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The Membrane Proximal Proline-Rich Region and Correct Order of C-Terminal Tyrosines on the Adaptor Protein LAT Are Required For TCR-Mediated Signaling and Downstream Functions

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Abstract

The primary activating receptor for T cells is the T cell receptor (TCR), which is stimulated upon binding to an antigen/MHC complex. TCR activation results in the induction of regulated signaling pathways vital for T cell differentiation, cellular adhesion and cytokine release. A critical TCR-induced signaling protein is the adaptor protein LAT. Upon TCR stimulation, LAT is phosphorylated on conserved tyrosines, which facilitates the formation of multiprotein complexes needed for propagation of signaling pathways. Although the role of the conserved tyrosines in LAT-mediated signaling has been investigated, few studies have examined the role of larger regions of LAT in TCR-induced pathways. In this study, a sequence alignment of 97 mammalian LAT proteins was used to identify several “functional” domains on LAT. Using LAT mutants expressed in Jurkat E6.1 cells, we observed that the membrane proximal, proline-rich region of LAT and the correct order of domains containing conserved tyrosines are necessary for optimal TCR-mediated early signaling, cytokine production, and cellular adhesion. Together, these data show that LAT contains distinct regions whose presence and correct order are required for the propagation of TCR-mediated signaling pathways.

Keywords

LAT; TCR; T cells; Signal transduction; Domain order; structure/function study

1. Introduction:

The activation of antigen-specific T cells is the central mechanism regulating the adaptive immune response. T cell activation results in the indirect modulation of the immune

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response by the release of effector cytokines that act upon immune and non-immune cell types or directly by killing cells infected with intracellular pathogens. The T cell antigen receptor (TCR) is the primary stimulatory receptor needed for the activation of antigen-specific T cells. The TCR expressed on an individual cell is stimulated by a small number of highly related protein antigens presented by MHC complex found on the surface of antigen presenting cells (1-3). Stimulation of the TCR induces intracellular signaling pathways that regulate gene transcription needed for the proliferation of antigen-specific T cells, the maturation of these T cells into armed effector cells, and the production of cytokines and cell surface receptors needed for modulation of the immune response or direct killing of infected cells (3-7).

Because of its importance in the activation of T cells, the intracellular signaling pathways downstream of the TCR have been extensively studied (3, 4). Stimulation of the TCR by the antigen/MHC complex leads to the increased phosphorylation of the intracellular domains of TCR subunits (3, 4). The tyrosine kinase ZAP-70 specifically binds phosphorylated TCR subunits, resulting in the recruitment of ZAP-70 to the TCR complex and its subsequent activation (5). ZAP-70 then phosphorylates several substrates, most notably LAT, an adaptor protein found in ordered lipid domains in the plasma membrane (5). Phosphorylated tyrosines on LAT then serve as binding sites for a small number of SH2 domain-containing proteins (8, 9). One such protein is GRB2, an adaptor protein that binds directly to LAT tyrosines 171, 191 and 226 (10-13). GRB2 serves a dual purpose of facilitating the formation of clusters containing 30 or more LAT proteins and their ligands (14-17), while simultaneously recruiting effector proteins to the LAT complex that drive downstream signaling. Additionally, LAT tyrosine 132 directly binds PLC-γ (10-13, 18, 19), a phospholipase that produces the second messengers diacyl glycerol and inositol 1,4,5 phosphate, needed for RAS-GRP/PKC activation and calcium influx, respectively (20, 21). Finally, LAT directly interacts with GADS, an adaptor protein related to GRB2 that recruits the adaptor protein SLP-76 to the LAT complex (10, 12, 13, 22). In addition to binding to GADS, SLP-76 also associates with PLC-γ forming a LAT/GADS/SLP-76/PLC-γ complex critical for actin rearrangement and downstream signaling (23, 24). Ultimately, the formation of the LAT “signalasome” regulates calcium influx, ERK1/ERK2 activation and other signaling pathways needed for altered transcription and actin cytoskeleton rearrangement required for T cell adhesion and migration (3, 4, 8).

Because of its central role in TCR-mediated signaling pathways, LAT is required for TCR signaling and T cell differentiation in both mice and humans (18, 25-27). Most structure/function studies of LAT have investigated the role of conserved tyrosines in the formation of multiprotein signaling complexes, the activation of downstream pathways, and T cell differentiation (8, 11-13, 28-31). Only a handful of studies have examined the role of larger regions on the activation and function of LAT. Aguado and coworkers found that a small patch of negatively charged amino acids associated with LCK and modulated LAT-mediated signaling events (32), while the Weiss laboratory found that deletion of the membrane proximal proline rich region on LAT abrogated the interaction of LAT with LCK and reduced TCR-mediated LAT and PLC-γ1 phosphorylation, and calcium influx (33). The amino acid sequence of LAT suggests that it is intrinsically unstructured with little to no secondary structure (19), but extensive studies on the function of LAT suggest that it may

have “functional” domains or regions that are required for the activation of downstream pathways. To date, no studies have determined the boundaries of the potential “functional” domains on LAT or if the presence and relative order of these domains is critical for the ability of LAT to transmit TCR-mediated signals. In this study, we used a sequence alignment of LAT proteins from 97 mammalian species to identify four potential “functional” domains. We then observed that the membrane proximal proline-rich region of LAT and the correct order of domains containing conserved tyrosines are required for optimal TCR-mediated early signaling, cytokine production, and cellular adhesion. This study provides further evidence that LAT contains discreet regions that facilitate TCR-induced signaling pathways.

2. Materials and Methods:

2.1 LAT sequence alignments:

The protein sequences for 97 mammalian species were downloaded from NCBI Gene website (https://www.ncbi.nlm.nih.gov/gene) and aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The aligned sequences with gaps were then analyzed for conservation using WebLogo 3 (http://weblogo.threeplusone.com/). We exclusively analyzed mammalian sequences since only 16 annotated LAT sequences are found in genomes from birds, fish, reptiles and amphibians. The annotated LAT proteins found from these limited number of non-mammalian species are poorly conserved compared each other and with human LAT; thus they were excluded from the analysis.

2.2 Jurkat E6.1 T cell growth and stimulation:

Jurkat E6.1 T cells were cultured at 37° C and 5% CO_2 in complete RPMI 1640 media (RPMI supplemented with 10% FBS (Atlanta Biologicals), 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (Gibco)). Lentiviral transduced cell lines were continually selected with 1 μg/mL puromycin. Prior to stimulation, the various T cell lines were grown to a concentration of 2-5 x 10^5 cells/mL, and then washed in RPMI 1640 without supplements. Cells were re-suspended to a concentration of 5 x 10^7 cells/mL in RPMI 1640 without supplements and rested for 10 min at 37° C. The cells were stimulated with soluble 10 μg/mL anti-CD3 (OKT3) for various times and lysed with a 4-fold excess of hot 2X lysis buffer (20mM Tris (pH 8.0), 2mM EDTA, 2mM Na_3VO_4, 20mM DTT, 2% SDS and 20% glycerol). Lysates were then heated to 95° C for 4 min and sonicated to reduce viscosity.

2.3 Vectors and shRNA design:

Lentiviral pLK4 vectors were a gift of Dr. Stephen Bunnell. The VSV-G construct was donated by Dr. Bruce Hostager. Packaging plasmids pCL-Eco and Pax2 were a kind gift from Drs. John Colgan and Dawn Quelle, respectively. ShRNA targeting sequences against human LAT (AAH11563.1) were developed utilizing a siRNA/shRNA based algorithm formulated by Dr. Sachidanandam and coworkers (http://katahdin.cshl.edu). The following sequences produced the most efficient suppression (LAT sense: 5’ ACTACTCTGTGTAATAGAATAA 3’, LAT anti-sense: 5’ TTATTCTATTACACAGAGTAGG 3’. This shRNA targeted the non-coding region of LAT;

therefore, the LAT WT and LAT 23 and LAT 132 mutations did not require mutations to make them shRNA resistant. The LAT sense and anti-sense sequences, hairpin loop, and terminal thymidines were inserted into pLK4 lentiviral vector alone or co-expressed with the gene for wild-type LAT (LAT WT), LAT 23 or LAT 132. The LAT WT, LAT 23 and LAT 132 genes were all synthesized by IDT before cloning into the pLK4 lentiviral vector. The LAT WT protein contained the entire coding region of LAT from amino acid 1-233. The LAT 23 protein removed amino acids 31-82, fusing the transmembrane region (a.a. 1-30) to the distal signaling domain of LAT (a.a. 83-233). The LAT 132 protein transferred amino acids 83-142 to the end of the protein (i.e. a.a. 1-82 followed by a.a. 143-233 followed by a.a. 83-142).

2.4 Production of lentiviruses and Transduction of Jurkat E6.1 T Cells:

To produce lentiviral particles, the shRNA containing pLK4 vectors were transfected into 293T cells with VSV-G envelope and packaging vector Pax2 using the calcium phosphate method as previously described (34). The viral supernatants were collected every 24 hours for 2 days and filtered through 0.45um Millipore filters. After collection, the viral supernatants were centrifuged at 4° for 1.5 hours at 48,000 x g. The viral pellets were resuspended in complete RPMI media and used for subsequent transductions. 3-5x10^5 Jurkat E6.1 cells were incubated and periodically mixed in 1.5 mL of concentrated viral supernatant in 25 cm^2 flasks with 8 μg/mL Hexadimethrine bromide (Polybrene) (Sigma-Aldrich). After 48 hours, the viral media was removed, and the cells were resuspended in complete RPMI 1640 with 1 μg/mL puromycin.

2.5 Immunoblotting and Immunoblot Analysis:

Cellular lysates were separated by polyacrylamide gel electrophoresis (SDS-PAGE) using 4-15% Criterion gels (Biorad) and transferred to PVDF-FL membrane (Millipore). To analyze site-specific phosphorylation the membranes were blocked for 1 hr at room temperature in a blocking buffer that was 1:1 1x PBS:SEA Block (Thermo Scientific). The PVDF membranes were incubated with primary antibodies against LAT pY132 (Biosource), LAT pY226 (BD Pharmingen), ZAP-70 pY319 (Cell Signaling Technologies), SLP-76 pY128 (BD Pharmingen), PLC-γ1 pY783 (Cell Signaling Technologies), ERK1/ERK2 pT185/pY187 (Thermo Scientific) and GAPDH (Meridian Life Sciences). IRDye 800CW or IRDye 680-conjugated secondary antibodies were diluted in SEA Block as above and incubated with the PVDF membrane for 30 min at room temperature. The membranes were then imaged using the Licor Odyssey. The intensity of the immunoblotting bands was determined using Odyssey's v3.0 software. Phosphorylated proteins were normalized to GAPDH and the relative amount of phosphorylation to the 2 minute (for LAT pY132, LAT pY226, ZAP-70, SLP-76 and PLC-γ1) or 5 minute (ERK1/ERK2) LAT WT bands were determined as described previously (35-37). The data from 4-5 independent replicates were compiled and then plotted using Prism (GraphPad Software).

2.6 Calcium Influx:

1 x 10^6 Jurkat E6.1 cells were suspended in RPMI 1640 without phenol red (Gibco). 5 μM Fluo-4 AM (Thermo Scientific) and 2.5 mM probenecid (Thermo Scientific) were added to the cell suspension, and then the cells were incubated at 37 °C and 5% CO_2 for 60 min.

Cells were washed in RPMI without phenol-red and resuspended at a concentration of 1 x 10^6 cells/mL. Probenecid was added to the cells again and incubated on ice for 15 min. The cells were warmed for 5 min and data was collected using the Accuri C6 flow cytometer for 35 s. The cells were stimulated with 2 μg/mL anti-CD3 (OKT3) and data was collected for an additional 5.5 min. The data was gated using the software provided by Accuri and the data from the gated cells was exported to Microsoft Excel. The average fluorescence of gated cells collected in a single second was calculated. The average baseline of the first 35 seconds was calculated and set to 1. The fold increase over baseline was calculated for every time point then plotted as fold increase over baseline vs. time. Fold increase was plotted due to differences in the baseline fluorescence of the individual mutants and substantial experiment-to experiment variation. The fold increase for four experiments was compiled and graphed using GraphPad Prism. Statistical analysis was performed as described below.

2.7 Total internal reflection fluorescence (TIRF) microscopy:

Jurkat E6.1 cells were stimulated with glass coverslips coated with 10 μg/mL anti-CD3 (OKT3), fixed with 4% paraformaldehyde, and permeabilized with 0.25% Triton-X. To detect micro-cluster formation of TCR signaling proteins, antibodies specific for LAT pY226 (BD Pharmingen), PLC-–1 pY783 (Cell signaling technologies), SLP-76 pY128 (BD Pharmingen) and anti-LAT (Millipore) were incubated with fixed and permeabilized cells overnight at 4 °C. Secondary antibodies Alexa Fluor 568 goat anti-mouse IgG1, Alexa Fluor 488 goat anti-mouse IgG1, Alexa Fluor 568 goat anti-rabbit IgG (Thermo Scientific) were incubated with the cells at room temperature for 2 hours. All images were captured by the Leica AM TIRF MC system using 100x magnification oil immersion objective lens at room temperature at the University of Iowa Central Microscopy Research Facility. TIRF images were processed and analyzed using ImageJ or FIJI Image J. Quantification of pixel intensity was performed for between 30-60 total cells from 2-3 independent experiments. To analyze the pixel intensity across the center of the cell, one line was drawn through the center of the cell on the original images and then the “Plot-Profile” analysis function was performed on ImageJ. Fuji ImageJ was used to measure the number of clusters in the cell. A ROI was drawn around all cells in an image that had adhered to the plate and were in focus on the DIC image. The background was subtracted using a 50 rolling ball radius and the count of clusters in an individual ROI was measured using the Find Maxima tool with prominence set at 10.

2.8 IL-2 cytokine production:

Jurkat E6.1 cells were washed in complete RPMI 1640 and then resuspended at 5 x 10^5 cells/mL. To stimulate the cells, 0.5mL of the cell suspension was added to 24 well plates coated with 2 μg/mL anti-CD3 (OKT3) or treated with 10 μM PMA and 1 μM ionomycin for 24 hours. IL-2 production was measured by standard TMB ELISA using an Epoch reader (Biotek) at 450nm. Three independent experiments were completed and the results averaged and plotted using GraphPad Prism.

2.9 Plate bound adhesion assay:

EIA/RIA plates were treated with various concentrations of anti-CD3 (OKT3) in PBS and incubated overnight at 4°C. The plates were washed three times with PBS and blocked with

1% BSA in PBS for 2 hours at 37°C. The cells were stained Licor CellVue Burgundy kit as previously described (38), washed three times with complete RPMI, resuspended in complete RPMI and then loaded on the plate in triplicate. After 30 minutes incubation at 37 C, the plates were washed two times with 1X PBS and 1X PBS was added to each well. The absorbance intensity was captured by the Licor Odyssey. The mean value ± SEM of 3 independent experiments are shown.

2.10 Statistical Analysis:

The p values for the statistical analysis of the 0, 5 and 15 minute timepoints for LAT pY132, LAT pY226, ZAP-70, SLP-76 and PLC-γ1 and the 0, 15 and 30 minute timepoints for the ERK1/ERK2 signaling experiments were determined in Excel using a two-tailed Students t test assuming unequal variance. The p values for the statistical analysis of the 2 minute timepoint for LAT pY132, LAT pY226, ZAP-70, SLP-76 and PLC-γ1 and the 5 minute timepoint for the ERK1/ERK2 signaling experiments were determined in GraphPad Prism using a one sample t test assuming a mean of 1. In both experiments, the level of phosphorylation in the WT LAT cells to phosphorylation levels in the other cell lines. The p values for the statistical analysis of individual timepoints for calcium influx and individual distance intervals for protein clustering were determined in Excel using Students t test. The values for LAT WT vs LAT KD, LAT 23 or LAT 132 were then plotted using GraphPad Prism. The p values for the statistical analysis of the data showing cluster per cell were determined in GraphPad Prism using the Brown-Forsythe and Welch ANOVA test using the Dunnett's multiple comparison test to calculate the individual p values. The p values for the statistical analysis of the adhesion and IL-2 release were determined in GraphPad Prism using ANOVA with multiple comparisons.

3. Results:

3.1 Organization of Functional Domains in Mammalian LAT:

Previous work suggests that LAT is largely unstructured and does not contain domains with stable secondary structures (19). Instead, LAT likely has "functional" domains that regulate its cellular localization, membrane positioning, and cellular function. To identify potential "functional" domains, we performed a protein alignment of LAT sequences from 97 mammalian species. As shown in Figure 1, we identified four distinct functional domains with varying levels of conservation between species. The N-terminal region of LAT contains a previously identified transmembrane domain that ends at human amino acid 24. This transmembrane is rich in leucine residues and contains a highly conserved proline residue that is likely required for membrane packing (orange shading; Fig.1A). There are also two highly conserved cysteines near the transmembrane domain that are palmitoylated and facilitate the interaction of LAT with ordered regions of the plasma membrane (Fig. 1A) (39).

The first functional domain in the intracellular portion of LAT encompasses amino acids 35-94. This region is poorly conserved between species but contains a high percentage of conserved prolines (~25%) and net basic charge (blue shading; Fig. 1A). The second functional intracellular domain encompasses two highly conserved tyrosines, Y110 and

Y132 in the human sequence (Y115 and Y170 in Fig. 1A, respectively). Tyrosine 132 binds to PLC-γ1 (11-13, 28, 30), while Y110 is a consensus GRB2 binding site that has been implicated in TCR-induced ERK1/ERK2 activation (11). This region is conserved between species, especially in amino acids surrounding the conserved tyrosines (green shading; Fig. 1a). This region is acidic with PI of ~3.8 due to a high percentage of aspartic and glutamic acids, especially in an acidic patch that precedes Y132. Several species, including many ruminants, sea lions, bats, and whales, have a poorly conserved 27-28 amino acid insert in this region. The third intracellular functional domain contains three conserved tyrosines Y171, Y191 and Y226 (Y214, Y234 and Y279 in Fig. 1A, respectively). These sites all bind to the GRB2 family of adaptor proteins and are critical for the downstream signaling and the clustering of LAT (10-13, 15). Similar to the second functional domain, this region is especially conserved between species around the conserved tyrosines. This region also has a high percentage of aspartic and glutamic acids, giving it overall average acidic PI of ~3.8 (red shading; Fig. 1A). Interestingly, there is an acidic patch proceeding Y226 that varies greatly in length between species.

3.2 LAT Mutations:

A recent study has suggested that the proline-rich, membrane proximal domain of the intracellular region of LAT contains prolines that bind to LCK and bridge LCK, ZAP-70 and LAT together (33). However, the net basic charge, which would allow it to interact with the plasma membrane, and the proline rich nature of this region suggests that it could also act as a “spacer” that regulates the placement of the more distal regions of LAT in comparison to the plasma membrane. The correct positioning of the regions of LAT with conserved tyrosines in relation to membrane could facilitate the phosphorylation of LAT and its interaction of cytoplasmic signaling proteins. In contrast, both the third and fourth functional intracellular domains of LAT have substantial net negative charge, suggesting that they are repelled from the negatively-charged plasma membrane. This would force the distal regions of LAT away from the membrane into the cytoplasm, which would facilitate the phosphorylation of conserved tyrosines and the subsequent formation of dynamic signaling complexes. In one of these complexes, PLC-γ1 binds to pY132 in the second domain and the adaptor protein GADS binds to pY171 and pY191 in the third domain and these proteins indirectly interact via other proteins, most notably the adaptor protein SLP-76 (23, 24). This suggests that the second and third domains of LAT need to have the correct spatial relationship to each other and the plasma membrane for the formation of the PLC-γ1/SLP-76/GADS complex.

3.3 Effects of LAT Mutations on the Activation of Proximal Signaling Events:

To test these hypotheses, we markedly reduced the levels of endogenous LAT in the Jurkat E6.1 T cell line by lentivirus-driven expression of a recombinant microRNA specific for endogenous, but not exogenously expressed, LAT. Four cell lines were produced: a LAT-deficient Jurkat E6.1 T cell line (LAT KD) or LAT-deficient cells with re-expression of wild-type LAT (LAT WT) or the mutant LAT molecules described below using a second promoter on the same lentivirus that expressed the LAT-specific microRNA. In the first of these mutants, the proline-rich domain of LAT was removed and the second and third functional domains of LAT were fused to the transmembrane domain (LAT 23; Fig. 1B). This mutant

allowed us to examine the importance of the proximal, proline-rich region of LAT in TCR-mediated functions. The second mutant swapped the position of the second and third functional domains of LAT (LAT 132; Figure 1B). This protein was chemically identical to wild-type LAT with the PLC-γ1 binding region of LAT now distal from the plasma membrane compared to the GRB2 family binding domain. We expressed these mutants in Jurkat E6.1 cells, while simultaneously expressing a microRNA specific for LAT that resulted in an ~50-90% reduction in LAT (Supplemental Figure 1A). As determined by immunoblotting, these mutant cell lines had similar levels of SLP-76 and ZAP-70, while also having similar or higher levels of LAT mutants compared to the WT LAT expressing cells. As assessed by flow cytometry, the expression of WT LAT, LAT 23 and LAT 132 mutants had similar levels of TCR expression, although the LAT 23 and LAT 132 mutant cell lines had a subset of cells that had reduced TCR expression (Supplemental Figure 1B). Surprisingly, reduced expression of LAT, without re-expression of some form of LAT, resulted in increased background fluorescence in Jurkat E6.1 cells when the TCR expression was assessed by flow cytometry. This increased background fluorescence occurred in several different lines of Jurkat E6.1 cells that were derived by different transductions. The mechanism or impact of the increased background fluorescence is unknown.

To begin to determine the impact of LAT mutations on T cell biology, we assessed the impact of the LAT mutations on the phosphorylation of key early signaling proteins. We first examined the activation of ZAP-70, which is the primary kinase for LAT. Previous work has shown that LAT expression can regulate the phosphorylation of ZAP-70 tyrosines Y111 and Y473 (29, 37). Reduced expression of LAT resulted in a statistically significant decrease in the TCR-induced phosphorylation of ZAP-70 Y319 compared to T cells that express LAT WT (Fig. 2A and 2B). T cells expressing the LAT 23 mutant had similar levels of ZAP-70 Y319 phosphorylation as cells expressing LAT WT, while expression of the LAT 132 mutant did not rescue the defect of ZAP-70 phosphorylation observed in LAT-deficient cells (Fig. 2A and 2B). These experiments suggest that the correct domain order in the C-terminal tail of LAT is critical for optimal ZAP-70 phosphorylation.

We next examined the effects of the LAT mutations on the phosphorylation of LAT Y132 and LAT Y226. Expression of the LAT-specific microRNA resulted in a significant near complete reduction in LAT Y132 and LAT Y226 phosphorylation upon TCR stimulation (Fig. 2A and 2B). This shows that the residual endogenous LAT in these cells does not result in significant amounts of phosphorylated LAT compared to WT LAT expressing cells. In contrast, LAT WT and LAT 132 mutant had similar levels of TCR-induced LAT Y226 phosphorylation and significantly enhanced LAT Y132 phosphorylation (Fig. 2A and 2B). This was surprising since the T cells expressing the LAT 132 mutant had reduced ZAP-70 phosphorylation of the activating Y319 residue. This suggests that even though ZAP-70 activation is reduced in LAT 132 expressing T cells, there is still sufficient levels of active ZAP-70 to facilitate optimal LAT phosphorylation. The LAT 23 mutant had robust phosphorylation of LAT Y132 and LAT Y226 after TCR induction (Fig. 2A and 2B). The approximately 3-fold increase in LAT Y226 phosphorylation in the LAT 23 compared to LAT WT was variable between replicates and likely due to quantification issues that arose from the unexpected banding pattern of the LAT 23 protein (Fig. 2A). Interestingly, the LAT 23 protein ran at the same molecular weight as LAT WT and LAT 132 proteins, even though

LAT 23 protein has a 51 amino acid deletion compared to these proteins. Endogenous LAT runs substantially higher on SDS-PAGE compared to its expected molecular weight (25 kDa expected vs. 36-38 kDa actual). Aguado and co-workers have recently shown that an acidic patch that precedes LAT Y127 is largely responsible for the higher apparent molecular weight of LAT in SDS-PAGE (32). The LAT 23 protein contains this acidic patch and other acidic residues in the C-terminal tail of LAT that allows it to migrate at a similar molecular weight to LAT WT and LAT 132.

We then assessed whether LAT mutations altered the phosphorylation of key signaling molecules recruited to the LAT complex. We found that LAT-deficient cells had significantly reduced levels of TCR-induced SLP-76 Y128 and PLC-γ Y783 phosphorylation compared to T cells expressing LAT WT (Fig. 2A and 2B). The low level of phosphorylation of SLP-76 Y128 and PLC-γ Y783 observed in the LAT-deficient cells is likely due to the residual LAT in these cells. T cells expressing the LAT 23 had similar levels of phosphorylation of SLP-76 Y128 and PLC-γ Y783 compared to those observed for LAT WT cells (Fig. 2A and 2B). T cells expressing LAT 132 had similar amounts of PLC-γ Y783 phosphorylation as the WT LAT cells, but had significantly reduced SLP-76 Y128 phosphorylation that was similar to LAT-deficient cells. LAT deficient T cells had significantly reduced ERK1/ERK2 phosphorylation compared to LAT WT expressing T cells. T cells with either the LAT 23 or LAT132 mutants having intermediate levels of ERK1/ERK2 phosphorylation (Fig. 2A and 2B), with the LAT 132 mutant having a significant reduction compared to LAT WT cells. A major signal produced upon LAT activation and clustering and PLC-γ1 phosphorylation is the increased influx of calcium into the cytoplasm of the T cells. We assessed TCR-induced calcium influx using flow cytometry and observed the T cells deficient in LAT orT cells expressing either the LAT 23 or LAT 132 mutants had markedly reduced calcium influx compared to T cells expressing LAT WT (Fig. 2C). This decrease in calcium influx in the LAT KD, LAT 23 and LAT 132 mutant T cells was statistically significant compared T cells expressing LAT WT (Fig. 2C). These experiments suggest that the proline rich region of LAT and the correct domain order in the C-terminal signaling tail of LAT is required for optimal activation of signaling proteins downstream of LAT activation and for the TCR-induced influx of cytoplasmic calcium.

3.4 Effects of LAT Mutations of the Intensity and Numbers of TCR-Induced Clusters of Proximal Signaling Proteins:

Upon phosphorylation, LAT forms large multiprotein complexes that are required for TCR-mediated intracellular signaling and calcium influx. Since the LAT 23 and LAT132 mutations result in reduced calcium influx and phosphorylation of proximal signaling proteins, we examined the intensity and numbers of the TCR-induced clusters of LAT and its ligands SLP-76 and PLC-γ1. For these studies, we stimulated LAT WT, LAT KD, LAT 23 and LAT132 T cells with anti-CD3 coated coverslips and examined intensity and number of protein clusters using immunofluorescence and TIRF microscopy. Quantification of images from at least 30 cells from two to three independent experiments showed that LAT WT expressing T cells had substantial numbers of clusters of both total LAT and phosphorylated LAT upon TCRstimulation (Fig. 3). LAT deficient cells had significantly reduced intensity and numbers of total and phosphorylated LAT clusters compared to LAT WT expressing

cells. LAT 23 and LAT 132 T cells had similar numbers and intensity of LAT clustering compared to LAT WT, except for the LAT 132 cells, which had slightly, but significantly, increased cluster intensity for total LAT and slightly reduced numbers of phosphorylated LAT clusters compared to LAT WT expressing cells (Fig. 3 and Supplemental Figure 2). The LAT clustering was due to CD3 stimulation, since stimulation of the WT LAT cells with anti-CD49d resulted in significantly reduced intensity of LAT pY226 clustering compared to anti-CD3 stimulated cells (Supplemental Figure 2A).

Similar to LAT clustering, we found that LAT KD and LAT 132 expression T cells had a similar, significant reduction in the intensity of TCR-mediated phosphorylated SLP-76 clusters compared to LAT WT T cells (Fig. 3 and Supplemental Fig. 2). LAT 23 expressing T cells had similar phospho-SLP-76 cluster intensity as LAT WT cells (Fig. 3 and Supplemental Fig. 2). There were no differences in the number of phosphorylated SLP-76 clusters between the cell lines, suggesting that the decreased intensity of phospho-SLP-76 clusters in the LAT KD and LAT 132 expressing cells lines is due to reduced recruitment of phosphorylated SLP-76 to the clusters rather than reduced numbers of clusters. Unexpectedly, we were unable to observe any difference in the intensity of TCR-mediated clusters of phosphorylated PLC-γ1 in any of the cell lines, even though LAT clustering was markedly reduced in LAT deficient T cells (Fig. 3 and Supplemental Fig. 2). However, the LAT deficient T cells and the LAT 132 expression cells had significantly reduced numbers of PLC-γ1 clusters compared to LAT WT and LAT 23 expressing cells (Fig. 3 and Supplemental Fig. 2). The presence of similarly intense PLC-γ1 clusters, even in LAT-deficient cells, was consistent across multiple derivations of stable LAT deficient cells and across at least 5 different imaging experiments (data not shown). Together, these imaging data show that the proline rich region of LAT or the correct domain order in the C-terminal tail of LAT is not required for LAT clustering. In contrast, the correct domain order, but not the proline rich region, is required for optimal SLP-76 clustering upon TCR stimulation.

3.5 Effects of LAT Mutations of the TCR-Induced Downstream Functions:

A subset of proximal signaling events are altered in LAT 23 and LAT 132 T cells compared to LAT WT T cells, but how these changes in early signaling alter downstream functions, such as T cell adhesion and cytokine production, is unresolved. To address this question, we examined TCR-mediated cellular adhesion using a plate-bound adhesion assay and cytokine production via ELISA. We found that LAT deficient T cells had reduced cellular adhesion compared to LAT WT T cells at anti-TCR doses of 1 ug/mL and above. The cells were treated with PMA and ionomycin to determine if there were differences in the potential to produce IL-2. Each cell line had a significant increase in IL-2 production upon PMA and ionomycin treatment (Supplemental Figure 1C). The LAT 132 mutant had reduced IL-2 production upon PMA and ionomycin treatment compared to the other cell lines but this decrease was not statistically significant (Supplemental Figure 1C). LAT 23 and LAT 132 T cells also had reduced cellular adhesion compared to LAT WT T cells at the same doses but only the 1 ug/mL anti-TCR dose was significantly reduced (Figure 4A). Similarly, LAT KD, LAT 23 and LAT132 expressing T cells all had significantly reduced TCR-mediated IL-2 production compared to LAT WT T cells. These studies show that the changes in proximal signaling events observed in Jurkat T cells expressing LAT 23 and LAT132 result in

significantly reduced downstream functions. This conclusively shows that the membrane proximal proline rich region of LAT and the correct domain order in the C-terminal signaling portion of LAT is required for the ability of LAT to facilitate effector functions upon TCR stimulation.

4. Discussion:

The consensus sequence for LAT shown was developed using the alignment of 97 distinct mammalian LAT sequences. We identified a transmembrane domain and three distinct intracellular “functional” domains on LAT. The transmembrane region contains conserved small hydrophobic residues, especially leucine, that form the membrane spanning alpha helix. The membrane spanning alpha helix is broken up by a conserved proline present in the middle of this domain. This proline likely changes the angle of the alpha helix and could facilitate the association of LAT with ordered membrane regions (40). In addition to the membrane spanning residues, this functional domain also contains two perfectly conserved cysteines. These cysteines are palmitoylated and are critical for the localization of LAT order membrane domains in the plasma membrane of T cells (39).

The first “functional” domain was a poorly conserved region between amino acids 35 and 95. There was some conservation of sequence in this region, but it was mainly identified by the high percentage of conserved prolines that made up ~25% of this region in all examined mammalian species. The proline present in this region could act as a “spacer” that regulates the placement of the more distal regions of LAT in comparison to the plasma membrane. The correct positioning of the regions of LAT with conserved tyrosines in relation to membrane could facilitate the phosphorylation of LAT and its interaction of cytoplasmic signaling proteins. To test this possibility, we deleted amino acids 31-81from human LAT, linking the membrane anchoring region with the tyrosine rich signaling domains. We observed that TCR-induced ZAP-70 or LAT Y132 or Y226 phosphorylation and the formation and number of large LAT clusters were identical between LAT WT and LAT 23 (Fig. 2 and Fig. 3). Similarly, the LAT WT and LAT 23 mutant had similar levels of PLC-γ1, ERK1/ERK2, and SLP-76 phosphorylation and SLP-76 clustering upon TCR stimulation (Fig. 2 and Fig. 3). In contrast, the LAT 23 mutant had a near absent influx of cytoplasmic calcium upon TCR activation (Fig. 2). Subsequently, the LAT 23 mutant had reduced TCR-induced cellular adhesion, especially at lower stimulatory concentrations, and cytokine production. Together, these data show that the proline rich region of the membrane proximal region of LAT is not critical for the ability of LAT to regulate ZAP-70 activation or for LAT, PLC-γ1, ERK1/ERK2, or SLP-76 phosphorylation/clustering, but is required for optimal induction of signaling events downstream of LAT, especially PLC-γ1 enzymatic activity, and TCR-induced effector functions (Fig. 5).

A recent paper from the Weiss laboratory has also examined the function of the membrane proximal proline rich region from LAT (33). In this paper, the authors used CRISPR to isolate a Jurkat E6.1 cell line that had a deletion of amino acids 40-107 in LAT. Similar to our studies, they found that deletion of this proline rich region had little effect on TCR-induced ZAP-70 and SLP-76 phosphorylation, while reducing TCR-mediated PLC-γ1 phosphorylation and calcium influx. In contrast to our findings, the Weiss group found that

deletion of the proline-rich region greatly reduced LAT Y132 and Y191 phosphorylation after TCR stimulation (33), while we observed that TCR-stimulated LAT Y226 phosphorylation was not affected in the LAT23 mutant (Fig. 2). There could be several reasons for this discrepancy. The Weiss group examined LAT Y132 and LAT Y191 phosphorylation, while we examined LAT Y226 phosphorylation. It is unlikely that this is the reason for the differences, since we have not observed substantial differences in the extent or timing of the phosphorylation of LAT Y191 and LAT Y226 (data not shown). In addition, the Weiss group used CRISPR to mutate LAT, so the cells used in their studies only expressed the mutated LAT. In contrast, our cells still had low levels of wild type LAT expression, since the LAT-specific miRNA reduced LAT expression by ~95%, which could impact LAT phosphorylation. The most likely reason is that our deletions are subtly but significantly different. The deletion used in this study encompasses amino acid 31-81, while the deletion in the cells used in by the Weiss group eliminated amino acids 40-107. Our protein still contained a portion of the LCK binding site identified by the Weiss group, potentially allowing for LCK and ZAP-70 binding that facilitated the phosphorylation of LAT. The Weiss group concluded that the major function of the proline rich region of LAT was to bind to LCK, thereby facilitating LAT phosphorylation (33). However, our LAT 23 mutant retains LAT phosphorylation, but still has reduced downstream functions compared to LAT WT. This suggests that there is still an LCK-independent function for the membrane proximal proline-rich region of LAT. We hypothesize that, along with the ability to interact with LCK, this region acts as a spacer region that correctly orients the membrane distal signaling domains from the plasma membrane.

The second and third “functional” domains contain the conserved tyrosines required for interactions with intracellular signaling proteins. The second region encompasses three conserved tyrosines, Y110, Y127 and Y132, while the third region contain the conserved tyrosines, Y171, Y191 and Y226. Interestingly, several mammalian species, including many ruminants, sea lions, bats, whales, and dolphins, contain a 27 a.a. insert between Y110 and Y127. There are several isoforms of human LAT that have been deposited in the NCBI data bases, including an isoform (isoform A NP055202) that has a 27 amino acid insert between Y110 and Y127. Work by Miazek and coworkers has shown that cows express only the mRNA for the larger isoform of LAT, while mice only express the mRNA for the shorter isoform of LAT (41). Humans, dogs, cats and pigs express varying ratios of the shorter and longer isoforms of LAT (41). It is unclear if the longer isoform of LAT found in the data base for other ruminants, sea lions, bats, whales, and dolphins is the solely expressed isoform of LAT in these species or if they also express an unreported shorter isoform. Miazek and coworkers also found that the colocalization and signaling functionality of the shorter and longer forms of LAT were identical, but that the longer isoform of LAT has a short protein half-life compared to the shorter isoform of LAT. Approximately 25% of the amino acids in the second and third functional domains of LAT are aspartic and glutamic acids, giving this region of LAT a PI of ~3.8. Thus, the C-terminal cytoplasmic tail of LAT responsible for the interaction with intracellular signaling proteins contains a large negative charge at physiological pH. The presence of this charge would repel the distal regions of LAT away from the membrane into the cytoplasm, which would facilitate the

phosphorylation of conserved tyrosines and the subsequent formation of dynamic signaling complexes.

Phosphorylation of conserved tyrosines found in the C-terminal tail of LAT result in the binding with key intracellular signaling proteins with LAT. The N-terminal SH2 domain of PLC-γ1 binds directly to phosphorylated LAT tyrosine 132 (10-13, 28, 30). Mutation of LAT Y132 to phenylalanine markedly reduces the phosphorylation of PLC-γ1 and its association with LAT. The related adaptor proteins GRB2 and GADS directly associate with phosphorylated LAT tyrosines 171, 191 and 226 (10-13). However, the maximal association between LAT and these signaling proteins requires the presence of multiple tyrosines (13, 42). Maximal binding of PLC-γ1 requires LAT Y132 and LAT Y191. Similarly, optimal binding between LAT and GADS requires the presence of LAT Y171, LAT Y191 and LAT Y226 together. One multiprotein complex that bridges the interaction motifs present on the second and third functional domains is the LAT/PLC-γ/SLP-76/GADS complex (23, 24). PLC-γ1 and GADS indirectly associate with each other while bound to LAT through the simultaneous binding of SLP-76 and/or other binding partners (23, 24). The presence of LAT Y132 and all three GADS binding tyrosines is required for calcium influx and NFAT activation, highlighting the importance of this complex for optimal PLC-γ activity.

Although the formation of the LAT/PLC-γ/SLP-76/GADS complex is functionally critical for signaling downstream of the TCR, no studies have examined if the relative order of the two domains in relation to each other and the plasma membrane are required for LAT function. To address this question, we expressed a mutant of LAT where the second and third functional domains of LAT were switched in order, such that the PLC-γ binding region was now C-terminal to the GADS/GRB2 binding domains. We found that the LAT KD and LAT 132 mutant had markedly reduced ZAP-70 phosphorylation compared to wild-type LAT (Fig. 2), suggesting that the LAT signaling complex regulates ZAP-70 phosphorylation. The impact of LAT expression on ZAP-70 phosphorylation is controversial. Samelson and coworkers found that ZAP-70 Y319 phosphorylation was reduced in LAT-deficient JCaM2.5 cells compared to Jurkat E6.1 cells (37), while Weiss and coworkers found no difference in total ZAP-70 phosphorylation between Jurkat E6.1 and JCaM 2.5 cells (18). Using quantitative mass spectrometry, Acuto and coworkers found ZAP-70 Y473 phosphorylation was enhanced in LAT-deficient JCaM2.5 cells compared to LAT-sufficient JCaM2.5 cells (29). Aguado et al found that ZAP-70 Y319 phosphorylation was reduced in LAT-sufficient JCaM2.5 cells compared to JCaM2.5 parental cells (43). There could be several reasons for the discrepancy between our current studies and the previously published work. First, all the previous studies used LAT deficient JCaM2.5 cells while our work utilized Jurkat E6.1 cells transduced with LAT-specific microRNAs. It is possible that the mutagenized JCaM2.5 cells and transduced Jurkat E6.1 cells have different regulation of ZAP-70 phosphorylation. Second, the sites of phosphorylation on ZAP-70 are different between the studies. This current study and previous study by the Samelson laboratory showing decreased ZAP-70 phosphorylation in JCaM2.5 cells both examined the phosphorylation of ZAP-70 Y319 (37), while the studies by Weiss and coworkers and Acuto and coworkers examined total ZAP-70 or ZAP-70 Y493 phosphorylation, respectively (18, 29). An argument against this hypothesis is that work by Aguado and coworkers observed increased ZAP-70 Y319 phosphorylation JCaM2.5 parental cells compared to LAT-sufficient JCaM2.5 cells (43). It is

possible that the phosphorylation of individual sites on ZAP-70 are differentially regulated by LAT expression. One potential mechanism for the ability of LAT to control ZAP-70 phosphorylation is the recent finding that LCK interacts with LAT (33), suggesting a potential regulatory mechanism. Further studies are required to fully understand the regulation of site-specific ZAP-70 phosphorylation by the LAT signaling complex.

Similar to the LAT 23 mutant, we found that TCR-mediated LAT Y226 phosphorylation and the formation of large LAT clusters were similar between LAT WT and LAT 132 (Fig. 2 and Fig. 3). Interestingly, LAT phosphorylation at Y132 was significantly enhanced in the LAT 132 mutant compared to the LAT WT cells. This suggests that the correct orientation of the domains of LAT are required for regulating LAT Y132 phosphorylation. However, in contrast to LAT 23, the LAT 132 mutant had reduced levels of SLP-76 phosphorylation and cluster intensity upon TCR stimulation compared to LAT WT cells, although the numbers of SLP-76 clusters were similar between the cell lines (Fig. 2 and Fig. 3). In fact, LAT KD and LAT 132 had similar amounts of SLP-76 phosphorylation and cluster intensity, indicating that the correct order of LAT tyrosines in comparison to the plasma membrane is required for the productive interaction between LAT and SLP-76. In contrast, the LAT 132 mutant had little to no reduction in the TCR-mediated phosphorylation of PLC-γ1 and reduced numbers of PLC-γ1 clusters, but had markedly reduced influx of cytoplasmic calcium upon TCR activation (Fig. 2). Subsequently, the LAT 132 mutant had reduced TCR-induced ERK1/ERK2 phosphorylation, cellular adhesion, and cytokine production. Together, these data show that the correct order of LAT tyrosines to each other and the plasma membrane is critical SLP-76 phosphorylation/clustering and optimal stimulation of signaling downstream of LAT (Fig. 5).

5. Conclusions:

These studies found that LAT has several conserved functional domains that are required for the ability of LAT to transmit TCR-induced intracellular signals. We found that the proline rich region of LAT is required for early TCR-induced signaling, cytokine production and cellular adhesion, suggesting that it acts as a spacer domain that correctly orients the membrane distal signaling domains LAT in comparison to the plasma membrane. We also observed that the orientation of LAT tyrosines to each other and the plasma membrane is also required for early TCR-induced signaling, cytokine production and cellular adhesion, indicating that the correct tyrosine order is vital for the formation of the LAT/PLC-γ/SLP-76/GADS complex and subsequent downstream signaling events. These studies show conclusively that LAT contains functional domains whose presence and correct order in comparison to the plasma membrane is required for the ability of LAT to facilitate signaling events downstream of TCR stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Alignment of 97 species of LAT identifies several “functional” domains in LAT
- LAT proline-rich region is required for optimal TCR-mediated signaling and function
- The order of tyrosines on LAT is vital for TCR-induced signaling and function
- LAT is unstructured and has functional domains critical for it’s signaling capacity

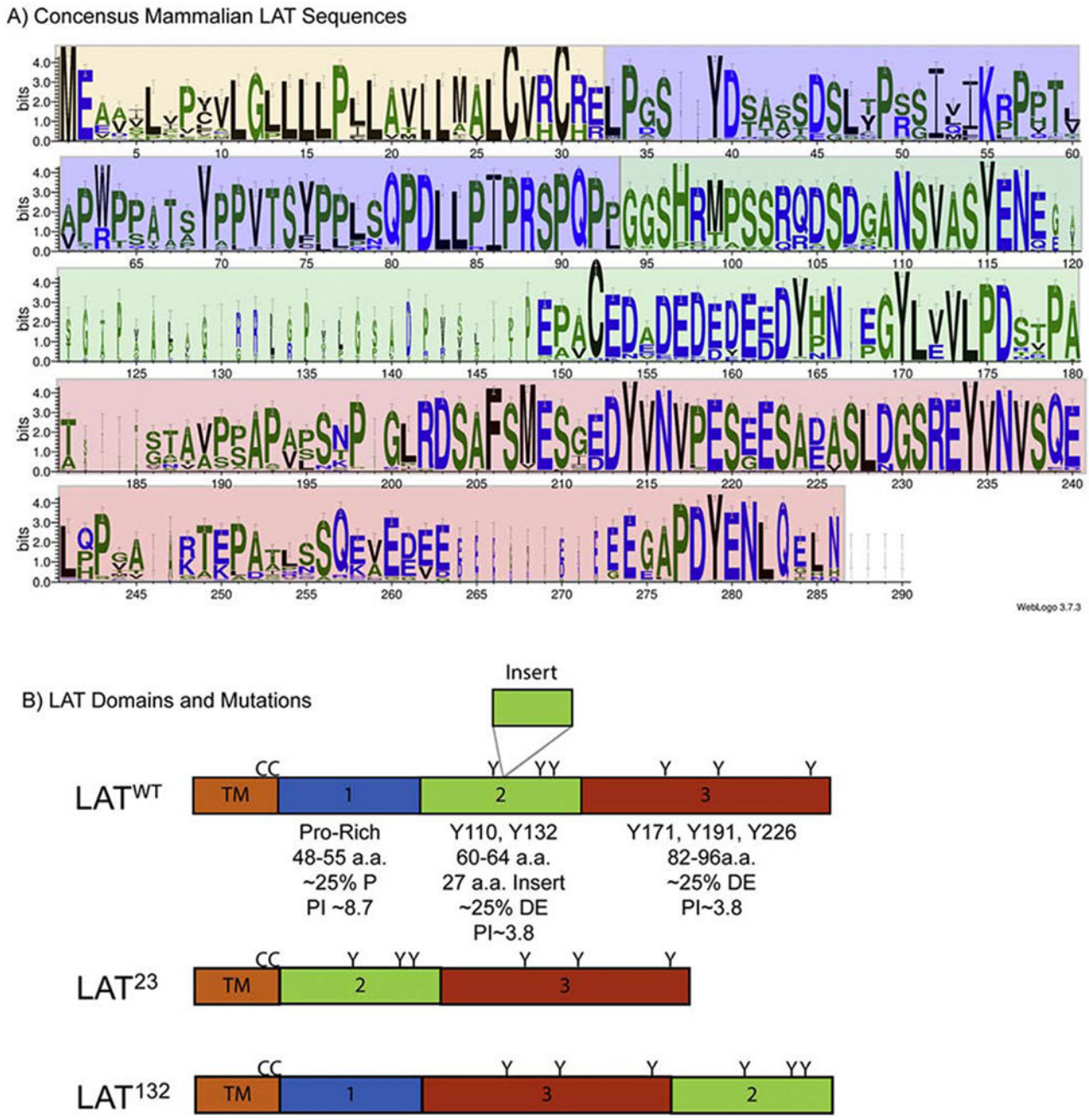

Figure 1: LAT has conserved functional domains in mammals.

A) Amino acid conservation for 97 mammalian LAT sequences. B) Cartoon of the domain structure of LAT based on functional domains identified from the alignment of mammalian LAT sequences and two LAT mutants used in this study. LAT 23 removes the membrane proximal proline-rich region and LAT 132 swaps the order of the two functional domains surrounding the conserved tyrosines.

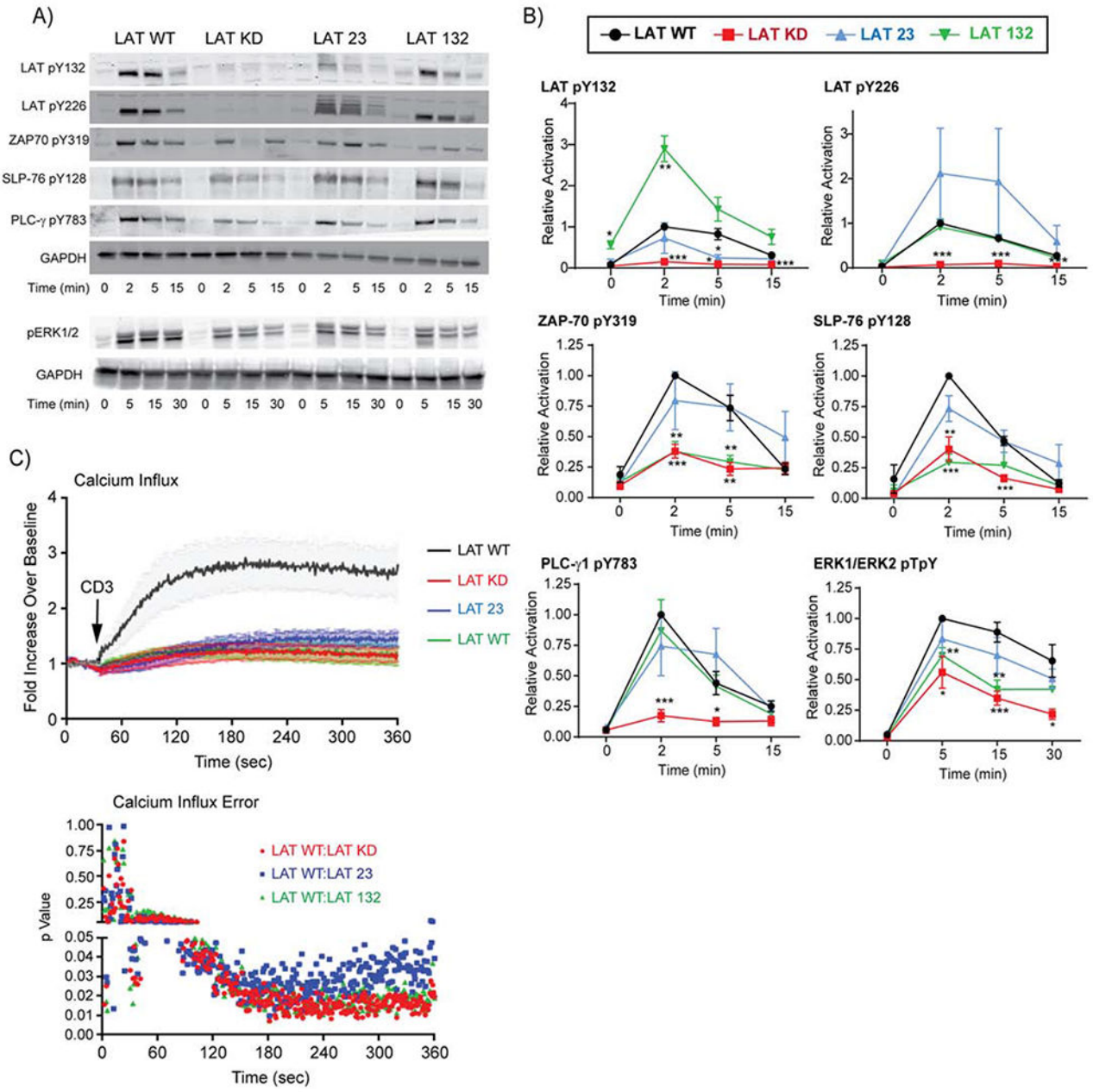

Figure 2: LAT 23 and LAT 132 mutants drive reduced proximal TCR-induced signaling and diminished calcium influx.

A) Jurkat T cells with reduced LAT expression or expressing LAT WT or LAT mutants were stimulated with anti-CD3 for various times. The site-specific phosphorylation of LAT pY132, LAT pY226, ZAP70 pY319, SLP-76 pY128, PLC-γ1 pY783 and pERK1/pERK2 pT185/pY187 were examined by immunoblotting. Data are representative of 4-5 independent experiments. B) The immunoblots were quantified and normalized to GAPDH expression. Data from 4-5 independent experiments were compiled and the results plotted as averages ± SEM. The results were statistically compared using Students t test and one sample t test. * $p<0.05$, ** $p<0.01$, ***$p<0.001$. C) Jurkat T cells with reduced LAT expression or expressing LAT WT or LAT mutants were stimulated with anti-CD3 and

calcium influx was measured by flow cytometry using Fluo-4. The data is shown as fold increase of fluorescent intensity over baseline. The data is plotted as the average ± SEM of 4 independent experiments in the upper panel. The lower panel shows a statistical analysis at all timepoints comparing LAT WT to LAT KD (red dots), LAT 23 (blue dots) and LAT 132 (green dots).

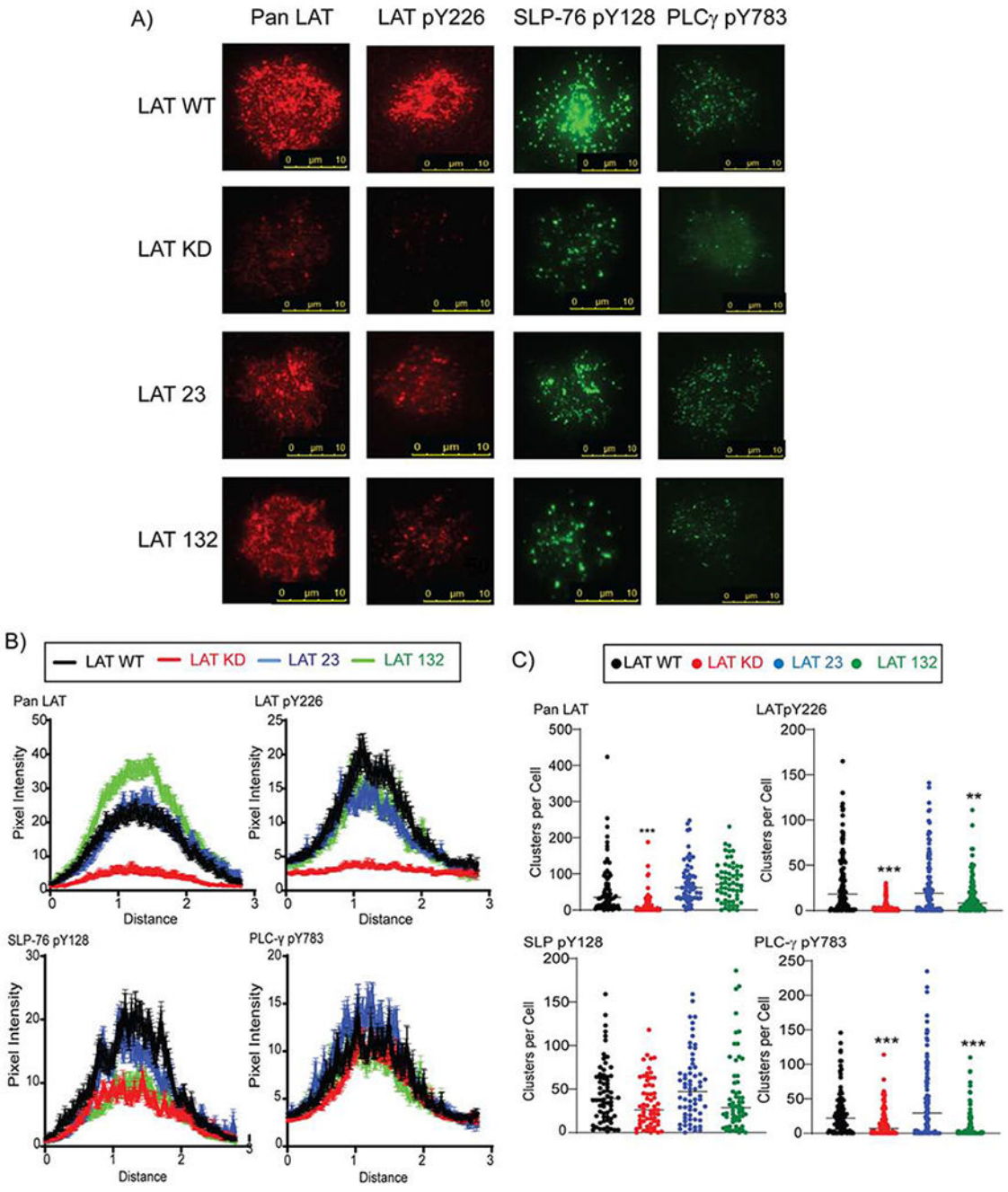

Figure 3: LAT 132 has reduced proximal TCR-induced protein clustering.

A) Jurkat T cells with reduced LAT expression or expressing LAT WT or LAT mutants were stimulated with plate bound anti-CD3 for 10 minutes. The TCR induced clustering of panLAT, LAT pY226, SLP-76 pY128, or PLC-γ1 pY783 were examined by TIRF microscopy. A representative cell from 3 independent experiments is shown. B) The fluorescence intensity across the axis of the 30-60 total cells from 2-3 independent experiments was measured using the Plot-Profile analysis function on ImageJ. The data is then plotted as the average ± SEM. C) The number of clusters per cell were measured for 30-40 total cells from 2-3 independent experiments as described in Materials and Methods.

The data is then plotted and statistically tested using Brown-Forsythe and Welch ANOVA test using the Dunnett's multiple comparison test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

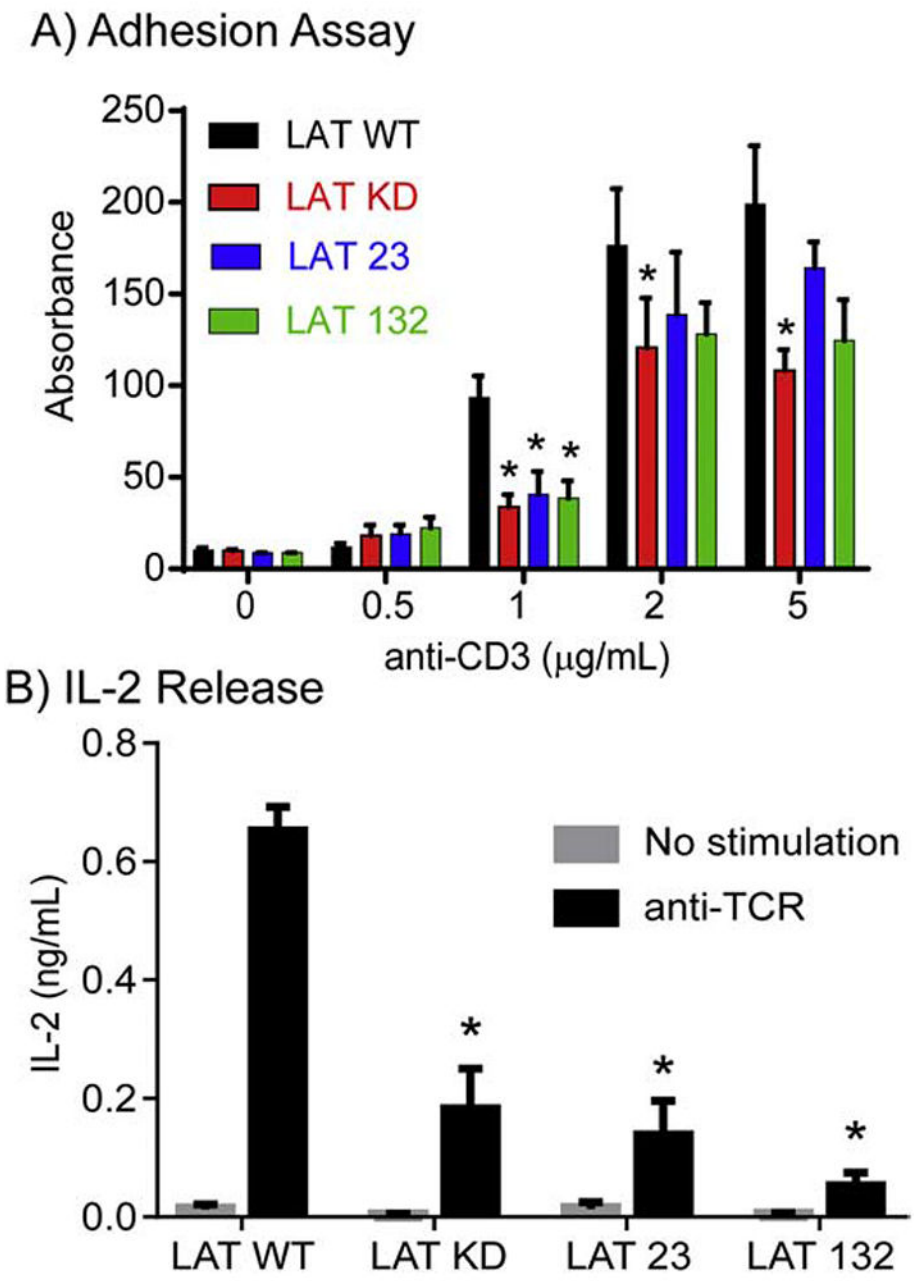

Figure 4. LAT 23 and LAT 132 mutants have decreased TCR mediated cellular adhesion and IL-2 release.

(A) Jurkat cells expressing LAT mutants were stained and then stimulated with various doses of plate-bound anti-CD3 for 30 minutes on a 96-well EIA/RIA plate. The intensity of the membrane dye was measured using a LiCor Odyssey imager to determine the relative number of cells bound to each well and intensity of triplicate wells were averaged. The average ± SEM of 3 independent experiments are shown. Statistical analysis was done using ANOVA with multiple comparisons. **(B)** Jurkat cells expressing LAT mutations were stimulated with 2 μg/mL plate-bound anti-CD3 for 24 h and IL-2 release was measured by

ELISA. The mean value ±SEM of 3 independent experiments are shown. The results were statistically compared using ANOVA with multiple comparisons. * $p<0.05$

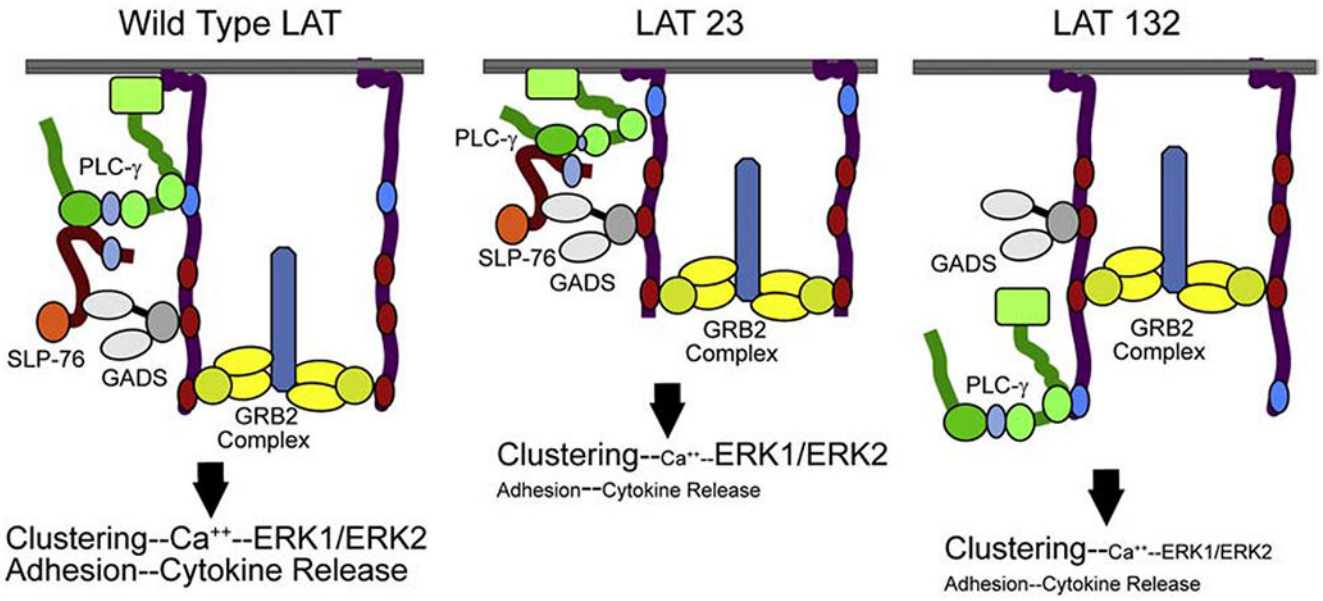

Figure 5. Model of the function of LAT mutants downstream of the TCR.

TCR stimulation of T cells expressing WT LAT results in the clustering LAT, SLP-76 and PLC-γ1 and the subsequent stimulation of calcium influx and intracellular signaling. These events induce T cell adhesion and cytokine release. T cells expressing LAT 23 mutant has a similar LAT, SLP-76 and PLC-γ1 clustering and phosphorylation of LAT, ZAP-70, SLP-76 and ERK1/ERK2. However, these cells have decreased TCR-induced calcium influx, leading to lower cellular adhesion and cytokine release. T cells expressing the LAT132 mutant have decreased SLP-76 clustering and phosphorylation, causing diminished calcium influx and ERK1/ERK2 phosphorylation and reduced cellular adhesion and cytokine release.