Selective interaction of LAT (linker of activated T cells) with the open-active form of Lck in lipid rafts reveals a new mechanism for the regulation of Lck in T cells

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In T cells, the lipid raft-associated Lck is strongly tyrosine phosphorylated and has reduced enzymic activity in contrast with the detergent-soluble pool, which has substantial activity. Lck tagged at the C-terminus (Lck/V5-His) was efficiently captured by epitope-specific reagents from the detergent-soluble fraction but not from lipid rafts. Binding was restored following urea denaturation, suggesting that Lck/V5-His is in a 'closed' conformation in these domains. In agreement with this hypothesis, the $Tyr^{505} \rightarrow Phe$/V5-His and $Arg^{154} \rightarrow Lys$/V5-His mutants, which disrupt the SH2-Tyr^{505} intramolecular interaction, were efficiently precipitated from lipid rafts. In contrast to Lck, Fyn/V5-His was precipitated equally well from both fractions. In the LAT(linker of activated T cells)-deficient J.CaM2 cells, Tyr^{505} phosphorylation of raft-associated Lck was reduced whereas its enzymic activity was elevated. This correlated with decreased levels of raft-localized Csk (C-terminal Src kinase) kinase. Increased tyrosine phosphorylation of Lck was restored in LAT-reconstituted J.CaM2 cells suggesting that LAT negatively regulates Lck activity in lipid rafts. Co-immunoprecipitation experiments from $Tyr^{505} \rightarrow Phe$/V5-His-expressing cells revealed that LAT preferentially interacts with the 'open' form of Lck in T cell raft domains. These results demonstrate that, unlike the non-raft pool, Lck in lipid rafts has a 'closed'-inactive structure, and that LAT plays a role in maintaining this conformation, possibly by facilitating critical associations within lipid rafts via its capacity to interact with the 'open' form of the kinase.

Key words: adapter, lipid rafts, signal transduction, T lymphocytes, tyrosine kinase.

INTRODUCTION

The multimeric T cell receptor (TCR) uses Src family tyrosine kinases to transduce signals generated at the cell surface [1–4]. Two members of this family of kinases, Lck and Fyn, have been implicated in phosphorylating a defined activation motif present in the cytoplasmic tails of the TCR chains called the immunoreceptor tyrosine-based activation motif (ITAM). The dual tyrosine phosphorylation in each ITAM is recognized by the tandem Src homology (SH) 2 domains of ZAP-70, a member of the Syk family of cytosolic tyrosine kinases [5]. Recruited ZAP-70 is itself tyrosine phosphorylated, activated and capable of instigating downstream signalling cascades [1,6,7]. A critical substrate of ZAP-70 is the adapter molecule LAT, which during TCR signalling plays the role of a plasma membrane scaffold, facilitating the assembly of numerous signalling complexes [8–10]. Genetic ablation of *Lck* and *Fyn* genes in the mouse have indicated that Lck plays a major role in TCR signalling, whereas Fyn has a smaller, more restricted, role [11,12]. The importance of Lck has been also demonstrated in a somatic mutant of the T cell line Jurkat, in which stimulation of the TCR completely fails to instigate intracellular signalling [13].

The molecular structure of Src-family kinases is conserved (reviewed in [14,15]). Distinct functional domains include an N-terminal motif responsible for targeting the protein to cellular membranes [16,17], followed by SH3, SH2 and kinase (SH1) domains [14,15]. Other important features are a C-terminal tyrosine (Tyr^{505} in mouse Lck protein) and a tyrosine residue present within the kinase domain (Tyr^{394} in Lck), which regulate enzymic activity in a negative and positive fashion respectively [18–21]. Intramolecular interaction of the C-terminal phosphotyrosine (pTyr) with the SH2 domain ('tail-bite' structure) induces folding of the enzyme into a low activity state [22,23]. This inactive conformation is further stabilized by interaction of the SH3 domain with the linker peptide that connects the SH2 and catalytic modules [21]. In contrast, phosphorylation of the tyrosine residue within the 'activation loop' of the kinase domain forces the molecule to adopt a conformation with increased enzymic activity [24].

Membrane localization of Lck and Fyn kinases, mediated by their dual acylation, involves their partitioning into lipid rafts, which are specialized microdomains within the plane of the plasma membrane. Lipid rafts, in contrast with the glycerophospholipid bilayer of the surrounding membrane, are rich in sphingolipids and cholesterol and are insoluble in cold non-ionic detergents [25–28]. In T cells, signalling proteins that are found to reside constitutively in lipid rafts, apart from Lck and Fyn, are the adapter proteins LAT (linker of activated T cells) and PAG/Cbp [protein associated with glycosphingolipid-enriched microdomains/C-terminal Src kinase (Csk) binding protein], GTP-binding proteins and certain surface receptors [26,29–31]. Accumulated evidence suggests an important role for lipid rafts in the initiation and propagation of TCR-generated signals in

Abbreviations used: Csk, C-terminal Src Kinase; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker of activated T cells; mAb, monoclonal antibody; MN buffer, 25 mM Mes, pH 6.5, 150 mM NaCl; MβCD, methyl-β-cyclodextrin; NRS, normal rabbit serum; PAG/Cbp, protein associated with glycosphingolipid-enriched microdomains/Csk-binding protein; pTyr, phosphotyrosine; RIPA buffer, modified radioimmunoprecipitation buffer; SH, Src homology; TCR, T cell receptor; WT, wild-type.

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T cells (reviewed in [32–35]). Following stimulation of the TCR, LAT in lipid rafts becomes tyrosine phosphorylated and many key components of signalling pathways that are regulated by the TCR translocate into rafts [29,36–38]. Examples of such proteins are chains of the TCR itself, ZAP-70, PLCγ1, Vav, SLP-76 and PKCθ [39–42]. Furthermore, TCR stimulation induces aggregation of lipid rafts and consequentially aggregation of Lck and LAT, which co-localize with the activated receptors [29,38–40,43,44].

Paradoxically, the *in vitro* enzymic activity of Lck in the lipid raft fraction is low [45,46], which seems to be at odds with its suggested action in these domains. Thus it is crucial to fully understand how Lck is regulated and the present study demonstrates that LAT negatively regulates Lck activity in lipid rafts and preferentially co-immunoprecipitates with the 'open'-active form of the kinase in these domains.

EXPERIMENTAL

Cells and reagents

The E6–1 clone of the human leukaemic T cell line Jurkat, its LAT-deficient somatic derivative J.CaM2 and the LAT-reconstituted J.CaM2-LAT#3 clone [37] were grown in RPMI 1640 supplemented with 5 % (v/v) foetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The J.CaM2 and J.CaM2-LAT#3 clones were a gift from Dr A. Weiss (Department of Medicine and the Howard Hughes Medical Institute, UCSF, San Francisco, CA, U.S.A.). COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS plus the aforementioned concentrations of L-glutamine and antibiotics. Anti-Lck monoclonal antibody (mAb; clone 3A5) and polyclonal antibodies to Lck, Fyn and Csk were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), anti-V5 mAb from Invitrogen (Carlsbad, CA, U.S.A.), antibodies specific for the phosphorylated Tyr^{505} of Lck (p505Lck) were from Cell Signaling Technology (Beverly, MA, U.S.A.), anti-LAT and the anti-pTyr mAb 4G10 from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and Ni^{2+}–agarose from Qiagen (Valencia, CA, U.S.A.). The anti-PAG/Cbp antibodies MEM-250 and MEM-255 were a gift from Dr B. Schraven (Institute for Immunology, Otto-von-Guericke-University, Magdeburg, Germany). Methyl-β-cyclodextrin (MβCD) was purchased from Sigma-Aldrich (Dorset, U.K.).

Construction of C-terminally tagged proteins

Murine wild-type (WT) Lck cDNA was amplified with PCR using the forward primer 5′-CAGGTACCATGGGCTGTGTCTG-CAGCTCA-3′ and the reverse primer 5′-TGACTCGAGGAAGG-CTGGGGCTG-3′, digested with *Kpn*I/*Xho*I restriction enzymes and inserted into the corresponding sites of the pcDNA6/V5-His(B) vector (Invitrogen, Carslbad, CA, U.S.A.) to generate pcDNA6/WT-Lck/V5-His. To subclone WT-Lck/V5-His into pREP3 expression vector, pcDNA6/WT-Lck/V5-His was digested with *Kpn*I/*Pme*I and the excised fragment was ligated into pREP3 vector, which was first linearized with *Xho*I, filled with Klenow fragment and subsequently digested with *Kpn*I to generate over-hanging/blunt ends. The Lck point mutants $Pro^{112} \rightarrow Leu$, $Arg^{154} \rightarrow Lys$, $Lys^{273} \rightarrow Ala$, $Tyr^{394} \rightarrow Phe$ and $Tyr^{505} \rightarrow Phe$ were amplified by PCR with the above primers, digested with *Kpn*I/*Xho*I and inserted into similarly digested pREP3/WT-Lck/V5-His construct to excise the WT-Lck gene and replace it with the desired point mutant. The new *Xho*I site present in the pREP3/WT-Lck/V5-His construct was transferred from the pcDNA6/V5-His(B) polylinker. The point mutants $Pro^{112} \rightarrow Leu$, $Arg^{154} \rightarrow Lys$, $Lys^{273} \rightarrow Ala$ and $Tyr^{394} \rightarrow Phe$ were generously provided by Dr M. Iwashima (Program in Molecular Immunology, Medical College of Georgia, Augusta, GA, U.S.A.) and the $Tyr^{505} \rightarrow Phe$ by Dr J. Ashwell (Laboratory of Immune Cell Biology, NCI, Bethesda, MD, U.S.A.).

To generate FynT/V5-His fusion protein the murine FynT cDNA (a gift from Dr K. Abraham, Department of Microbiology and Immunology, University of Maryland, MD, U.S.A.) was amplified by PCR using the forward primer 5′-TCAGGTACCATGGGCTGTGTGCAATG-3′ and the reverse primer 5′-AGACTCGAGGACAGGTTTTCACCAGG-3′. The PCR product was digested with *Xho*I and ligated to pcDNA6/V5-His(B) that was digested with *Eco*RV/*Xho*I. Subcloning into pREP3 vector was done by excising FynT/V5-His with *Pme*I and ligating it into pREP3 vector that was *Nhe*I linearized and subsequently Klenow treated. FynT/V5-His cDNA inserted in the correct orientation in the vector was identified by restriction enzyme digestion and sequencing.

Cell transfections

Jurkat cells (20×10^6) were washed once in FCS-free RPMI 1640 medium, resuspended in 250 μl of the same medium and transferred into a 4 mm gap electroporation cuvette (BioRad, Hercules, CA, U.S.A.). A portion (10 μg) of the desirable expression DNA construct was added to the cell suspension and cells were electroporated in a Gene Pulser electroporator (BioRad) set at 270 V/960 μF, after which cells were allowed to recover for 10 min at room temperature and then cultured in FCS-containing medium. Oligoclonal populations of stably transfected cells were obtained following selection in 250 μg/ml hygromycin B (Life Technologies). For transient transfections, COS-7 cells were grown to 75 % confluence in 175 cm^2 flasks (Nucleon). On the day of the experiment, cells were collected by trypsinization, washed and 5×10^6 cells were electroporated, as described above, with 5 μg of each of the indicated expression constructs. The LAT expressing construct was a gift from L. Samelson (Laboratory of Cellular and Molecular Biology, NCI, Bethesda, MD, U.S.A.) [29]. Electroporated cells were cultured in 100×15 cm Petri dishes (Nuclon) with DMEM/10 % (v/v) FCS medium for 48 h before harvest.

Preparation of detergent-insoluble lipid rafts

Cells were washed once in RPMI 1640 medium without FCS and lysed in ice-cold MN buffer [25 mM Mes, pH 6.5, 150 mM NaCl] containing 1 % (v/v) Triton X-100 plus protease inhibitors (5 μg/ml of each chymostatin, pepstatin A, leupeptin, aprotinin and 1 mM 4-(2-aminoethyl)benzenesulphonylfluoride (AEBSF) hydrochloride, all from Calbiochem, La Jolla, CA, U.S.A., and 10 mM sodium orthovanadate). Cell lysates were adjusted to 40 % (w/v) sucrose by addition of equal volume of 80 % (w/v) sucrose in MN buffer and vigorous mixing, and then overlaid with an equal volume of 30 % (w/v) sucrose followed by 1 ml of 5 % (w/v) sucrose solution. Isopycnic equilibrium was achieved by centrifugation at 200 000 ***g*** for 16 h at 4 °C using an SW41 rotor (Beckman). Lipid rafts were collected from the 30–5 % interface, the sucrose content was diluted by addition of MN buffer and lipid rafts were pelleted in a microcentrifuge at 14 000 ***g*** for 20 min. The 40 % heavy sucrose cell lysate was collected from the bottom of the tube.

Immunoprecipitations and Western blot analysis

Lipid rafts were dissolved in modified radioimmunoprecipitation (RIPA) buffer [150 mM NaCl, 50 mM Tris, pH 8.0, 1 % (w/v) NP-40, 0.5 % deoxycholic acid and 0.1 % SDS] containing 20 mM MβCD plus protease and phosphatase inhibitors and used for immunoprecipitations with antibodies or Ni^{2+}–agarose, as indicated in the Figure legends. Similarly, immunoprecipitations from the heavy sucrose lysate fraction were carried out after the detergent and MβCD concentrations were adjusted as in the raft fraction. In the case where Ni^{2+}–agarose was used to precipitate His-tagged proteins, 20 mM of imidazole was included in the buffer to reduce non-specific binding and bound proteins were eluted with 500 mM imidazole. In the cases where Ni^{2+}–agarose binding was preceded by denaturation of proteins, 8 M urea in RIPA buffer was added and the samples were heated at 56 °C for 5 min before being subjected to Ni^{2+}–agarose binding. Immunoprecipitations from transiently transfected COS-7 cells were performed following their lysis in 1 ml of RIPA buffer containing 20 mM MβCD plus protease and phosphatase inhibitors. Precleared lysates were divided into three aliquots for immunoprecipitations with normal rabbit serum (NRS), anti-Lck or anti-LAT antibodies. For Western analysis, total cell lysates or immune complexes were resolved by SDS/PAGE using 10 % (w/v) polyacrylamide, transferred on to PVDF membrane and immunoblotted with the indicated antibodies, followed by appropriate HRP-conjugated secondary antibodies. Development of the Western blots was done by enhanced chemiluminescence.

In vitro kinase assay

In vitro kinase assays were performed as described previously [46]. Briefly, lipid rafts were disrupted in RIPA buffer containing 20 mM MβCD plus protease and phosphatase inhibitors and aliquots were used for immunoprecipitations with control NRS or anti-Lck antibodies (clone 3A5) for 4 h at 4 °C. Immune complexes were washed 6 times with lysis buffer and once with kinase buffer (50 mM PIPES, pH 6.5, 2 mM $MnCl_2$, 5 mM dithiothreitol, 0.1 mg/ml BSA and 10 μM ATP). The reaction was performed in 25 μl of kinase buffer containing 1 mM of C peptide (NH_2-GAEEEIYAAFFAKKK-COOH; one-letter symbols are used to denote amino acid residues) and 1 μCi [γ^{32}P]ATP for 45 min at room temperature. The reaction was terminated by the addition of 25 μl of 40 mM EDTA, bound to P81 paper, washed extensively and ^{32}P-incorporation to C peptide was assessed by Cerenkov counting.

RESULTS

C-terminally tagged Lck reacts poorly with epitope-specific reagents in lipid rafts

In an attempt to isolate Lck-interacting proteins from T cell lipid rafts, murine WT Lck and a collection of point mutants, which inactivate critical domains of the protein, were tagged at the C-terminus with the V5 and 6 × His epitopes (Figure 1A). The Lck mutants used were the SH3 and SH2 inactivating point mutations $Pro^{112} \rightarrow$ Leu and $Arg^{154} \rightarrow$ Lys respectively, the kinase-dead $Lys^{273} \rightarrow$ Ala, the activation-defective $Tyr^{394} \rightarrow$ Phe and the dominant-active $Tyr^{505} \rightarrow$ Phe mutant [47,48]. For comparison purposes, a V5-His-tagged version of murine FynT protein was also constructed (Figure 1A). Western analysis with anti-V5 antibodies of lysates prepared from E6.1 Jurkat cells transfected with the panel of Lck constructs or WT-Fyn showed comparable expression of the transfected proteins (Figure 1B). Tagged proteins partitioned to both detergent-insoluble lipid raft and soluble non-raft fractions (approx. 20–25 % present in the raft fraction, results not shown).

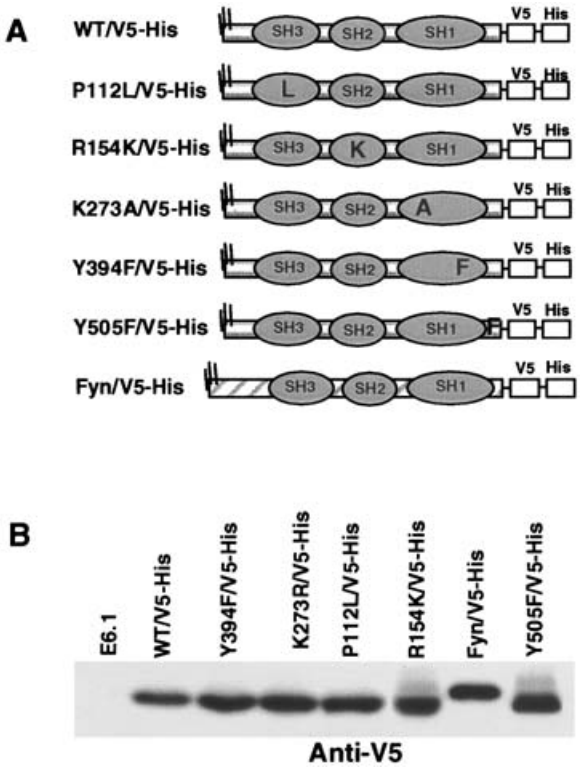


Figure 1 V5-His tagged proteins

(**A**) Schematic depiction of the V5-His tagged proteins used in this study. The single letter amino acid designation is used to indicate point mutations. (**B**) Aliquots of cell lysate equivalent to 1.5×10^5 cells from the parental E6.1 Jurkat cell line or its transfected derivatives were analysed by Western blotting with anti-V5 antibodies to assess expression of transfected proteins.

To capture expressed Lck fusion proteins, purified lipid rafts and sucrose heavy lysate from Lck-WT/V5-His expressing cells were dissolved in RIPA buffer containing 20 mM MβCD and aliquots were incubated with anti-V5 antibodies, Ni^{2+}–agarose or the anti-Lck mAb 3A5 (Figure 2A). MβCD extracts cholesterol, an essential structural component of lipid rafts, and accelerates their complete disruption. Comparative experiments showed that RIPA buffer in the absence of MβCD is also capable of disrupting raft membranes, although incubation for 5–10 min at room temperature may be required. Anti-V5 blotting of the immunoprecipitates showed that only anti-Lck antibodies could efficiently precipitate tagged Lck from lipid rafts, whereas the two epitope-specific reagents reacted poorly with the transfected protein (Figure 2A). In contrast, all three reagents were effective in capturing WT/V5-His Lck from the detergent-soluble fraction (Figure 2A). Inaccessibility of the C-terminus of the fusion protein could indicate that in lipid rafts, Lck adopts a different conformation compared with the non-raft fraction.

Denaturation of lipid-raft-attached Lck renders it susceptible to binding by tag-specific reagents

To investigate the possibility that epitope inaccessibility is due to altered Lck conformation, the lipid raft and heavy lysate fractions from WT/V5-His-expressing cells were left untreated or treated with 8 M urea-containing buffer before being subjected to incubation with Ni^{2+}–agarose. Binding of Ni^{2+} to the 6 × His

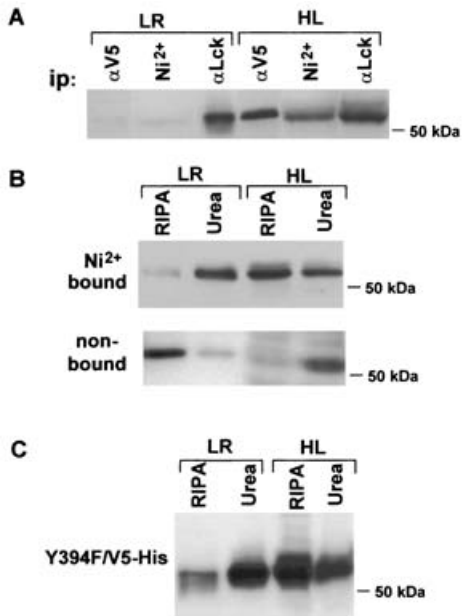


Figure 2 Differential reactivity of tag-specific reagents reveals a conformational difference between lipid raft and non-raft localized Lck

(**A**) Equal aliquots from detergent-insoluble lipid raft (LR) and detergent-soluble heavy lysate (HL) fractions from WT/V5-His-transfected cells were used for immunoprecipitations (ip) with anti-V5 (αV5), Ni^{2+}–agarose (Ni^{2+}) or anti-Lck (αLck) antibodies. Precipitated proteins were resolved and probed with anti-V5 in Western blots. (**B**) Lipid rafts (LR) and heavy lysate (HL) fractions from WT/V5-His-transfected cells were resuspended in lysis buffer with or without the addition of 8 M urea. Urea-containing samples were heated at 56 °C for 5 min before being subjected to Ni^{2+}–agarose binding. Ni^{2+}-bound (upper panel) and non-bound (lower panel) proteins were detected with anti-V5 antibodies. (**C**) LR and HL fractions from $Tyr^{394} \rightarrow$ Phe/V5-His (Y394F/V5-His)-expressing cells were used for Ni^{2+}–agarose precipitations in the presence or absence of urea and captured proteins were probed with anti-Lck antibodies.

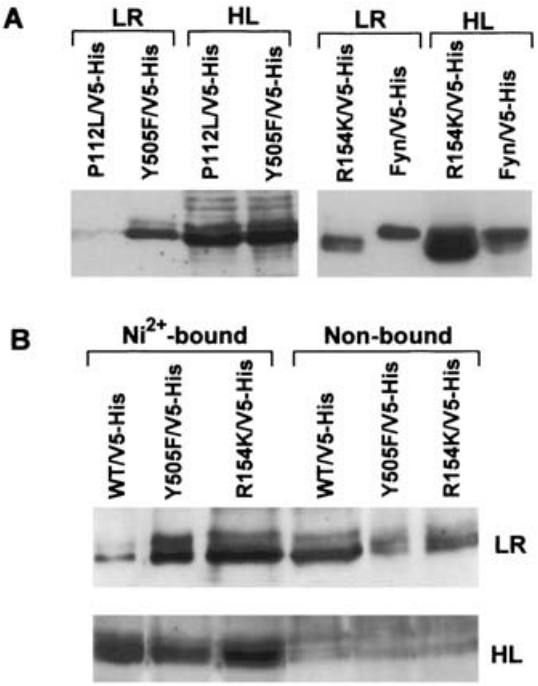


Figure 3 The SH2/pTyr505 interaction determines the conformation of Lck in lipid rafts

(**A**) Aliquots from lipid rafts (LR) and heavy lysate (HL) fractions from cells transfected with $Pro^{112} \rightarrow$ Leu/V5-His (P112L/V5-His), $Arg^{154} \rightarrow$ Lys/V5-His (R154K/V5-His) and $Tyr^{505} \rightarrow$ Phe/V5-His (Y505F/V5-His) Lck mutants or with Fyn/V5-His were adjusted with RIPA buffer without urea and subjected to Ni^{2+}–agarose binding. Bound proteins were analysed with anti-V5 antibodies to detect both Lck and Fyn transfected proteins. (**B**) Lipid rafts (upper panel) or heavy lysate (lower panel) from WT/V5-His, Y505F/V5-His and R154K/V5-His expressing cells were used for Ni^{2+}–agarose precipitations and bound and non-bound fusion proteins were detected by blotting with anti-V5 antibodies.

epitope should be preserved in 8 M urea solution. Transfected WT/V5-His protein was readily precipitated from heavy lysate and analysis of equivalent aliquots from bound and unbound material indicated that the vast majority of the tagged protein was captured by Ni^{2+}–agarose (Figure 2B, lane 3, compare upper and lower panels). In contrast, the WT/V5-His protein pool that distributes to lipid rafts bound poorly to Ni^{2+}–agarose and the majority of the protein was eluted in the unbound fraction (Figure 2B, lane 1, compare upper and lower panels). Capture of WT/V5-His from lipid rafts was strongly increased when proteins were denatured in urea before Ni^{2+}–agarose binding (Figure 2B, lane 2, compare upper and lower panels). Therefore disruption of the conformation of raft-attached Lck exposes the C-terminally fused epitopes to binding by specific reagents. In the heavy lysate, there was a slight decrease in the amount of tagged Lck captured following urea treatment, indicating that in such conditions binding of Ni^{2+} to 6 × His epitope may not be optimal (Figure 2B, lanes 3 and 4). Nevertheless, the increase in the levels of captured Lck from the lipid raft fraction after urea treatment is indicative of the distinct conformation that Lck adopts in raft domains.

To investigate whether the intrinsic kinase activity of the tagged Lck is needed for the protein to adopt its distinct conformation in lipid rafts, the kinase-dead $Tyr^{394} \rightarrow$ Phe/V5-His (Figure 2C) and the activation-deficient $Lys^{273} \rightarrow$ Ala/V5-His (results not shown) mutants were expressed in Jurkat cells and the lipid raft and non-raft pools of Lck were analysed for their ability to bind to Ni^{2+}–agarose. Figure 2(C) shows that $Tyr^{394} \rightarrow$ Phe/V5-His mutant protein from lipid rafts reacted poorly to Ni^{2+}–agarose; however, there was a strong increase in the amount of protein captured from an equivalent lipid raft aliquot that was pre-treated with urea. Therefore, in this respect, the $Tyr^{394} \rightarrow$ Phe/V5-His mutant (and $LysK^{273} \rightarrow$ Ala/V5-His, results not shown) resembles that of WT/V5-His, suggesting that the conformation that Lck adopts in lipid rafts does not depend on its kinase activity. In the detergent-soluble lysate $Tyr^{394} \rightarrow$ Phe /V5-His Lck readily bound to Ni^{2+}–agarose and treatment with urea somewhat decreased the amount of protein captured, as seen with WT/V5-His-expressing cells, confirming that 8 M urea does not represent optimal conditions for Ni^{2+} binding.

Lck conformation in lipid rafts is determined by the intramolecular interaction of its SH2 domain with the C-terminal regulatory tyrosine

To determine which Lck domains are involved in its distinct conformation in lipid rafts, the point mutants $Arg^{154} \rightarrow$ Lys/V5-His, $Pro^{112} \rightarrow$ Leu/V5-His and $Tyr^{505} \rightarrow$ Phe/V5-His were analysed for binding to Ni^{2+}–agarose. Since FynT, in contrast with Lck, has been shown to be active in lipid rafts [46,49], a Fyn/V5-His construct was analysed in parallel with the Lck mutants. Similarly to WT-Lck and to the kinase-inactive mutants, $Pro^{112} \rightarrow$ Leu/V5-His from lipid rafts bound poorly to Ni^{2+}–agarose (Figure 3A). In contrast, $Tyr^{505} \rightarrow$ Phe/V5-His and $Arg^{154} \rightarrow$ Lys/V5-His proteins were clearly captured by Ni^{2+}–agarose, as was Fyn/V5-His, which correlates with its high

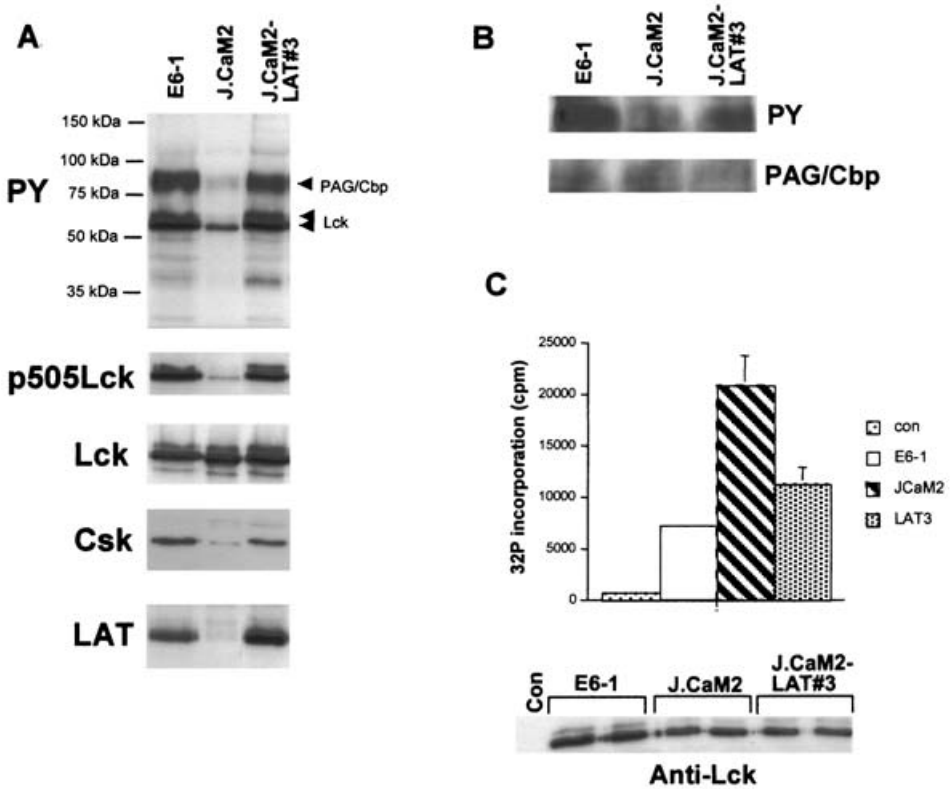


Figure 4 Reduced tyrosine phosphorylation and increased kinase activity of lipid raft-associated Lck in LAT-deficient cells

(**A**) Lipid rafts prepared from the E6-1, J.CaM2 and J.CaM2-LAT#3 clones were sequentially immunoblotted with the anti-pTyr mAb 4G10 (PY), antibodies specific to the phosphorylated form of Lck Tyr^{505} (p505Lck), anti-Lck, anti-Csk and anti-LAT antibodies. (**B**) Lipid rafts purified from the E6-1, J.CaM2 and J.CaM2-LAT#3 cell lines (150×10^6 cells) were used for immunoprecipitations with the anti-PAG/Cbp MEM-250 antibody and immune complexes were analysed in Western blots with anti-PY, stripped and then re-blotted with the MEM-255 anti-PAG/Cbp antibody. (**C**) Lipid rafts from 50×10^6 cells from the indicated clones were used to immunoprecipitate Lck and its *in vitro* enzymic activity was assessed by incorporation of ^{32}P into a peptide substrate following a 45 min incubation at room temperature. Control immunoprecipitations (Con) were performed with normal rabbit serum. Results are the mean of duplicate immunoprecipitations for each cell line and are normalized for the levels of Lck immunoprecipitated from each sample, as determined by Western blotting shown in the lower panel.

activity in lipid rafts (Figure 3A). All fusion proteins were efficiently precipitated from heavy lysate. These results indicate that the SH2-pTyr505 intramolecular interaction is responsible for the 'closed' conformation of Lck in lipid rafts, which is consistent with the classical 'tail-bite' mechanism described for Src-kinase inactivation. In addition, a correlation between conformation and enzymic activity was observed, since the activity of $Tyr^{505} \rightarrow$ Phe/V5-His mutant in lipid rafts was found to be dramatically elevated when compared with WT Lck immunoprecipitated from the parental E6-1 cells (results not shown), again suggesting that the activity of Lck in lipid rafts is kept at low levels due to the SH2-pTyr505 interaction.

To directly compare the amounts of captured protein, lipid rafts and heavy lysate from WT/V5-His-, $Tyr^{505} \rightarrow$ Phe/V5-His- and $Arg^{154} \rightarrow$ Lys/V5-His-expressing cells were subjected to Ni^{2+}–agarose binding, and the levels of bound and unbound proteins were detected with anti-V5 antibodies. Densitometric analysis showed that 18 %, 85 % and 75 % of the lipid raft-localized WT/V5-His, $Tyr^{505} \rightarrow$ Phe/V5-His and $Arg^{154} \rightarrow$ Lys/V5-His protein respectively was captured by Ni^{2+}–agarose (Figure 3B, upper panel). The fact that somewhat less $Arg^{154} \rightarrow$ Lys/V5-His protein is captured compared with $Tyr^{505} \rightarrow$ Phe/V5-His, may reflect the incomplete inactivation of the SH2 domain by this point mutation. From the heavy lysate, 78 %, 76 % and 84 % of WT/V5-His, $Tyr^{505} \rightarrow$ Phe/V5-His and $Arg^{154} \rightarrow$ Lys/V5-His protein was captured respectively (Figure 3B, lower panel).

LAT expression regulates tyrosine phosphorylation of Lck in lipid rafts

In order to understand the genetic requirements for Lck regulation, lipid rafts from a collection of somatic mutants of the Jurkat T cell line were analysed. The predominant tyrosine phosphorylated proteins detected in lipid raft preparations from T cells migrate on SDS/PAGE at approx. 55–60 and 75–80 kDa molecular mass. Previous work has established that the vast majority of the 55–60 kDa bands correspond to Lck, since they can be immunodepleted from raft membrane preparations using Lck antibodies (results not shown). The diffused 75–80 kDa bands could represent differentially phosphorylated species of the adapter protein PAG/Cbp [30,31]. Western blotting with anti-pTyr antibodies revealed that in the LAT-negative J.CaM2 cells, tyrosine phosphorylation of Lck was reduced when compared with the parental E6-1 clone, as was tyrosine phosphorylation of the 75–80 kDa molecular mass bands (Figure 4A). Reduced reactivity with antibodies specific for the phosphorylated form of Tyr^{505} indicated that at least part of this reduction is due to lower levels of Tyr^{505} phosphorylation (Figure 4A). The above difference is not due to differential expression of Lck protein between the two cell lines, but most likely due to decreased levels of Csk kinase present in the J.CaM2 lipid rafts (Figure 4A). Csk phosphorylates the C-terminal regulatory tyrosine of Src-family kinases and hence

inhibits their activity. Re-expression of LAT in the J.CaM2-LAT#3 clone restored the levels of Lck and 75–80 kDa proteins phosphorylation, which correlated with increased recruitment of Csk into rafts, indicating that LAT expression mediates this event (Figure 4A). Immunoprecipitation of PAG/Cbp from E6-1, J.CaM2 and J.CaM2-LAT#3 lipid rafts using specific antibodies [30], showed reduced tyrosine phosphorylation of this adapter molecule in J.CaM2 lipid rafts, suggesting that some of the 75–80 kDa phosphoproteins seen in the raft preparations represent PAG/Cbp (Figure 4B). A possible interpretation of these results is that LAT determines, at least in part, the levels of Lck Tyr^{505} phosphorylation by regulating PAG/Cbp phosphorylation and subsequent increase in Csk recruitment into lipid rafts. Another possibility is that LAT directly recruits Csk; however, repeated attempts by co-immunoprecipitation, failed to show interaction of LAT with Csk in lipid rafts.

To see whether the reduced phosphorylation of Lck-Tyr^{505} in J.CaM2 lipid rafts correlates with elevated enzymic activity, Lck from E6-1, J.CaM2 and J.CaM2-LAT#3 lipid rafts was immunoprecipitated and its *in vitro* activity was determined. In three independent experiments, Lck from J.CaM2 lipid rafts was found to have 2–3-fold higher activity when compared with that from E6-1, whereas Lck activity in the J.CaM2-LAT#3 cells was comparable with E6-1 (Figure 4C). The lower panel of Figure 3(B) shows that comparable levels of Lck were immunoprecipitated from the three cell lines.

LAT preferentially interacts with the 'open'-active form of Lck in lipid rafts

The above finding led to the investigation of possible interactions between LAT and Lck. To this end, lipid rafts from the parental E6-1 clone or the $Tyr^{505} \rightarrow$ Phe/V5-His-expressing cells were used as a source of Lck protein in closed or open configuration respectively. They were disrupted with RIPA buffer containing 20 mM MβCD and aliquots were used for Lck and LAT immunoprecipitations. Following electrophoresis, the immune complexes were analysed by Western blots, with antibodies to Lck or LAT, to detect whether these proteins can co-imunoprecipitate. Two different anti-Lck antibodies (a polyclonal and the 3A5 monoclonal) were used for Lck immunoprecipitations. In some experiments a weak association between Lck and LAT was detected in lipid rafts from the E6-1 clone following prolonged exposure of the blot (Figure 5A). However, when the same experiment was performed with lipid rafts from $Tyr^{505} \rightarrow$ Phe-expressing cells, a clear interaction between Lck and LAT was revealed, which was detectable with all the immunoprecipitating antibodies used (Figure 5B). The detected levels of LAT which co-immunoprecipitated with the Lck antibodies was approx. 15 % of that precipitated with specific anti-LAT antibodies. When the detergent-soluble fraction was used for the immunoprecipitations, only a weak association between Lck and LAT was seen after prolonged exposure of the blots (Figure 5C). Finally, no association of LAT with Fyn was detected in lipid rafts from E6-1 cells (Figure 5D), despite the fact that Fyn has an open conformation in this fraction. These experiments suggest that LAT preferentially interacts with the 'open'-active form of Lck and this interaction predominantly takes place in lipid raft domains.

To characterize further the LAT/Lck interaction, COS-7 cells were transiently co-transfected with the $Tyr^{505} \rightarrow$ Phe/V5-His Lck and LAT expression constructs and association of the expressed proteins was analysed by co-immunoprecipitation as above. As shown in Figure 6(A) (upper and middle panels), association

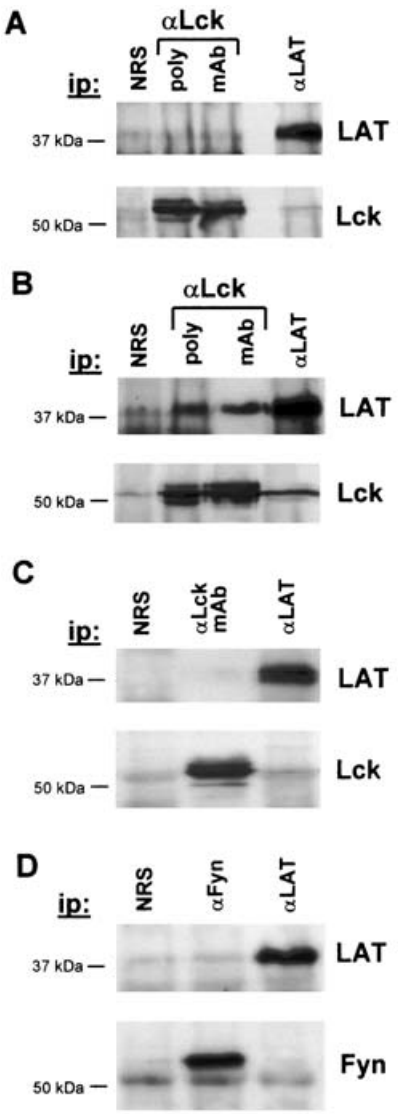


Figure 5 LAT preferentially associates with the 'open'-active form of Lck in T cell lipid rafts

(**A**) Lipid rafts were prepared from Jurkat cells and equal aliquots were used for immunoprecipitations (ip) with normal rabbit serum (NRS), polyclonal (poly) and monoclonal (mAb) anti-Lck antibodies, or anti-LAT antibodies (αLAT). Immune complexes were resolved by SDS/PAGE and Western blotting with antibodies to Lck or LAT, as indicated to the right of the panels. (**B**) Lipid rafts were prepared from cells expressing the $Tyr^{505} \rightarrow$ Phe/His-V5 (Y505F/His-V5) mutant and immunoprecipitations and Western analyses were performed as in (**A**). (**C**) The detergent-soluble lysate from Y505F/His-V5-expressing cells was used for immunoprecipitations and Western analysis as in (**A**). (**D**) Lipid rafts prepared from E6-1 cells were used for immunoprecipitations with NRS, anti-Fyn or anti-LAT antibodies and Western analysis was performed with antibodies to LAT or Fyn.

of $Tyr^{505} \rightarrow$ Phe-Lck with LAT was clearly detectable in COS-7 cells, indicating that their interaction does not require the presence of lymphoid-specific intermediate protein(s). The anti-Lck mAb was more effective in detecting the Lck/LAT association when compared with the anti-LAT polyclonal, possibly reflecting the higher potency of the 3A5 clone as an immunoprecipitating antibody. Expression of transfected proteins was determined with Western blotting of total cell lysate aliquots using Lck and LAT antibodies (Figure 6A, lower panel). Antibodies to Lck did not precipitate LAT from COS-7 cells transfected only with the LAT construct, and vice versa, anti-LAT antibodies did not

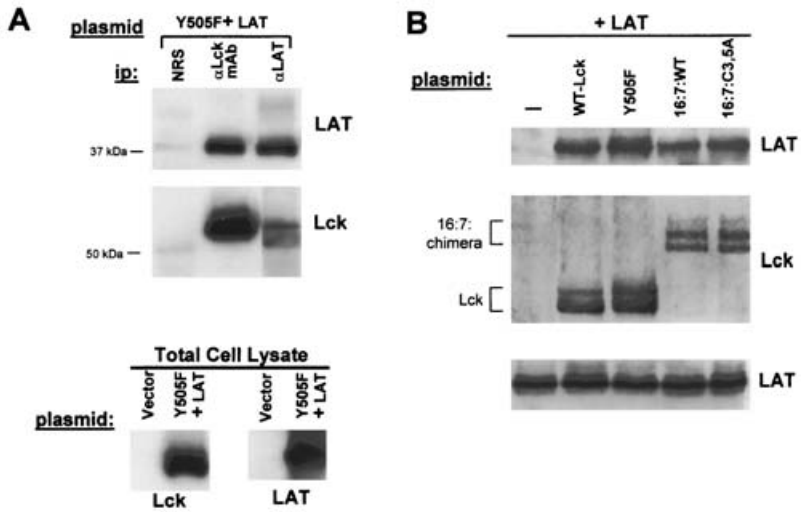


Figure 6 Interaction of Lck and LAT in non-lymphoid cells

(**A**) COS-7 cells were transiently co-transfected with $Tyr^{505} \rightarrow$ Phe (Y505F)-Lck- and LAT-expressing plasmids, or with empty vector, and cell lysates were used for NRS, anti-Lck mAb (αLck) and anti-LAT (αLAT) immunoprecipitations (ip). Proteins in the immune complexes were resolved by electrophoresis and analysed by Western blotting with anti-LAT (upper panel) and anti-Lck (middle panel) antibodies. Expression levels of transfected proteins were determined by Western blotting equivalent aliquots of total cell lysate with Lck and LAT antibodies (lower panel). (**B**) COS-7 cells were transfected with LAT-expressing plasmid only or co-transfected with LAT plus one of the WT-Lck, Y505F mutant, 16:7:WT-Lck or 16:7:C3, 5A-Lck chimaeras. Cell lysates were used for immunoprecipitations with the Lck mAb. Co-immunoprecipitated LAT (upper panel), levels of immunoprecipitated Lck (middle panel) and expression levels of LAT in total cell lysates (lower panel) were determined by Western analyses with specific antibodies.

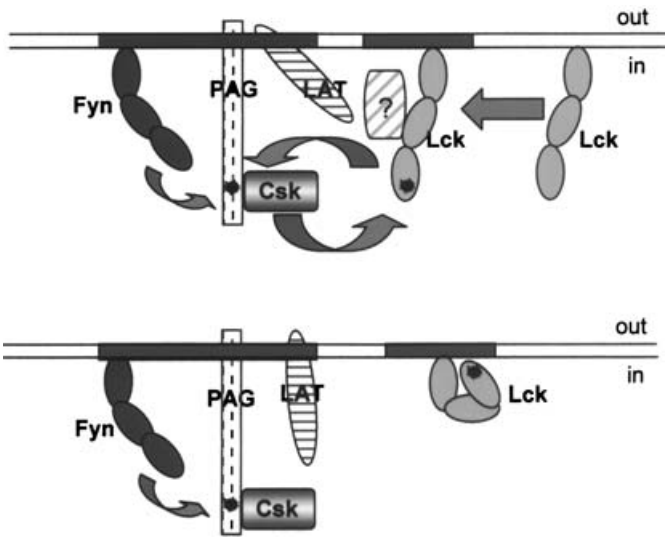
precipitate Lck from cells transfected only with the Lck construct, demonstrating the specificity of the antibodies and the complete solubilization of lipid rafts in the RIPA/20 mM MβCD buffer (results not shown). In contrast with what was observed in T cells, LAT was also able to interact with WT-Lck in COS-7 cells, as shown in Figure 6(B). This may suggest that Lck expressed in COS-7 cells is not regulated the same way as when it is localized in T cell lipid rafts, and hence is able to interact with LAT. To investigate whether the Lck N-terminal dual cysteine motif, which is a target for palmitoylation, is involved in its interaction with LAT, the 16:7:WT and 16:7:C3,5A Lck chimaeras [17] were co-transfected with LAT in COS-7 cells. As shown in Figure 6(B), both the 16:7:WT and the 16:7:C3,5A proteins associated with LAT equally well, excluding a role for this motif in the Lck/LAT interaction.

DISCUSSION

It has been reported previously that Lck in T cell lipid rafts possesses low activity when compared with the non-raft pool [45,46,49], which could be due to the exclusion of the CD45 tyrosine phosphatase from these domains [43,45]. The results obtained with the C-terminally tagged Lck constructs corroborate this observation by revealing that the majority of Lck in lipid rafts is in the 'tail-bite' configuration mediated by the SH2/pTyr505 interaction. In contrast, the detergent-soluble pool has an 'open' structure, possibly maintained by the action of CD45. In agreement with this model, disruption of T cell lipid rafts with cyclodextrin induces transient increase in tyrosine kinase activity and phosphorylation of protein substrates. This was dependent on the expression of Lck and CD45, suggesting that Lck, once released from the lipid raft environment, is susceptible to the positive action of the CD45 phosphatase [46]. Crystallographic evidence show that, in the folded state, the SH3 domain of Src kinases interacts with an internal polyproline stretch present in the linker region that connects the kinase and SH2 domains [15]. In the presented experiments, however, the closed configuration is maintained in the SH3-deficient mutant P112L, but not in the $Tyr^{505} \rightarrow$ Phe mutant, suggesting that, at least in lipid raft domains, the pTyr505/SH2 interaction by itself is sufficiently strong to promote the 'tail-bite' configuration of Lck.

In addition to CD45, other molecules are also reported to have a role in regulating Lck. In lipid rafts the 'closed' Lck configuration could be induced by the action of the adapter molecule PAG/Cbp, a palmitoylated transmembrane protein which is a resident of these domains [30,31]. PAG/Cbp is tyrosine phosphorylated in resting T cells and one of the tyrosine residues in its cytoplasmic tail (Tyr^{317} in the human protein) recruits the Csk kinase into lipid rafts via its SH2 domain. Brdicka et al. [30] have demonstrated that PAG/Cbp is a substrate for Lck and Fyn but not for ZAP-70 or Syk, and that in the Lck-negative Jurkat cells J.CaM1.6 tyrosine phosphorylation of PAG/Cbp was dramatically reduced. Furthermore, studying lymph node T cells and thymocytes from Fyn null mice, Yasuda et al. [49] showed that optimal PAG/Cbp phosphorylation requires the expression of Fyn. Collectively, these results most probably indicate that both of these Src-family kinases contribute to the steady-state phosphorylation of PAG/Cbp in resting T cells (Scheme 1).

In the present study, it is suggested that LAT also plays a role in the regulation of Lck activity in lipid rafts, which could be mediated by its ability to selectively associate with the 'open' form of Lck. At present, it is not known whether the LAT/'open' Lck interaction is direct or via intermediate proteins; however, it is preserved in non-lymphoid cells albeit in contrast with T cells, WT Lck expressed in COS-7 cells was also able to associate with LAT. A model that addresses the involvement of LAT in the regulation of Lck is shown in Scheme 1. The dynamic nature of lipid rafts allows shuttling of molecules between membrane regions and it is conceivable that Lck in the 'open' form that enters lipid rafts from the surrounding membrane interacts with LAT molecules residing in the same or possibly adjacent rafts facilitating their transient aggregation. Although PAG/Cbp phosphorylation is occurring in raft domains due to the action of Fyn, the LAT/Lck-mediated



Scheme 1 A mechanistic model for the regulation of Lck by LAT in T cell lipid rafts

'Open'-active Lck entering lipid rafts from non-raft membrane could associate with LAT molecules resident within the same or adjacent raft domains. This type of interaction could facilitate aggregation of different rafts or protein complexes, increased phosphorylation of PAG/Cbp, possibly by both Fyn and Lck kinases, and enhanced recruitment of Csk into lipid rafts. Csk could then convert Lck to its folded Tyr^{505} phosphorylated state, inducing its dissociation from LAT-containing protein complexes.

aggregation could contribute to the optimal phosphorylation of PAG/Cbp and enhanced recruitment of Csk, which in turn inactivates Lck (Scheme 1). Recent evidence suggests that LAT and Lck could reside in separate raft domains in human T cells [50]. Interestingly, in the same report TCR stimulation induced coalescence of LAT- and Lck-containing rafts. The molecular mechanism shown in Scheme 1 provides an auto-regulatory circuit which, in the absence of CD45, is capable of maintaining Lck in a low activity state in raft domains. Despite the fact that Lck in the soluble fraction is in the 'open' form, its interaction with LAT is minimal. An explanation for this could be that in non-raft membrane Lck and/or LAT preferentially interact with other protein partners, which may exclusively localize outside lipid rafts. A pertinent recent publication [51] describing another interaction between Src kinases and adapter molecules demonstrates the direct interaction of c-Src with the P66 and P52 isoforms of the adapter protein Shc. However, in this case, Shc binding induces activation of the kinase both *in vitro* and *in vivo*, as determined in A431 human epidermal carcinoma cells [51], rather than down-regulation, as is the case with LAT and Lck in T cell raft membranes.

In contrast to Lck, Fyn has an 'open'-active configuration in lipid rafts. The reason for the differential regulation of these two very similar kinases is unknown, but a possible explanation could be that Fyn is less accessible to the inhibitory action of the PAG/Cbp-Csk molecular complex. Alternatively, Fyn may interact via its SH2 and/or SH3 domains with other proteins that prevent it from folding into the inactive state. Nevertheless, the differential regulation of Lck and Fyn in lipid rafts may allude to the importance for stringent control of Lck activity in order to avoid inappropriate signalling, whereas Fyn could have a minor role in TCR signalling and instead be involved predominantly in other networks.

Since Lck is found predominantly in an inactive form in lipid rafts, tyrosine phosphorylation in these domains following TCR stimulation may require a transient increase in its catalytic activity. This can be achieved through the action of a tyrosine phosphatase which can de-phosphorylate PAG/Cbp, resulting in the shedding of Csk from lipid rafts and/or de-phosphorylation of Lck-Tyr^{505}. A new phosphorylation of PAG/Cbp by Lck can promote the re-attachment of Csk and cessation of signal transmission. In accordance with this hypothesis, TCR triggering of human peripheral blood T cells induces the transient de-phosphorylation of PAG/Cbp and release of Csk from lipid rafts [30,52]. Also, dominant-negative mutants of Csk when over-expressed in Jurkat T cells augment activation-induced TCRζ phosphorylation and NFAT (nuclear factor of activated T cells) production [52]. The above sequence of events implies the existence of a protein tyrosine phosphatase acting upstream of Lck, the activity or topology of which is regulated by the TCR. Identification of a phosphatase that fulfils this role will shed new light into the intracellular signalling events that take place proximal to the TCR.

Recent data by Drevot et al. [53] show that lipid rafts in T cells represent a heterogeneous group of membrane domains, which are differentially extracted by various detergents. Specifically, Drevot et al. report the existence of two subsets with differential sensitivity to Triton X-100, the detergent used in the present study. The Triton X-100-sensitive (but Brij 98-resistant) subset was implicated in TCR signal initiation and contained TCR and CD4 but low levels of the GPI-anchored receptor Thy1, whereas the Triton X-100-insensitive subset responsible for signal propagation was rich in Thy-1 and LAT but had low levels of TCR [53]. Therefore it is possible that by preparing T cell rafts in Triton X-100, a TCR-rich subset of lipid rafts and its associated Lck, is lost. The activity of Lck in these TCR-rich rafts has yet to be elucidated. It will be interesting in the future to isolate lipid rafts in Brij 98 detergent, as described by Drevot et al. [53] to investigate the conformation of Lck and its ability to interact with LAT in these preparations. Nevertheless, the results presented in this report demonstrate that the T cell membrane contains lipid raft domains in which resident Lck molecules are predominantly in a 'closed' conformation with reduced kinase activity. Another possibility is that transient aggregation of distinct lipid raft subsets mediated by the Lck/LAT interaction, provide a link through which the TCR-generated signal is amplified.

Two crucial questions that are currently being investigated deal with characterizing the nature of the LAT/Lck association and whether it is regulated by the TCR. It will be intriguing if TCR stimulation does promote this association, since this could provide a molecular mechanism for the tyrosine phosphorylation of proteins (i.e. PLCγ1) which are recruited by LAT after stimulation [9,37].

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