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LAT Palmitoylation: Its Essential Role in Membrane Microdomain Targeting and Tyrosine Phosphorylation during T Cell Activation

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Summary

The linker molecule LAT is a critical substrate of the tyrosine kinases activated upon TCR engagement. Phosphorylated LAT binds Grb2, PLC-γ1, and other signaling molecules. We demonstrate that human LAT is palmitoylated and that palmitoylated LAT predominantly localizes into glycolipid-enriched microdomains (GEMs). Although the LAT transmembrane domain is sufficient for membrane localization, palmitoylation at C26 and C29 is essential for efficient partitioning into GEMs. LAT palmitoylation is necessary for its tyrosine phosphorylation. After T cell activation, most tyrosine-phosphorylated LAT molecules and a fraction of PLC-γ1 and other signaling molecules are present in GEMs. LAT is central to T cell activation and is a novel linker molecule shown to require targeting to membrane microdomains for signaling.

Introduction

Engagement of the T cell antigen receptor (TCR) by specific antigen–MHC complexes or antibodies that bind TCR subunits results in activation of multiple biochemical pathways, eventually leading to cytokine production and effector function (Weiss and Littman, 1994; Chan and Shaw, 1996; Wange and Samelson, 1996). Activation of protein tyrosine kinases (PTKs) and tyrosine phosphorylation of intracellular proteins are early events in this process. The Src family PTKs Lck and Fyn phosphorylate the paired tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAM) of the TCRζ and CD3 chains. ZAP-70 then binds to the phosphorylated ITAMs via its tandem SH2 domains and is activated by Lck- or Fyn-mediated tyrosine phosphorylation. These activated PTKs subsequently phosphorylate many intracellular protein substrates.

Tyrosine phosphorylation of intracellular enzymes such as ZAP-70 (Chan et al., 1992; Wange et al., 1995), PLC-γ1 (Rhee and Bae, 1997), and Vav (Crespo et al., 1997) is required for enzymatic activation. Tyrosine phosphorylation of other proteins, including the linker or adaptor molecules LAT, SLP-76, and Cbl, creates binding sites for SH2 domain-containing proteins capable of binding phosphotyrosine in a specific sequence context (Pawson, 1995; Wange and Samelson, 1996; Koretzky, 1997).

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The recently identified molecule LAT is a 36–38 kDa integral membrane protein that is tyrosine phosphorylated after T cell activation on multiple tyrosine residues, at least in part, by ZAP-70 (Weber et al., 1998; Zhang et al., 1998). LAT phosphorylation results in its interactions with Grb2, SLP-76, Vav, Cbl, PLC-γ1, and the p85 subunit of PI-3 kinase, either directly or indirectly. Expression of a mutant form of LAT with Y→F mutations at positions 171 and 191, which is not capable of binding Grb2 and other associated molecules, inhibits T cell activation as measured by a block in the transcriptional activity of NFAT and AP-1. Recruitment of proteins and formation of signaling complexes required for downstream signaling events after T cell activation are thus induced by LAT tyrosine phosphorylation.

Phosphorylation of proteins, as an example of post-translational modification, is critical to signaling induced by many receptor systems. Lipid modifications such as myristylation, palmitoylation, and farnesylation have also been found to be very critical in the functions of several families of signaling molecules such as the Gα subunits of G proteins, Ras, and Src family PTKs (Resh, 1996). Gα subunits are both myristylated and palmitoylated (Wedegaertner et al., 1995). Extensive mutagenesis studies have shown that both of these acylations are required for optimal targeting of Gα subunits to the plasma membrane. Farnesylation and palmitoylation of Ras are well known and both are required for membrane targeting and transforming activity (Clark, 1992; Willumsen et al., 1996). The membrane localization and function of many members of the Src family tyrosine kinases also are regulated by dual acylation (Resh, 1994). Lck mutants that lack either myristate or palmitate moieties do not localize to the plasma membrane and do not function properly (Kabouridis et al., 1997).

The posttranslational addition of lipids has been shown to target proteins such as Src family PTKs to subdomains of the plasma membrane known as glycolipid-enriched microdomains (GEMs) or detergent-insoluble rafts (Brown and London, 1997; Simons and Ikonen, 1997). This membrane heterogeneity is caused by the self-association of sphingolipid, cholesterol, and protein components. Many GPI-linked proteins also localize to GEMs. Cross-linking these GPI-anchored proteins leads to activation of T cells, as measured by an influx of extracellular calcium, production of IL-2, or cell proliferation (Lublin, 1992). A few studies suggest that membrane microdomains and the proteins targeted to them are involved in activation of immunoreceptors. Analysis of the activation of FcεRI in the rat mast cell line RBL-2H3 indicates that antigen-induced receptor aggregation leads to activation of a subset of receptors in low density, plasma membrane microdomains (Field et al., 1995, 1997). Activated receptors that are phosphorylated on tyrosines are detected in these microdomains.

Analysis of the deduced amino acid sequence of both human and murine LAT reveals two cysteine residues in the juxtamembrane region. Such cysteines are frequently the sites for palmitoylation of integral membrane

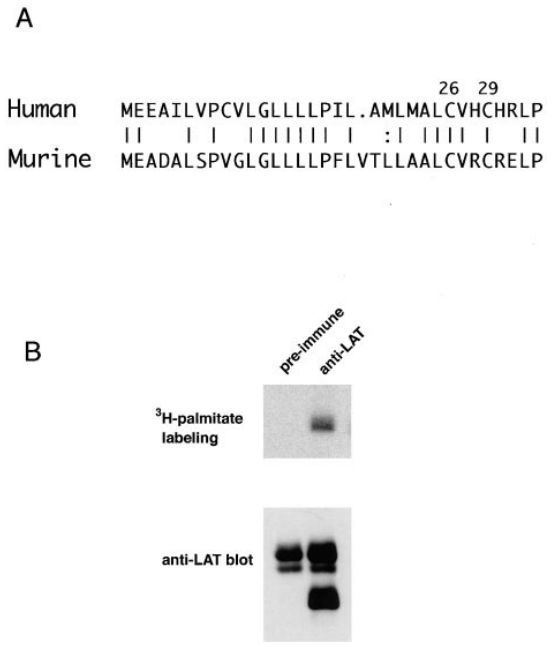

Figure 1. Palmitoylation of LAT

(A) Sequence alignment of human (top) and murine (bottom) LAT proteins showing the conserved cysteine residues. LAT has a very short extracellular domain (residues 1–4), a putative transmembrane domain (residues 5–27), and a long cytosolic tail (residues 28–233; data not shown). Cysteine 26 and 29 in human LAT are conserved between human and murine LAT.

(B) Metabolic labeling of LAT with [^{3}H]palmitate. Jurkat cells (2×10^7) were labeled with [^{3}H]palmitate for 3 hr, lysed in 1% Brij, and immunoprecipitated with either preimmune or anti-LAT antisera. Samples were analyzed by SDS-PAGE and fluorography (top) or anti-LAT blot (bottom).

and membrane-associated proteins. Our present studies were undertaken to determine whether LAT undergoes palmitoylation and whether lipid modification of LAT affects its membrane localization and function. Our results show that LAT is indeed palmitoylated and that mutations of these two cysteines (C26 and C29 in human LAT) abolish the palmitoylation. We also demonstrate that LAT localizes to GEMs and that palmitoylation is required for targeting of LAT to GEMs. LAT mutants that do not localize to GEMs are poorly tyrosine phosphorylated by PTKs upon TCR engagement. Further evidence for the significance of these results is the observation that PLC-γ1 and other signaling molecules also migrate to GEMs upon T cell activation.

Results

LAT Is Palmitoylated and Primarily Localizes to Glycolipid-Enriched Microdomains

In our previous study, we demonstrated that the human LAT protein exists as two forms that migrate on SDS-PAGE at apparent molecular weights of 36 and 38 kDa (Zhang et al., 1998). Transfection of the cDNA encoding the human *LAT* mRNA resulted in expression of both of these forms, suggesting that the heterogeneity is likely due to posttranslational modification. Human and murine LAT contain two conserved cysteines, C26 and C29, near the putative transmembrane domain (Figure 1A). These features suggest that LAT could be a member of a class of transmembrane proteins modified by palmitate at juxtamembrane cysteine residues. To examine whether LAT is palmitoylated, Jurkat cells were metabolically labeled with [^{3}H]palmitate. LAT was immunoprecipitated with anti-LAT antiserum, and immunoprecipitates were analyzed by SDS-PAGE and fluorography. [^{3}H]palmitate was found to be incorporated into LAT (Figure 1B). When an identical gel was treated with 1 M hydroxylamine, the ^{3}H signal was almost completely lost (data not shown), indicating that the addition of palmitate to LAT was through S-acylation.

Many palmitoylated proteins, such as the Src family kinases Lck and Fyn, are targeted into GEMs of the plasma membrane (Alland et al., 1994; Shenoy-Scaria et al., 1994). GEMs are operationally characterized by resistance to Triton extraction at 4°C and are found in low-density fractions of a sucrose gradient (Simons and Ikonen, 1997). Since LAT is palmitoylated, we next examined whether LAT localizes to GEMs. Jurkat cells were solubilized with 1% Triton, and lysates were subjected to overnight ultracentrifugation over a sucrose gradient (5%/30%/40% step gradient). Among a total of 12 fractions collected, fraction 3, at the interface between 5% sucrose and 30% sucrose, contained the low-density membrane fraction, the GEMs (Brown and Rose, 1992). Fractions 8–12 from the 40% sucrose section were the Triton-soluble fractions (Figure 2A). LAT was detected by immunoblotting and was found in both GEM and Triton-soluble fractions. LAT in the GEM fractions was predominantly the p36 form. Two prominent forms of LAT, p36 and p38, were detected in Triton-soluble fractions. The exposure of the autoradiograph shown in Figure 2A did not clearly demonstrate the abundance of LAT protein in the third fraction. When this sample was diluted 16-fold, the amount of LAT still exceeded that found in the undiluted fraction 10. As controls for GEM purification, we immunoblotted the same membrane with anti-Lck and anti-ZAP-70 antibodies. Lck was observed in both GEM and Triton-soluble fractions, but ZAP-70 was found only in Triton-soluble fractions (Figure 2A).

To study the relationship between palmitoylation and GEM localization, Jurkat cells were labeled with [^{3}H]palmitate for 3 hr, lysed in 1% Triton, and subjected to ultracentrifugation on a sucrose gradient. LAT was then immunoprecipitated from GEM and Triton-soluble fractions, resolved by SDS-PAGE, and analyzed by both fluorography and anti-LAT immunoblotting. As shown in Figure 2C, the anti-LAT immunoblot showed that an approximately equal amount of LAT was precipitated from these two fractions. However, LAT in the GEM fraction was clearly more palmitoylated, indicating that palmitoylated LAT predominantly localizes to GEMs.

Distribution of Signaling Molecules into GEMs in Activated Jurkat T Cells

Since LAT is present in both GEM and Triton-soluble fractions, we next examined in which fraction tyrosine-phosphorylated LAT can be found after stimulation of Jurkat cells. We also studied whether activation of T

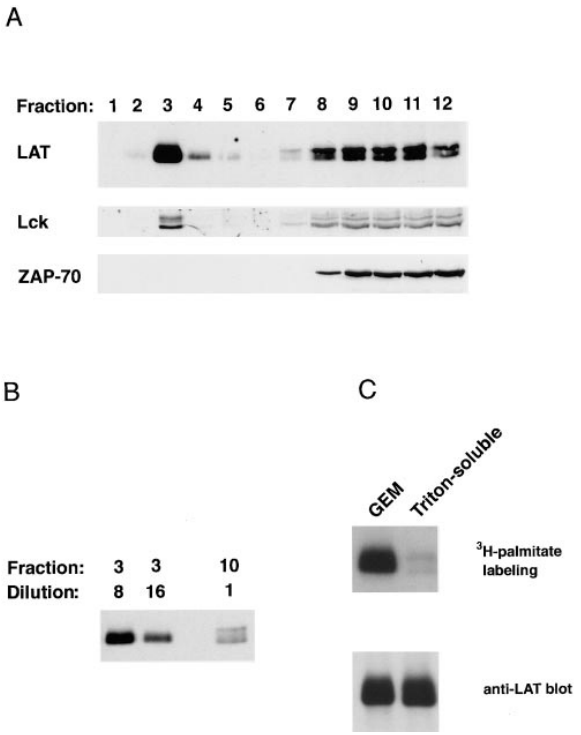

Figure 2. Partition of LAT into GEMs as Analyzed by Sucrose Gradient

Cells (5×10^7) were lysed in 1 ml 1% Triton lysis buffer and mixed with 1 ml 80% sucrose. Lysates were then transferred to a centrifuge tube and overlaid sequentially with 2 ml 30% sucrose and 1 ml 5% sucrose. These preparations were subjected to ultracentrifugation for 16–18 hr at 4°C. Gradient fractions in 0.4 ml aliquots were collected from the top of the gradient, and these aliquots were mixed with equal volume of 2× SDS sample buffer and analyzed by SDS-PAGE.

(A) The distribution of LAT was analyzed by immunoblotting with anti-LAT antibody. The distribution of Lck and ZAP-70 was assayed by Western blotting with specific antibodies.

(B) An aliquot of fraction 3 was subjected to serial dilution before blotting with anti-LAT antibody.

(C) Jurkat cells were labeled with [^{3}H]palmitate for 3 hr before separation of GEM fractions from Triton-soluble fractions. Fractions 2–4 were combined, as were fractions 8–12. LAT was immunoprecipitated from these two pooled fractions and analyzed by fluorography and an anti-LAT immunoblot.

cells induces redistribution of other signaling molecules into GEMs. Cross-linking with the anti-CD3ϵ antibody OKT3 leads to tyrosine phosphorylation of many cellular proteins and activation of T cells. Unstimulated or OKT3-stimulated Jurkat lysates were subjected to sucrose gradient centrifugation to separate GEM fractions from Triton-soluble fractions. Each fraction was analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody and antibodies against each individual protein. As shown in Figure 3A, LAT and Lck were basally tyrosine phosphorylated in both GEM and Triton-soluble fractions from unstimulated cells. A tyrosine-phosphorylated 80 kDa diffuse band was also observed in the GEM fractions. While the majority of Vav localized to the Triton-soluble fractions, some tyrosine-phosphorylated Vav was detected in the GEM fractions (Figure 3A) and fractions 5–7, as shown by anti-Vav blot in Figure 3C.

Upon stimulation with OKT3, most tyrosine-phosphorylated LAT appeared in the GEM fractions, although some was in the Triton-soluble fractions (Figure 3B). There was no obvious change in Lck tyrosine phosphorylation, but the 60 kDa form of Lck, well known to accompany TCR activation (Marth et al., 1989), was increased in Triton-soluble fractions upon stimulation, as shown by the anti-Lck blot (Figure 3C). Other prominent tyrosine-phosphorylated proteins present in the GEM fractions upon stimulation were PLC-γ1 and Vav (Figure 3B). Blotting with antibodies to particular signaling molecules supported the conclusion that certain molecules redistribute to GEMs upon TCR activation (Figure 3C). The amount of PLC-γ1 increased in the GEM fractions and fractions 5–7 upon stimulation. Like PLC-γ1, the amount of Vav in those fractions also increased after stimulation. The distribution of the p85 subunit of PI-3 kinase was similar to Vav (data not shown). There was also some increase of Grb2 in the GEM fractions after stimulation (Figure 3C). Cbl and ZAP-70 were present in Triton-soluble fractions, but they did not redistribute into GEMs after stimulation (Figure 3C). We used the detergent octyl-glucoside to solubilize GEMs and Triton-soluble fractions in order to assess LAT-associated proteins in the two pools. This detergent is capable of solubilizing Triton-insoluble GEMs (Brown and Rose, 1992). Following extraction with this detergent, we observed by co-immunoprecipitation that more PLC-γ1 and Vav associated with LAT in GEMs than LAT in Triton-soluble fractions (data not shown), suggesting that LAT in GEMs interacts with these signaling molecules following activation.

C26 and C29 Are the Major Sites for LAT Palmitoylation

To test whether two cysteines, C26 and C29, in human LAT are the sites for addition of the palmitate moiety (Figure 1A), we made Myc-tagged LAT mutants with single cysteine-to-alanine mutations (C26A, C29A) and a double mutant (C26/29A). We also made a mutant LAT (Δtm) with a deletion of the N-terminal region (residues 1–22), including the transmembrane domain, to investigate the role of the transmembrane domain in LAT palmitoylation and function. T antigen–transformed human kidney fibroblasts (293T cells) were transiently transfected with these constructs and metabolically labeled with [^{3}H]palmitate. LAT was then immunoprecipitated with anti-Myc antibody from lysates of these transfected cells. As seen in Figure 4 (top panel), two forms of LAT labeled with [^{3}H]palmitate were detected in immunoprecipitates of wild-type (wt) LAT. For the C26A mutant, only the p38 form was labeled with [^{3}H]palmitate, and the amount of ^{3}H labeling in C26A was less than in wt. Two forms of LAT labeled with [^{3}H]palmitate were also observed with the C29A mutant, but compared with wt the amount of ^{3}H incorporated into the p38 form was reduced and ^{3}H labeling in the p36 form was barely detectable. A similar trace amount of ^{3}H was present in the LAT double cysteine mutant (C26/29A) and in the transmembrane domain deletion mutant (Δtm).

The same samples from the above labeling experiment were loaded on another gel and analyzed by blotting with anti-Myc antibody. As shown in Figure 4 (lower

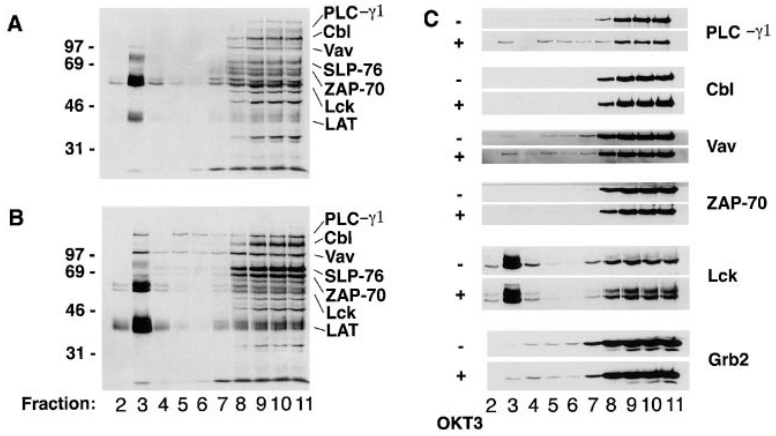

Figure 3. Redistribution of Signaling Proteins into GEMs upon T Cell Activation

(A) Fractions from a sucrose gradient separating a 1% Triton lysate of unstimulated Jurkat cells were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (4G10).

(B) Fractions from a sucrose gradient separating a 1% Triton lysate of OKT3-stimulated Jurkat cells were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody.

(C) The same membrane from (A) and (B) was stripped and blotted with anti-PLC-γ1, Cbl, Vav, ZAP-70, Lck, and Grb2 antibodies. Twelve fractions were collected and fractions 2–11 were analyzed.

panel), similar amounts of proteins were expressed in each lane. Note that only the p38 form of LAT was detected by blotting, indicating that in transient transfections the p36 form was rare. These results also indicate that C26 and C29 are the major sites for LAT palmitoylation. The trace amount of ^{3}H in C26/29A and Δtm mutant LAT is most likely due to metabolic conversion of [^{3}H]palmitate into [^{3}H]amino acids, although we could not totally exclude the possibility that LAT might be palmitoylated at a low level at another site.

Effects of Cysteine Mutations on LAT Membrane Localization and GEM Targeting

Because mutations of two cysteines, C26 and C29, affected the palmitoylation of LAT, we next tested whether these mutations also altered membrane localization and the partition of LAT into GEMs. We examined the membrane localization of wt and mutant LAT by immunofluorescence staining of transiently transfected 293T cells. There was no significant difference in localization between wt LAT and any of the cysteine mutants. However, deletion of the transmembrane domain of LAT resulted in a predominantly cytosolic localization (Figure 5A). We also transiently transfected Jurkat/TAg cells (SV40 large T antigen–transformed Jurkat cells; Clipstone and Crabtree, 1992) with LAT constructs and analyzed the subcellular distribution of LAT by fractionation of transfected cells into cytosolic and particulate fractions. Myc-tagged wt LAT, C26A, C29A, and C26/29A mutants predominantly localized to the particulate fraction, while the LAT Δtm was detected mainly in the cytosolic fraction (Figure 5B).

Although C26A, C29A, and C26/29A LAT mutants all localized to the plasma membrane, it was possible that these mutations might affect the targeting of LAT into GEMs. Transiently transfected Jurkat cells were lysed in 1% Triton, and GEM fractions were purified using a sucrose gradient. Samples from fractions 3 and 10, as representatives of the GEM and Triton-soluble fractions, respectively, were analyzed on SDS-PAGE and immunoblotted with anti-Myc antibody. The wt LAT and all of the LAT mutants were expressed equally in Triton-soluble fractions (Figure 5C). Two forms of wt LAT and C29A were observed in these fractions, though the p36 form of C29A was barely detectable. Strikingly, only the wt LAT and a small amount of C29A were present in the GEM fractions. The plasma membrane localization of C26A and C26/29A and the palmitoylation of the C26A mutant were not sufficient for GEM targeting. The cytosolic LAT Δtm mutant was not seen in the GEM fraction, as expected. These results indicate that, while the transmembrane domain alone suffices for LAT plasma membrane localization, palmitoylation at C26 is essential for GEM targeting. Palmitoylation only at C29 is not sufficient for GEM targeting, but it does increase the efficiency of C26-mediated targeting.

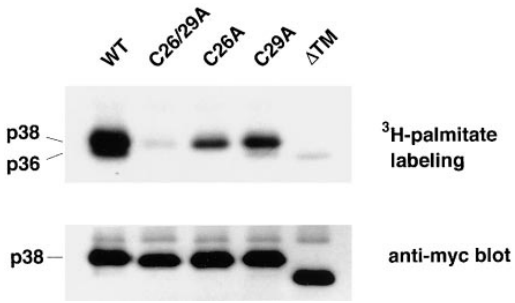

Figure 4. Metabolic Labeling of Wt and Mutant Forms of LAT with [^{3}H]palmitate

293T cells were transiently transfected with different LAT constructs and metabolically labeled with [^{3}H]palmitate for 3 hr. Cells were lysed and immunoprecipitated with anti-Myc antibody. Anti-Myc immunoprecipitates were analyzed by SDS-PAGE and fluorography (top) or anti-Myc blot (bottom). Myc-tagged human LAT migrated on SDS-PAGE as two forms with apparent molecular weights of 38 and 40 kDa. However, we chose to refer to them as p36 and p38 to remain consistent with our description of the endogenous forms of LAT.

Partition of LAT into GEMs Is Required for Efficient Tyrosine Phosphorylation

We next addressed whether palmitoylation and targeting into GEMs were necessary for the phosphorylation of LAT by tyrosine kinases. Jurkat cells were transfected with mutant LAT constructs and stimulated with OKT3 at 36 hr after transfection. The p36 form of wt Myc-tagged LAT was rapidly tyrosine phosphorylated upon OKT3 stimulation (Figure 6). The p36 form of the C29A mutant was also tyrosine phosphorylated, though

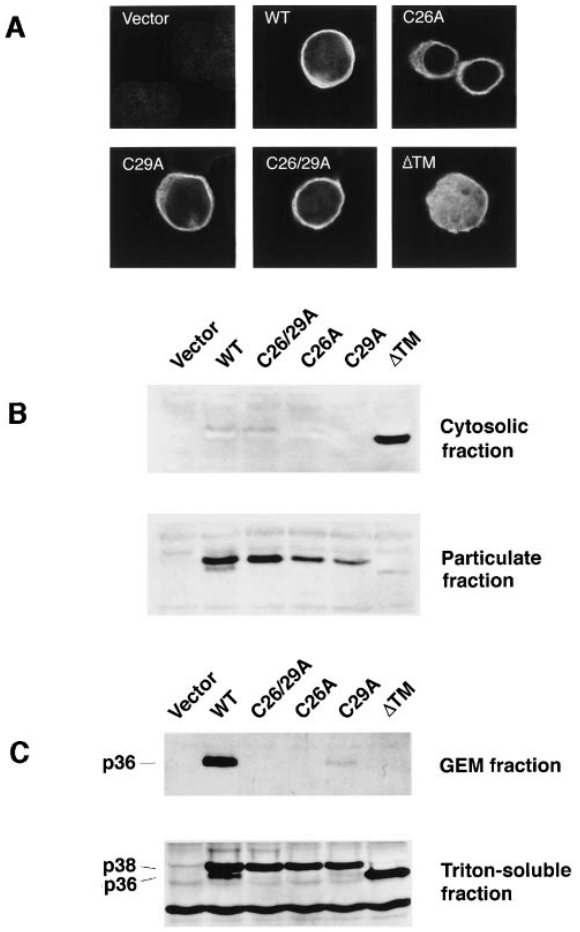

Figure 5. Localization of Wt and Mutant LAT to the Plasma Membrane and Glycolipid-Enriched Microdomains

(A) Transfected 293T cells were fixed, permeabilized, labeled with anti-LAT antibody, stained with Texas red conjugated goat anti-rabbit antibody, and then examined by confocal microscopy.

(B) Cytosolic and particulate fractions of Jurkat/TAg cells transiently transfected with different LAT constructs were prepared by dounce homogenization in hypotonic buffer and ultracentrifugation. Samples from each cytosolic and particulate fractions were resolved on SDS-PAGE and immunoblotted with anti-Myc antibody.

(C) Jurkat/TAg cells (4×10^7) transiently transfected with different LAT constructs were lysed in 1% Triton and lysates were subjected to a sucrose gradient ultracentrifugation to purify GEM fractions from Triton-soluble fractions. Fraction 3 (GEM fraction) and fraction 10 (from the Triton-soluble fractions) from each transfection were analyzed by SDS-PAGE and immunoblotting with anti-Myc antibody (9E10).

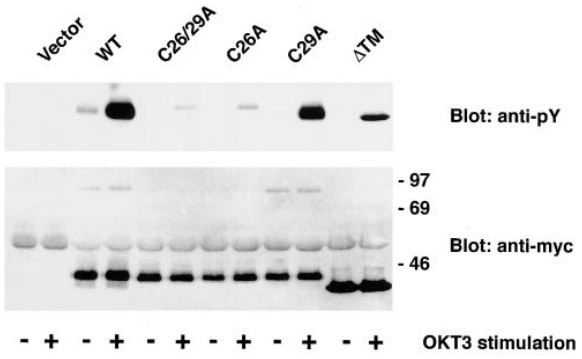

Figure 6. Tyrosine Phosphorylation of Wt and Mutant LAT

Jurkat/TAg cells were transiently transfected with wt and mutant LAT constructs. Thirty-six hours after transfection, cells were either left unstimulated or stimulated with OKT3 for 2 min, lysed in 1% Brij lysis buffer, and immunoprecipitated with anti-Myc antibody. Immunoprecipitates were then resolved on SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (4G10) or anti-Myc antibody.

less than wt. Only very weak tyrosine phosphorylation of the p38 form of C26A or C26/29A was observed. Surprisingly, LAT Δtm was also tyrosine phosphorylated to some extent. This could be explained by the presence of activated ZAP-70 in the cytosol following stimulation (W. Z., unpublished data). These activated ZAP-70 molecules might phosphorylate LAT Δtm in the cytosol. Endogenous LAT, however, must be in GEMs to become optimally tyrosine phosphorylated during activation. Blotting the same membrane with anti-Myc antibody showed that the amount of LAT was nearly equal in all lanes. This protein blot also revealed the presence of a band at 80 kDa in the wt and C29A lanes. This band could be detected with anti-LAT antibody (data not shown). This 80 kDa band could be a dimer of LAT, resistant to treatment with SDS and DTT. In conclusion, our results clearly show that LAT palmitoylation at C26 and C29 not only is required for LAT palmitoylation, but also is necessary for efficient tyrosine phosphorylation by tyrosine kinases during T cell activation.

Discussion

Acylation of proteins with the 16 carbon fatty acid palmitate is a well known posttranslational modification of many proteins (reviewed in Resh, 1996). A number of transmembrane viral and cellular proteins are acylated at juxtamembrane cysteines. Several membrane-associated proteins with important functions in signaling are also palmitoylated. These include α subunits of many G proteins, Ras, and members of the Src family of protein tyrosine kinases such as Lck and Fyn. Here we report that the critical linker molecule LAT, which is tyrosine phosphorylated during T cell activation, is palmitoylated at two cysteines in the juxamembrane region. While the palmitoylation of LAT is not required for membrane localization, it is essential for targeting of LAT to GEMs. Palmitoylation of LAT is also required for its tyrosine phosphorylation by tyrosine kinases during T cell activation. Upon stimulation, most tyrosine-phosphorylated LAT molecules are in GEMs. Other signaling molecules such as PLC-γ1, Vav, and Grb2 shift to GEMs probably through binding to LAT, suggesting that many signaling events occur in GEMs. Our results clearly demonstrate the importance of palmitoylation for specific GEM targeting and function and indicate an additional complexity in TCR-mediated signaling.

The deduced amino acid sequence of LAT predicts a short extracellular domain, a hydrophobic transmembrane domain, and a long cytosolic domain containing the multiple tyrosine residues that are targets for tyrosine kinases. A plasma membrane localization for this

protein was predicted from earlier biochemical studies and confirmed by immunofluorescence studies (Sieh et al., 1994; Zhang et al., 1998). By both immunofluorescence and biochemical analysis in this study, we show that a LAT mutant lacking the transmembrane domain was detected in the cytosol. The LAT C26/29A mutant, which was not palmitoylated, localized to the plasma membrane. Thus, the transmembrane domain of LAT alone is enough for plasma membrane targeting.

The fact that LAT exists in two forms, p36 and p38, and the presence of two cysteine residues near the transmembrane domain suggested that LAT could be posttranslationally modified through S-acylation at these residues. Metabolic labeling studies with [^{3}H]palmitate in Jurkat cells and transiently transfected 293T cells confirmed that LAT is palmitoylated. When either of these sites was mutated, LAT palmitoylation was decreased but still present. Mutations of both C26 and C29 resulted in a nearly complete inhibition of palmitate incorporation into LAT. These studies demonstrate that C26 and C29 are the major sites for palmitoylation.

It is still not entirely clear how the two different forms of LAT are generated in vivo. Our mutagenesis studies indicated that the nonpalmitoylated form of LAT migrated on SDS-PAGE as the p38 form. However, some of the p38 form is palmitoylated, as is the p36 form. The C29A single mutant existed as both p36 and p38 forms, suggesting that the difference in the number of palmitate molecules incorporated might not explain the generation of the two forms of LAT. Mutation of C26 or both C26 and C29 affected the generation of the p36 form. Since these two mutants were not present in GEMs, it is possible that an additional posttranslational modification might occur in GEMs to process p38 into p36. Thus, though the generation of the two forms of LAT seems to be related, in part to palmitoylation, we do not understand the details of the generation of these forms.

Heterogeneity of lipid membranes has been studied extensively in model membrane systems, and recent analysis of cellular plasma membranes has further revealed their complexity (Brown and London, 1997; Harder and Simons, 1997; Brown, 1998). It is thought that self-association of sphingolipids and cholesterol induces the formation of microdomains that separate from the more abundant glycerophospholipids. One significant property of these sphingolipid-cholesterol domains is their insolubility in Triton X-100 at 4°C. Many palmitoylated proteins, including members of the Src family kinases, become targeted to these microdomains (GEMs). Our results show that LAT is also enriched in these GEMs and that palmitoylation is required for GEM targeting. We also showed that palmitoylated LAT predominantly localizes to the GEMs. These results indicate that palmitoylation has a role in targeting proteins to these microdomains and not just to the plasma membrane.

Although both C26 and C29 could be sites for palmitoylation, these two cysteines are not equally important for targeting to GEMs. The C26A mutant failed to appear in GEMs, whereas the C29A mutant could be targeted into GEMs, though less efficiently compared with wt. These results are similar to those obtained in mutagenesis studies of Lck and Fyn (Alland et al., 1994; Kabouridis et al., 1997; van't Hof and Resh, 1997). Both C3 and C5 in Lck are the sites for palmitoylation. Mutation at C3 greatly reduces Lck membrane localization, while mutation at C5 only has a slight effect. Similar results were obtained in studies of Fyn. From our studies on LAT and others on Lck and Fyn, it appears that the membrane-proximal cysteine is more important than the distal cysteine in palmitoylation and targeting. One possibility is that the membrane insertion mediated by palmitate at the membrane-proximal cysteine might bring the cytosolic portions of these molecules closer to the membrane, so that the second palmitate acylated at the other cysteine can be inserted into the membrane. Deletion of the transmembrane domain of LAT abolished its palmitoylation, supporting the hypothesis that palmitoylation of proteins occurs at the plasma membrane (Berthiaume and Resh, 1995; Resh, 1996).

Previous studies on two immunoreceptor systems suggested the importance of GEM targeting. In the case of the FcεRI, incubation with IgE and specific antigen induces aggregation of the receptor, followed by activation of PTKs (Field et al., 1995, 1997). The relevant Src family kinase Lyn, a subset of receptors, and a protein likely to be LAT can be detected in GEMs following receptor ligation, whereas other PTK substrates were detected only in the detergent-soluble fractions. A recent study on the role of Lck palmitoylation in TCR-mediated activation indicates that optimal activation of T cells requires the targeting of Lck to GEMs (Kabouridis et al., 1997). Our results clearly demonstrate the functional consequences of LAT palmitoylation and GEM localization. Mutant LAT molecules that failed to partition into GEMs were not tyrosine phosphorylated upon T cell activation, suggesting that LAT might be tyrosine phosphorylated in GEMs. Since LAT is a substrate of ZAP-70 and activation of ZAP-70 requires Lck, ZAP-70 might have to be present in GEMs or near GEMs, where it is activated by Lck and phosphorylates LAT. In unstimulated Jurkat cells, LAT showed some level of basal tyrosine phosphorylation. Since LAT colocalizes with Lck in GEMs, this basal tyrosine phosphorylation might be by Lck. This speculation is also supported by our previous data that LAT could be tyrosine phosphorylated weakly by Lck when Lck and LAT were coexpressed in 293 cells (Zhang et al., 1998).

Stimulation through the TCR induces phosphorylation of many intracellular proteins. Prominent substrates are PLC-γ1, Cbl, Vav, SLP-76, ZAP-70, and LAT. Our data show that most of tyrosine-phosphorylated LAT was in GEMs. There was only a slight increase in LAT phosphorylation in Triton-soluble fractions. A small fraction of PLC-γ1, Vav, and Grb2 also shifted into GEMs after activation. These molecules were associated with LAT in GEMs (data not shown). The functional effect of this molecular redistribution still remains to be determined. Since many signaling molecules, such as trimeric G proteins (Li et al., 1995), Ras (Song et al., 1996), and phosphoinositides (Pike and Casey, 1996), localize to these microdomains, one can postulate that the efficiency of signaling is markedly enhanced by the increased concentration of signaling molecules in such microdomains. Signaling pathways, like Ca^{2+}, Ras, and Rac pathways, might be initiated through recruiting of PLC-γ1, Vav, and Grb2 into GEMs.

While this paper was under review, Xavier et al. published a paper showing that localization of signaling proteins into glycolipid-enriched microdomains is required for T cell activation (Xavier et al., 1998), and our data certainly support their conclusion. However, they observed that many more proteins such as Cbl, Syk, Vav, ZAP-70, PLC-γ1, TCRζ chains, and others are enriched in GEMs. In contrast, we only observed that a very small amount of PLC-γ1, Vav, p85 of PI-3K, and TCRζ chain (data not shown) localized to GEMs. This discrepancy is likely due to different methods used to analyze the relative enrichment of proteins into GEMs. Since only a very small fraction of cellular proteins are present in GEMs, Xavier et al. overrepresented GEM proteins by comparing equal amounts of protein from cytosol, membrane, and GEM fractions. Nevertheless, their results showed that many signaling proteins could be detected in GEMs after T cell activation.

The central role of LAT tyrosine phosphorylation was amply demonstrated in our earlier studies, wherein mutations of critical tyrosine residues blocked critical binding to other signaling molecules and inhibited T cell activation. The data presented in this paper provides further evidence for the central role of this protein in signaling and indicates that it must be appropriately targeted to the plasma membrane microdomains in order to be tyrosine phosphorylated for its function. The role of these membrane microdomains in TCR signaling must be more thoroughly studied in the future.

Experimental Procedures

Antibodies, Immunoprecipitation

The following antibodies were used: rabbit polyclonal anti-LAT antiserum (Zhang et al., 1998), anti-ZAP-70 (Wange et al., 1995), anti-Lck (Samelson et al., 1990), anti-CD3ε (OKT3; Kung et al., 1979), monoclonal anti-Myc (9E10), monoclonal anti-PLC-γ1 (a gift from Dr. S. G. Rhee, NHLBI, NIH), anti-Cbl and anti-Grb2 antibodies from Santa Cruz Biotechnology, and anti-Vav and anti-phosphotyrosine (4G10) from Upstate Biotechnology.

Jurkat T cells were removed from culture, washed, and resuspended in RPMI 1640 at 10^8 cells/ml. Cells were either stimulated with OKT3 for 2 min or left unstimulated. Cells were lysed in ice-cold 1% Brij lysis buffer. Separation of the cytosolic fraction and the particulate fraction was performed as described (Weissman, 1996).

Purification of GEM Fractions and Subcellular Fractionation

Cells (5×10^7) were lysed on ice in 1 ml 1% Triton in MNE buffer (25 mM MES [pH 6.5], 150 mM NaCl, 5 mM EDTA), dounced 10 times, and mixed with 1 ml 80% sucrose made with MNE buffer. After transfer of the lysate to the centrifuge tube, 2 ml 30% sucrose in MNE buffer was overlaid, then 1 ml 5% sucrose in MNE was overlaid. After centrifugation for 16–18 hr at 200,000 g in a Beckman SW55Ti, 0.4 ml gradient fractions were collected from the top of the gradient. For purification of GEMs from OKT3-stimulated cells, cells were spun down quickly after stimulation and then lysed in 1 ml 1% Triton in 25 mM Tris-Cl [pH 7.6], 150 mM NaCl, 5 mM EDTA, 30 mM pyrophosphate, 10 mM glycerol phosphate, and 1 mM sodium orthovanadate. Lysates were prepared for sucrose gradient ultracentrifugation as above.

Mutagenesis and Subcloning

Cysteine to alanine mutations (positions 26 and 29) of LAT cloned into the pCEFL expression vector (a gift from Dr. S. Gutkind, NIDR, NIH) were made with the Stratagene Quickchange kit. The LAT transmembrane domain deletion mutant (residues 1–22) was made by cloning a double-stranded linker annealed with two oligonucleotides (AATTCGCCGCCATGGCACTGTGTG and TGCACACACAGTGCCATGGCGGCG) and the ApaL1/XbaI fragment from pEF/LAT-Myc into pCEFL EcoRI/XbaI sites.

Transfection, Labeling of Jurkat Cells and 293T Cells, and Immunofluorescence

Transient and stable transfection of Jurkat cells were performed as described (Zhang et al., 1998). For labeling with [^{3}H]palmitate, 2×10^7 Jurkat cells were removed from culture and resuspended in 1 ml RPMI 1640 containing 5% dialyzed fetal calf serum (FCS), 5 mM sodium pyruvate, and 0.5 mCi [^{3}H]palmitate for 3 hr. 293T cells were transfected with LAT constructs using the calcium phosphate method in 6-well plates. Twenty-four hours after transfection, 293T cells were labeled with 0.5 mCi [^{3}H]palmitate in 1 ml DMEM containing 5% dialyzed FCS and 5 mM sodium pyruvate for 3 hr. The gels were fixed, treated with Enlightning (Dupont) for 30 min, dried, and exposed to film for 3–4 weeks. Immunofluorescence staining and confocal microscopy were done with 293T cells transfected with LAT constructs as described (Zhang et al., 1998).

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