THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 9, Issue of February 28, pp. 6771–6778, 2003

Printed in U.S.A.

T Cell Receptor Can Be Recruited to a Subset of Plasma Membrane Rafts, Independently of Cell Signaling and Attendantly to Raft Clustering*

Received for publication, October 22, 2002, and in revised form, December 9, 2002
Published, JBC Papers in Press, December 22, 2002, DOI 10.1074/jbc.M210758200

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The constitutive/inducible association of the T cell receptor (TCR) with isolated detergent-resistant, lipid raft-derived membranes has been studied in Jurkat T lymphocytes. Membranes resistant to 1% Triton X-100 contained virtually no CD3ϵ, part of the TCR complex, irrespective of cell stimulation. On the other hand, membranes resistant either to a lower Triton X-100 concentration (*i.e.* 0.2%) or to the less hydrophobic detergent Brij 58 (1%) contained (i) a low CD3ϵ amount (approximate 2.7% of total) in resting cells and (ii) a several times higher amount of the TCR component, after T cell stimulation with either antigen-presenting cells or with phytohemagglutinin. It appeared that CD3/TCR was constitutively associated with and recruited to a raft-derived membrane subset because (i) all three membrane preparations contained a similar amount of the raft marker tyrosine kinase Lck but no detectable amounts of the conventional membrane markers, CD45 phosphatase and transferrin receptor; (ii) a larger amount of particulate membranes were resistant to solubilization with 0.2% Triton X-100 and Brij 58 than to solubilization with 1% Triton X-100; and (iii) higher cholesterol levels were present in membranes resistant to either the lower Triton X-100 concentration or to Brij 58, as compared with those resistant to 1% Triton X-100. The recruitment of CD3 to the raft-derived membrane subset appeared (i) to occur independently of cell signaling events, such as protein-tyrosine phosphorylation and Ca^{2+} mobilization/influx, and (ii) to be associated with clustering of plasma membrane rafts induced by multiple cross-linking of either TCR or the raft component, ganglioside GM_1. We suggest that during T cell stimulation a lateral reorganization of rafts into polarized larger domains can determine the recruitment of TCR into these domains, which favors a polarization of the signaling cascade.

The plasma membranes (PM)[1] of many cell types contain domains rich in cholesterol and sphingolipids, which have come to be referred to as lipid rafts (1, 2). It is thought that rafts may form because of the segregation of their components from the bulk of the glycerol-based phospholipid PM because of the orientation and tight packing of the long, largely saturated acyl chains of the sphingolipids. This phase separation of the membrane results in patches of molecules, which form the rafts, existing in the liquid-ordered phase (l_o) but surrounded by and co-existing with the phospholipids in the bulk PM that are in the liquid disordered phase (l_d). In many cell types rafts are organized into structurally distinct invaginations of the PM called caveolae (3, 4). However in other types, including lymphocytes, the rafts are thought to exist as islands of tightly packed sphingolipid and cholesterol-based structures that, like caveolae, can be isolated from the rest of the PM by purification methods based on their detergent insolubility at low temperatures (5, 6). These PM domains have been therefore called detergent-resistant membranes (DRMs), detergent-insoluble glycolipid-enriched complexes, and Triton-insoluble floating fractions (reviewed in Refs. 2 and 7).

The comparative analysis of the detergent-insoluble domains, caveolae, and the bulk PM has provided an inventory of proteins apparently residing in each (6, 8). However, doubts have been raised as to whether the detergent treatment itself may modify the lipid rafts or destabilize certain proteins resident therein (7). On the other hand, a recent report (9) strongly suggests that use of different detergents can result in different DRMs, which contain different proteins and likely correspond to different cholesterol-based PM lipid rafts.

In T lymphocytes, many proteins involved in signal transduction have been constitutively or inducibly recovered in DRMs (reviewed in Ref. 10). Among these are glycosylphosphatidylinositol-anchored proteins, the Src family protein-tyrosine kinases Lck and Fyn (10, 11), the transmembrane adapter protein linker for activation of T cells (12), and a variety of co-stimulatory and co-receptor proteins (Refs. 10 and 13–16 and the references therein).

A large body of evidence supports a crucial role for rafts in the signaling events activated by the T cell receptor (TCR) engagement (see Ref. 17 for a recent review). Uncertainties, however, still exist concerning the constitutive or inducible association of TCR to rafts/DRMs, as well as to the mechanisms underlining the possible recruitment of the receptor complex to rafts upon T cell stimulation. Neither a constitutive nor an inducible (after treatment with antibodies to CD3) association of the TCR/CD3 complex to DRMs was found in Jurkat T cells

* This work was supported by grants from the Italian Ministry of Instruction, University and Scientific Research Cofin 2001 (to A. B.), University of Siena Progetto Giovani Ricercatori (to E. G.), and the Italian Space Agency (to A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PM, plasma membrane; TCR, T cell receptor; DRM, detergent-resistant membrane; Lck, lymphocyte-specific protein-tyrosine kinase; PP2, 4-amino-5-(4-cholrophenyl)-7-(t-butyl)pirazolo[3,4-d]pyrimidine; SEE, staphylococcal enterotoxin E; CTB, cholera toxin B subunit; PHA, phytohemagglutinin; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

(18, 19). No constitutive association to DRMs of TCRζ has been also found both in Th1 and Th2 lymphocytes, whereas TCRζ was recruited to DRMs only in Th1 cells challenged with antigen-presenting cells (20). In a murine T cell hybrydoma, it has been demonstrated that the TCR complex is excluded from DRM domain before and after TCR stimulation, although a portion of the TCRζ component appeared to be constitutively associated with DRMs (21). Another report (22) has shown the recruitment of TCR/CD3 to DRMs, which is dependent on both receptor engagement and the activity of Src family kinases. While this work was being completed, evidence was been provided for the constitutive presence of a minor portion of TCR/CD3 in a subset of DRMs prepared from splenic and thymic T lymphocytes (23). On the other hand, microscopical evidence has been provided for the recruitment of TCR to PM rafts as a consequence of raft clustering (18).

Here we have preliminarily assessed a suitable experimental protocol to prepare cholesterol-based DRMs in Jurkat T lymphocytes. Taking advantage of this assessment, we have then investigated on the possible constitutive/inducible association of TCR/CD3 with DRMs. As a main result, we report that TCR/CD3 can be recruited to DRMs/rafts upon T cell stimulation. Moreover, we provide evidence that clustering of TCR/rafts can be a determinant of the recruitment, independently of cell signaling activation.

EXPERIMENTAL PROCEDURES

Cells—Jurkat cells, Jurkat-derived JCaM 1.6 cells (purchased from the American Type Culture Collection, Manassas, VA) and the Epstein-Barr virus transformed human B cells (EBV-B, kindly supplied by Chiron-Biocine, Siena, Italy) were grown in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma). In the case of EBV-B cells, 50 μM 2-mercaptoethanol was also included in the culture medium. The cells were harvested 48–60 h after transplantation.

Cell Treatments—To stimulate Jurkat cells with antigen-presenting cells, 12.5×10^6 Jurkat cells (in 0.5 ml of RPMI 1640 at 37 °C) were mixed with 12.5×10^6 EBV-B cells (in 1 ml of RPMI 1640 at 37 °C), the mixture was rapidly centrifuged at $400 \times g$, and pelletted cells (in the presence of supernatant) were incubated for 30 min at 37 °C. Prior to adding to Jurkat T cell suspension, EBV-B cells were preincubated (5×10^6/ml, in RPMI 1640) with or without 1 μg/ml of staphylococcal enterotoxin E (SEE) for 1.5 h at 37 °C and then washed twice with RPMI 1640 to remove free SEE. Phytohemagglutinin (PHA) stimulation was performed by treating cells with 10 μg/ml of the lectin in RPMI 1640 supplemented with 0.1% fetal calf serum for 30 min at 37 °C. Treatment with anti-CD3 antibodies was performed by incubating cells (10×10^6/ml, in RPMI 1640 supplemented with 0.1% bovine serum albumin) in the presence of 5 μg/ml of the anti-CD3 antibody, TR66, for 5 min at 37 °C. To cross-link CD3-TR66 complexes (18), TR66-treated cells (washed and resuspended in fresh RPMI 1640) were subsequently treated (10 min at 37 °C) with an antibody to TR66 (anti-mouse IgG, 8 μg of protein/ml). To induce GM_1 cross-linking, cells (10×10^6/ml in RPMI 1640 supplemented with 0.1% bovine serum albumin) were treated with cholera toxin B subunit (CTB; 0.1 μg/ml) for 1 min at 37 °C, washed twice with RPMI 1640, and subsequently treated with an anti-CTB antibody for 5 min at 37 °C.

Preparation of Detergent-resistant Membrane Fractions (DRMs)—Cells (25×10^6) were washed twice with ice-cold RPMI 1640 and homogenized in 1.5 ml of ice-cold MBS (0.15 M NaCl, 25 mM Mes, pH 6.5) containing the detergent (*i.e.* 1% Triton X-100, 0.2% Triton X-100, or 1% Brij 58) and a mixture of protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The homogenates were incubated for 1 h on ice under gentle shacking and then centrifuged for 5 min at $400 \times g$ to remove nuclei and debris. The supernatants were then adjusted to 45% sucrose by the addition of an equal volume of 90% sucrose/MBS, placed in the bottom of ultracentrifuge tubes, and overlaid with 5 ml of 35% sucrose and 4 ml of 5% sucrose (24). The gradients were centrifuged at $187{,}000 \times g$ in a SW41 rotor (Beckman) for 20 h at 4 °C. Ten fractions (1 ml each) were collected from the top of the gradients (fractions 1–10), and the residual volume of the centrifuge tube (1.5–1.7 ml) was recovered as fraction 11. The protein content of fractions was determined by a modified Lowry assay (Bio-Rad). Aliquots (50–100 μl) of the sucrose gradient fractions were withdrawn to measure light scattering and cholesterol content (see below). Because fractions 9–11 contained the bulk of solubilized cell materials, they were subsequently pooled for futher analysis. The proteins contained in fractions 1–8, as well as in the pooled fractions 9–11, were recovered by trichloroacetic acid/deoxycholic acid precipitation as reported in Ref. 25. The proteins were then dissolved in SDS-PAGE buffer, and half (fractions 1–8) or 1/12 (pooled fractions 9–11) of the solutions were loaded onto 5–15% gradient polyacrylamide gels and blotted onto nitrocellulose. The immunoblots were probed with the different antibodies and analyzed by enhanced chemiluminescence (Amersham Biosciences). Scanning densitometry was performed within the linear range of preflashed x-ray film with a Bio-Rad VERSADOC mod.1000 imaging densitometer.

Tyrosine Phosphorylation Assay—The cells were lysed for 30 min at 4°C in 1% Nonidet P-40 buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA) in the presence of protease and phosphatase inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM $NaVO_4$). The samples were centrifuged (at $13{,}000 \times g$ for 10 min) and postnuclear supernatants were subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting.

Light Scattering Assay—Aliquots (0.1 ml) of the sucrose gradient fractions (see above) were diluted with 1.9 ml of 10 mM K-Pipes, pH 7.0, containing 1 mM EGTA. Light scattering intensity of each fraction was measured at 400 nm at right angles to the incoming light beam (whose intensity was 80% reduced with the aid of a grid) using a fluorimeter (Perkin-Elmer model 650-10S) equipped with a temperature-controlled cuvette holder (22 °C) (26, 27).

Cholesterol Determination—The cholesterol content of sucrose gradient fractions was measured enzymatically, essentially as reported in Ref. 28. Briefly, 50–100 μl of the fractions were reacted (for 30 min at 37 °C in the dark) in 1.5 ml of KPi buffer (0.1 M, pH 7.4) containing 2 mM sodium cholate, 0.66 mg/ml of *p*-hydroxyphenilacetic acid, 0.1 UI/ml of cholesterol oxidase, and 1 UI/ml of horseradish peroxidase. Parallel samples without cholesterol oxidase were also run as blanks. The final product of the coupled reactions, oxidized *p*-hydroxyphenilacetic acid derivative, was measured fluorimetrically (excitation and emission wavelengths, 325 and 415 nm, respectively).

$[Ca^{2+}]_i$ Measurements—The cells were loaded with fura-2 (acetoxymethyl ester), and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as described in Ref. 29. To minimize the leakage of intracellular fura-2, the assay temperature was 30 °C, and 200 μM sulfinpyrazone was included in the medium (29).

Microscopical Analysis—Cells suspensions (2×10^6 cell/ml in serum-free RPMI 1640) were treated with 0.15 μM BODIPY FL-labeled C5-ganglioside for 2 min at 22 °C. The cells were rapidly harvested by centrifuging at $1000 \times g$ for 15 s, resuspended in 0.1 ml of serum-free RPMI 1640, placed on a coverslip, and immediately observed with a real time confocal microscope (Bio-Rad DCV 250 mounted on a Nikon Eclipse 300 inverted microscope). The images were acquired with a cooled CCD camera (Princeton Inst.) and a Metamorph® imaging system.

Materials—Triton X-100, PHA, polyclonal antibodies to cholera toxin B subunit and horseradish peroxidase (type 4A) were obtained from Sigma. Brij 58 was obtained from Fluka. 4-Amino-5-(4-cholrophenyl)-7-(t-butyl)pirazolo[3,4-d]pyrimidine (PP2) and cholesterol oxidase were from Calbiochem. Fura-2 (acetoxymethyl ester) and BODIPY FL-labeled C5-ganglioside GM_1 were from Molecular Probes. Polyclonal antibodies to CD3ε and monoclonal antibodies to Lck and CD45 were obtained from Santa Cruz Biotechnology. Monoclonal antibodies to transferrin receptor and the anti-phosphotyrosine fragment, directly conjugated with horseradish peroxidase (RC20:HRP), were from BD Transduction Laboratories. SEE was obtained from Toxin Technology. Anti-CD3 (clone TR66) and CTB were a gift from Chiron-Biocine (Siena, Italy). All other chemicals were of analytical grade.

RESULTS

Characterization of DRMs Prepared under Different Detergent Solubilization Conditions—A classic method to prepare DRMs is based on the disruption of cells with 1% Triton X-100 at 0–4 °C. However, alternative detergents/conditions have been also used to prepare DRMs, particularly in lymphocytes (13, 20, 22, 23, 30–37). As a prelude to examining the constitutive or inducible association of CD3/TCR to DRMs, we have characterized DRMs prepared from (unstimulated) Jurkat cells

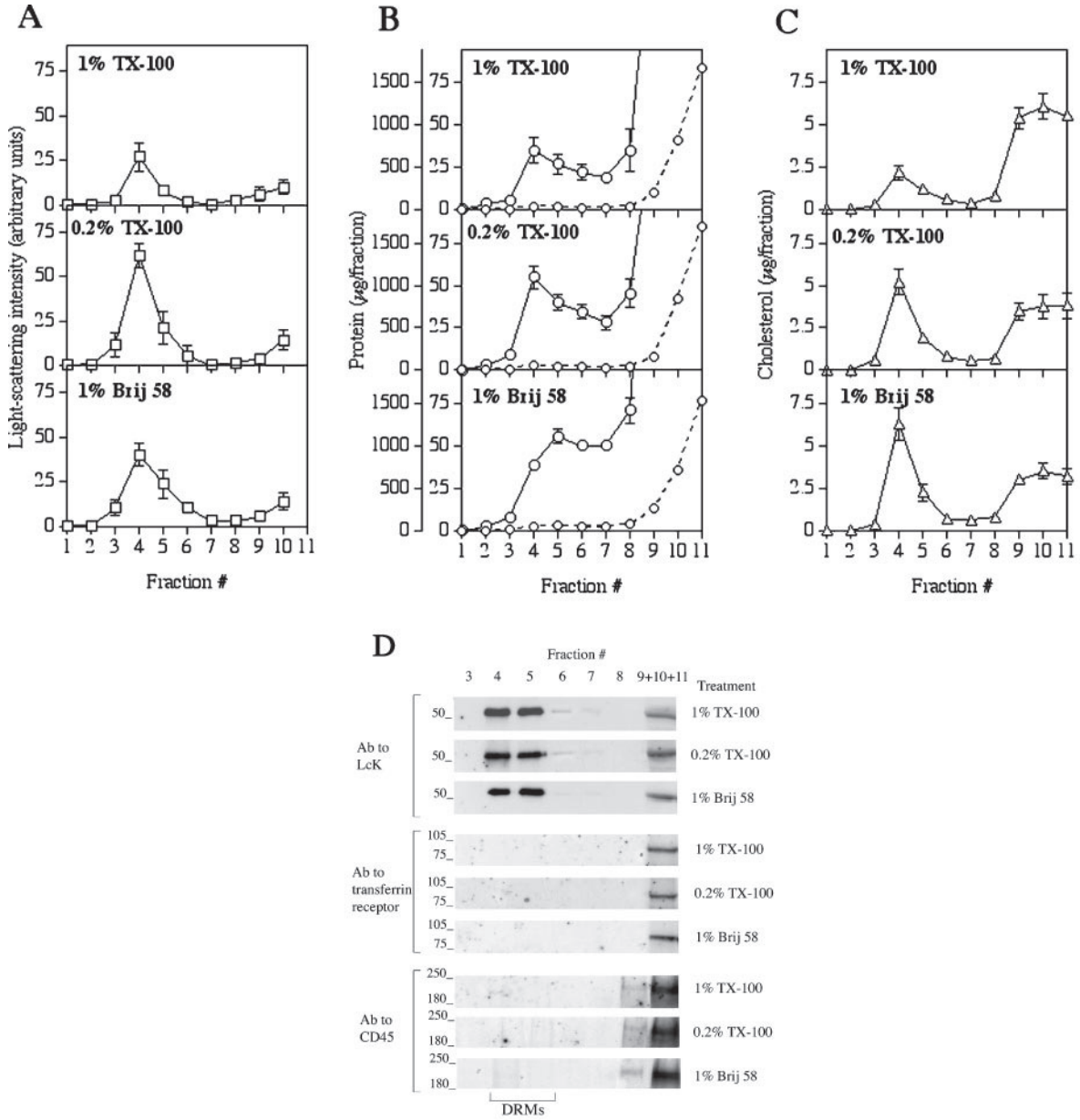

FIG. 1. **Characterization of DRMs prepared under different detergent solubilization conditions.** Jurkat cells (25×10^6 cells) were lysed in a medium containing 1% Triton X-100, 0.2% Triton X-100, or 1% Brij 58, the lysates were then fractionated by sucrose density centrifugation, and 11 fractions were collected from the top of the gradients as described under "Experimental Procedures." Light-scattering intensity (*A*), protein (*B*), and cholesterol content (*C*) of each fraction were determined as detailed under "Experimental Procedures." *D*, proteins derived from 12.5×10^6 or 2×10^6 Jurkat cells, in the case of fractions 3–8 or the solubilized materials (pooled fractions 9–11), respectively, were analyzed by SDS-PAGE and Western blotting and probed with antibodies recognizing the indicated proteins; the positions of molecular mass markers (in kilodaltons) are shown; fractions 1 and 2, which showed no immunoreactivity, are not shown for clarity. In *B*, protein levels represented by *continuous* and *dotted lines* correspond to the *wider* and *smaller abscissa scales*, respectively. In *A–C* data are the means ± S.E. of four to six different experiments. In *D* a representative experiment of three is shown. *TX-100*, Triton X-100; *Ab*, antibody.

treated with 1% Triton X-100, 0.2% Triton X-100, or 1% Brij 58 at 0–4 °C and separated by sucrose gradient ultra centrifugation. Fig. 1 shows the distribution of particulate membranes (evaluated by light scattering), total proteins, and cholesterol content across the density gradient in the three experimental conditions of membrane solubilization. Particulate membranes were largely recovered in fractions 4 and 5 (Fig. 1*A*) in all cases, indicating that these fractions contain DRMs. Actually fractions 4 and 5 contained an opaque band, which equilibrated by flotation at 10–25% sucrose (data not shown), independently of the detergent treatment employed. A relatively high protein (Fig. 1*B*) and cholesterol (Fig. 1*C*) content was also found in fractions 4 and 5. Notably, in the cases of both 0.2% Triton X-100 and 1% Brij 58, the amount of protein and cholesterol in the DRM-containing fractions was significantly higher than in the case of cell solubilization with 1% Triton X-100 (Fig. 1, *B* and *C*). Also the content in particulate membranes was apparently higher in DRMs prepared from cells treated with the lower concentration of Triton X-100 or Brij 58. However, light-scattering intensity may be influenced by factors other than the concentration of membranes, such as, for example, the size of the membrane particles (26, 27). The fact that not only the protein but also the cholesterol content is higher in DRMs prepared with 0.2% Triton X-100 or Brij 58, indicates that these DRMs can be considered cholesterol-based and can be regarded as raft-derived. Consistently, either the transferrin receptor or CD45 proteins, which are assumed to be located in the conventional lipid environment of the PM (22, 36, 38), were

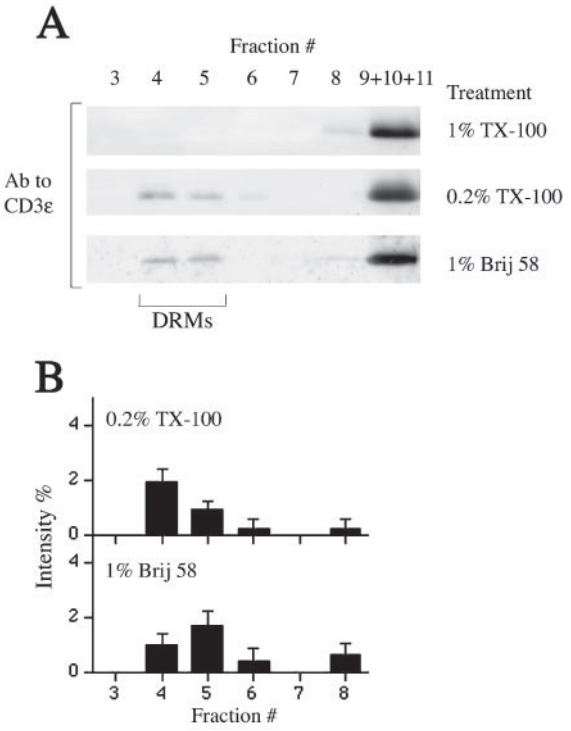

FIG. 2. **Associations of CD3ϵ with DRMs prepared by different detergent treatments of resting Jurkat cells.** The cells (25×10^6 cells) were lysed in a medium containing 1% Triton X-100, 0.2% Triton X-100, or 1% Brij 58, and the lysates were then fractionated by sucrose density centrifugation as described under "Experimental Procedures." *A*, proteins derived from 12.5×10^6 or 2×10^6 Jurkat cells, in the case of fractions 3–8 or the solubilized materials (pooled fractions 9–11), respectively, were analyzed by SDS-PAGE and Western blotting and probed with antibodies recognizing the CD3ϵ protein; fractions 1 and 2, which showed no immunoreactivity, are not shown for clarity. *B*, Western blots like those shown in *A* were quantified using scanning densitometry. Normalized densitometry data are presented as the percentages of total intensity, and they represent the means ± S.E. of four different experiments; the values for the pooled fractions 9–11 are not shown for clarity. *TX-100*, Triton X-100; *Ab*, antibody.

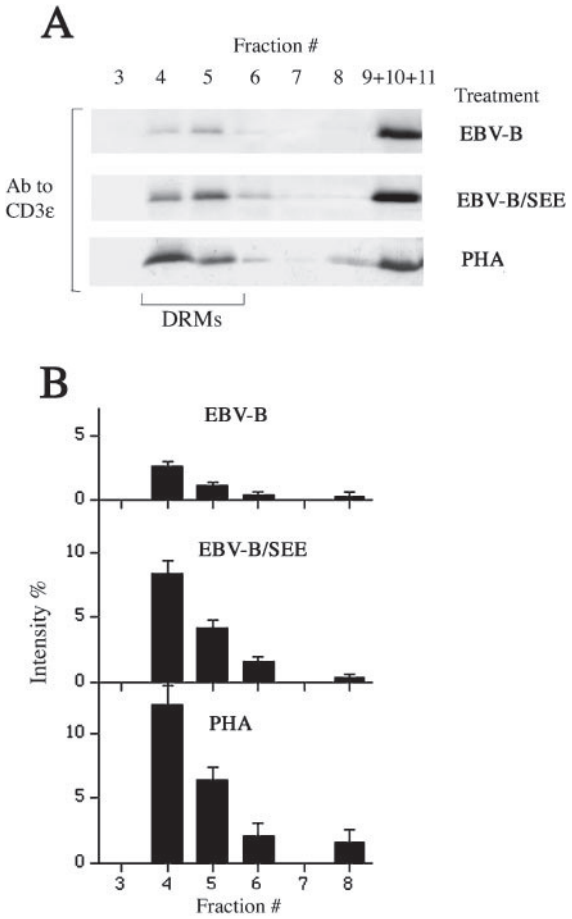

FIG. 3. **Recruitment of CD3ϵ to DRMs in Jurkat cells stimulated with SEE-prepulsed EBV-B cells or with PHA.** Jurkat cells were treated with control EBV-B cells, SEE-prepulsed EBV-B cells or PHA, and lysed in a medium containing 0.2% Triton X-100, and the lysates were then fractionated by sucrose density centrifugation as described under "Experimental Procedures." *A*, proteins derived from 12.5×10^6 or 2×10^6 Jurkat cells, in the case of fractions 3–8 or the solubilized materials (pooled fractions 9–11), respectively, were analyzed by SDS-PAGE and Western blotting and probed with antibodies recognizing the C CD3ϵ protein. *B*, Western blots like those shown in *A* were quantified using scanning densitometry. Normalized densitometry data are presented as the percentages of total intensity, and they represent the means ± S.E. of three to five different experiments; the values for the pooled fractions 9–11 are not shown for clarity. *Ab*, antibody.

found to be virtually undetectable in the DRM-containing fractions (Fig. 1*D*). On the other hand, the Lck protein that is known to be largely localized in DRMs from Jurkat cells (10, 11) was found to be associated with the DRM-containing fractions at a very similar extent in all of the solubilization protocols employed (Fig. 1*D*).

A Minor Portion of TCR/CD3 Is Constitutively Associated with 0.2% Triton X-100- and Brij 58-resistant Membranes—In the case of cell membrane solubilization with 1% Triton X-100, virtually no CD3ϵ protein was detected in DRMs (Fig. 2*A*), which is in agreement with previous observations (19). However, a minor portion of total CD3ϵ protein was immunorevealed in DRMs prepared from Jurkat cells upon solubilization with 0.2% Triton X-100 and Brij 58 (Fig. 2). The percentages of the CD3ϵ protein in DRM-containing fractions (fractions 4 and 5) were 2.8 ± 0.4 and 2.7 ± 0.4 (mean ± S.E.), in the case of cell solubilization with 0.2% Triton X-100 and 1% Brij 58, respectively. It was previously observed that cell membrane solubilization with 1% Brij 58 at 0–4 °C (22) or with 1% Brij 98 at 37 °C (23) resulted in the recovery of a portion of cell CD3/TCR in DRMs.

Recruitment of TCR/CD3 to 0.2% Triton X-100 and Brij 58-resistant Membranes—To investigate whether or not TCR/CD3 can be dynamically recruited to DRMs as a consequence of T cell stimulation, Jurkat cells have been treated with the mitogenic lectin PHA or EBV-B cells prepulsed with SEE as a model for antigen-presenting cells. Both treatments did result in a marked increase in the amount of CD3ϵ protein associated with DRMs prepared with 0.2% Triton X-100 (compare Fig. 3 with Fig. 2). The percentages of the CD3ϵ protein present in DRMs of cells stimulated with PHA or SEE-pulsed EBV-B cells (fractions 4 and 5) were 18.7 ± 2.2 and 12.6 ± 0.9 (means ± S.E.). The amount of CD3ϵ protein recovered in DRMs of Jurkat cells treated with control EBV-B cells (without SEE treatment) was very similar to that of resting Jurkat cells (compare Fig. 3 with Fig. 2). Experiments with Brij 58, which were performed in the case of PHA stimulation (not shown), gave analogous results; the percentage of CD3ϵ protein associated with DRMs (fraction 4 + fraction 5) was 19.7 ± 1.7 (mean ± S.E., $n = 4$). In both above stimulatory conditions, virtually no immunodetectable CD3ϵ protein was found in DRMs prepared by cell membrane solubilization with 1% Triton X-100 (data not shown).

Recruitment of TCR/CD3 to 0.2% Triton X-100-resistant Membranes Does Not Require Activation of Cell Signaling—Challenging T cells with antigen-presenting cells or PHA results in the ligation of TCR and of a variety of co-stimulatory proteins as well. A downstream key event is the activation of

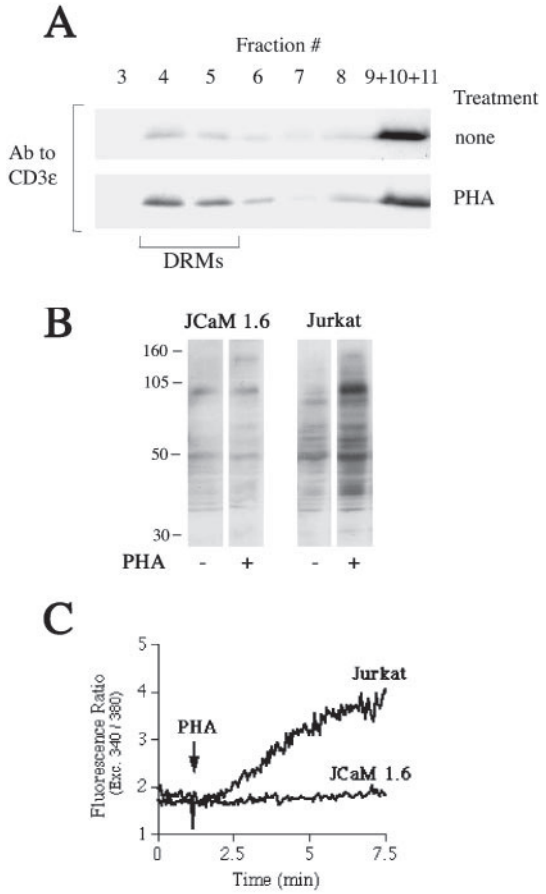

FIG. 4. **Recruitment of CD3ε to DRMs (*A*), cell protein-tyrosine phosphorylation (*B*), and Ca^{2+} signaling (*C*) in JCaM 1.6 cells stimulated with PHA.** *A*, JCaM 1.6 cells were treated with PHA and lysed in a medium containing 0.2% Triton X-100, and the lysates were then fractionated by sucrose density centrifugation, as described under "Experimental Procedures." proteins derived from 12.5×10^6 or 2×10^6 cells, in the case of fractions 3–8 or the solubilized materials (pooled fractions 9–11), respectively, were analyzed by SDS-PAGE and Western blotting and probed with antibodies recognizing the CD3ε protein. *B*, total lysated of JCaM 1.6 and Jurkat cells were analyzed by SDS-PAGE and Western blotting and probed using an anti-phosphotyrosine antibody, as described under "Experimental procedures"; the positions of molecular mass markers (in kilodaltons) are shown. *C*, variations in $[Ca^{2+}]_i$ induced by PHA addition (10 μg/ml) to JCaM 1.6 and Jurkat cells were measured as described under "Experimental Procedures." *Ab*, antibody.

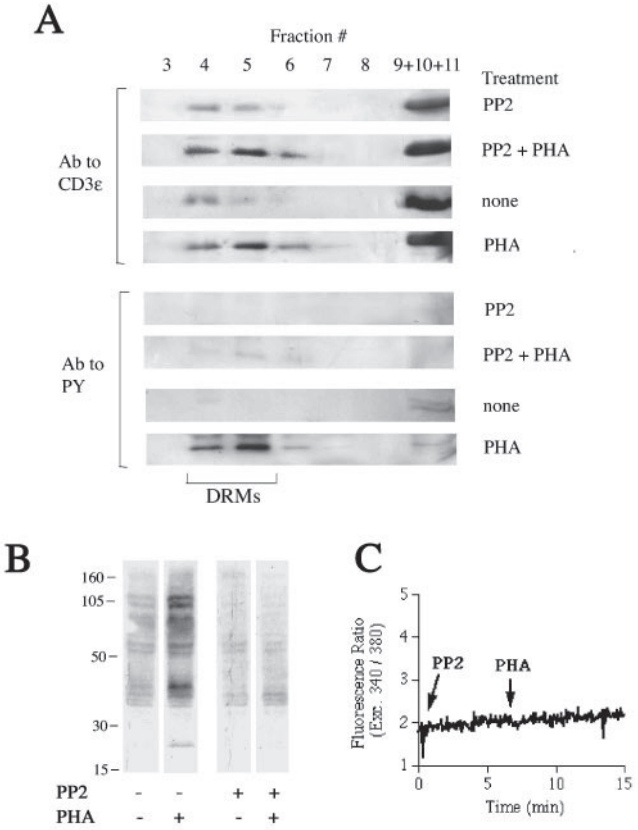

FIG. 5. **Recruitment of CD3ε to DRMs (*A*), cell protein-tyrosine phosphorylation (*B*), and Ca^{2+} signaling (*C*) in Jurkat cells pretreated with the Src kinase inhibitor PP2 and stimulated with PHA.** *A*, cells were pretreated with 10 μM PP2 (for 10 min at 37 °C), stimulated with PHA, and lysed in a medium containing 0.2% Triton X-100 (also including 50 mM NaF, 10 mM $Na_4P_2O_7$ and 1 mM $NaVO_4$, to allow phosphotyrosine detection), and the lysates were then fractionated by sucrose density centrifugation, as described under "Experimental Procedures"; proteins derived from 12.5×10^6 or 2×10^6 Jurkat cells, in the case of fractions 3–8 or the solubilized materials (pooled fractions 9–11), respectively, were analyzed by SDS-PAGE and Western blotting; the blot membranes were probed with an anti-phosphotyrosine antibody (antibody to PY) and then reprobed with antibodies to the CD3ε protein. *B*, total lysated of Jurkat cells were analyzed by SDS-PAGE and Western blotting and probed using an anti-phosphotyrosine antibody, as described under "Experimental Procedures"; the positions of molecular mass markers (in kilodaltons) are shown. *C*, variations in $[Ca^{2+}]_i$ induced by PHA addition (10 μg/ml) to PP2-pretreated Jurkat cells were measured as described under "Experimental Procedures." *Ab*, antibody.

the Src kinase Lck, which in turn causes tyrosine phosphorylation and Ca^{2+} signaling. To investigate on the role of cell signaling in the recruitment of TCR/CD3 to DRMs, we employed the Jurkat-derived cell line JCaM 1.6, which lacks the activity of the Lck tyrosine kinase (39). As shown in Fig. 4*A*, PHA treatment of JCaM 1.6 cells resulted in a recruitment of the CD3ε protein to DRMs (prepared with 0.2% Triton X-100), at an extent that was comparable with that observed in PHA-treated Jurkat cells (Fig. 3*A*). As expected, PHA stimulation caused virtually no increase in tyrosine phosphorylation and Ca^{2+} signaling in JCaM 1.6 cells (Fig. 4, *B* and *C*), whereas it resulted in a robust increase in tyrosine phosphorylation and Ca^{2+} signaling in Jurkat cells (Fig. 4, *B* and *C*). Ca^{2+} signaling was presumably due to both mobilization of cell Ca^{2+} stores and influx of extracellular Ca^{2+}, because it was evaluated in the presence of (1 mM) extracellular Ca^{2+} (29).

Src kinases other than Lck, such as Fyn, may also be involved in T cell signaling (40–42). In further experiments, we therefore investigated whether or not the CD3ε protein is recruited to DRMs obtained from Jurkat cells pretreated with the selective inhibitor of Src kinases, PP2 (43). As shown in Fig. 5*A*, an evident PHA-induced recruitment of the CD3ε protein to DRMs was also present in PP2-treated Jurkat cells. As expected, PHA stimulation caused little or no increase in tyrosine phosphorylation and Ca^{2+} signaling in PP2-treated cells (Fig. 5, *B* and *C*). In addition, the CD3ε protein was recruited to DRMs irrespective of its phosphorylation status. Indeed, the CD3ε protein associated with DRMs (after PHA stimulation) was apparently not phosphorylated in PP2-treated cells, whereas it was phosphorylated in PHA-treated control cells (Fig. 5*A*).

In the experiments shown in Figs. 3–5, the cells were stimulated with PHA for 30 min (see "Experimental Procedures"). However, comparable amounts of CD3ε protein were found associated with DRMs either in control Jurkat cells or in PP2-treated Jurkat and JCaM 1.6 cells, at later times of PHA treatment (60–90 min) of PHA stimulation (data not shown). This suggests that the stability of the association of CD3/TCR with DRMs over time does not require cell signaling activation,

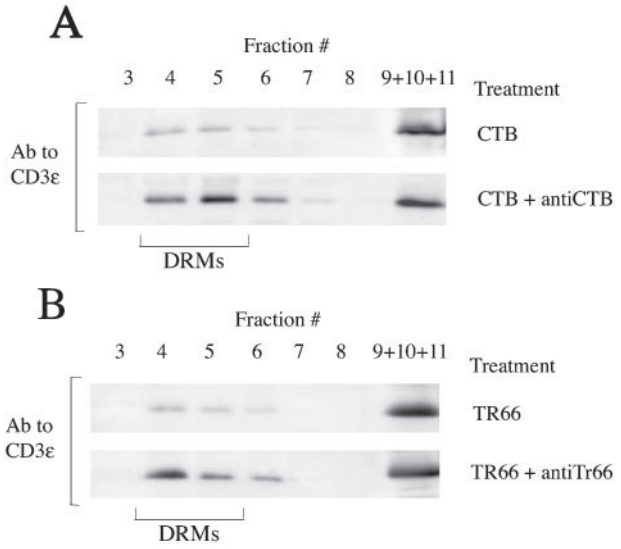

FIG. 6. **Multiple cross-linking of GM_1 (*A*) or CD3 (*B*) results in the recruitment of CD3ε to DRMs.** *A*, Jurkat cells were pretreated with the GM_1 ligand, CTB, and then treated with or without antibodies to CTB to cross-link GM_1/CTB complexes as described under "Experimental Procedures." *B*, Jurkat cells were pretreated with the antibody to CD3, TR66, and then treated with or without antibodies to TR66, as described under "Experimental Procedures." The cells were lysed in a medium containing 0.2% Triton X-100, and the lysates were then fractionated by sucrose density centrifugation, as described under "Experimental Procedures"; proteins derived from 12.5×10^6 or 2×10^6 cells, in the case of fractions 3–8 or the solubilized materials (pooled fractions 9–11), respectively, were analyzed by SDS-PAGE and Western blotting and probed with antibodies recognizing the CD3ε protein. *Ab*, antibody

at least in the case of PHA stimulation.

In all of the experimental conditions described above, no immunodetectable CD3ε protein was found in DRMs prepared with 1% Triton X-100 (data not shown).

Antibody-mediated Multiple Cross-linking of GM_1 and CD3 Can Determine the Recruitment of TCR/CD3 to 0.2% Triton X-100-resistant Membranes—It is well known that clusters of TCR are formed at the site of contact between T cells and antigen-presenting cells (44), as well as on the PM surface of lectin-treated T cells (45). It also has been shown that cross-linking of either the raft component GM_1 or TCR results in the co-clustering of the ganglioside and the receptor (18). Therefore, in subsequent experiments, we investigated whether or not the cross-linking of either GM_1 or CD3 can induce the recruitment of TCR/CD3 to DRMs.

To induce GM_1 cross-linking, the cells were treated with CTB as a ligand for GM_1, and then the GM_1-CTB complex was cross-linked with an antibody to CTB (18, 46). This treatment resulted in a marked increase in the amount of the CD3ε protein associated with DRMs (Fig. 6*A*). In the cells treated with CTB alone, the amount of the CD3ε protein associated with DRMs was comparable with that observed in control (unstimulated) cells (Fig. 2*A*).

Cross-linking of TCR/CD3 was performed by treating the cells with the antibody to CD3, TR66, and subsequently with antibodies to TR66. As can be seen in Fig. 6*B*, cross-linking of CD3 resulted in a marked increase in the amount of the CD3ε protein associated with DRMs. In the cells treated with TR66 alone, the amount of the CD3ε protein present in DRMs was comparable with that observed in control (unstimulated) cells (Fig. 2*A*).

In the two experimental conditions as above, virtually no immunodetectable CD3ε protein was found in DRMs prepared with 1% Triton X-100 (data not shown).

Recruitment of TCR/CD3 to 0.2% Triton X-100-resistant Membranes Is Associated with Clustering of PM Rafts—In a final set of experiments, we investigated whether or not the recruitment of CD3 to DRMs (resistant to 0.2% Triton X-100) is paralleled by lipid raft clustering by microscopical observation of PM rafts probed with a fluorescent analogue of GM_1. Indeed, previous microscopic observations have shown that fluorescent GM_1 probes uniformly label the PM of resting (unstimulated) Jurkat cells but selectively stain PM patches in cells treated with cross-linking antibodies to GM_1 or CD3 (18). The logical explanation is that the GM_1 analogue inserts in PM lipid rafts; lipid rafts in resting cells, however, are too small (≤70 nm in diameter (4–6)) to be resolvable by light microscopy, whereas their aggregates (patches) are resolvable by light microscopy (18, 46, 47).

As demonstrated in Fig. 7, clusters of rafts were formed in the experimental conditions, in which we observed the recruitment of the CD3ε protein to DRMs. This appeared to be the case independently of activation of cell signaling. Indeed, PHA treatment of JCaM 1.6 or PP2-pretreated Jurkat cells resulted in both the recruitment of CD3ε protein to DRMs (Figs. 4 and 5) and raft clustering (Fig. 7) but in no evident activation of tyrosine phosphorylation and Ca^{2+} signaling (Figs. 4 and 5). On the other hand, treating Jurkat cells with the antibody to CD3, TR66, caused neither the recruitment of the CD3ε protein to DRMs (Fig. 6*A*) nor clustering of PM rafts (Fig. 7). Instead, as expected on the basis of previous reports (22, 48), TR66 stimulation caused a marked increase in tyrosine phosphorylation and Ca^{2+} signaling also in the present experimental conditions (data not shown).

DISCUSSION

Although previous microscopical evidence suggest that the TCR is present in PM rafts (18), a variety of biochemical studies gave conflicting results with respect to the constitutive/inducible association of the receptor with DRMs (18–23). The present data show that a relatively low amount of the TCR component CD3ε is constitutively associated with a DRM subset and that this amount can be largely increased as a result of T cell stimulation in a cell signaling-independent manner.

These data were gained by using 0.2% Triton X-100 or 1% Brij 58 to solubilize "conventional" nonraft membranes. On the other hand, we observed that DRMs prepared with the "classic" concentration of Triton X-100, *i.e.* 1%, did not contain any detectable CD3 amount. It could be argued that 1% Triton X-100, but not the less hydrophobic detergent Brij 56 or a lower concentration of Triton X-100 itself, simply extracts CD3/TCR from rafts. However, solubilization with either Brij 58 or the lower concentration of Triton X-100 also resulted in a higher recovery of membranes as well as of cholesterol and proteins in the DRM-containing fractions. Therefore, a logical explanation is that the CD3/TCR complex is contained in a subset of cholesterol-enriched membranes that are not resistant to 1% Triton X-100 but are resistant to a lower Triton X-100 concentration or to Brij 58. The idea that heterogeneity in cholesterol-based DRMs and/or PM raft domains exists is not unprecedented. For example, the co-existence within a membrane domain, such as the apical plasma membrane, of different cholesterol-based lipid rafts has recently been proposed (9). Moreover, evidence for structural diversity of the PM domains occupied by functionally different glycosylphosphatidylinositol-anchored proteins has been previously forwarded (49).

It should be noted that a variety of previous studies on the association of signaling proteins to rafts/DRMs in T cells has been based on the use of detergents other that Triton X-100 (13, 14, 20, 22, 23, 31–35) or of Triton X-100 concentrations lower than 1% (14, 30, 36, 37). The aim of these studies, however, was not related to the possible heterogeneity in DRMs/rafts; pre-

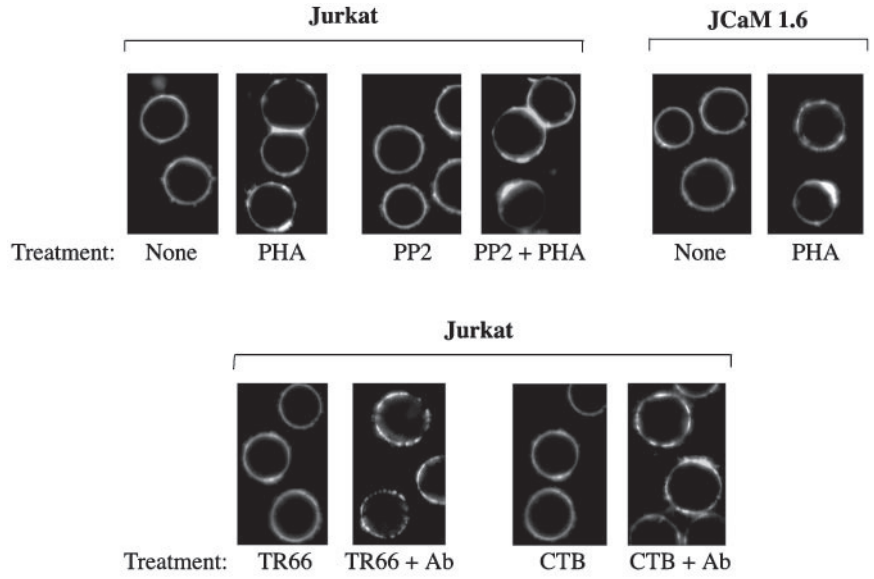

FIG. 7. **Clustering of PM rafts upon treatment with different stimulators and/or inhibitors in Jurkat and JCaM 1.6 cells.** The cells were treated with the different compounds as indicated in the legend to Figs. 3–6. After treatments, the cells were labeled with 0.15 μM BODIPY FL-labeled C5-ganglioside (for 2 min at 22 °C), rapidly harvested by centrifugation, placed on a coverslip, and immediately observed with a real time confocal microscope, as detailed under "Experimental Procedures." *Ab*, antibody.

sumably, the more convenient/efficient detergent type/concentration was merely used. With respect to the previous conflicting results on the association of TCR with DRMs/rafts, the use of different solubilization protocols, in addition to other experimental differences such as the T cell type investigated, may account for by the discrepancies. For example, no CD3 has been found in DRMs by using 1% Triton X-100 (19), whereas some CD3 was recovered in membranes resistant to a lower concentration (0.5%) of Triton X-100 (36), as well as to 1% Brij 58 (35). While this work was being completed, evidence for the constitutive association of TCR to a subset of DRMs, prepared with 1% Brij 98 at 37 °C, was reported (23).

The recruitment of TCR to PM rafts may favor a role for these domains as platforms coordinating activation/polarization of signaling pathways. It is known that TCR is recruited to the site of contact between T cells and antigen-presenting cells (so-called immunosynapse) (43, 50), as well as to the capped PM regions after treatment with mitogenic lectins (45). It is possible, therefore, that these polarized PM regions contain raft domains including the recruited TCR. Indeed, both at the immunosynapse level (51) and in the capped PM regions (present study), the raft marker GM_1 is concentrated. On the other hand, the direct activation of TCR signaling by antibodies to receptor components (*e.g.* OKT3 or TR66) can promote (unpolarized) signaling events, which are apparently independent of the recruitment of CD3/TCR to rafts (Fig. 6*B*). This is consistent with our very recent data (52) showing that cell protein phosphorylation and Ca^{2+} signaling, induced by direct stimulation of the TCR, are not inhibited by T cell raft disassembly.

Cell signaling events, such as protein phosphorylation, Ca^{2+} mobilization/influx, cytoskeleton rearrangement, and phosphorylation of TCR components, might be necessary determinants for the recruitment of CD3/TCR to DRMs. The present data, however, indicate that the recruitment of CD3/TCR to DRMs can occur in the absence of signaling events, such as increase in tyrosine phosphorylation and Ca^{2+} mobilization/influx. Moreover, we did not observe any CD3 recruitment in Jurkat cells stimulated with the anti-CD3 antibody TR66 (Fig. 6*B*), a treatment that induces a marked increase in tyrosine phosphorylation and cytosolic free Ca^{2+} levels (52).

On the other hand, multiple cross-linking of either the TCR or of the raft component GM_1 can result in a recruitment of the receptor to DRMs/rafts. This is the case of the exposure of cells pretreated with the anti-CD3 antibody TR66 to antibodies to TR66 (Fig. 6*B*). Clustering of GM_1 (by treating Jurkat cells with CTB and anti-CTB antibodies) also resulted in an evident recruitment of CD3/TCR to DRMs (Fig. 6*A*). As mentioned above, GM_1 and TCR clusters have been shown to be present both in the PM caps induced by PHA treatment (Ref. 44; see also Fig. 7) and in the PM of the T cells at the immunosynapse level (43, 50, 51). Moreover, GM_1 clusters can be formed independently of cell signaling activation (Fig. 7), which is consistent with previous observation by others in JCaM 1.6 cells (46).

The fact that multiple cross-linking of TCR results in raft coalescence and in recruitment of the receptor to DRMs/rafts is consistent with a variety of previous results discussed in Ref. 7. For example, it has been proposed (7) that clustering of a protein that has an affinity for rafts could either cause small, dispersed rafts containing the protein to coalesce into larger rafts or increase the overall raft affinity of the protein cluster enough to recruit it to rafts. The fact that multiple cross-linking of GM_1 results not only in raft coalescence but also in the attendant recruitment of the (unligated) receptor to DRMs/rafts is consistent with the previous observation that GM_1 clustering results in co-clustering of CD3/TCR (18). A clear mechanistic reason for this phenomenon cannot be presently forwarded; one could argue that if CD3 is loosely associated with lipid rafts, then the aggregation of small rafts into larger ones increases and stabilizes them in the raft domain, making them more resistant to detergent extraction. We should also consider that the overall picture is likely more complex, because of the heterogeneity in the physicochemical structure of rafts. In addition, several co-stimulatory molecules in the T cell PM have been reported to become dynamically associated with DRMs/rafts upon multiple cross-linking of the component itself as well as of other (raft) components. Examples are CD2 (15), CD26 (53), and CD28/GM_1 (48).

In any event, the multiple cross-linking of molecules in the PM of the T cell facing the PM of the antigen-presenting cell may result *per se* in a local recruitment of the TCR to raft structures/clusters. This mechanism, however, does not exclude the participation of (subsequent) cell signaling events in the formation and/or in dynamic evolution of the immunosynapse. Consistently, it has been observed that in the immunosynapse some protein distribution patterns may arise directly from the physicochemical properties of molecules bound to ligands on an opposing cell membrane (54), although synapse formation also requires participation of the actin cytoskeleton and signaling from the initial pool of engaged TCR (54–56).

Acknowledgment—We are grateful to Antonella Viola for helpful discussion.

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