Selective Accumulation of Raft-associated Membrane Protein LAT in T Cell Receptor Signaling Assemblies

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Abstract. Activation of T cell antigen receptor (TCR) induces tyrosine phosphorylations that mediate the assembly of signaling protein complexes. Moreover, cholesterol-sphingolipid raft membrane domains have been implicated to play a role in TCR signal transduction. Here, we studied the assembly of TCR with signal transduction proteins and raft markers in plasma membrane subdomains of Jurkat T leukemic cells. We employed a novel method to immunoisolate plasma membrane subfragments that were highly concentrated in activated TCR–CD3 complexes and associated signaling proteins. We found that the raft transmembrane protein linker for activation of T cells (LAT), but not a palmitoylation-deficient non-raft LAT mutant, strongly accumulated in TCR-enriched immunoisolates in a tyrosine phosphorylation–dependent manner. In contrast, other raft-associated molecules, including protein tyrosine kinases Lck and Fyn, GM1, and cholesterol, were not highly concentrated in TCR-enriched plasma membrane immunoisolates. Many downstream signaling proteins coisolated with the TCR/LAT-enriched plasma membrane fragments, suggesting that LAT/TCR assemblies form a structural scaffold for TCR signal transduction proteins. Our results indicate that TCR signaling assemblies in plasma membrane subdomains, rather than generally concentrating raft-associated membrane proteins and lipids, form by a selective protein-mediated anchoring of the raft membrane protein LAT in vicinity of TCR.

Key words: membrane protein assemblies • signal transduction • membrane rafts • palmitoylation • cholesterol

Introduction

The T cell antigen receptor (TCR)[1] is activated by engagement with a cognate peptide–MHC complex expressed on the surface of an antigen-presenting cell (APC). This triggers tyrosine phosphorylation cascades leading to a physiological response of activated T lymphocytes (Weiss and Littman, 1994; Chan and Shaw, 1996; Wange and Samelson, 1996). After TCR/CD3 engagement, tyrosine-based activating motifs (ITAMs) of the CD3-subunits and TCR ζ-chains become phosphorylated by Lck and, to lesser extent, by Fyn protein tyrosine kinases (PTKs). PTK ZAP-70 binds, via dual SH2 domains, to phosphorylated ITAMs (Chan and Shaw, 1996) and phosphorylates the transmembrane protein linker for activation of T cells (LAT; Zhang et al., 1998a). LAT forms complexes with many signaling proteins (for example GADs, Grb2, and PLC-γ1) and, therefore, links TCR triggering to the Grb2/SOS/ras pathway and the induction of Ca^{2+} fluxes (Finco et al., 1998; Zhang et al., 1998a; Zhang and Samelson, 2000).

In addition, a role of membrane microdomains (rafts) has been implicated in TCR signaling (Horejsi et al., 1999; Xavier and Seed, 1999; Ilangumaran et al., 2000; Janes et al., 2000). Raft membrane domains are envisioned as lateral assemblies of sphingolipids and cholesterol that form liquid-ordered membrane phases (Simons and Ikonen, 1997; Brown and London, 1998). A detergent-resistant membrane fraction (DRM) is currently used to define rafts biochemically (Parton and Simons, 1995; Brown and London, 1997). After TCR stimulation phosphorylated TCR ζ-chain and cytoplasmic signaling proteins including Grb2 and PLC-γ were shown to partition into DRMs (Montixi et al., 1998; Xavier et al., 1998; but see also Zhang et al., 1998b; Janes et al., 1999). Src kinases Lck and Fyn and the linker protein LAT are recovered from DRMs independently of TCR triggering, suggesting that they are constitutively associated to raft membrane domains in T cells (Zhang et al., 1998b; Resh, 1999). DRM association of LAT and Lck depends on S-acylation, most probably by palmitic acid moieties, which are believed to

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[1]*Abbreviations used in this paper:* Ab, antibody; APC, antigen presenting cell; DRMs, detergent resistant membranes; GM1, ganglioside GM1; ITAM, immunoreceptor tyrosine-based activating motif; LAT, linker for activation of T cells; mAb, mouse monoclonal antibody; MβCD, methyl β cyclodextrin; MHC, major histocompatibility complex; PM, plasma membrane; PTK, protein tyrosine kinase; SH2, Src homology 2 domain; TCR, T cell antigen receptor; TfR, transferrin receptor.

insert into the cytoplasmic leaflet of raft membrane domains (Rodgers et al., 1994; Shenoy-Scaria et al., 1994; Zhang et al., 1998b). Lck and LAT require membrane anchoring via palmitoylation to transduce TCR-elicited signals (Kabouridis et al., 1997; Lin et al., 1999; Zhang et al., 1999). Moreover, cross-linking of immunoreceptors like TCR and FcεRI by soluble antibodies or multivalent antigens leads to the formation of patches that are in a highly ordered membrane phase and accumulate raft-associated molecules and signaling proteins (Thomas et al., 1994; Janes et al., 1999; Sheets et al., 1999b). Similar large stabilized raft patches form in Jurkat T leukemic cells by cross-linking the raft lipid ganglioside GM1 using choleratoxin B-subunit. These GM1 patches accumulate tyrosine-phosphorylated proteins, LAT, Lck, as well as TCR/CD3, suggesting that they form foci of TCR signal transduction (Harder and Simons, 1999; Janes et al., 1999). Hence, it was proposed that coalescence of large raft domains in the vicinity of ligand–cross-linked TCR leads to activation by concentrating raft-associated signaling proteins while excluding negatively regulating proteins such as the CD45 phosphatase (for reviews see Horejsi et al., 1999; Xavier and Seed, 1999; Janes et al., 2000).

To specifically address the spatial organization of raft-associated signaling proteins in the plasma membrane (PM) environment of TCR, we employed a novel method to immunoisolate PM fragments highly enriched in activated TCR. Analysis of the immunoisolates showed a selective accumulation of the raft-associated transmembrane protein LAT, but not a nonpalmitoylatable LAT variant, in the plasma membrane environment of TCR. In contrast, other raft-associated molecules, including Lck/Fyn PTKs and glycosphingolipid GM1, were not strongly concentrated. Our findings indicate a selective protein-mediated formation of LAT-TCR signaling scaffolds and are counter to the view that raft lipid–mediated interactions stably couple raft-associated signaling proteins to activated TCR.

Materials and Methods

Cells and Reagents

Jurkat T leukemic cells were obtained from American Type Culture Collection; ANJ3 and JCaM2 LAT–deficient Jurkat derivatives were provided by Laurence Samelson (National Institutes of Health, Bethesda, MD) and Arthur Weiss (University of California San Francisco [UCSF], San Francisco, CA), respectively. Jurkat cells and their derivatives were grown in RPMI medium, 7% FCS, penicillin (100 μg/ml)/streptomycin (100 U/ml), and 2 mM glutamine (all from GIBCO BRL) at 37°C in 5% CO_2 in a humidified atmosphere.

Anti-CD3 mouse monoclonal antibody (mAb) TR66 was from Antonio Lanzavecchia (Institute of Research in Biomedicine, Bellinzona, Switzerland). Antibodies and reagents were purchased from the following sources: mAb against TCR ζ-chain and rabbit antibodies (Ab) against CBL were from Santa Cruz Biotechnology, Inc.; Grb2, PLC-γ, p85, SHC, SLP 76, ZAP-70, and Ha-ras antibodies were purchased from Transduction Laboratories. PE-conjugated α-human CD3 Ab and α-Lck were purchased from PharMingen, and α-LAT rabbit antisera was from Upstate Laboratories. α-TfR mAbs were from Roche Molecular Biochemicals and Zymed, and choleratoxin B subunit and α-choleratoxin rabbit Ab were from Sigma Chemical Co. Secondary HRP-conjugated anti-mouse and anti-rabbit antibodies were obtained from Bio-Rad Laboratories, and FITC-conjugated anti-rabbit antibodies were from PharMingen. Bacterial toxin proaerolysin and chicken α-aerolysin antibodies were obtained from Gisou van der Goot (University of Geneva, Geneva, Switzerland; Abrami et al., 1998).

Immunoisolation of PM fragments

M-450 goat α-mouse magnetic beads (Dynal) were coated with TR66 α-CD3 mAb or B3/25 α-TfR mAb following the instructions of the manufacturers. 1–4 × 10^7 Jurkat cells per data point were incubated with α-CD3 beads (1:2 ratio of beads/cells) in 200 μl RPMI and 1% FCS at 0°C for 2 min and subsequently pelleted with the beads at 2,000 rpm for 10 s at 4°C in an Eppendorf tabletop centrifuge. The bead–cell conjugates were incubated at 37°C for 0, 3, and 7 min, respectively. Cell conjugates were subsequently washed once in H buffer (10 mM sodium Hepes, pH 7.2, 250 mM sucrose, 2 mM $MgCl_2$, 10 mM NaF, and 1 mM vanadate) and suspended in 1 ml H buffer containing CLAP protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin [Sigma Chemical Co.] 100 μM each) and 0.2 mM pervanadate. The cells were nitrogen-cavitated using a nitrogen cavitation bomb (model 4639; Parr Instrument Company) equilibrated at 4°C, 50 bar for 10 min. The homogenate was filled to 10 ml with H buffer, and the beads were subsequently retrieved with a magnet (Dynal) and washed three times in 10 ml of H buffer for 1 min each at 0°C. Homogenates were pelleted by centrifugation at 100,000 *g* at 4°C for 20 min in a TL100 tabletop ultracentrifuge (Beckman Coulter). Beads and cell pellets were analyzed by Western blotting using ECL chemiluminescence (Amersham Pharmacia Biotech). In some experiments, a nonreducing SDS-PAGE was used. ECL-exposed X-ray films were scanned and images were mounted using Adobe Photoshop™ software. For metabolic labeling, cells were kept for 16 h in low Met/Cys (1.5 mg/liter Met and 5 mg/liter Cys) RPMI and 7% FCS supplemented with 0.5 mCi/ml ^{35}S-Cys, ^{35}S-Met (Promix; Amersham Pharmacia Biotech).

Immunofluorescence and DiIC16 Loading

For immunofluorescence analysis, Jurkat cell bead conjugates were adhered to poly-L-lysine (Sigma Chemical Co.)–coated microscope slides for 30 s and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were permeabilized for 1 min in 0.1% saponin in PBS at room temperature, blocked with PBS, 0.2% BSA, and incubated with the antibodies diluted in PBS and 0.2% BSA for 1 h at room temperature. After the antibody incubations, the cells were rinsed twice for 5 min in PBS, 0.2% BSA and mounted in fluoromount G (Southern Biotechnologies). For α-CD3 patching, 10^6 Jurkat cells were suspended and incubated in 100 μl RPMI, 10 mM Hepes (RPMI-Hepes) medium, pH 6.95, at 4°C with 2 μg/ml TR66 α-CD3 mAb. Subsequently, cells were washed twice and incubated in 100 μl RPMI-Hepes with 5 μg/ml FITC-conjugated goat α-rabbit at 37°C for 5 min.

DiIC16 labeling of Jurkat cells was performed as described previously for RBL 2H3 cells (Thomas et al., 1994). In brief, 5 × 10^6 cells were washed twice and suspended in 1 ml RPMI-Hepes. 4 μl of a 1 mg/ml DiIC16 (Molecular Probes) methanolic stock solution was added and the suspension was rapidly mixed. After washing in RPMI-Hepes, DiIC16-labeled cells were conjugated to Ab-coated beads, incubated at 37°C for 7 min, plated on poly-L-lysine–coated coverslips and directly inspected using rhodamine optics. Epifluorescence microscopy was performed on a Zeiss Axiophot fluorescence microscope. Digital images were obtained using a 12-bit Hamamatsu C4742-95 CCD camera. Images were mounted using Openlab™ (Improvision) and Adobe Photoshop™ software.

[^{3}H]Cholesterol Labeling of Cells and Cholesterol Depletion

2 × 10^7 cells were washed in serum-free RPMI and incubated for 3 h at 37°C in 20 ml of serum-free RPMI with 85 μCi 1α, 2α [^{3}H]cholesterol (47 Ci/mmol; Amersham Pharmacia Biotech) and added 1:200 as an ethanolic solution. Subsequently, cells were washed in RPMI 7% FCS and incubated for 16 h at 37°C in full medium. ^{3}H radioactivity was measured by liquid scintillation counting using a 200CA Packard liquid scintillation analyzer.

Before extraction with methyl β cyclodextrin (MβCD; Sigma Chemical Co.), the Jurkat cells were washed twice with RPMI-Hepes and 1 mg/ml BSA. 10^7 cells/ml were extracted with 10 mM and 4 mM MβCD for the indicated times in RPMI-Hepes and 1 mg/ml BSA at 37°C on a rocking platform. Viability of the cells was tested by trypan blue (GIBCO BRL) exclusion. The extent of cholesterol depletion was measured using [^{3}H]cholesterol-labeled Jurkat cells and expressed as mean ± SD from three measurements.

Transfection and Generation of Stable JCaM2 Cell Lines

LAT expression constructs in pcdef3 for wt LAT, Lck-LAT, and C26/29S LAT were provided by Joseph Lin and Arthur Weiss (UCSF, San Francisco, CA). The plasmids were purified using a QIAGEN column and linearized with ScaI. 1.8×10^7 JCaM2 cells were electroporated at 250V, 960 μF with 50 μg of the respective plasmid DNA in PBS, 2 mM ATP, 5 mM reduced glutathione (both Fluka) in 0.4-cm cuvettes using a Gene pulser™ electroporation device (Bio-Rad Laboratories). Resistant clones were selected in 96-well plates (10,000 cells/well) in full medium containing 2 mg/ml G418 (GIBCO BRL). Clones with equal LAT and CD3 surface expression, which was determined by FACS analysis, were chosen for subsequent analysis.

PP2 Treatments and Biotinylation

1.5×10^7 Jurkat cells were washed once in RPMI-Hepes and treated with PP2 (100 μM; 1:350 dilution from stock in DMSO; Calbiochem) at 37°C for 1 h in 1.4 ml serum-free RPMI-Hepes medium. 5×10^6 cells per data point were used for immunoisolation.

Jurkat cells were biotinylated in PBS^{2+} (PBS, 1 mM $CaCl_2$, 5 mM KCl, and 2 mM $MgCl_2$) at 4°C using 0.5 mg/ml Sulfo-NHS-biotin (Pierce Chemical Co.) for 30 min at 4°C following the instructions of the manufacturer. PM was detected using HRP-avidin (Pierce Chemical Co.) by Western blot analysis and ECL detection (Amersham Pharmacia Biotech) following the instructions of the manufacturer.

Detergent-insoluble Membrane Fractions

2×10^7 cyclodextrin- or mock-treated Jurkat cells were homogenized in 300 μl ice-cold HNE (10 mM Hepes, pH 7.0, 150 mM NaCl, and 5 mM EDTA) and 1% Triton X-100 by passage through a 22G syringe and incubated for 15 min on ice. The extract, adjusted to 40% Optiprep™ (Nycomed Pharma), was transferred to an SW55 centrifuge tube (Beckman Coulter) and overlaid with 1 ml of 35, 30, 25, and 0% Optiprep in HNE, and 1% Triton X-100. The gradients were spun at 40,000 rpm for 7 h at 4°C. 600-μl fractions were TCA-precipitated and analyzed by Western blotting.

Results

We developed an immunoisolation procedure for PM subfragments that are highly enriched in activated TCR and associated signaling proteins. Molecular analysis of these immunoisolates served as an assay for the accumulation of signaling molecules in the vicinity of activated TCR.

Enrichment of TCR and Signaling Proteins in TCR/CD3 Immunoisolates

To define PM fragments enriched in activated TCR, we formed conjugates between α-CD3 antibody–coated magnetic beads and Jurkat cells at 0°C. These were warmed to 37°C for 0, 3, and 7 min, inducing TCR signaling as described previously (Hashemi et al., 1996; Lowin-Kropf et al., 1998). Subsequently, the conjugates were homogenized using nitrogen cavitation, and the beads were retrieved and washed. The immunoisolated membrane fragments bound to the antibody-coated beads were analyzed biochemically (Fig. 1).

Western blot analysis showed a strong accumulation of TCR ζ-chain (and CD3ε; data not shown) in the immunoisolates in the course of the incubation at 37°C (Fig. 1), possibly reflecting an active transport of TCR to the bead–cell interface or increased mobility of TCR in the PM at 37°C. Very low amounts of TCR and signaling proteins were recovered at 0°C incubation, showing that the amount of TCR that binds to α-CD3 beads after homogenization is negligible. Approximately 1/10 of the TCR ζ-chain present in the 100,000-*g*–pelleted homogenate was recovered after a 7-min incubation (Fig. 1). We determined an ~50-fold enrichment of TCR ζ-chain over protein of the 100,000-*g* homogenate pellet (Table I). Importantly, the raft-associated transmembrane protein LAT was equally strongly enriched in the immunoisolates as TCR ζ-chain. Other TCR signaling proteins are enriched in immunoisolates, most probably as a consequence of binding to TCR and LAT. Accordingly, the enrichment of ZAP-70 is due to its association to phosphorylated ITAMs in the TCR complex (Chan and Shaw, 1996). A strong representation of Shc and Cbl may be caused by interaction with CD3/TCR subunits and ZAP-70, respectively (Ravichandran et al., 1993; Fournel et al., 1996; van Leeuwen and Samelson,

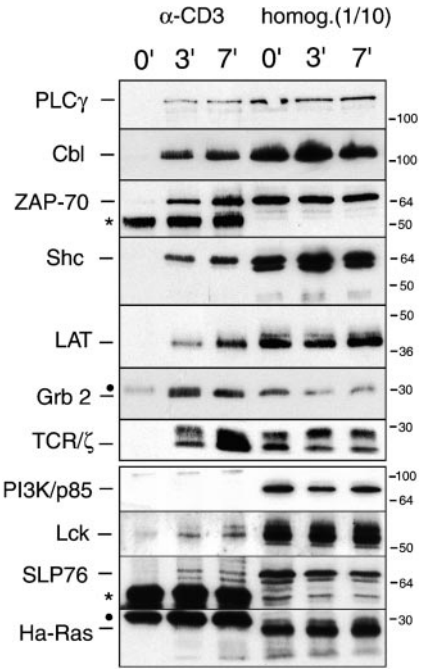

Figure 1. Immunoisolation of TCR-containing PM fragments. α-CD3 immunoisolates obtained after 0, 3, and 7 min of incubation at 37°C and 1/10 of the respective pelleted homogenates were analyzed by Western blots using the antibodies against the indicated proteins. The positions of molecular mass M_w markers (in kilodaltons) are shown. Asterisks mark the position of the Ab heavy chain; closed circles mark the position of the Ab light chain.

Table I. Quantitive Analysis of Immunoisolates

	Percent protein	Percent biotinylated cell surface	Percent cholesterol	Percent TCR ζ-chain and LAT
α-CD3 immunoisolate	0.2	1–2	0.74 ± 0.17 SD	10
α-TfR immunoisolate	0.15	1–2	0.62 ± 0.18 SD	<2

Yields of immunoisolated protein, biotinylated cell surface, cholesterol, and TCR ζ-chain in percentage of total pelleted homogenate after 7 min at 37°C incubation. Protein amounts were determined by measuring sulfur-35 metabolically labeled protein. Recovery of cholesterol was determined by [^{3}H]cholesterol labeling of the cells. The amount of TCR/LAT and biotinylated cell surface recovered in the immunoisolates was estimated by comparison with dilutions of the total homogenate (Figs. 1 and 2). Each determination was repeated at least three times. Cholesterol yields are determined from six measurements each.

1999). In addition, we observed a strong enrichment of signaling molecules PLC-γ and Grb2, which both bind to phosphorylated LAT (Zhang et al., 1998a). Thus, LAT coenriches with TCR in the α-CD3 immunoisolates together with the signaling proteins involved in TCR-evoked responses such as ras activation and induction of Ca^{2+} fluxes. In contrast to the raft-transmembrane protein LAT, raft-associated Lck and Fyn tyrosine kinases as well as Ha-ras did not appear in high amounts in the isolated TCR signaling complexes. Likewise, SLP-76 as well as the p85 regulatory subunit of PI3 kinase, both recovered from immunoprecipitations of LAT from detergent-solubilized cells (Zhang et al., 1998a), were not highly enriched in the immunoisolated membrane fractions.

LAT Is Specifically Concentrated in α-CD3–immunoisolated Plasma Membrane Fragments

LAT and TCR are enriched ~50-fold over total pelletable protein in the α-CD3 immunoisolated PM fragments (Table I). Next, we tested whether they specifically accumulate in the α-CD3 isolated PM fragments. As a measure for PM recovery, we used biotinylated cell surface probed on a Western blot with avidin-HRP (Fig. 2). α-Transferrin receptor (TfR) antibody–coated beads were employed to isolate control PM fragments. The α-TfR beads did not elicit Ca^{2+} fluxes, whereas α-CD3 beads efficiently triggered a sustained rise in intracellular Ca^{2+} (data not shown). The amounts of PM retrieved by α-CD3 and α-TfR beads after different times of incubation at 37°C were similar, corresponding to 1–2% of the total biotinylated cell surface (Fig. 2, Table I). As expected, TfR was enriched in α-TfR bead isolates. The presence of TfR in the α-CD3 isolates indicates a background that corresponded to the amount of isolated PM. Likewise, the α-TfR beads contained background amounts of LAT. However, the amounts of LAT, PLC-γ, and Grb2 in α-CD3 immunoisolates was much higher, demonstrating that these proteins specifically accumulate in α-CD3–immunoisolated PM fragments together with the TCR ζ-chain. In contrast, α-CD3, α-TfR immunoisolates, and dilutions of cell homogenate, normalized to comparable amounts of PM, contained similar amounts of Lck and of Fyn (Fig. 2). Thus, Lck and Fyn were not detectably concentrated in the α-CD3 immunoisolated PM fragments. Next, we assayed recovery of raft glycolipid GM1 and cholesterol in the immunoisolates using an overlay with the choleratoxin B subunit or [^{3}H]cholesterol-labeled cells, respectively. Neither GM1 nor cholesterol significantly accumulated in PM fragments immunoisolated with α-CD3 beads (Fig. 2 and Table I). Moreover, we did not detect an enrichment of GPI-anchored proteins using an overlay with the GPI probe bacterial toxin proaerolysin (data not shown; Abrami et al., 1998).

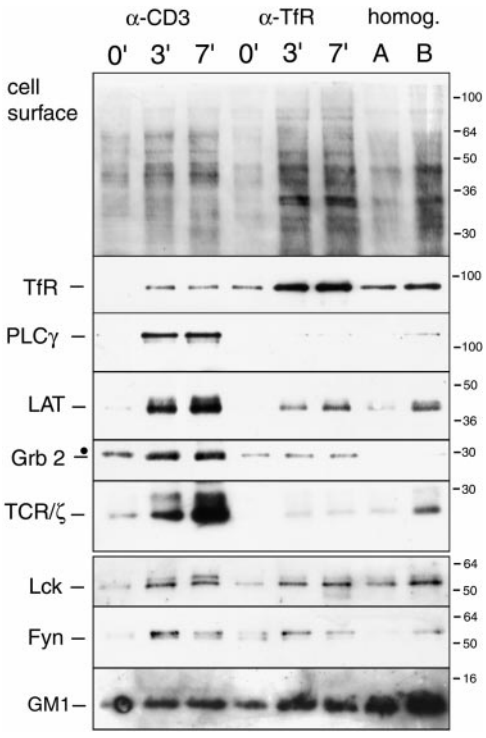

Figure 2. Signaling proteins are concentrated in α-CD3–isolated PM fragments. α-CD3 and α-TfR immunoisolates were analyzed by Western blot using antibodies against the indicated proteins. Recovery of biotinylated cell surface was monitored by Western blot with HRP-avidin. 1:100 (A) and 1:50 (B) dilutions of pelleted homogenate were loaded. The positions of molecular mass markers (in kilodaltons) are shown. Closed circles mark the position of the Ab light chain.

The biochemical analysis was complemented by fluorescence microscopy of bead–cell conjugates (Fig. 3). Immunofluorescence microscopy showed a specific LAT accumulation at the α-CD3 bead–cell contact area (Fig. 3 A, arrows) but not at the α-TfR control beads. In contrast, Lck was not specifically concentrated in the PM region contacting α-CD3 beads (Fig. 3 C). Increased Lck staining was visible in membrane ruffles and microvilli forming at the cell pole facing the α-CD3 bead (Fig. 3 C, arrows). This strongly supports our biochemical data that Lck is, if at all, only weakly concentrated in the PM region contacting the TCR activating beads.

The fluorescent lipid dye DiIC16 preferentially partitions into membrane domains in a highly ordered lipid state, which form in patches of FcεRI IgE receptor cross-linked by soluble antigen (Thomas et al., 1994). Therefore, we followed the distribution of DiIC16 lipid dye in α-CD3 and α-TfR bead–cell conjugates by fluorescence microscopy. DiIC16 staining was neither detectably increased nor excluded in the Jurkat PM in contact with α-CD3 beads or α-TfR beads (Fig. 3, E and F), whereas antibody–cross-linked TCR clusters were strongly labeled with DiIC16 (Fig. 3, G and H). The PM fragments recovered by the beads are shown in Fig. 3 (E and F) and are ~0.5–1 μM in size. Taken together, these data show that α-CD3 beads do not generate large raft-like patches in the PM resembling those that form upon cross-linking of FcεRI and TCR using soluble antigens or antibodies, respectively.

Accumulation of LAT in TCR PM Environment Depends on Tyrosine Phosphorylation

We examined whether tyrosine phosphorylation by Lck/Fyn is required for accumulation of LAT and signaling proteins in the TCR vicinity. We performed α-CD3 immunoisolation using Jurkat cells treated with PP2, a specific and potent inhibitor of protein tyrosine kinases Lck and Fyn (Fig. 4; Hanke et al., 1996). This treatment had little effect on the enrichment of TCR ζ-chain in the immu-

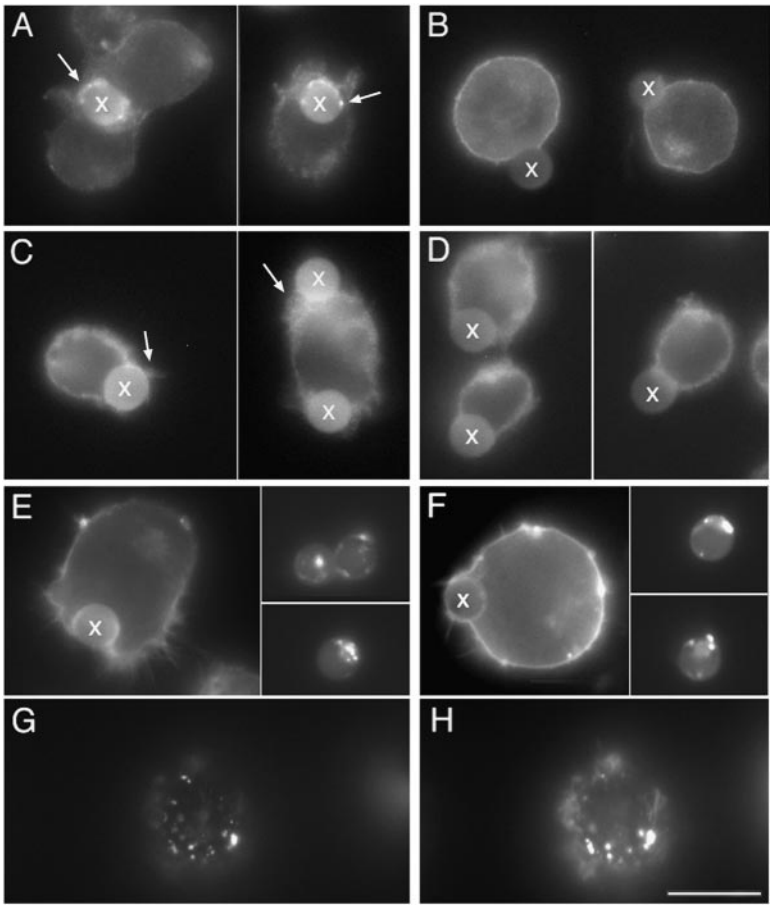

Figure 3. Fluorescence microscopy of Jurkat cells. Jurkat cells were conjugated with α-CD3–coated (A, C, and E) and α-TfR–coated (B, D, and F) beads (marked by x) and incubated for 7 min at 37°C. A and B show immunofluorescence staining of LAT. Arrows indicate LAT accumulating at α-CD3–coated beads. C and D show distribution of Lck. Arrows in C indicate membrane ruffles and microvilli stained with α-Lck antibodies. In E–H, Jurkat cells were stained with the lipid dye DiIC16 before conjugate formation. Insets in E and F show DiIC16-stained PM fragments after the immunoisolation procedure. Exposure times of DiIC16-labeled immunoisolates were 10 times longer than DiIC16-labeled whole cells. G shows distribution of α-CD3 patches visualized by FITC-conjugated secondary antibodies; H shows distribution of lipid dye DiIC16. Bar, 10 μM.

noisolates. However, recovery of LAT as well as other signaling proteins, ZAP-70, Grb2, and PLC-γ, was strongly inhibited. Thus, accumulation of LAT and other signaling proteins in the α-CD3 immunoisolates depended on tyrosine phosphorylations mediated by the PTKs Lck/Fyn.

LAT Requires Palmitoylation To Be Anchored in the TCR Environment

Next, we tested whether LAT requires raft association for its accumulation in the TCR microenvironment. We used the LAT-deficient Jurkat derivative ANJ3 cells either reconstituted with wild-type LAT or with LAT lacking palmitoylation sites, i.e., a non-raft LAT mutant (Fig. 5 A; Zhang et al., 1998b; Lin et al., 1999). α-CD3 immunoisolates from ANJ3 contained TCR ζ-chain, albeit at a reduced immunoisolation efficiency, but lacked LAT and associated PLC-γ and Grb2. Importantly, LAT, PLC-γ, and Grb2 were efficiently coisolated in α-CD3–immunoisolated PM from wt LAT-reconstituted ANJ3 cells. In contrast, a LAT variant mutated in the S-palmitoylation sites Cys 26 and Cys 29 was not recruited into the α-CD3 isolates and failed to reconstitute PLC-γ and Grb2 recruitment into α-CD3 immunoisolates. Palmitoylation-deficient LAT mutant as well as wild-type LAT are both located in the PM (Zhang et al., 1998b). Thus, in addition to Lck/Fyn tyrosine kinase activity, LAT requires palmitoylation for its accumulation in the vicinity of triggered TCR.

Lck is anchored in the inner leaflet of the PM via an NH_2-terminal myristoyl group and dual S-acylation, whereas LAT has a transmembrane domain and two S-acylation sites. It is possible that these different modes of raft anchoring cause the distinct behavior of Lck and LAT in our immunoisolation experiments. Therefore, we

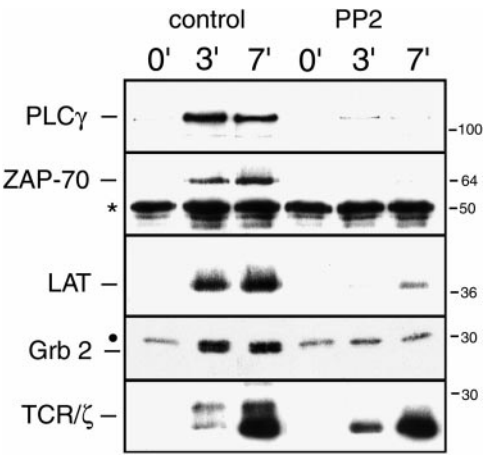

Figure 4. Assembly of TCR signaling complexes depends on Lck/Fyn activity. Control cells or cells treated with specific Lck/Fyn inhibitor PP2 were conjugated with α-CD3 beads. After incubation at 0, 3, and 7 min, α-CD3 immunoisolation was performed as described, and the isolates were analyzed by Western blot with antibodies against the indicated proteins. Asterisks mark the position of heavy chain; closed circles mark the position of the light chain of the antibody used for the immunoisolation. The positions of molecular mass markers (in kilodaltons) are shown.

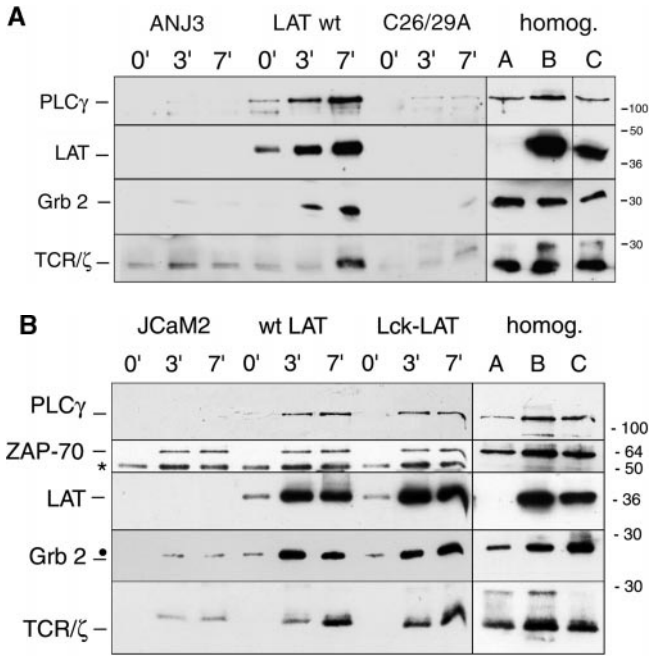

Figure 5. (A) TCR-LAT assembly formation requires palmitoylation of LAT. LAT-deficient ANJ3 cells and ANJ3 cells reconstituted with WT or palmitoylation-deficient C26/29A LAT were used for α-CD3 immunoisolation. Immunoisolates were analyzed by Western blot using antibodies against the indicated antigens. A, B, and C show relative amounts of proteins in 1/10 of pelleted homogenates of ANJ3, ANJ3 wt LAT, and ANJ3 C26/29A derivatives, respectively (exposure times were 1/3 of the immunoisolates). (B) LAT with a Lck raft membrane anchor accumulates in TCR immunoisolates. LAT-deficient JCaM2 cells, JCaM2 expressing wt LAT, and Lck-LAT were subjected to α-CD3 immunoisolation. A, B, and C show relative amounts of proteins in 1/10 of pelleted homogenates of JCaM2, JCaM2 wt LAT, and Lck-LAT, respectively. Asterisks mark the position of Ab heavy chain; closed circles mark the position of the Ab light chain. The positions of molecular mass markers (in kilodaltons) are shown.

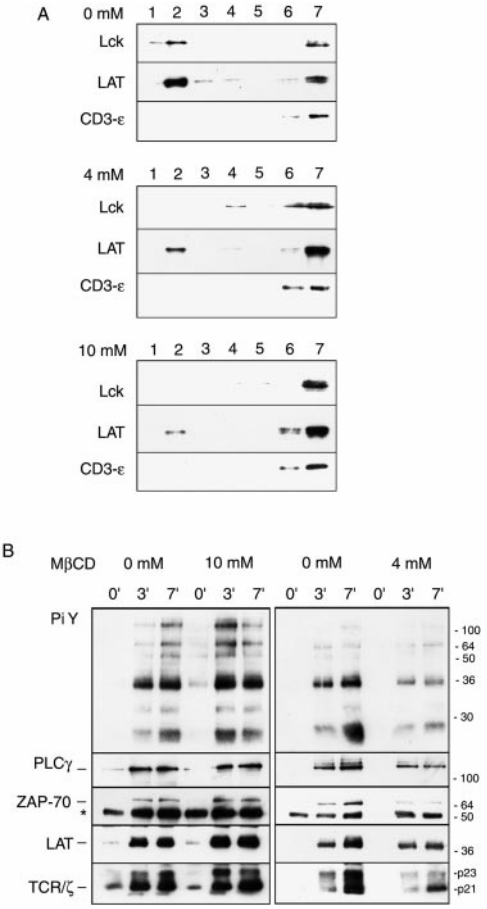

Figure 6. α-CD3 immunoisolation from cholesterol-depleted Jurkat cells. The conditions of MβCD depletion depicted as 0 mM, mock depletion; 4 mM, 4 mM MβCD for 40 min; and 10 mM, 10 mM MβCD for 20 min. Western blots were probed with Abs against the indicated proteins. (A) Association with Triton X-100–insoluble membranes: DRMs were recovered in fraction 2, whereas fractions 6 and 7 contained Triton X-100–soluble proteins. (B) Effect of cholesterol depletions on recovery of signaling protein in α-CD3 immunoisolates. α-CD3 immunoisolates from MβCD-extracted cells were analyzed by Western blot using antibodies against the indicated antigens. Asterisks mark the position of heavy chain; closed circles mark the position of the light chain of the antibody used for the immunoisolation. The positions of the p21 and phosphorylated p23 form of TCR ζ-chain are shown. Positions of molecular mass markers (in kilodaltons) are indicated.

tested whether a Lck-LAT chimera that is anchored in the PM via Lck's NH_2-terminal membrane region (Lin et al., 1999) concentrates like wt LAT in α-CD3 immunoisolates (Fig. 5 B). Starting from the JCaM2 Jurkat derivative that, like ANJ3 cells, essentially lacks LAT expression (Finco et al., 1998), we generated stable cell lines that express wt LAT and a LAT mutant that harbors the NH_2-terminal membrane–anchoring domain of Lck (Lin et al., 1999). We found that the LAT construct with an Lck anchor was equally strongly enriched in α-CD3 immunoisolates as WT LAT. Immunoisolation efficiencies from JCaM2 and ANJ3 cells that did not express signaling-competent LAT were slightly reduced, as indicated by the lower TCR ζ-chain recovery. However, the reduction of LAT mutants and signaling proteins in immunoisolates from these cells is much more pronounced and, therefore, is not caused by the slightly reduced efficiency of immunoisolation. Taken together, our results show that the different behavior of Lck and LAT in our experiments are not caused by their distinct raft anchors but are functions of their respective cytoplasmic domains.

Effects of Cholesterol Depletion on α-CD3 Immunoisolations

Disruption of raft domains using cholesterol depletion has been used to study the role of raft domains in TCR signaling (Xavier et al., 1998; Kabouridis et al., 2000). Importantly, Kabouridis et al. (2000) showed that cholesterol depletion of Jurkat cells with 10 mM MβCD abrogated DRM association of Lck and LAT within the first 20 min of extraction and, in parallel, stimulated tyrosine phosphorylations and the ras pathway (Kabouridis et al., 2000). Therefore, we studied the effects of these cholesterol depletion conditions on the recovery of TCR signaling proteins in our immunoisolation procedure. Extraction with 10 mM

MβCD for 20 min reduced the amount of cholesterol in Jurkat cells to 35 ± 2% SD of the control value. Moreover, it strongly reduced DRM association of Lck and LAT in Triton X-100 Jurkat lysates, suggesting a reduction of raft association (Fig. 6 A). Interestingly, the α-CD3 immunoisolates of Jurkat cells treated for 20 min with 10 mM MβCD contained the same set of tyrosine-phosphorylated signaling proteins as the isolates from the control cells. Indeed, the recovery of TCR-LAT signaling assemblies from these cells was slightly enhanced (Fig. 6 B). This showed that TCR signaling protein assemblies form after MβCD extraction when raft domains are disrupted but tyrosine phosphorylations are increased and deregulated. This strongly supports the view that LAT-TCR assemblies are stably connected via tyrosine phosphorylation–dependent protein–protein interactions and are not coupled by cholesterol-dependent raft lipid domains.

Extraction with 10 mM MβCD for longer than 30 min resulted in significant cell death during conjugate formation and incubation at 37°C. Therefore, we tested lower MβCD concentrations and extended the extraction times. We found that depletion with 4 mM MβCD for 40 min reduced the cholesterol to 45 ± 4% SD and led to reduced DRM association of Lck and LAT (Fig. 6 A). Jurkat cells, extracted under these conditions, are viable and fully recover in medium containing 10% FCS. Retrieval of TCR and signaling proteins in the α-CD3 immunoisolates from cells treated with 4 mM MβCD for 40 min was clearly reduced. The tyrosine-phosphorylated p23 form of TCR ζ-chain tyrosine phosphorylation was relatively reduced over the less phosphorylated p21 form. We also observed a strong reduction of ZAP-70 amounts in the isolates, which is consistent with an inhibition of TCR ζ ITAMs phosphorylation and reduced recruitment of ZAP-70. A shorter (20 min) extraction with 4 mM MβCD had little effect on the recovery of signaling proteins in α-CD3 immunoisolates (not shown). Therefore, an inhibition of tyrosine phosphorylation was observable only after longer cholesterol extraction times.

Using our novel immunoisolation procedure, we describe signaling protein assemblies in TCR-enriched PM subdomains. These assemblies accumulated the raft-associated transmembrane protein LAT and signaling proteins, including ZAP-70, Grb2, PLC-γ, and Shc, which mediate and regulate biochemical reactions leading to TCR-evoked signal transduction (Wange and Samelson, 1996; van Leeuwen and Samelson, 1999). In contrast, we did not detect an accumulation of other raft-associated markers tested, including Lck/Fyn tyrosine kinases, raft-lipid GM1, and cholesterol and raft DiIC16 lipid dye showing a selective enrichment of LAT over other raft-associated molecules.

Discussion

To define the role of raft membrane domains in TCR signaling, it is important to understand how rafts, raft-associated signal transduction proteins, and TCR are spatially organized in the plane of the plasma membrane. We addressed this question using a novel immunoisolation procedure for PM subdomains highly enriched in activated TCR complexes. By this approach, we found that the raft-associated transmembrane linker protein LAT selectively accumulated in the membrane environment of activated TCR, whereas other raft markers were not detectably concentrated. Taken together, our observations question the view that assemblies of membrane proteins involved in TCR signaling form by coalescence of raft domains and strongly indicate that protein-mediated interactions mediate a selective accumulation of the raft-associated membrane protein LAT in the PM environment of activated TCR.

Protein- and Raft Lipid–mediated Interactions in TCR-LAT Assembly

We found that selective LAT accumulation in TCR-enriched PM immunoisolates required tyrosine phosphorylations and, therefore, is most likely mediated by protein–protein interactions. This raft lipid–independent element of TCR-LAT assembly is corroborated by cholesterol depletion experiments. Using the cholesterol extraction conditions described by Kabouridis et al. (2000), at which DRM association of LAT and Lck were abolished but tyrosine phosphorylations were strongly induced, LAT was efficiently recovered in TCR-enriched immunoisolates. The molecular mechanism of LAT accumulation in TCR's PM environment is currently under investigation. Possibly, LAT becomes laterally cross-linked by signaling molecules and/or it is anchored by interaction with cytoskeletal elements.

Moreover, we show that a nonpalmitoylatable LAT mutant does not accumulate in the PM environment of TCR, suggesting the importance of LAT's raft association in the formation of TCR-LAT assemblies. Importantly, raft-targeting sequences of Lck are sufficient for mediating an accumulation of LAT in the membrane environment of TCR. This enforces the view that the distinct behavior of Lck and LAT in our immunoisolation experiments is not due to their different raft-membrane anchor but is a function of their cytoplasmic domains. This is in line with the finding that LAT requires membrane anchoring via S-palmitoylations for its ability to transduce TCR-evoked signals (Zhang et al., 1998b, 1999; Lin et al., 1999).

It is possible that S-palmitoylation and raft targeting of LAT is directly required for its interaction with the TCR environment. Similar to our observations for LAT–TCR interactions, the association of Fyn with TCR ζ-chain ITAMs required, in addition to Fyn's acylations, SH2 domain–phosphotyrosine interactions (van't Hof and Resh, 1999). These findings as well as our observations may be explained by relatively transient palmitoylation-dependent encounters with TCR or TCR-associated targets, followed by phosphorylation-dependent protein-mediated stabilization. Alternatively, it is possible that LAT does not directly require S-palmitoylation for its interaction with TCR but for an interaction with another raft-associated regulatory protein or for the formation of LAT homo-oligomers. Last, the involvement of an adaptor protein that specifically interacts with S-palmitoyl groups cannot be excluded.

Tyrosine phosphorylation induced by cholesterol depletion (Kabouridis et al., 2000) as well as the efficient immunoisolation of TCR/LAT signaling assemblies from cholesterol-depleted cells, described here, suggest a negative regulatory role of raft domains. Possibly, the segregation

of nonengaged TCR and raft-associated signaling proteins in distinct membrane phases suppress tyrosine phosphorylations and signaling.

Enrichment of Signaling Proteins in α-CD3 Immunoisolates

Enrichment of cytoplasmic signaling proteins ZAP-70, grb2, and PLC-γ in our α-CD3 immunoisolates indicate the formation signaling protein complexes around a scaffold of TCR-LAT. Surprisingly, some proteins that have been recovered from immunoprecipitations of LAT, such as SLP-76 and p85 PI3 kinase subunit (Zhang et al., 1998a), were not detectably enriched in our α-CD3 immunoisolates. It is possible that these proteins interact with LAT molecules that are not part of TCR-LAT assemblies isolated here. Moreover, protein complexes of low stoichiometry may not be detectable as enrichment in our immunoisolates. This may occur when signaling proteins compete for binding sites in the isolates. For example binding of Fyn, via its SH2 domains, to phosphorylated TCR ζ-chain ITAMs of the TCR complex (van't Hof and Resh, 1999) may be reduced by the occupation of these ITAMs by ZAP-70. Last, it cannot be excluded that some cytoplasmic proteins, which may, for example, be anchored to the actin cytoskeleton, are selectively shorn from the signaling complexes during the homogenization procedure.

Structure of Membrane Domains Generated by Soluble Ligand and α-CD3 Beads

We showed that Jurkat PM domains bound by α-CD3–coated dynabeads do not strongly accumulate raft lipid dye DiIC16 and, except for LAT, other raft markers. In contrast, high affinity IgE receptor FcεRI and TCR, cross-linked by soluble multivalent ligands, formed membrane patches in a highly ordered phase that accumulate DiIC16 and raft-associated PTKs (Thomas et al., 1994; Janes et al., 1999; Sheets et al., 1999a; and this study). This difference could be due to higher densities of TCR or FcεRI in clusters formed by soluble oligomeric ligands leading to the formation of large continuous patches of highly ordered membrane domains that concentrate raft markers. It is important to note that a low level of oligomerization into dimers or trimers is a sufficient stimulus for TCR signaling (Boniface et al., 1998; Cochran et al., 2000). Moreover, under physiological conditions, the number of cognate peptide–MHC ligands for TCR on an APC is low (10–100/APC; Germain, 1997). Therefore, the formation of micrometer scale patches of cross-linked TCR are unlikely to be required for TCR triggering.

Raft Involvement in T Cell Activation: Accessory Molecular Interactions

LAT and Lck associate with CD4 and CD8 T cell coreceptors for MHC (Weiss and Littman, 1994; Bosselut et al., 1999) and are, thus, concentrated in the proximity of MHC-engaged TCR. However, this CD4 or CD8-mediated concentration is not required for TCR triggering and T cell activation using α-CD3 antibodies or potent peptide–MHC complexes (Viola et al., 1997). Raft may also be involved in CD4/CD8 function as suggested by their DRM association (Cerny et al., 1996; Parolini et al., 1996; Xavier et al., 1998; Horejsi et al., 1999; Millan et al., 1999). Moreover, CD8β is palmitoylated and this is required for its targeting to DRMs and for the association of the CD8 complex with Lck (Arcaro et al., 2000).

T cell activation is supported by coengagement of raft molecules in the contact zone between a T cell and an APC or TCR-activating beads (Moran and Miceli, 1998; Leyton et al., 1999; Viola et al., 1999). Moreover raft lipid GM1 was shown to polarize towards beads coated with TCR and CD28-activating antibody (Viola et al., 1999), possibly mediated by an actin/myosin-mediated plasma membrane transport that, likewise, depends on CD28 costimulation (Wülfing and Davis, 1998). Formation of larger raft-like membrane domains may be required for sustained TCR signaling and the activation of resting T lymphocytes. Likewise, the formation of defined of plasma membrane domains in the immunological synapse at the APC/T cell contact zone may support compartmentalizing principles required for T cell activation (Monks et al., 1998; Grakoui et al., 1999; van der Merwe et al., 2000). However, the relationship of these membrane domains and liquid-ordered raft membrane phases remain to be to defined.

Our observations point to the important role of protein–protein interactions in the formation of TCR-LAT signaling assemblies. Using our novel approach it will be interesting to analyze how signaling proteins assemble into multimolecular complexes and to define how their structure links to TCR signaling. Moreover, our results indicate that the role of raft domains may go beyond the formation of TCR signaling platforms. The key challenge is to understand how rafts behave upon TCR engagement and how potentially highly dynamic raft lipid–dependent interactions control TCR signal transduction.

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