2(CY 6) could be perceived, although they were easily detected by immunoblotting. C, Flow cytometry analysis of rat CD2 expression in E6.1-CD2 (left) and E6.1-CD2(CY 6) cells (right).

FIGURE 4. Analysis of raft localization of different rat CD2 cytoplasmic deletion mutants. Fractions of sucrose gradient density centrifugation of lysates from E6.1 cells expressing rat CD2 cytoplasmic tail deletion mutants were immunoblotted for the localization of CD2, Lck, and LAT. Fractions recovered were numbered 1 to 9 from the top of the gradient. Cells analyzed were E6.1-CD2, E6.1-CD2(CY 97), E6.1-CD2(CY 81), E6.1-CD2(CY 66), E6.1-CD2(CY 40), and E6.1-CD2(CY 6), expressing full-length CD2 and truncation mutants with the membrane proximal 97, 81, 66, 40, or 6 aa residues, respectively. All mutants except CD2(CY 6) partition within lipid rafts.

Raft localization of rat CD2 mutants correlates with their association with Lck

To determine the molecular basis for the constitutive presence of a large fraction of rat CD2 in the rafts, we focused on the cytoplasmic domain, determining which segments could play a role. We used previously characterized E6.1 Jurkat clones stably expressing cytoplasmic domain truncations of rat CD2 retaining 97, 81, 66, 40, and 6 aa of the cytoplasmic tail (12, 17, 19, 38). All CD2 variants were expressed at similar levels at the membrane (data not shown). The location of each mutant was analyzed following sucrose gradient centrifugation and immunoblotting of rat CD2 with OX-55. As shown in Fig. 4, the deletion mutants CD2(CY 97), CD2(CY 81), CD2(CY 66), and CD2(CY 40), like the full-length CD2 molecule, are mostly raft-resident, whereas rat CD2(CY 6) is totally excluded from lipid rafts and found in the soluble fractions. This finding suggests that the first
and does not target to lipid rafts.

**FIGURE 5.** The CD2(Δ7–40) mutant looses the ability to bind to Lck, and does not target to lipid rafts. A, Flow cytometry analysis of E6.1 Jurkat cells expressing rat CD2 with a deleted sequence between aa 7 and 40 of the cytoplasmic tail, CD2(Δ7–40). B, Cell lysates from E6.1-CD2 and from E6.1-CD2(Δ7–40) cells were run on SDS-PAGE under reducing conditions, and immunoblotted using OX-55. CD2(Δ7–40) had the expected molecular mass compatible with its slightly shorter tail (Fig. 5A). Strikingly, when we assessed the membrane localization of CD2(Δ7–40), we determined that this mutant does not localize within lipid rafts, but rather in the soluble fractions (Fig. 5B).

We then tested whether CD2(Δ7–40) was incompetent for interacting with Lck, performing immunoprecipitations followed by kinase assays of the immune complexes of CD2 from E6.1-CD2(Δ7–40), as well as from E6.1-CD2 cells for comparison. Among the proteins coprecipitated with CD2 from E6.1-CD2 cell lysates, a prominent phosphoprotein of 56 kDa was clearly visible, whereas no corresponding protein band was detected in the immune complexes from E6.1-CD2(Δ7–40) cells, although a number of other phosphoproteins with similar sizes are present (Fig. 5D). To confirm that the putative 56-kDa protein was indeed phosphorylated Lck, we performed a reprecipitation of Lck using a polyclonal Ab and confirmed the identity of p56 as Lck. No protein was detected in the reprecipitation of Lck from E6.1-CD2(Δ7–40) cells, suggesting that CD2(Δ7–40) looses the ability to bind to Lck, therefore strengthening our assumption that CD2 is trapped in lipid rafts fractions due to its association with the kinase.

We checked whether having CD2 associated with Lck in rafts, as presented in E6.1-CD2 cells, would result in an increase of the Lck activity associated with CD2 in activated cells. In Fig. 5E, stimulation of CD2 with mAb triggers a sharp increase of the kinase activity associated with CD2 as shown by the increased autophosphorylation of Lck. By contrast, triggering CD2 in E6.1-CD2(Δ7–40) cells did not induce any detectable changes.

**A physical association of rat CD2 with Lck is sufficient for the raft targeting of CD2**

To obtain definitive proof that Lck is the key determinant in the addressing of rat CD2 to lipid rafts, we resorted to the Lck-deficient J.CaM1 cellular model. Rat CD2 was stably expressed in J.CaM1 cells and its inclusion in the membrane fractions analyzed. Supporting our prediction, rat CD2 expressed in J.CaM1 cells localizes to the soluble fractions, whereas the kinase is absent in these cells as expected (Fig. 6A). Further evidence that Lck could couple rat CD2 to lipid rafts was obtained through co-capping experiments in which we induced capping of CD2. Following fixation of the cells, we analyzed whether rafts would colocalize with polarized CD2 that would demonstrate a functional association.
FIGURE 6. Lck is required for the translocation of CD2 into lipid rafts. A, Sucrose density centrifugation and immunoblotting with OX-35 confirm the exclusion of rat CD2 from the raft fractions of J.CaM1-CD2, an Lck-deficient cell line expressing rat CD2. Rat CD2 labeled with OX-34-FITC could be visualized (Fig. 6B, red) conjugated to Abs recognizing raft-resident LAT. Whereas in untreated cells no polarization of CD2 or rafts were induced (Fig. 6B, E6.1-CD2 cells, upper row; J.CaM1-CD2 cells, data not shown), in E6.1-CD2 cells, capping of CD2 induced very clear co-capping of LAT (Lck) detected in green, and LAT shown in red. The top row shows E6.1-CD2 cells had no capping induced. The differential interferential contrast (DIC) view is provided. C, Quantitative analysis from two independent experiments show that LAT co-capped very neatly with CD2 in nearly 80% of E6.1-CD2 cells. By contrast, in J.CaM1-CD2 cells, co-capping was less well defined and detected in ~31% of cells. Data shown are mean percentage ± SE.

re-expressed Lck, was by large restored (Fig. 7). To determine whether a functional Lck kinase was required for addressing CD2 to the rafts, we transfected J.CaM1-CD2 cells with an Lck cDNA encoding a mutation at the ATP binding site (K273A), and so referred to as kinase defective. J.CaM1/Lck(KD)-CD2 cells were lysed and subjected to sucrose gradients, SDS-PAGE, and immunoblotting with OX-35. As can be seen in the respective panels in Fig. 7, both Lck(KD) as well as CD2 still localize in lipid rafts, discarding the Lck kinase activity as required for the lipid raft-C2 localization.

Nonactivated cells were lysed in Triton X-100 lysis buffer, Lck or CD2 were immunoprecipitated from the lysates, and immune complexes were subjected to in vitro kinase assays. CD2 immunoprecipitates from J.CaM1/Lck(WT)-CD2 and J.CaM1/Lck(C3A)-CD2 cells displayed substantially less kinase activity when compared with CD2 immunoprecipitates from J.CaM1/Lck(WT)-CD2 (Fig. 8A). Because raft-resident
Lck(WT) and raft-excluded Lck(C3A) displayed comparable kinase activities and autophosphorylation efficiency, this suggested that the association between CD2 and Lck is preferentially held in the rafts, and that association in the soluble phase is much reduced, albeit still detectable.

Given that the T cell-specific Src-like kinase Fyn has also been shown to contribute to some of the CD2-associated kinase activity, we tested whether Fyn could also associate with CD2 in J.CaM1/Lck(WT)-CD2 and J.CaM1/Lck(C3A)-CD2 cells. The primary CD2 immune complexes were disrupted by heat and SDS, and Fyn as well as Lck were reprecipitated. As can be seen from the lower panels of Fig. 8A, both Lck and Fyn are clearly present in CD2 complexes from J.CaM1/Lck(WT)-CD2 cells, but mostly absent from CD2 immunoprecipitates from J.CaM1/Lck(C3A)-CD2 cells, indicating that in nonactivated cells CD2 associates with both kinases mostly within lipid rafts.

Using the set of Lck mutants expressed in J.CaM1 cells, we tested the signaling ability of CD2 molecules dispersed in the soluble phase of the membrane vs CD2 molecules in rafts. J.CaM1/Lck(WT)-CD2 and J.CaM1/Lck(C3A)-CD2 cells, as well as Lck as well as Lck were reprecipitated. As can be seen from the lower panels of Fig. 8A, both Lck and Fyn are clearly present in CD2 complexes from J.CaM1/Lck(WT)-CD2 cells, but mostly absent from CD2 immunoprecipitates from J.CaM1/Lck(C3A)-CD2 cells, indicating that in nonactivated cells CD2 associates with both kinases mostly within lipid rafts.

FIGURE 8. The association between CD2 and Lck and the signaling capacity of CD2 are held preferentially within lipid rafts. A, J.CaM1/Lck(WT)-CD2 and J.CaM1/Lck(C3A)-CD2 cells were lysed and Lck and CD2 were immunoprecipitated (IP). Immune complexes were subjected to kinase assays, run on SDS-PAGE, and exposed to autoradiography. Lck and Fyn were reprecipitated (Rep) from the original CD2 immunoprecipitations and exposed to autoradiography (bottom blots). B, J.CaM1-CD2, J.CaM1/Lck(WT)-CD2, and J.CaM1/Lck(C3A)-CD2 cells were stimulated with OX-54 plus OX-55 mAb, and after the times indicated cells were lysed. Equal volumes of lysates were run on SDS-PAGE and immunoblotted with anti-phosphotyrosine 4G10.

We established that CD2 localizes to rafts dependently of its association with Lck and that within rafts also interacts with Fyn, both protein tyrosine kinases deeply involved in signal transduction in T cells. Thus, we investigated whether upon cell activation CD2 could shift through different membrane microenvironments, and what would be the role of CD2 reallocation in the regulation of kinase activity in the different membrane phases.

Splenocytes from 3-mo-old Wistar rats were stimulated through CD2 cross-linking for 15 min at 37°C or left unstimulated. Cells

FIGURE 9. A rat CD2/PAG chimera does not address to lipid rafts. A chimeric variant of CD2 with the transmembrane and cysteine motifs of PAG substituting the corresponding sequences of CD2 was expressed in J.CaM1 cells, as detected by FACS (A) and immunoblotting (B), with CD2(Δ7–40) shown for size comparison. C, Sucrose density centrifugation and immunoblotting with OX-55 demonstrate that despite containing the raft-addressing motifs from PAG, the CD2/PAG chimera does not localize within rafts.
were lysed in Triton X-100-based lysis buffer and lysates subjected to sucrose gradient centrifugation and the recovered fractions were analyzed for the presence of CD2, Lck, and Fyn. Densitometric analysis of the immunoblots illustrated in A. Data show densitometry values of CD2, Lck, and Fyn subcellular distributions, representing the proportion of the total amount of each molecule detected in the raft and soluble fractions. In vitro kinase assays were performed in immune complexes of CD2 (C) and Lck (D), prepared from the fractions indicated by in A (arrowheads). Following SDS-PAGE, phosphorylated products were visualized by exposure of dried gels at −70°C to CL-Xposure films. Immune complexes were disrupted, and Lck and Fyn were repropped, subjected to SDS-PAGE, and exposed to autoradiography. IP, immunoprecipitation; Rep, reprecipitation.

FIGURE 10. Rat CD2 redistribution and molecular associations upon CD2 stimulation. A, Rat splenocytes were stimulated through CD2 for 15 min at 37°C or left unstimulated. Lysates were fractioned by sucrose gradient centrifugation and the recovered fractions were analyzed for the presence of CD2, Lck, and Fyn. B, Denstometric analysis of the immunoblots illustrated in A. Data show densitometry values of CD2, Lck, and Fyn subcellular distributions, representing the proportion of the total amount of each molecule detected in the raft and soluble fractions. In vitro kinase assays were performed in immune complexes of CD2 (C) and Lck (D), prepared from the fractions indicated by in A (arrowheads). Following SDS-PAGE, phosphorylated products were visualized by exposure of dried gels at −70°C to CL-Xposure films. Immune complexes were disrupted, and Lck and Fyn were repropped, subjected to SDS-PAGE, and exposed to autoradiography. IP, immunoprecipitation; Rep, reprecipitation.

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Discussion

It is increasingly clear that lipid rafts play an important role in T cell signaling because the assembly of the TCR signaling machinery takes place within this membrane microenvironment enriched in glycosphingolipids, sphingomyelin, and cholesterol (30, 45, 46). Moreover, cross-linking the TCR with accessory proteins such as CD2, CD5, CD9, and CD28 readily increases the association of the TCR with lipid rafts (31–33). The conventional view is that the TCR and other signaling molecules translocate to these platforms that facilitate signal transduction mechanisms. However, given that MHC-engaging T cell receptors that originate activation and signaling, together with accessory molecules binding to the respective counter receptors, are polarized toward the sites of cell contact with APCs, a fairer assessment is that lipid rafts coalesce into the interface of TCR-Ag recognition (47, 48).

Therefore, rather than considering individual TCRs or other signaling molecules being cumulatively trapped by the raft, depending on certain characteristics such as intrinsic affinity for the raft lipids or interactions with raft-peripheral proteins, a TCR-centered perspective should consider instead how the whole of the raft is captured at the sites of Ag recognition. There are currently many open theoretical possibilities, but a simple one advances that lipid rafts need not be fairly large or complex structures: homotypic FRET studies have shown that some individual rafts can form high-density clusters of nanometer size (4–5 nm), containing as few as four GPI-anchored molecules (49). Moreover, Douglass and Vale (50) showed that the raft-associated molecule LAT, as well as Lck, had a much larger diffusion coefficient than non-raft CD2, suggesting that lipid rafts can be highly mobile and diffuse to the TCR activation spots. The study also suggests that, as Lck and LAT change from a highly mobile to a motionless state when they encounter a signaling cluster, it is possible that protein-protein interactions, rather than any sort of raft-addressing labels, can play a major role in establishing specific interactions between raft and of CD2 was translocated to lipid rafts. Quantification of the immunoblots by densitometry indicates that the percentage of CD2 in the rafts increased over 4-fold from 8 to 36% (Fig. 10B).

To analyze the kinase activity associated with CD2 in the different fractions, we selected fraction 2 as representative of rafts, fraction 9 as a soluble fraction, and also a control fraction 6 (Fig. 10A, arrowheads), where no CD2, Lck, or Fyn are present. CD2 was immunoprecipitated from fractions 2, 6, and 9, both from resting as well as from activated cells, and immune complexes were subjected to in vitro kinase assays. Immune complexes of Lck were processed in parallel. As can be seen in Fig. 10C, phosphoproteins of 55–60 kDa were present in CD2 immune complexes already in resting cells, and were better defined in the rafts than in the soluble fraction, with no signal in the control fraction 6. Upon cellular activation, increased phosphorylation of the CD2-associated 55–60 kDa proteins was detected in the rafts and the soluble fractions. These phosphoproteins corresponded mostly to phosphorylated Lck and also Fyn, as can be seen in the reprecipitations of the kinases from the original CD2 immunoprecipitates.

In resting cells, the kinase activity of Lck reflects the proportion of the protein in and out of rafts. However there is a clear enhancement of the activity of raft-based Lck upon CD2 stimulation that does not require any translocation of Lck to the rafts (Fig. 10D, refer to Fig. 10, A and B). Interestingly, Fyn, which is not known for associating specifically to Lck, coprecipitates with Lck only in rafts, and displays higher activity following CD2 stimulation (Fig. 10D, bottom blots). This suggests that Lck and Fyn may interact in lipid rafts of activated cells, and that this association may be mediated by CD2.
non-raft proteins. We demonstrate that the association of rat CD2 with membrane lipid rafts is in fact mostly determined by the physical interaction established with the protein tyrosine kinase Lck.

CD2 has been shown to associate to both Lck and Fyn protein tyrosine kinases (20, 21), and deletion of the cytoplasmic tail eliminates these interactions and abolishes CD2-mediated signaling (17, 51). Given that our results, using several mutant molecules of CD2 and Lck and different cells lines, show an absolute correlation between the capacity of rat CD2 to interact with raft-resident Lck and its ability to be found in lipid rafts, we can conclude that it is Lck, and not Fyn, that retains CD2 at the rafts in nonactivated cells. Moreover, the levels of Fyn expression in the J.CaM1 cells used in this study were fair (data not shown), so it should not be attributed to Fyn a relevant role in the primary addressing of CD2 to lipid rafts, although it may play a part in retaining CD2 within rafts following cell stimulation.

The interaction between Lck and CD2 has been extensively characterized, and it has been shown that a GST/Lck-SH3 fusion protein could bind, in solution, to peptides containing different proline-rich sequences of the cytoplasmic domain of rat CD2 (21). However, SH3-domain/proline-rich contacts do not fully account for the total of the CD2-Lck association, as the first 40 amino acids of the proline-rich sequences of the cytoplasmic domain of rat CD2 (21, 22), but other CD2 partners compete for these sequences. The CD2 proline-rich motif PPPGHR is complexed with the GYF domain of CD2BP2, but only outside lipid rafts, as upon translocation, the SH3 domain of Fyn displaces CD2BP2 (22). The penultimate CD2 proline motif PPLPRPR has been reported to associate to the SH3 domains of both Lck and CD2BP1 (21, 52), and so it is possible that the association between CD2 and Lck may be similarly regulated and confined to lipid rafts as well. As the amounts of Lck and Fyn, in and outside the rafts, remain unchanged following activation, the increase of the phospho-Fyn signal coprecipitated with Lck (Fig. 10C, bottom blot) has to be due to an increase in the amount of Fyn associating with Lck or to the phosphorylation of Fyn induced by Lck (29). Conversely, the augmented Lck signal in the raft fraction of activated cells can be caused by an increase in the phosphorylation of Lck, possibly catalyzed by Fyn (Fig. 10D, bottom). Therefore, a central role of CD2 in the reorganization of lipid rafts upon cellular activation could be the facilitation of the association between Lck and Fyn.

Finally, what can then be the role of the two membrane proximal cysteine residues of rat CD2? It is intriguing that even using a chimeric CD2 molecule where we have inserted the relevant sequences of PAG, known to address very effectively the adaptor to lipid rafts (53), we failed to link CD2 to lipid rafts (Fig. 9). Palmitoylation of integral proteins can be a very dynamic and reversible process, sometimes dependent on activation signals (54). For different transmembrane receptors, palmitoylation can occur at the plasma membrane or already in the endoplasmic reticulum (55, 56), so it is possible that CD2 is not available to lipid modifications depending on its own biosynthetic pathway, or that CD2 palmitoylation may depend on very specific activation signals, or even that the ectodomain may interact with other proteins that in some form can prevent the assembly of the complexes involved in lipid modifications to integral proteins. Alternatively, CD2 cysteines may perhaps help establishing protein-protein interaction with raft-resident molecules, although it is unlikely that they mediate the actual association with Lck. The cysteine residues of CD2 known to interact with CD4 and CD8 are localized further away from the membrane (57) and thus unable to contact to the CD2 membrane-juxtaposed residues. The determination of the function of these two amino acid residues will no doubt require further investigation.

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