Association of Grb2, Gads, and Phospholipase C-γ1 with Phosphorylated LAT Tyrosine Residues

EFFECT OF LAT TYROSINE MUTATIONS ON T CELL ANTIGEN RECEPTOR-MEDIATED SIGNALING*

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The linker for activation of T cells (LAT) is a critical adaptor molecule required for T cell antigen receptor (TCR)-mediated signaling and thymocyte development. Upon T cell activation, LAT becomes highly phosphorylated on tyrosine residues, and Grb2, Gads, and phospholipase C (PLC)-γ1 bind LAT via Src homology-2 domains. In LAT-deficient mutant Jurkat cells, TCR engagement fails to induce ERK activation, Ca^2+ flux, and activation of AP-1 and NF-AT. We mapped the tyrosine residues in LAT responsible for interaction with these specific signaling molecules by expressing LAT mutants with tyrosine-to-phenylalanine mutations in LAT-deficient cells. Our results showed that three distal tyrosines, Tyr171, Tyr191, and Tyr226, are responsible for Grb2-binding; Tyr171, and Tyr226, are necessary for Gads binding. Mutation of Tyr132 alone abolished PLC-γ1 binding. Mutation of all three distal tyrosines also abolished PLC-γ1 binding, suggesting there might be multiple binding sites for PLC-γ1. Mutation of Tyr132 affected calcium flux and blocked ERK and NF-AT activation. Since Grb2 binding is not affected by this mutation, these results strongly suggest that PLC-γ1 activation regulates Ras activation in these cells. Mutation of individual Grb2 binding sites had no functional effect, but mutation of two or three of these sites, in combination, also affected ERK and NF-AT activation.

The integral membrane adaptor protein, linker for activation of T cells (LAT), is a substrate of the protein tyrosine kinases (1, 2). Upon LAT tyrosine phosphorylation, multiple adaptor molecules and enzymes critical to T cell activation bind to LAT directly by virtue of the SH2 domains that these proteins contain. These proteins can also bring other associated molecules to these LAT-nucleated complexes. Co-immunoprecipitation experiments have demonstrated that following TCR binding, phosphorylated LAT associates with Grb2, Gads, SOS, PLC-γ1, Vav, SLP-76, Cbl, and the p85 subunit of phosphatidylinositol 3-kinase. The enzymes recruited by LAT can themselves be activated in these complexes by tyrosine phosphorylation and find higher concentrations of their substrates in the plasma membrane. In particular, phospholipase-Cγ is activated by tyrosine phosphorylation to cleave phosphoinositides into diacylglycerol, an activator of protein kinase C, and inositol-1,4,5-trisphosphate (IP3), which induces intracellular calcium elevation (3). Tyrosine phosphorylation of Vav, the guanine nucleotide exchange factor for the small G proteins Rac, Rho, and cdc42, also activates its enzymatic activity (4). Phosphatidylinositol 3-kinase and SOS, the guanine nucleotide exchange factor for Ras, are not activated by tyrosine phosphorylation in T cells but are recruited to the membrane following receptor engagement via interaction with adaptor proteins (5, 6).

The central role of LAT in T cell activation has also been demonstrated in several other experiments. The LAT gene has been disrupted by gene targeting experiments, and intrathymic development in the resulting mice is completely blocked at an early stage (7). Thus receptor-mediated events in immature T cells are dependent on the presence of LAT, and presumably, the molecules LAT recruits in these cells. In Jurkat T cells, overexpression of a mutant form of LAT lacking two tyrosine residues predicted to bind Grb2 had a dominant negative effect on T cell signaling (1). Following TCR activation of cells expressing this mutant, a number of signaling molecules failed to bind to the mutant LAT molecule, and TCR-coupled transcriptional events, AP-1 and NF-AT activation, were partially disrupted. The functional significance of the LAT molecule in signaling events coupled to the TCR was clearly demonstrated in the study of two independently derived Jurkat T cell variants (8, 9). Both of these lines were shown to be deficient in LAT expression. TCR engagement in these two lines failed to induce tyrosine phosphorylation of PLC-γ1, calcium elevation, Ras and Erk activation, or transcriptional activation of AP-1 and NF-AT. These defects were corrected by expression of wild-type LAT.

The importance of this molecule in regulating both intrathymic T cell development and activation of mature T cells, as modeled by the Jurkat cell line, led us to investigate the specificity of binding to LAT. Can the interaction of multiple signaling molecules be mapped to particular LAT tyrosine resi-
dues? The existence of the LAT-deficient cell lines enabled us to perform such a structure-function analysis of the LAT protein. In this study we stably express LAT tyrosine mutants to test the role of individual tyrosines and combinations of tyrosine residues in the cytosolic tail of LAT. We demonstrate that specific tyrosine residues do bind distinct signaling molecules, showing that one particular tyrosine residue (Tyr132) is critical for LAT function and thus for T cell activation. Other individual tyrosines when mutated alone have no effect on LAT binding and signaling, but combinations of these mutations have a deleterious effect on signaling initiated through the TCR.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids—The antibodies used in our studies are: rabbit polyclonal anti-LAT (1), the C305 monoclonal antibody binding the Jurkat TCR (10), anti-Gads (11), and anti-Erk2 (Santa Cruz Bio-technology), monoclonal anti-SLP-76 (Transduction Laboratory), anti-CD3ε (OKT3), anti-Myc (9E10), and monoclonal anti-PLC-y1 and anti-phosphotyrosine (4G10) from Upstate Biotechnology. Mutant LAT constructs in the mammalian expression vector pCEP7 were made by site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene). Mutations were confirmed by automated sequencing. This vector contains the EF promoter and the neo gene.

Cell Culture, Transfection, and Immunoprecipitation—Jurkat cells (E6.1) and LAT-deficient cells (J.CaM2.5) (8) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. To establish stable cell lines expressing different LAT mutants, J.CaM2.5 cells were electroporated with 10 μg of plasmid under conditions described previously (1). Stable clones were selected in the presence of 1.2 mg/ml G418. All of the transfectants were screened for TCR expression by fluorescence-activated cell sorter analysis and for LAT expression by anti-LAT immunoblotting. For immunoprecipitation, Jurkat cells (106 cells/ml) were either stimulated with C305 (1:50 tissue culture supernatant) for 1.5 min or left untreated. Cells were lysed in ice-cold lysis buffer (1% Brij, 25 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4) with protease inhibitors. Protein samples were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with monoclonal antibody or rabbit polyclonal antiserum. Immunoreactive proteins were detected with horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech) followed by detection with ECL (Amersham Pharmacia Biotech).

Ca2+ Flux, Luciferase Assay, and Erk Kinase Assay—The measurement of intracellular free Ca2+ was done using Fluo-3-AM (6 μM/ml) and Fura-Red (10 μM/ml) (Molecular Probes). Cells were preloaded with the dyes for 30 min at 37 °C and then kept at room temperature for 20 min. Cells preloaded with dyes were washed with RPMI without fetal bovine serum once and analyzed by flow cytometry (FACScan, Becton Dickinson). OKT3 ascites (1:100) were used to induce Ca2+ flux in those stable transfectants. Ca2+ levels were indicated by Fluo-3/Fura-Red fluorescence intensity ratio determined using PCSAsistant software. For the luciferase assay, 1.6 × 106 cells were transfected with 1.5 μg of pNFAT-luciferase plasmid and 0.5 μg of pCEFL-LAT plasmid. 24 h after transfection, the cells were stimulated with OKT3 coated on microtiter plates and phorbol 12-myristate 13-acetate (10 ng/ml) plus ionomycin (1.5 μM) or left untreated for 6 h. Luciferase activity was assayed according to the manufacturer's protocol (Promega). For the Erk kinase assay, Jurkat cells were stimulated with C305 for 10 min or left untreated. Anti-Erk2 immunoprecipitates were resuspended in kinase reaction buffer (20 mM Tris-C1, pH 7.6, 13 mM MgCl2, 1.5 mM EGTA). The kinase reaction was performed using 10 μg myelin basic protein as a substrate and 5 μCi of [γ-32P]ATP per reaction.

RESULTS

Transfection of LAT Mutants into LAT-deficient Jurkat Cells—The LAT-deficient Jurkat cell line, J.CaM2.5, is defective in Ras-MAPK activation and Ca2+ flux in response to anti-TCR or anti-CD3 antibody stimulation (8). We used this cell line to establish stable transfectants expressing different LAT mutants containing substitutions of critical tyrosines with phenylalanines. Based on the extensive characterization of SH2 binding preferences, one can determine that sequences around tyrosines 171 (YYVN), 191 (YYVN), and 226 (YENL) form classical Grb2-binding motifs (YXN), whereas Tyr132 (YLVV) is in a potential PLC-γ1-binding motif (12). To make constructs expressing mutant forms of LAT, each of these tyrosines was mutated to phenylalanine either separately or in different combinations. LAT 2YF, with mutations at Tyr171 and Tyr191, was previously shown to inhibit NF-AT- and AP-1-mediated transcription when overexpressed in wild-type Jurkat cells (1). 3YF, with mutations at Tyr171, Tyr191, and Tyr226, was made to remove these three potential Grb2-binding motifs to test the effect of these mutations on the LAT-Grb2 interaction. We also made a mutant, 4YF, with mutations at Tyr132, Tyr171, Tyr191, and Tyr226. These constructs were introduced into J.CaM2.5 cells by electroporation. Stable clones were selected with G418. All clones were screened for LAT expression, as well as for CD3 surface expression as determined by flow cytometry using an anti-CD3 antibody (OKT3). Multiple clones for each mutant were obtained and analyzed biochemically and functionally. Although we did extensive screening of many clones for each mutant, it was very difficult to match TCR and LAT expression exactly among different stable transfectants. In these experiments, we show that all but one of the cell lines expressing mutant LAT are well matched for expression of the transfected cDNA (Fig. 1). The conclusions that we draw about LAT function in TCR signaling take these clonal variations into consideration.

Tyrosine Phosphorylation of LAT Mutants—To test whether mutant LAT molecules from these stable transfectants could be tyrosine phosphorylated upon TCR ligation, we activated these transfectants with an anti-TCR antibody (C305), immunoprecipitated the Myc-tagged LAT with anti-Myc antibody, and detected LAT phosphorylation by anti-phosphotyrosine blotting. As shown in Fig. 2, all of the mutant LAT molecules were tyrosine phosphorylated following activation. The tyrosine phosphorylation of the Y226F protein appeared to be low compared with other mutant LAT molecules, but this was probably due to the low level of expression of this mutant, as shown in Figs. 1 and 2. Surprisingly, the LAT 4YF mutant, which had mutations at four tyrosines, was still tyrosine phosphorylated after TCR stimulation, suggesting that tyrosines other than Tyr132, Tyr171, Tyr191, and Tyr226 are also phosphorylated. Endogenous LAT in E6.1 Jurkat cells was not detected in these anti-Myc immunoprecipitates.

Association of LAT with Grb2 Adaptor Protein—LAT has several potential Grb2-binding sites. To see whether the association of LAT with Grb2 was affected by mutations at the distal three, Tyr171, Tyr191, and Tyr226 or at the Tyr132 site, we immunoprecipitated Grb2 from activated Jurkat cells and detected the presence of LAT with an anti-phosphotyrosine immunoblot. As shown in Fig. 3, Grb2 could associate with most LAT mutants except 3YF and 4YF. The amount of LAT asso-
Association of Grb2 with LAT correlated with the amount of LAT expression (as shown in Fig. 1) for WT, Y132F, Y171F, Y191F, and Y226F. The fact that mutation of these tyrosines individually did not abolish Grb2-binding indicates that there are multiple binding sites for Grb2. The amount of 2YF mutant associated with Grb2 was significantly reduced compared with Y191F (Y191F and 2YF had similar amounts of LAT expression), suggesting that Tyr171 is one of the Grb2-binding sites. There was no LAT-Grb2 association in 3YF, indicating that Tyr226 is also involved in LAT-Grb2 association. We can conclude that at least Tyr171 and Tyr226 bind Grb2 upon phosphorylation. Since Tyr191 has the same binding motif as Tyr171, YVNY, Tyr191 might also be a Grb2-binding site. Other potential Grb2-binding sites seem not to be involved in Grb2 binding.

Association of LAT with Gads Adaptor Protein—Gads is a newly identified Grb2-like adaptor protein exclusively expressed in hematopoietic tissues. It has been isolated independently by a number of investigators (13–17). The SH2 domain of Gads was shown to have a similar binding specificity as that of the Grb2 SH2 domain (13). It was reported that the SH2 domain of Gads binds LAT and its C-terminal SH3 domain binds the proline-rich region of SLP-76. It was thought that Gads bridges the association of SLP-76 with LAT. We immunoprecipitated Gads from lysates of C305-stimulated transfectants. The association of Gads with LAT was detected using anti-phosphotyrosine antibody. As shown in Fig. 4, this antibody precipitated similar amounts of Gads detected by anti-Gads blotting and of SLP-76 detected by anti-phosphotyrosine blotting. LAT was present in Gads immunoprecipitates from C305-stimulated Jurkat cells using rabbit anti-Gads antiserum. SLP-76 was identified with an anti-SLP-76 antiserum. Gads protein was immunoprecipitated from resting or C305-stimulated Jurkat cells lysed with 1% Brij lysis buffer. The presence of LAT in the Gads immunoprecipitate was detected with an anti-phosphotyrosine (anti-pY) blot. Equal amounts of Grb2 were immunoprecipitated from each cell as indicated by the anti-Grb2 blot.

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between SLP-76 and LAT, we also examined the association of LAT with SLP-76 (Fig. 2). SLP-76 is present in anti-LAT immunoprecipitates from WT transfectants. The amount of SLP-76 associated with LAT was reduced slightly in Y191F and was abolished in the 2YF, 3YF, and 4YF mutants. Y226F had a similar amount of SLP-76-associated LAT even though its expression was relatively low. It appeared that more SLP-76 was associated with Y171F compared with WT. However, since the expression level of Y171F was the highest among all these stable clones, the mutation of Tyr171 alone might not have any effect on LAT association with SLP-76. These results confirm the above conclusion that Tyr171 and Tyr191 are the only two sites involved in Gads and thus in SLP-76 binding.

Association of LAT with PLC-γ1—We also examined the association of LAT with PLC-γ1. Previous studies showed that LAT was required for PLC-γ1 tyrosine phosphorylation and subsequent Ca2+ flux (18). LAT has one predicted PLC-γ1-binding site at Tyr132 (YLV). PLC-γ1 was immunoprecipitated from the wild-type and mutant LAT transfectants activated with C305. As shown in Fig. 5, mutation of this tyrosine completely abolished the association of LAT with PLC-γ1, indicating that Tyr132 is involved in the binding of PLC-γ1. However, mutations at other tyrosines also affected the LAT-
PLC-γ association. Compared with WT, the associations of the Y171F and Y226F mutants with PLC-γ were not affected. In Y191F, this association was obviously reduced. When both Tyr171 and Tyr191 were mutated as in 2YF, a similar amount of 2YF LAT was associated with PLC-γ as was bound in the Y191F mutant, suggesting that Tyr171 might not be involved in LAT-PLC-γ interaction. Furthermore, when Tyr171, Tyr191, and Tyr226 were all mutated (3YF), the LAT-PLC-γ association was abolished, suggesting that phosphorylation at Tyr226 might also contribute to LAT-PLC-γ interaction. We also examined whether the tyrosine phosphorylation of PLC-γ is affected by these mutations. The tyrosine phosphorylation of PLC-γ was restored in WT and in those mutants (Y171F, Y191F, Y226F, and 2YF) in which LAT interacted with PLC-γ. Phosphorylation of PLC-γ in Y132F and 3YF transfectants was only slightly increased compared with that in J.CaM2.5. In the 4YF transfectant, PLC-γ was not tyrosine phosphorylated at all after TCR ligation as in J.CaM2.5.

**LAT in Erk Kinase Activation**—It has been proposed that phosphorylated LAT recruits the Grb2Sos complex to the plasma membrane where Sos activates Ras followed by activation of the MAPK pathway (1). In LAT-deficient cells, Erk activation after TCR ligation was defective, and transfection of LAT into these cells reconstituted Erk activation. We next tested Erk kinase activation in the LAT stable transfectants using myelin basic protein as a substrate. As shown in Fig. 6, the activation of Erk extracted from J.CaM2.5 was greatly reduced compared with E6.1 cells. Expression of WT, Y171F, Y191F, and Y226F forms of LAT reconstituted the TCR-mediated Erk activation. This could be explained by the observation that mutation at Tyr171, Tyr191, or Tyr226 did not result in a significant loss of Grb2 association. However, Y132F, 2YF, 3YF, and 4YF all failed to restore Erk activation. The failure of Erk activation in these transfectants was not due to a kinetic difference in Erk activation (data not shown). The failure of these mutants to restore Ras-MAPK activation could be due to two different mechanisms. As shown in Fig. 3, the binding of LAT to Grb2 was not affected by the Y132F mutation, suggesting that the recruitment of the Grb2Sos complex to other sites was not sufficient for Erk activation. Activation of PLC-γ following interaction at the Tyr132 site and subsequent activation of PKC could be necessary for Erk activation mediated via the TCR. The effect of Tyr171, Tyr191, and Tyr226 mutations as shown in 2YF and 3YF could be due to reduced Grb2 binding to LAT and thus less recruitment of the Grb2Sos complex to the plasma membrane. In this model, the recruitment of Grb2Sos and the activation of PLC-γ and PKC are required for activation of Erk after T cell activation. Additionally, the decrease in Erk activation in these mutants could also reflect, to some extent, decreases in PLC-γ activation as discussed above.

**Ca2⁺ Flux in Different LAT Mutant Transfectants**—LAT is required for Ca2⁺ flux following T cell activation as demonstrated in LAT-deficient cells (8, 9). We further analyzed the LAT transfectants to see whether the LAT mutations affected Ca2⁺ flux initiated via the TCR. Mutation at either Tyr171, Tyr191, or Tyr226 had no effect on Ca2⁺ flux after addition of OKT3 (data not shown). Mutations at both Tyr171 and Tyr191 or at Tyr171, Tyr191, and Tyr226 also had no obvious effect on Ca2⁺ mobilization (Fig. 7A). Mutation of all four tyrosines, 4YF (Tyr132, Tyr171, Tyr191, and Tyr226) led to defective Ca2⁺ flux in this transfectant. Mutation of Tyr132 alone had a marked effect on Ca2⁺ flux compared with WT transfectants. In this particular transfectant, Ca2⁺ flux in the initial phase was normal or elevated, whereas in the sustained phase calcium levels were much reduced (Fig. 7B). To confirm this result, we also tested several different clones of Y132F with different levels of LAT expression. All of these clones showed a similar phenotype (data not shown). A number of conclusions follow from these data. The results suggest that the association of LAT with PLC-γ is not essential for the initial Ca2⁺ flux. In both Y132F and 3YF, the interaction of PLC-γ and LAT was abolished by these mutations. However, both of these transfectants flux Ca2⁺, although the interaction of PLC-γ with Y132 appears necessary for normal calcium dynamics. In addition, optimal phosphorylation of PLC-γ might not be necessary for Ca2⁺ flux initiated via the TCR. The tyrosine phosphorylation of PLC-γ in Y132F and 3YF was much less than in WT (Fig. 5). Nevertheless, the observation that 4YF fails to flux Ca2⁺ like LAT deficient-J.CaM2.5 demonstrates that interactions mediated by some combination of these residues is critical to the calcium response.

**LAT in NF-AT Transcription Activation**—Since many of the tyrosine to phenylalanine mutations affected Erk activation and Ca2⁺ flux, we next tested whether these mutations affected NF-AT-mediated transcription following T cell activation. We transiently transfected the NFAT/luciferase reporter construct with different LAT mutant constructs separately. The luciferase expression is driven by a promoter consisting of tandem AP-1 and NF-AT binding sites. As shown in Fig. 8, cotransfection of WT LAT into the J.CaM2 cells restored the NF-AT-mediated transcription following OKT3 stimulation, whereas
cotransfection of the empty vector (pCEFL) had no effect. Transfection with the Y132F LAT mutant failed to reconstitute NF-AT transcription. In contrast, expression of the other single tyrosine to phenylalanine mutants restored NF-AT activation. Compared with WT, NF-AT activation in Y226F was reduced even though Erk activation and Ca$^{2+}$ flux appeared to be normal. This assay may be sensitive to the lower levels of LAT protein seen in this clone (as above). NF-AT activation in 2YF was not restored to normal levels. No NF-AT activation over base-line levels was observed following activation of the 3YF or 4YF mutants. The NF-AT reporter construct contains a composite binding site for both NF-AT and AP-1. Thus, a defect in either AP-1 or NF-AT activation would lead to failure of transcriptional activation as indicated by the luciferase reporter. The failure of Erk activation demonstrated in the Y132F and the 3YF mutants as well as Ca$^{2+}$ flux abnormalities in Tyr$^{132}$ and 4YF explain the failure of NF-AT activation detected in this assay.

**DISCUSSION**

The importance of LAT for T cell signaling has been documented using various experimental approaches. Extensive co-immunoprecipitation studies before and after the cloning of LAT demonstrated that this molecule, after phosphorylation on its multiple tyrosines, interacts with several critical signaling proteins including both enzymes and adaptor proteins (1, 6, 19, 20). Gene-targeting experiments demonstrated that the absence of LAT expression had a profound effect on T cell development (7). No mature T cells were observed in mice without LAT. The block in thymocyte development occurred at a stage at which cells bear the pre-TCR and require intact signaling pathways to induce TCR $\alpha$-chain rearrangement and expression of CD4 and CD8 co-receptor molecules.

In the absence of normal T cells lacking LAT, the study of the role of LAT in mature T cells has depended on the Jurkat tumor line model. Two independently derived LAT-deficient Jurkat cell lines have been shown to be defective in many signaling events dependent on TCR cross-linking, including Ras-MAPK activation, Ca$^{2+}$ flux, and NF-AT transcription, although the activation of the protein tyrosine kinases that function in TCR signaling was normal in these cells (8, 9). All of these defects were corrected by re-expression of LAT following transfection. These cells have been successfully used to study the importance of two cysteine residues for LAT palmitoylation, intracellular localization, and signaling (9, 21).

We used LAT-deficient Jurkat T cells in this study to probe the contribution to the TCR signaling pathway of individual
and sets of tyrosine residues in the cytosolic tail of LAT (Fig. 9). The three most distal tyrosine residues (residues 171, 191, and 226) are in the sequence context YXNX, which is a perfect motif for Grb2 SH2 binding after phosphorylation. Independent mutation of each of these YXNX sites showed only slight quantitative effects in biochemical and functional studies. However, because of the difficulty of exactly matching LAT and TCR-CD3 levels among those transfected, and because of other unexplained clonal variations, it is difficult to be certain of the significance of these subtle defects.

The significance of at least two of these tyrosine residues, Tyr171 and Tyr191, was shown in our previous study in which overexpression of mutant LAT with the double Y171F/Y191F mutation resulted in inhibition of LAT association with other signaling molecules and reduced activation of AP-1 and NF-AT following TCR cross-linking (1). In the current study, the double Y171F/Y191F mutation had a significant effect on molecular binding and function. A decrease in Grb2 and PLC-γ1 and a loss of Gads binding were observed. The interaction of SLP-76 with LAT was also affected in these mutants. The loss of SLP-76 binding that occurred with the 2YF could be explained by previous studies showing that Gads mediates the association of SLP-76 with LAT (11, 16, 17). The defects observed with the 2YF mutant might explain the functional inhibition caused by overexpression of this mutant in WT Jurkat cells. The triple 3YF failed to bind Grb2 or Gads. From these data, we conclude that there is a subtle difference in the binding of the Grb2 and Gads SH2 domains. Grb2 can bind Tyr171, Tyr191, and Tyr226 when they are phosphorylated, and Gads can only bind Tyr171 and Tyr191 but not Tyr226.

An unexpected result was the loss of binding of PLC-γ1 in the 3YF (Y171F/Y191F/Y226F) mutant. Neither of the PLC-γ1 SH2 domains would be predicted to bind these three sites with high affinity, although it is possible that one of these SH2 domains binds a more favorable site (see below), the other would tolerate a lower affinity interaction. Alternatively, the interaction of PLC-γ1 with the 171, 191, and 226 sites could be indirect and mediated, for example, via Grb2 or Gads. Whatever the explanation of this binding data, the functional effect of these three mutations was considerable, although not complete. The loss of Grb2 binding correlated with a loss of Erk and NF-AT activation. It is surprising that this mutant still mediated TCR-induced Ca2+ flux, although the binding of PLC-γ1 with LAT was abolished and there was no significant increase of PLC-γ1 tyrosine phosphorylation. More extensive studies need to be performed to explain this result.

The single mutation at Y132 had a profound and unexpected effect on signaling. The phosphorylated sequence YLVV was predicted to bind both PLC-γ1 N-SH2 domain, although in the original characterization the PLC-γ1 SH2 domain favors leucine in the +1 position (12). It was showed previously, prior to the cloning of the LAT, that the N-SH2 domain of PLC-γ1 binds to LAT and that this interaction is critical for TCR-induced tyrosine phosphorylation of PLC-γ1 (18). In this model, the C-SH2 domain might interact with one of the sites deleted in 3YF, as discussed above. Our studies on the Y132F mutant support and extend this result. Tyr183 is required for both PLC-γ1 binding and tyrosine phosphorylation.

The Y132F mutant demonstrated abnormal calcium flux in response to TCR cross-linking. The initial peak of calcium...
Itk has been found to bind to tyrosine phosphorylated SLP-76, which, as described above, is associated with abnormal calcium flux (26). The Y132F mutation does not inhibit Gads binding, but a slight decrease in SLP-76 binding to LAT was detected (27), perhaps because PLC binding normally stabilizes SLP-76 at LAT. Itk might bind LAT by multiple mechanisms (27), but both a loss of PLC and a decrease in SLP-76 binding to LAT would likely result in a deleterious effect on Itk binding and function.

The lack of PLC-γ1 binding in the Y132F mutant is also likely to contribute to the failure of Erk activation in cells expressing this mutant. A similar finding has been observed in B cells lacking PLCγ (28). The authors of this study attribute the failure of Erk activation not to a failure of calcium flux but to a lack of PKC activation, caused by the PLCγ dependence. In B cells lacking Btk and in T cells lacking Itk, PKC phosphorylation at Tyr191 (1). In ongoing studies, a set of mutants containing only this mutant no reconstitution of the LAT null phenotype is observed in calcium, ERK, or NF-AT assays. Nonetheless, some level of TCR-induced tyrosine phosphorylation is detected on this protein.

In this study, we have shown that the four distal tyrosine residues of LAT are important for association with critical signaling molecules and thus for LAT function. An important question that remains is whether these four tyrosines are indeed phosphorylated in vivo. In our initial purification of LAT from activated Jurkat cells, mass spectroscopy revealed phosphorylation at Tyr191 (1). In ongoing studies, a set of mutants has been made in which all tyrosines in LAT have been mutated to phenylalanine except for, individually, Tyr132, Tyr171, Tyr191, or Tyr226. Upon expression of each of these individual mutants in LAT-deficient cells, TCR cross-linking results in phosphorylation in LAT mutants containing tyrosine only at position 171, 191, or 226. The mutant containing only Tyr132 is not phosphorylated, although all of the results of the mutation at this site presented in this study strongly suggest that phosphorylation occurs on this tyrosine. Additional studies are directed at uncovering the requirements for phosphorylation at this site. Future studies will need to define further all of the sites of phosphorylation. Characterization of other, as yet undetermined interactions and of the role of all of these sites on normal T cell activation and development are in progress.

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