A New Type of Membrane Raft-Like Microdomains and Their Possible Involvement in TCR Signaling

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Membrane rafts and signaling molecules associated with them are thought to play important roles in immunoreceptor signaling. Rafts differ in their lipid and protein compositions from the rest of the membrane and are relatively resistant to solubilization by Triton X-100 or similar detergents, producing buoyant, detergent-resistant membranes (DRMs) that can be isolated by density gradient ultracentrifugation. One of the key signaling molecules present in T cell DRMs is the transmembrane adaptor protein LAT (linker for activation of T cells). In contrast to previous results, a recent study demonstrated that a LAT construct not present in the buoyant DRMs is fully able to support TCR signaling and development of T cells in vivo. This finding caused doubts about the real physiological role of rafts in TCR signaling. In this study, we demonstrate that these results can be explained by the existence of a novel type of membrane raft-like microdomains, producing upon detergent solubilization "heavy DRMs" containing a number of membrane molecules. At a moderate level of expression, LAT supported TCR signaling more efficiently than constructs targeted to the microdomains producing heavy DRMs or to nonraft membrane. We suggest that different types of membrane microdomains provide environments regulating the functional efficiencies of signaling molecules present therein. ***The Journal of Immunology*****, 2010, 184: 3689–3696.**

Membrane rafts (also less correctly called lipid rafts), specific microdomains of the plasma membrane, are distinguished from the rest of the membrane by their relative resistance to solubilization by Triton X-100 or similar detergents (1, 2). Compared to the rest of the plasma membrane, rafts appear to be enriched in cholesterol, sphingomyelins, and glycosphingolipids, containing long saturated acyl residues. These sphingolipids form a liquid-ordered phase further stabilized by cholesterol (3). Detergent-solubilized rafts (also called detergent-resistant membranes [DRMs] or detergent-insoluble glycosphingolipid complexes) can be conveniently isolated by density gradient ultracentrifugation, because they float to the low-density fractions of the gradient and therefore can be separated from completely solubilized membrane proteins and their complexes present in the bottom high-density fractions (4). Membrane rafts apparently participate in a number of biologically important phenomena, such as in immunoreceptor (TCR, BCR, and FcR) signaling, because they concentrate several critical signaling molecules and after initiation of immunoreceptor signaling they associate with the ligated immunoreceptors (5–7). In TCR-mediated signaling pathways, one such raft-associated protein is a transmembrane adaptor protein, linker for activation of T cells (LAT) (8, 9), which after TCR-induced phosphorylation serves as a scaffold for organizing membrane-proximal multicomponent complexes of cytoplasmic signaling proteins. An early report indicated that palmitoylation of LAT is critical for its raft association and for its functionality (10).

However, despite multiple sources of evidence indicating important roles for rafts in immunoreceptor signaling, not only the details of the raft-based mechanisms but even their very existence remain a topic of debate (11, 12). The main problem is that membrane rafts are difficult to visualize as distinct morphological structures. Therefore, most of the structural and functional data are based on (or inferred from) results of biochemical experiments based on detergent extraction of membranes. Such an approach may however almost inevitably induce various artifacts, especially due to selective extraction of some raft components. Other methods often used to demonstrate involvement of membrane rafts in receptor signaling and other phenomena are based on depletion of cholesterol; however, this can also produce artifacts due to generalized alterations in the physical properties the membrane (13). Also, low temperature used during membrane solubilization and production of DRMs may induce artifacts due to phase separations in the mixtures of membrane lipids; however, DRMs can be readily obtained also at 37°C (14), and membrane fragments resembling DRMs can be obtained even by detergent-free membrane disintegration (15, 16).

One of the strongest arguments for the real functional involvement of membrane rafts in TCR signaling has been based on the correlation between loss of (biochemically defined) raft association of palmitoylation-deficient mutants of Lck (17) and LAT (10) and loss of their signaling functionality. Furthermore, inhibition of Lck, Fyn, and LAT palmitoylation led to their raft displacement and inhibition of TCR signaling (18). Therefore, it came as a surprise when a mutant LAT construct was described that did not contain any palmitoylation motif and was not associated with buoyant DRMs but was fully functional in TCR signaling (19). This construct was composed of the extracellular and transmembrane domains of another transmembrane adaptor protein,

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Abbreviations used in this paper: DRM, detergent-resistant membrane; LAT, linker for activation of T cells; LAX, linker for activation of X; LM, laurylmaltoside; LNGFR, a truncated form of human nerve growth factor receptor; MbCD, methyl β-cyclodextrin; OFP, orange fluorescent protein; RV, retrovirus.

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linker for activation of X (LAX) (which is not present in conventionally defined membrane rafts), and the cytoplasmic domain of LAT. Moreover, recently it was shown that the palmitoylation-deficient cysteine mutant of LAT is actually not effectively incorporated into plasma membrane (20). A simple interpretation was that membrane rafts may not be, after all, important in the signaling process.

In this study, we set out to clarify this issue. We hypothesized that the LAX–LAT construct (as well as LAX) may be actually present in a novel type of raft-like microdomains that upon detergent solubilization produce nonbuoyant DRMs but are able to support, at least partially, the TCR signaling. Our results indeed support such a hypothesis, and we describe such DRMs resembling in some respects those corresponding to "conventional rafts." Furthermore, we found that targeting of LAT intracellular domain into different membrane microenvironments (putative conventional rafts, novel raft-like microdomains producing heavy DRMs, and nonraft membrane) affects signaling performance of the molecule, especially at low levels of expression.

Materials and Methods

Plasmids, cells, and Abs

The CD25–LAT construct was created by fusion of sequences encoding the extracellular and transmembrane parts of human CD25 (aa 1–259), the submembrane peptide of human CD3ε (aa 153–160), and the intracellular part of human LAT (aa 35–233); this construct was cloned into the pcDNA3 vector. Constructs encoding fluorescently tagged proteins were made by in-frame fusion of sequences encoding LAT, LAX–LAT, and CD25–LAT with orange fluorescent protein (OFP) (21) using Myc tag as a spacer. All of these constructs were subsequently cloned into the pMXsIN retroviral vector (kindly provided by Dr. A. Cerwenka) (22), which expresses a truncated form of human nerve growth factor receptor (LNGFR) from an internal ribosomal entry site sequence.

Jurkat cells were from the American Type Culture Collection (Manassas, VA), LAT-negative J.CaM2 cells were kindly provided by Dr. A. Weiss, and J.CaM2 cells expressing the LAX–LAT fusion protein (19) were kindly provided by Dr. W. Zhang (LAX–LAT fusion protein comprises the extracellular and transmembrane domains of human LAX [aa 1–68] and the intracellular part of human LAT [aa 34–233]).

J.CaM2 cells expressing the CD25–LAT fusion protein were created by electroporation with plasmid DNA and subsequent selection in the presence of 1 mg/ml G418.

Rabbit antiserum to human LAT (8) was kindly provided by Dr. L. Samelson (National Institutes of Health, Bethesda, MD), rabbit antiserum to human Lck was kindly provided by Dr. A. Veillette (Clinical Research Institute of Montreal, Montreal, QC, Canada), mAb HC10 to HLA class I was kindly provided by Dr. H. Ploegh (Whitehead Institute for Biomedical Research, Cambridge, MA), mAb βF1 to TCRβ was kindly provided by Dr. M. Brenner (Harvard Medical School, Boston, MA), mAb C305 to Jurkat cell TCR was kindly provided by Dr. A. Weiss (University of California, San Francisco, CA), mAb to LAX was kindly provided by Dr. W. Zhang (Duke University Medical Center, Durham, NC), and mAb C33 to CD82 was kindly provided by Dr. O. Yoshie (Kinki University School of Medicine, Osaka, Japan).

Abs to the following molecules were obtained from the indicated commercial sources: LAT (Dy647-labeled; Exbio, Prague, Czech Republic), LNGFR (FITC-labeled; Miltenyi Biotec, Bergisch Gladbach, Germany), CD69 (FITC-labeled; BD Biosciences, San Jose, CA), Myc tag (Cell Signaling Technology, Danvers, MA), CD28 (eBioscience, San Diego, CA), CD71 (Zymed, San Francisco, CA), TCR-ζ (Santa Cruz Biotechnology, Santa Cruz, CA), CD3ε (Becton Dickinson), actin (Sigma-Aldrich, St. Louis, MO). mAbs to CD5 (MEM-32), CD45 (MEM-28), TRIM (TRIM-4), H-Ras (RAS-01), and SIT (SIT-02) are commercially available from Exbio.

Cell and membrane solubilization

A total of 5×10^7 Jurkat cells or various Jurkat transfectant cells, or corresponding amounts of membrane preparations, were lysed in 1 ml lysis buffer (20 mM Tris [pH 8.2], containing 100 mM NaCl, 5 mM iodoacetamide, Protease Inhibitor Cocktail III [Calbiochem/Merck, Darmstadt, Germany], 10 mM EDTA, 50 mM NaF, and 10 mM $Na_4P_2O_7$), containing as detergents either 1% Brij-98 (polyoxyethylene 20 oleyl ether; Sigma-Aldrich) or 1% laurylmaltoside (LM) (*n*-dodecyl-β-D-maltoside; Calbiochem), for 30 min on ice. This lysate was either used directly for density gradient ultracentrifugation (without removing insoluble components) or spun at $25,000 \times g$ for 3 min to remove nuclei and other insoluble materials. Where indicated, the cells were pretreated with 10 mM methyl-β-cyclodextrin (MbCD; 20 min, 37°C), washed with PBS, and solubilized as described above.

Plasma membrane preparation

Cells (2×10^8) were resuspended in 1 ml ice-cold hypotonic buffer (10 mM HEPES [pH 7.4], 42 mM KCl, 5 mM $MgCl_2$, and protease inhibitor mixture), incubated on ice for 15 min, and then passed 10× through the 30-gauge needle (23). The suspension was centrifuged 5 min at $580 \times g$ and 0°C to remove nuclei. The ice-cold postnuclear supernatant was centrifuged 10 min at $25,000 \times g$ and 2°C to pellet the membranes.

Density gradient ultracentrifugation

Cells (5×10^7) were solubilized in 0.5 ml lysis buffer containing 1% Brij-98 (30 min on ice), then 0.5 ml ice-cold 80% (w/v) sucrose in lysis buffer was added, and the mixture was placed at the bottom of a 5.2 ml polyallomer centrifuge tube (Beckman Coulter, Brea, CA), then carefully overlaid with 1.8 ml 30%, 0.8 ml 20%, 0.8 ml 10%, and 0.7 ml 5% sucrose in lysis buffer (with the detergent) and finally with 0.1 ml lysis buffer. Centrifugation was performed at 2°C in Beckman Optima MAX-E ultracentrifuge, using the MLS50 swing-out rotor (18 h, 50,000 rpm). Nine 0.57 ml fractions (and sediment as 10th fraction) were collected gradually from the top of the gradient; proteins were separated by SDS–PAGE and analyzed by immunoblotting.

In some experiments, selected fractions were diluted with six volumes of sucrose-free buffer (20 mM Tris [pH 8.2] and 100 mM NaCl) and centrifuged at 2°C and $50,000 \times g$ for 2 h to recover any large complexes. The sediment was analyzed by SDS–PAGE and immunoblotting.

Gel filtration

A total of 0.1 ml of the cell lysate (after nuclei removal by 3 min centrifugation at $25,000 \times g$) was applied at the top of a 1 ml Sepharose 4B column (in the lysis buffer with detergent) and washed with the lysis buffer; 0.1 ml fractions were collected (all performed at 4°C) and analyzed by SDS–PAGE and immunoblotting. In some experiments, the detergent lysates were supplemented with 0.6 M KI (24) before gel filtration. The void volume fractions of this highly porous gel (fraction 4) contain very large complexes or particles (approximately $>10^7$ Da); maxima of m.w. standards IgM and IgG elute in fractions 7 and 9, respectively (25).

Other biochemical methods (SDS–PAGE and Western blotting) were performed essentially as described previously (26).

Retroviral infection and MACS sorting

Retroviruses (RVs) were prepared by transfection of Phoenix-Ampho cells (Origene, Rockville, MD) with plasmid DNA using Lipofectamine (Invitrogen, Carlsbad, CA) in six-well plates. RV-containing supernatant was centrifuged to remove debris and then used to spin-infect ($1200 \times g$ for 90 min at room temperature) J.CaM2 cells in the presence of Polybrene (Sigma-Aldrich; 10 μg/ml). Cells were allowed to expand in culture for 3 d, and then the infected cells were enriched by magnetic bead sorting because all RVs express the surface marker LNGFR from an internal ribosomal entry site. Infected J.CaM2 cells were stained with FITC-labeled Ab to LNGFR on ice, cells were then washed and incubated with anti-FITC beads (Miltenyi Biotec), and labeled cells were then sorted on AutoMACS cell sorter (Miltenyi Biotec).

Microscopy

J.CaM2 cell transfectants were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min on ice. Cells were then incubated with anti-Myc Ab followed with Alexa Fluor 488-labeled goat anti-mouse IgG secondary Ab (Invitrogen). Nuclei were visualized with Hoechst 33258 dye. Images were acquired on Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Cell stimulation and cytofluorometry

To measure upregulation of CD69, J.CaM2 cells were stimulated overnight with C305 Ab (purified, 1 μg/ml) or left unstimulated, and cells were then washed with FACS buffer (PBS containing 2% FCS and 0.1% sodium azide) and stained on ice with FITC-labeled anti-CD69 Ab. The level of CD69 expression was analyzed by gating on Hoechst 33258-negative (live)

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cells. To measure calcium response to anti-TCR activation, cells expressing LAT chimeras fused with OFP were first loaded with 5 μM Fura-Red (Invitrogen) in loading buffer (1× HBSS, 2% FCS, without Ca^{2+} or Mg^{2+}) for 30 min at 37°C. After being washed, cells were resuspended in loading buffer supplemented with Ca^{2+} and Mg^{2+} and kept on ice. Before the calcium response was imaged, cells were warmed for 10 min at 37°C and then stimulated with C305 mAb (50 μg/ml). For MbCD treatment, cells were incubated for 30 min at 37°C in loading buffer containing 5 μM Fura-Red with or without 1 mM MbCD. After being washed, cells were then resususpended in calcium-free loading buffer containing 1 mM EGTA, stimulated with C305 mAb (50 μg/ml), and analyzed for 2 min until the intracellular calcium stores were depleted. Then, calcium was added to a final concentration of 2 mM, and cells were again analyzed for 2 min to determine the magnitude of the extracellular calcium flux. Cytofluorometry was carried out on a LSRII instrument (Becton Dickinson) in a ratiometric setup using 405/480 nm excitation. Analysis of the data was performed using the FlowJo software (Tree Star, Ashland, OR). To determine the levels of expression of LAT by cytofluorometry, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with cold methanol on ice for 30 min, and nonspecific staining was blocked with 1% BSA in PBS containing 0.01% Triton X-100. Subsequently, cells were incubated with Dy647-labeled anti-LAT Ab, washed, and analyzed on a LSRII flow cytofluorometer.

Results

To explain the results of Zhu et al. (19), we first hypothesized that (a fraction of) the LAX–LAT construct might be actually raft-associated but more sensitive to detergent extraction. Thus, we tested a milder detergent Brij-98 instead of the standard Triton X-100. However, even in the presence of this milder detergent, a large majority of the LAX–LAT protein was absent from the buoyant fractions of the sucrose gradient corresponding to DRMs, whereas almost all LAT was present in the buoyant fractions under the same conditions (Fig. 1*A*).

Next, we tested the hypothesis that the LAX–LAT chimera might be present in a sort of atypical heavy DRM originating from microdomains different from conventional rafts (i.e., large, nonbuoyant complexes) of a similar detergent sensitivity and resistance as the classical DRMs (resistant to Brij-98 but sensitive to LM).

Thus, we performed gel filtration of 1% Brij-98 or LM detergent lysates of the LAX–LAT transfectants on minicolumns of the highly porous gel Sepharose 4B. As shown in Fig. 1*B*, a substantial fraction of LAX–LAT was indeed found in large complexes in the presence of the raft-preserving detergent Brij-98 but not in the presence of the raft-breaking detergent LM. As expected, typical raft molecules LAT and Lck exhibited similar behaviors. Similar results were obtained using Brij-58. When using 1% Triton X-100, only ~30–50% of typical raft molecules (LAT, Lck, and CD55) floated to the low-density fractions of the gradient, and these, as well as LAX–LAT, were found not in the void volume fractions of the Sepharose 4B gel filtration columns but were spread in retarded fractions 6–8, corresponding to lower-m.w. complexes (data not shown).

Alternatively, we isolated the heavy DRMs also from the high-density fractions of the sucrose gradient by simple dilution followed by high-speed centrifugation when Brij-98 lysate (but not LM) was used (Fig. 1*C*).

Next, we compared the sensitivities of the heavy and classical (light) DRMs to cholesterol depletion by MbCD. As shown in Fig. 1*D*, both types of DRMs exhibited similar sensitivities to the pretreatment of the cells by MbCD before Brij-98 solubilization, because both LAT and LAX–LAT largely shifted to fractions corresponding to smaller complexes after the treatment.

Thus, we concluded that the majority of the LAX–LAT construct is apparently present in previously undetected membrane structures resembling in their detergent sensitivity and sensitivity to cholesterol extraction classical membrane rafts but differing from them in the higher buoyant density of the corresponding DRMs. The difference in the densities of the heavy and classical (light) DRMs could be obviously due to a higher protein-to-lipid ratio in the former, and therefore the integrity of the heavy DRMs could be more dependent on protein–protein interactions. Thus, we tested the resistance of both types of DRMs to the chaotropic agent 0.6 M KI (24), which is known to be a mild disruptor of protein–protein interactions (27). As shown in Fig. 1*E*, heavy but not light DRMs were disrupted under these conditions, indicating potentially a more important role for protein–protein interactions in the former.

This result might indicate potential involvement of F-actin in maintaining the heavy DRMs. However, distribution of actin in the gel filtration fractions of the Brij-98 cell lysate corresponded to fully solubilized molecules and was very different from the distribution of LAX–LAT (Fig. 2); the same result was obtained also when using Brij-98–solubilized membranes (data not shown).

Next, we tested which other molecules might be present in the membrane microdomains yielding heavy DRMs. Although several well-known classical raft resident molecules (LAT, Lck, Fyn, and CD55) almost quantitatively floated up in the sucrose gradients containing 1% Brij-98 (Fig. 1*A* and data not shown), a number of others (e.g., CD3ε, CD5, CD28, CD45, CD71(TfR), CD82, TRIM, SIT, LAX, H-Ras, HLA class I, ζ-chain, TCRβ-chain, PLCγ, ZAP70, SLP76, Csk, and tubulin) were found almost exclusively in the bottom fractions of the gradient (data not shown). Of these, under the conditions of gel filtration, LAX, CD5, CD45, H-Ras, and CD28 behaved similarly to LAX–LAT (i.e., were present mostly in large complexes resistant to Brij-98 and sensitive to LM). CD71 (transferrin receptor), TRIM, HLA class I, and TCRζ-chain were distributed almost homogeneously in all of the fractions obtained by gel filtration in the

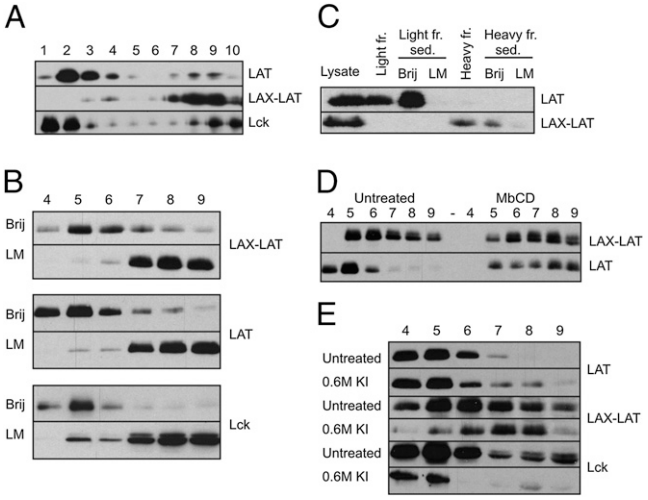

FIGURE 1. Properties of the detergent-resistant membrane complexes containing the LAX–LAT protein. *A*, Brij-98 lysate of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT, Lck) were subjected to density gradient ultracentrifugation, and the separated fractions were analyzed by SDS–PAGE and immunoblotting. Polyclonal Ab to LAT was used to detect both LAT and LAX–LAT. The fractions are numbered from the top. No. 10 represents the sediment. *B*, Brij-98 or LM lysates of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT, Lck) were subjected to gel filtration on Sepharose 4B, and the separated fractions were analyzed by SDS–PAGE and immunoblotting. *C*, Brij-98 or LM lysates of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT) were subjected to density gradient ultracentrifugation as in *A*, and combined fractions 2 and 3 (light) and 8 and 9 (heavy) were diluted and centrifuged to recover large complexes as described in *Materials and Methods*. Samples were analyzed by SDS–PAGE and immunoblotting. *D*, Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT) were pretreated with MbCD or left untreated and solubilized by Brij-98, and the lysates were subjected to gel filtration as in *B*. *E*, Brij-98 lysates of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT, Lck) were treated with KI or left untreated and subjected to gel filtration as in *B*.

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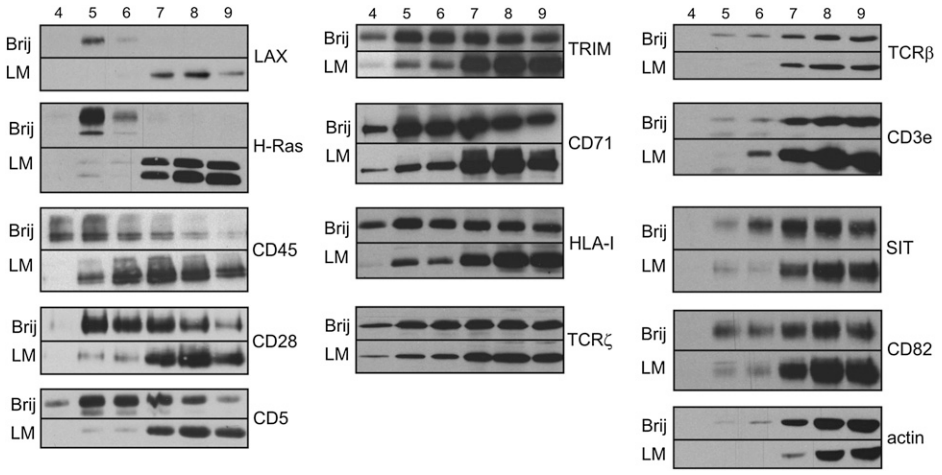

FIGURE 2. Gel filtration of specific membrane proteins solubilized in the presence of Brij-98 or LM. Brij-98 or LM lysates of LAX–LAT J.CaM2 transfectants were subjected to gel filtration on Sepharose 4B as in Fig. 1*B*, and the indicated proteins were visualized by immunoblotting.

presence of Brij-98 and largely shifted to fractions corresponding to small complexes or molecules in the presence of LM. However, TCR/CD3, CD82, SIT, actin, and cytoplasmic molecules, such as PLCγ, Csk, ZAP70, SLP76, and tubulin, were mostly or exclusively present in low-m.w. fractions in the presence of both Brij-98 and LM detergents (Fig. 2 and data not shown). As expected, the molecules known to be present in classical buoyant DRMs (e.g., LAT, Lck, Fyn, and CD55) were also detected almost exclusively in void volume fractions of the Sepharose 4B gel filtration columns in the presence of Brij-98 and shifted markedly to low-m.w. fractions in the presence of LM (data not shown).

To exclude the possibility that almost all of the membrane proteins are present in some sort of Brij-98–resistant complexes simply because the detergent poorly solubilizes membranes, detergent (Brij-98 or LM) lysates of partially purified plasma membranes were subjected to gel filtration on Sepharose 4B, and the protein profiles of the fractions were compared for both of these detergents. As shown in Fig. 3, a large majority of the plasma membrane proteins are present in low-m.w. fractions even after Brij-98 solubilization.

Next, we tried to prepare a LAT construct that would not be targeted to heavy DRMs to be able to compare signaling capacities of the LAT versions targeted to rafts, the newly described microdomains, and nonraft membrane. To this end, we prepared a construct containing the extracellular and transmembrane domains of CD25,

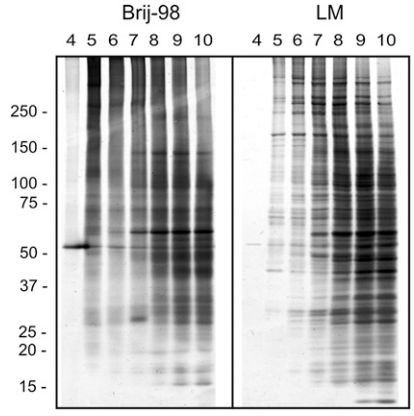

FIGURE 3. Distribution of detergent-solubilized total membrane proteins in gel filtration fractions. Brij-98 or LM lysates of LAX–LAT J.CaM2 transfectant plasma membranes were subjected to gel filtration on Sepharose 4B as in Fig. 1*B*, separated fractions were subjected to 5–15% gradient gel SDS–PAGE, and proteins were visualized by silver staining. Positions of m.w. standards are indicated.

a short submembrane segment coming from CD3ε, and the major cytoplasmic part of LAT (*Materials and Methods* and Fig. 4*A*). This construct was transfected into LAT-deficient J.CaM2 cells, and the stable transfectants were subjected to Brij-98 solubilization followed by gel filtration on Sepharose 4B. As shown in Fig. 4*B*, in contrast to LAT and LAX–LAT, the CD25–LAT chimeric protein was found in the fractions corresponding to solubilized molecules (or small complexes) and therefore was apparently targeted to nonraft membrane sensitive to Brij-98 solubilization.

Finally, we wished to compare LAT, LAX–LAT, and CD25–LAT chimeras as to their abilities to support TCR signaling when expressed in LAT-deficient J.CaM2 cells. The results could be obviously affected by the level of expression of these molecules, and therefore we prepared all of these constructs with C-terminal OFP tags to be able to compare signaling events in cell populations with comparable expression of these constructs. This approach was considered safer than selection of multiple clones with similar expression, because individual clones might exhibit various poorly defined idiosyncratic anomalies. Therefore, for measurement of proximal (calcium flux) and distal (CD69 expression) effects of TCR activation, we compared the cell populations of comparable levels of expression of the constructs, as determined by the OFP fluorescence.

As can be seen in Fig. 5*A*, all three constructs were properly expressed in the plasma membrane and supported TCR signaling equally well if the cells expressed high amounts of the constructs. However, at moderate levels of expression (corresponding to the expression level of endogenous LAT; Fig. 5*B*), LAT (present mostly in classical rafts) supported signaling better than LAX–LAT (present in the novel type of microdomains), whereas CD25–LAT was the least effective one (Fig. 5*C–E*). Therefore, the differences in signaling potencies of these LAT chimaeras become significant only when the amount of LAT available to initiate the TCR signaling cascade is limiting. The functional differences among the three constructs become even more pronounced when calcium release from intracellular stores and from extracellular medium is measured separately (Fig. 5*D*, −MbCD). These data also demonstrate that the major functional difference between LAT and LAX–LAT constructs is in how they support the extracellular calcium influx rather than calcium release from intracellular stores.

Signaling potency of LAT (present in conventional rafts) is obviously more sensitive to partial cholesterol depletion by MbCD as compared with that of LAX–LAT (Fig. 5*D*). This is in agreement with the observed higher resistance of the LAX–LAT-containing microdomains to cholesterol depletion (Fig. 1*D*).

We also tried to compare the LAT constructs as to their redistribution to the immunological synapse formed between Jurkat

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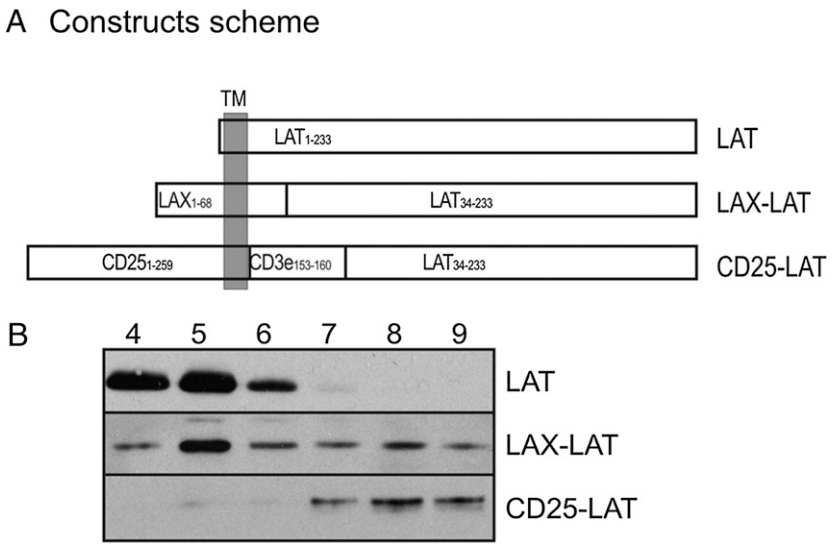

FIGURE 4. Sensitivity of CD25–LAT chimeric protein to Brij-98 solubilization. *A*, Scheme of the LAT constructs used. *B*, Brij-98 lysates of Jurkat (LAT), LAX–LAT J.CaM2, or CD25–LAT transfectants were subjected to gel filtration on Sepharose 4B as in Fig. 1*B*.

cell transfectants and Raji B cells treated with staphylococcal enterotoxin E superantigen. However, even the control wild-type LAT was not significantly redistributed (data not shown).

Discussion

The finding that an apparently nonraft form of LAT (the LAX–LAT chimera) was able to support TCR signaling both ex vivo and in vivo (19) was a serious blow to the widely accepted hypothesis on the importance of membrane rafts in immunoreceptor signaling. In the present paper, we demonstrate that this result can be plausibly explained by the existence of a so far rather overlooked type of membrane microdomain. These appear to be similar to the classical rafts in yielding DRMs resistant to solubilization by polyoxyethylene type detergents, such as Brij-98, but sensitive to LM and to cholesterol extraction (Fig. 1).

These heavy DRMs (and also the classical, buoyant DRMs) can be conveniently separated from fully soluble proteins by gel chromatography on highly porous gel Sepharose 4B. However, this method is essentially not able to distinguish between classical and heavy DRMs, because both these types of large (and probably heterogeneous) complexes are eluted in or near the void volume fractions of the Sepharose 4B column. Nevertheless, it is possible to separate them conveniently by ultracentrifugation in a sucrose density gradient followed by simple dilution of the respective fractions (to decrease density) and sedimentation of the large complexes by ultracentrifugation (Fig. 1*C*).

The heavy DRMs are apparently more dependent on protein–protein interactions because they are, in contrast to the light DRMs, sensitive to treatment with the chaotropic agent 0.6 M KI (Fig. 1*E*). This is in agreement with their higher density, probably due to a higher protein-to-lipid ratio. Interestingly, these structures are apparently not dependent on F-actin, because the distribution of actin in gel filtration corresponds to small molecules (Fig. 2). We have not yet examined the lipid composition of these assemblies to compare it with that of the classical, light DRMs. Nevertheless, it seems likely that lipid-based interactions (in addition to protein–protein interactions) also play an important role in maintaining the integrity of the heavy DRMs, because the detergent LM (which is known to generally preserve protein–protein interactions) as well as cholesterol extraction effectively disrupted them (Fig. 1*C*, 1*D*). The heavy DRMs were less sensitive to cholesterol depletion by MbCD treatment (Fig. 1*D*), and also the signaling functionality of the corresponding microdomains was less sensitive to cholesterol depletion (Fig. 5*D*), whereas signaling capacity of nonraft CD25–LAT was essentially unchanged. The heavy DRMs obviously did not contain a characteristic marker of classical DRMs, GM1, because this glycolipid was detected in many published studies exclusively in the buoyant fractions of the density gradient.

It should be noted that under the conditions used there is not a perfect separation of the components of the heavy and light DRMs (e.g., minor amounts of LAX–LAT can be detected in the floating fractions and minor amounts of LAT or Lck can be detected in the bottom fractions of the gradient [Fig. 1*A*]). It is difficult to determine to what extent it reflects a real situation in the corresponding membrane microdomains before the detergent solubilization. However, the presence of small amounts of LAX–LAT in the classical rafts could obviously contribute to the functional effects of this construct, especially at high levels of expression (see also further discussion).

As stated above, instead of the more commonly used Triton X-100 detergent, we used Brij-98 because the results were more clear-cut and more reproducible; when using Triton X-100, large fractions of typical raft molecules (Lck, LAT, and GPI-anchored proteins) were fully solubilized and found outside the large buoyant detergent-resistant complexes. In our hands, Brij-98 appears to be an optimal detergent for reproducible preparation of DRMs. We are of course aware that the use of any detergent is connected with a certain degree of artefactuality; however, it seems reasonable to assume that the milder detergents, such as Brij-98, preserve the structures corresponding to native membrane rafts better than more stringent ones (e.g., Triton X-100).

We demonstrated that the newly observed type of DRMs (and presumably the membrane microdomains from which they originate) contains not only the artificial construct LAX–LAT but also (as could be expected) endogenous LAX and a number of other membrane proteins (Fig. 2). These membrane proteins include a number of functionally important ones, and therefore this new type of membrane microdomains may be of special functional importance. At the moment, it is not clear how heterogeneous these DRMs (and the presumed corresponding native microdomains) are (i.e., whether one type is rich in LAX only, another in CD28, etc. or whether various mixed DRMs [and microdomains] exist, containing various combinations of these molecules).

It is also unclear what structural motifs are responsible for targeting membrane proteins into this type of membrane environment. Our preliminary unpublished results indicate that the submembrane basic motif present in LAX is of importance, but this question has yet to be studied in more detail. It should be noted that even in the case of

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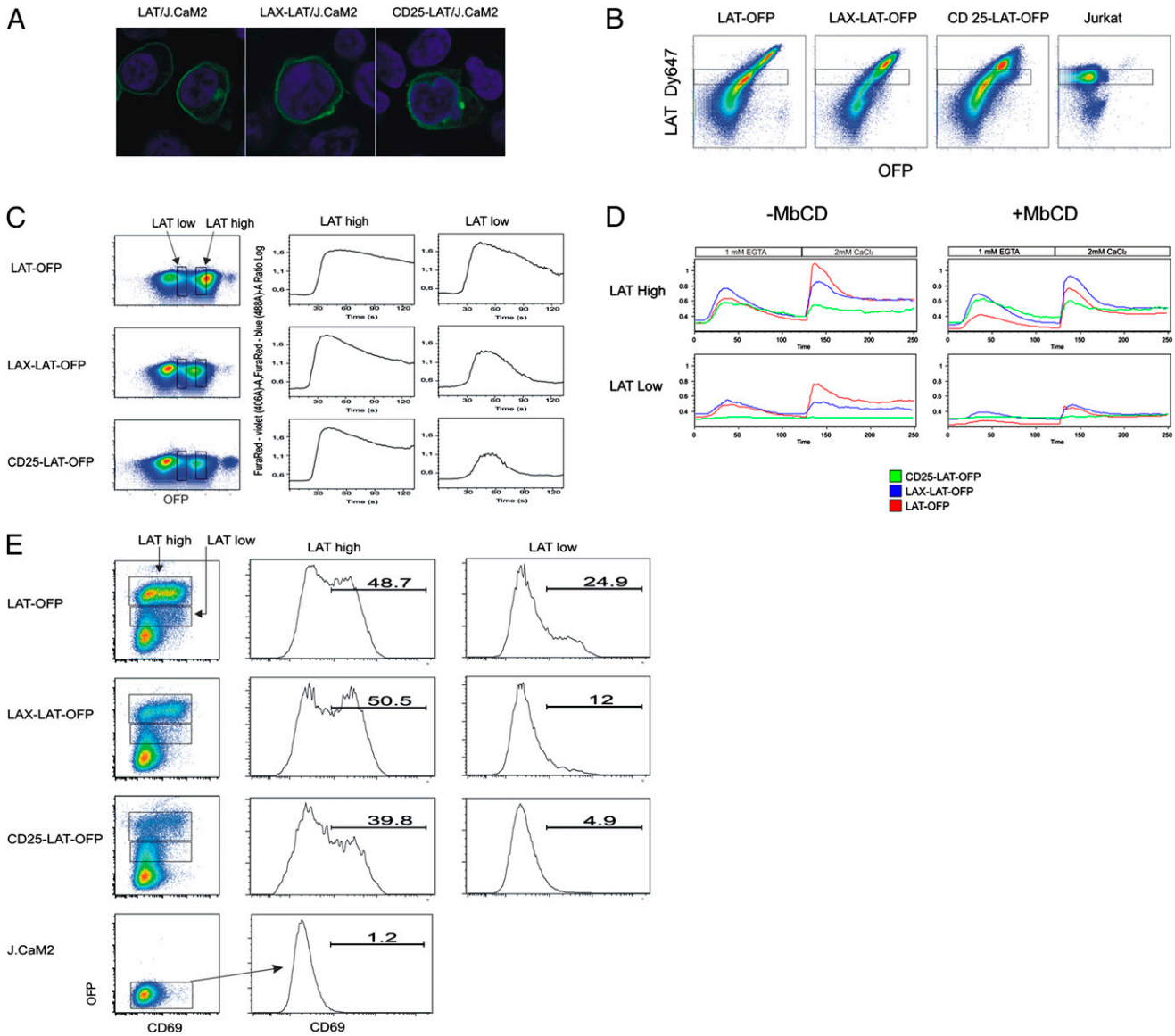

FIGURE 5. Activation responses of J.CaM2 transfectants expressing various LAT constructs containing Myc and OFP tags. *A*, The indicated fixed and permeabilized J.CaM2 cell transfectants were stained with anti-Myc Ab followed by Alexa Fluor 488-labeled goat anti-mouse IgG secondary Ab, nuclei were stained with Hoechst 33258 dye, and images were acquired on a Leica SP5 confocal microscope (original magnification ×800). *B*, The indicated fixed and methanol-permeabilized cells were stained with Dy647-labeled anti-LAT Ab and analyzed by flow cytometry. *C*, The indicated transfectants of J.CaM2 were stimulated with C305 Ab, and dynamics of their cytoplasmic calcium response were measured in populations gated for high or low LAT construct expression, respectively, as described in *Materials and Methods*. *D*, To determine the effect of MbCD (1 mM) treatment on TCR signaling, cells were treated with MbCD as described in *Materials and Methods*, and the magnitude of intracellular and extracellular calcium response was determined following stimulation with C305 Ab. *E*, The indicated transfectants of J.CaM2 were stimulated overnight with C305 Ab, and surface expression of the activation marker CD69 was measured by cytofluorometry in populations gated for high or low LAT construct expression, respectively.

conventional rafts (or classical DRMs) the motifs targeting proteins into them are not entirely clear; palmitoylation appears to be important, but there are cases of raft-associated nonpalmitoylated proteins as well as palmitoylated nonraft molecules (28).

It is also obvious that some molecules are present in these complexes almost completely (e.g., LAX, TRIM, CD5, CD45, H-Ras, and CD28), whereas others (CD71, HLA class I, and TCR) only partially (Fig. 2). Importantly, most of the membrane proteins are fully solubilized even by Brij-98, thus excluding the possible objection that the mild detergent used simply did not solubilize the membrane (Fig. 3).

It should be noted that similar DRMs corresponding to some sort of raft-like membrane microdomains (called heavy rafts by the authors) were already described previously in other cell types (29–33). These heavy DRMs did, somewhat in contrast to those described in our present study, clearly float in the density gradient but less than the conventional ones (i.e., into intermediate density fractions).

We are of course aware of the fact that the biochemical results based on detergent solubilization must be judged with caution because of possible artifacts caused by detergents (selective extraction of some components from the microdomains originally present in the native membrane, possible detergent-induced fusion of native microdomains, etc.).

Therefore, probably the most significant finding of this study is that at a moderate level of expression, similar to the physiological level of the endogenous protein, LAT (present mostly in conventional rafts) supported signaling better than LAX–LAT (present in the newly described raft-like microdomains), whereas CD25–LAT was the least effective one (Fig. 5); it should be noted that the lack of functionality of the nonpalmitoylated cysteine mutant of LAT as described in previous

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papers (10, 34) is actually mostly due to its defective plasma membrane targeting (20). In contrast, all types of LAT constructs, targeted either to the conventional rafts (wild-type LAT), novel raft-like microdomains (LAX–LAT), or nonraft membrane (CD25–LAT) supported the TCR signaling almost equally at high levels of expression (Fig. 5). Interestingly, targeting of LAT outside conventional lipid rafts most profoundly impaired calcium influx from extracellular medium. In B cells, it has been shown that functionality of CRAC channels is dependent on the presence of Src and Syk family kinases (35). Because LAT is a substrate of Syk family kinases, it may be somehow involved in the regulation of CRAC current; however, the detailed mechanism still remains to be elucidated.

Our results indicate that different microdomains do exist in the native membrane and that the environment corresponding to conventional rafts may be under certain conditions (moderate level of expression) optimal for signaling functions of LAT (and perhaps also some other molecules). We therefore propose that conventional rafts (producing upon detergent solubilization buoyant DRMs) might provide an optimal environment for sensitive signaling, which may be important (e.g., in the case of weak stimuli). Nevertheless, the putative novel type of raft-like microdomains (detected here as heavy DRMs) are apparently also able to communicate with TCR complex and participate in the signaling processes.

In conclusion, we believe that our results plausibly explain the startling results by Zhang and colleagues (19). We suggest that their failure to detect any differences in TCR signaling outcome between LAT and LAX–LAT transfectants of LAT-negative T cell lines was due to a relatively high level of expression in these transient transfectants. Their in vivo results demonstrating rescue of LAT-negative thymocyte development by stable transfection with the LAX–LAT construct may simply indicate that even a suboptimal form of LAT (the LAX–LAT construct) can be sufficient [especially if its level of expression may be higher than that in the WT cells, as seems to be the case in Fig. 2*B* of their paper (19)]. It may be speculated that the use of a suboptimal LAX–LAT (and even more CD25–LAT) construct instead of the optimal LAT could lead to (subtle) changes in TCR repertoire (selection for T cells expressing higher-affinity TCRs during development in thymus).

A recent study (36) addressed also a similar problem (i.e., whether a nonraft mutant of LAT is able to support TCR signaling). The authors used another chimeric, nonpalmitoylated LAT construct consisting of the plasma membrane-targeting N-terminal sequence of Src kinase and the LAT cytoplasmic domain (Src–LAT) localized as a peripheral membrane protein in the plasma membrane but outside lipid rafts. This Src–LAT construct restored T cell development and activation, just as the LAX–LAT construct in the earlier study (19). Using the Src–LAT construct kindly provided by M. Hundt and A. Altman, we confirmed its non-DRM localization (similar to our CD25–LAT) even under our mild detergent conditions (1% Brij-98) (data not shown). We suggest that even the suboptimal signaling capacity of the Src–LAT construct, if expressed at its proper level, is sufficient to support TCR signaling and T cell development, similarly to the previously described LAX–LAT construct. Nevertheless, our data suggest that such constructs are less effective than wild-type LAT; the optimal efficiency of wild-type LAT can be best observed at low levels of expression. In fact, some of the data presented in the study by Hundt at al. (36) also suggested that compared with wild-type LAT higher expression levels of Src–LAT were required to complete thymic development, which is in good agreement with our conclusions.

Our findings not only explain the results of Zhu et al. (19) but also point to yet another level of plasma membrane heterogeneity. It will be important to determine the mechanisms responsible for targeting various proteins into the different types of membrane microdomains and clarify basic properties and biological roles of the putative raft-like microdomains described in this study.

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Disclosures

The authors have no financial conflicts of interest.

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