Localization of LAT in Glycolipid-enriched Microdomains Is Required for T cell Activation*

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LAT, a transmembrane adapter protein found in glycolipid-enriched microdomains (GEMs), is essential for T cell activation. In this study, we have utilized a LAT-deficient mutant of the Jurkat T cell line, J.CaM2, to explore various requirements for LAT function. First, we demonstrate that LAT must be present in GEMs for coupling T cell receptor (TCR) engagement to activation of the Ras signaling pathway, increases in intracellular Ca\(^{2+}\), and induction of the transcription factor nuclear factor of activated T cells (NF-AT). Second, we show that the extracellular and transmembrane domains of LAT are dispensable for these TCR-mediated events once LAT has localized to GEMs. These results provide important insights into both the structural domains of LAT and its subcellular localization that are required for effective TCR signaling.

Engagement of the T cell receptor (TCR) with either antigen or antibodies that bind TCR subunits results in the initiation of an intracellular signaling cascade, a complex series of biochemical events that culminate in T cell proliferation, differentiation, and gain of effector functions (1, 2). The earliest of the signal transduction processes that occur following TCR stimulation include the activation of Src (Lck, Fyn) and Syk (ZAP-70, Syk) families of tyrosine kinases. These kinases subsequently phosphorylate downstream substrates allowing for a continuation of the signaling cascade. Phosphorylation of substrates by Src and Syk kinases can mediate the induction of enzymatic activity, for example with Vav and phospholipase C\(\gamma\)-1 (PLC\(\gamma\)-1) (3–6), and may also facilitate protein-protein interactions, such as with SLP-76 and Cbl (7–12). Both of these outcomes of tyrosine phosphorylation are critical for effective T cell activation.

One molecule that becomes heavily tyrosine phosphorylated in response to TCR engagement is LAT (Linker for Activation of T Cells), a transmembrane protein whose expression is limited to T, NK, and mast cells. Although LAT lacks intrinsic enzymatic activity, tyrosine phosphorylation at multiple tyrosine residues within LAT facilitates its association with a number of signaling molecules that may contribute to T cell activation, including Grb2, GADS, the p85 subunit of phosphatidylinositol 3-kinase, PLC\(\gamma\)-1, Vav, SLP-76, and Cbl (13–18). An essential role for LAT in T cell activation has been revealed through the characterization of J.CaM2, a LAT-deficient derivative of the Jurkat T cell line (19). J.CaM2 cells fail to produce many of the TCR-derived signaling events required for T cell activation, including activation of the Ras pathway, elevation in intracellular Ca\(^{2+}\), and induction of the transcription factor NF-AT. Importantly, re-expression of wild-type LAT in J.CaM2 restores all of these processes (19). More recently, the analysis of mice that lack LAT expression has also revealed a necessary role for this molecule in T cell development (20).

LAT contains a very short extracellular region, a transmembrane domain, and a tyrosine-rich cytoplasmic tail. Consistent with these structural features, LAT is found predominantly in the plasma membrane of T cells (14, 15, 17). Furthermore, LAT is palmitoylated on two conserved cysteines (amino acids 26 and 29), and this modification localizes LAT to glycolipid-enriched microdomains (GEMs) within the plasma membrane (21). GEMs, sometimes referred to as detergent-insoluble lipid rafts, have been proposed to function as platforms for the formation of multi-component signaling complexes (22). Interestingly, other molecules in addition to LAT, including Lck, Vav, Grb2, PLC\(\gamma\)-1, and Ras, are also either constitutively associated with GEMs or redistribute into GEMs following TCR engagement (21, 23–25). However, it remains unclear whether the localization of specific molecules to GEMs is actually necessary for their function in T cell signaling.

In this report, we demonstrate that LAT must be present in GEMs for the activation of the Ras pathway, an increase in intracellular Ca\(^{2+}\), and the stimulation of NF-AT-dependent transcriptional activity in response to TCR engagement. Also, we show that the extracellular and transmembrane domains of LAT are not required for these processes once LAT has localized to GEMs.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The LAT-deficient Jurkat T cell derivative J.CaM2 (26) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin. Cells were stimulated with C305, an anti-Jurkat T cell chain mAb (27). The anti-phosphotyrosine mAb, 4G10, and the anti-phospho-ERK2 antibody were obtained from Upstate Biotechnology, Inc. and New England Biolabs, respectively. Anti-Myc mAb was derived from the 9E10 hybridoma. Antibodies for LAT (17), Lck (28), and ZAP-70 (19) have been previously described. For use in transfections, a myc-tagged form of LAT was excised from pBluescript with EcoRI and XhoI and inserted into the expression vector, pcd3f. C26/29S-LAT, also in pcd3f, was created with the QuickChange site-directed mutagenesis kit (Stratagene). The Lck-LAT chimera was made by digesting pcd3f-LAT with EcoRI and ApaLI to remove the extracellular and transmembrane domains of LAT and replacing this region, in frame, with an oligo encoding the N terminus 10 amino acids of Lck. Erk2 and CD8 were expressed from pcdef3. C26/29S-LAT, also in pcdef3, was created with the QuickChange site-directed mutagenesis kit (Stratagene). The Lck-LAT chimera was made by digesting pcd3f-LAT with EcoRI and ApaLI to remove the extracellular and transmembrane domains of LAT and replacing this region, in frame, with an oligo encoding the N terminus 10 amino acids of Lck. Erk2 and CD8 were expressed from pcdef3. C26/29S-LAT, also in pcd3f, was created with the QuickChange site-directed mutagenesis kit (Stratagene).
a combination of protease and phosphatase inhibitors. All subsequent steps were performed at 4 °C. Lysates were mixed with 80% sucrose in TNE, transferred to centrifuge tubes, and overlaid with 2 ml of 30% sucrose in TNE and then 1 ml of 5% sucrose in TNE. Samples were centrifuged for 20–22 h at 45,000 rpm in a Beckman SW55Ti at 4 °C, and twelve 400-µl fractions were collected from the top of each gradient.

Transfection, Stimulation, and Luciferase Assay—For transient transfections, 2 x 10^7 J.CaM2 cells in 400 µl of RPMI 1640 were electroporated at 250 V, 960 microfarads with 30–50 µg of DNA total. Unless otherwise noted, 2.5–10 µg of the various LAT vectors were used to obtain equal protein expression. TCR stimulation for analysis of LAT and ERK2 phosphorylation involved incubating PBS-washed cells (8 x 10^5 cells/ml) at 37 °C for 15 min followed by addition of anti-TCR mAb (1:500) or PBS as a control for 5 min. Cells were immediately pelleted and lysed. For TCR stimulation in luciferase assays, cells were either left untreated or were incubated with immobilized anti-TCR mAb or PMA (25 ng/ml) and ionomycin (1 µM) for 5 h. Cells were then harvested, lysed, and assayed for luciferase activity (29).

Preparation of Lysates, Immunoprecipitation, and Western Blotting—Cells were lysed at 10^6 cells/150 µl in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.6, 2 mM EDTA, and protease and phosphatase inhibitors). After a 10-min incubation on ice, samples were clarified by centrifugation at 14,000 rpm for 10 min. For immunoprecipitations, lysates were incubated with primary antibody for at least 1 h and then protein G-Sepharose beads for 1 h. Beads were then washed three times in 500 µl of lysis buffer. Samples were resuspended in reducing SDS sample buffer, heated at 95 °C for 5 min, and separated by SDS-PAGE, and proteins were transferred to Immobilon-P (Millipore) for analysis by Western blotting. Membranes were blocked with 3% bovine serum albumin, incubated with the indicated primary antibodies followed by the appropriate secondary antibody conjugated to horseradish peroxidase. Reactive proteins were subsequently visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Measurement of Intracellular Ca^{2+} Levels—J.CaM2 cells were transfected with the various LAT constructs and 20 h later cells were lysed. A, half of the cells were lysed in 1% Nonidet P-40, separated by SDS-PAGE, and analyzed by Western blot with an anti-Myc mAb to detect LAT (the slightly faster mobility band detected in empty-vector-transfected cells is because of nonspecific cross-reactivity). Blots of fraction 3 and 11 were also probed with anti-Lck and anti-ZAP-70 antibodies respectively to verify equal loading of samples. wt LAT, wild-type LAT.

RESULTS
LAT Localization to GEMs Is Required for Function—Although palmitoylation of LAT on cysteines 26 and 29 is not necessary for LAT localization to the plasma membrane, it is essential for its distribution into GEMs (21). To determine whether the presence of LAT within GEMs is required for coupling TCR engagement to downstream signaling events, an expression vector was created encoding a form of LAT containing serines instead of cysteines at these palmitoylation sites (C26/29S-LAT). Although C26/29S-LAT was expressed at levels similar to wild-type LAT (WT-LAT) following transfection of vectors encoding either protein into LAT-deficient J.CaM2 T cells (Fig. 1A), C26/29S-LAT was completely absent in GEM fractions and, instead, was found exclusively in the Triton-soluble fraction (Fig. 1B). Conversely, a majority of transfected WT-LAT was found in GEMs with little detected in the Triton-soluble fraction (Fig. 1B). To ensure that our method of GEM isolation yielded fractions devoid of cross-contamination, we also assayed for the presence of markers that specifically localize to GEM and Triton-soluble fractions, namely the ganglioside GM1 (using cholera toxin) and ZAP-70, respectively. Consistent with other studies (21, 25), we detected the ganglioside GM1 in the GEM fraction and ZAP-70 only in the Triton-soluble fraction of unstimulated cells (data not shown).

Following TCR engagement, LAT becomes heavily tyrosine phosphorylated. To determine whether LAT localization to GEMs is required for its inducible tyrosine phosphorylation, J.CaM2 cells were transfected with vectors encoding either WT-LAT or C26/29S-LAT and subsequently stimulated through the TCR followed by the analysis of LAT phosphorylation. Surprisingly, C26/29S-LAT was constitutively phospho-
phosphorylation of Erk2, which occurs as a consequence of Ras activation in T cells following TCR engagement (30). Consistent with previous results (19), J.CaM2 cells receiving WT-LAT, but not empty vector, demonstrated Erk2 phosphorylation following TCR engagement (Fig. 3A). Interestingly, cells expressing C26/29S-LAT failed to exhibit Erk2 phosphorylation in response to TCR stimulation (Fig. 3B). A similar pattern of results was seen when the levels of intracellular Ca^{2+} and induction of NF-AT were analyzed; cells receiving WT-LAT but not C26/29S-LAT restored intracellular Ca^{2+} mobilization (Fig. 3B) and activation of NF-AT (Fig. 4) in response to TCR engagement. Together these results indicate that LAT localization to GEMs is required for coupling TCR stimulation with downstream signaling events.

The Extracellular and Transmembrane Domains of LAT Are Dispensable for Function—As an initial step toward identifying regions of LAT required for function, we focused on its extracellular and transmembrane domains. Because our results suggested that LAT must localize to GEMs to facilitate activation of downstream signaling pathways, we studied the role of these domains in the context of GEMs. A vector was created that encoded a form of LAT where the extracellular and transmembrane domains were replaced with the first 10 amino acids from the N terminus of Lck (Lck-LAT). This Lck motif provides the necessary signals for myristoylation and palmitoylation and when attached to a heterologous protein can target it to GEMs (31). Thus, Lck-LAT should localize to GEMs but will lack both the extracellular and transmembrane domains of LAT. As shown in Fig. 1, A and B, respectively, Lck-LAT was expressed at levels comparable with WT-LAT and localized primarily to the GEM fraction of cell lysates.

We next analyzed Lck-LAT phosphorylation, and in a manner similar to WT-LAT, Lck-LAT became heavily tyrosine phosphorylated after engagement of the TCR (Fig. 2). Furthermore, cells expressing Lck-LAT demonstrated TCR-mediated activation of the Ras signaling pathway (Fig. 3A), mobilization of intracellular Ca^{2+} (Fig. 3B), and induction of NF-AT (Fig. 4). Taken together, the results obtained with Lck-LAT demonstrated that once LAT localizes to GEMs, both its extracellular and transmembrane domains were dispensable for coupling TCR engagement to the activation of downstream signaling pathways.

DISCUSSION

Glycolipid-enriched microdomains (GEMs) have increasingly been implicated as critical components of intracellular signaling cascades. In T cells for instance, a number of important signaling molecules have been shown to localize to GEMs, either constitutively or following TCR stimulation. In addition, treatment of T cells with various agents that disrupt GEM structure can inhibit effective TCR signaling (25). Thus, the formation of GEM-dependent, higher order signaling complexes may play a central role in T cell activation.

The contribution of LAT to T cell activation likely derives from its TCR-mediated tyrosine phosphorylation, a process which facilitates LAT association with multiple signaling proteins, including Grb2, GADS, SLP-76, Vav, PLCγ-1, Cbl, and the p85 subunit of phosphatidylinositol 3-kinase. The interaction of these molecules with LAT may result in their activation and/or may promote protein-protein interactions that are crucial for downstream signaling events. Here we demonstrate that a form of the adaptor molecule LAT (C26/29S-LAT) that fails to localize to GEMs cannot support TCR-mediated signal transduction. These results are consistent with other studies that have suggested that GEMs play an important role in T cell activation. More importantly however, our results strongly suggest that LAT function may depend on its ability to recruit multiple signaling proteins to GEMs, where proper protein
LAT Function Requires Localization to GEMs

interactions can form and/or where essential substrates may be located. Relevant to this idea is the observation that C26/29S-LAT displayed a high level of basal tyrosine phosphorylation yet could not support productive TCR signaling. One conceivable explanation for this result is that, although C26/29S-LAT binds critical signaling molecules, it is unable to couple TCR engagement to downstream signaling processes because of its failure to localize into GEMs. Alternatively, because LAT contains nine sites of potential tyrosine phosphorylation, it is possible that the constitutive sites of tyrosine phosphorylation in C26/29S-LAT are not those that mediate the recruitment of appropriate signaling molecules. Of note, previous analysis of a GEM-excluded LAT variant containing alanines in place of cysteines at residues 26 and 29 did not reveal a high constitutive level of LAT tyrosine phosphorylation (21). Although an explanation for this discrepancy is presently lacking, variations in results may reflect the different cell types utilized (Jurkat versus J.CaM2) or experimental approaches employed (transient versus stable transfection of LAT variants).

In this study we also addressed which domains of LAT are critical for function once the protein has localized to GEMs. In particular, we focused on the potential role of the extracellular and transmembrane domains. A comparison of LAT protein sequences from mouse, human, and rat revealed the presence of a conserved, α-helix disrupting proline within the transmembrane domain, which could potentially destabilize LAT insertion into lipid bilayers (17, 18). Often, proteins which contain such atypical amino acids within the transmembrane region are stabilized through the interaction of this domain with other molecules. It was therefore possible that LAT interacted with another protein via its transmembrane domain and that this interaction may be critical for LAT function. It was also possible that the extracellular and transmembrane domains were required for LAT function by some other mechanism. However, our results clearly show that both the extracellular and transmembrane domains are dispensable in coupling TCR engagement to the activation of the Ras signaling pathway, intracellular Ca2+ mobilization, and NF-AT activation. It is possible that these domains are required for other functions of LAT. For example, the transmembrane region may initially localize LAT to the plasma membrane, permitting palmitoylation of LAT on cysteine residues 26 and 29 and thereby resulting in subsequent LAT insertion into GEMs. Alternatively, the extracellular and transmembrane domains may be required for coupling TCR engagement to downstream pathways that were not examined in the present study or that are required for functions independent of signaling. Future studies will hopefully address these alternative possibilities.