Identification of the Minimal Tyrosine Residues Required for Linker for Activation of T Cell Function*

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Received for publication, March 13, 2001, and in revised form, May 16, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.M102221200

The linker for activation of T cells (LAT) is essential for signaling through the T cell receptor (TCR). Following TCR stimulation, LAT becomes tyrosine-phosphorylated, creating docking sites for other signaling proteins such as phospholipase C-γ1 (PLC-γ1), Grb2, and Gads. In this study, we have attempted to identify the critical tyrosine residues in LAT that mediate TCR activation-induced mobilization of intracellular Ca\(^{2+}\) and activation of the MAP kinase Erk2. By using the LAT-deficient Jurkat derivative, J.CaM2, stable cell lines were established expressing various tyrosine mutants of LAT. We show that three specific tyrosine residues (Tyr\(^{132}\), Tyr\(^{171}\), and Tyr\(^{181}\)) are necessary and sufficient to achieve a Ca\(^{2+}\) flux following TCR stimulation. These tyrosine residues function by reconstituting PLC-γ1 phosphorylation and recruitment to LAT. However, these same tyrosines can only partially reconstitute Erk activation. Full reconstitution of Erk requires two additional tyrosine residues (Tyr\(^{110}\) and Tyr\(^{226}\)), both of which have the Grb2-binding motif YXX. This reconstitution of Erk activation requires that the critical tyrosine residues be on the same molecule of LAT, suggesting that a single LAT molecule nucleates multiple protein-protein interactions required for optimal signal transduction.

Engagement of the T cell receptor (TCR)\(^1\) triggers a complex cascade of events culminating in T cell proliferation, differentiation, and increased gene transcription (1, 2). The initial steps of this process are carried out by the Src and Syk families of tyrosine kinases (3). Targets of the Syk family of tyrosine kinases include an emerging class of proteins known as adaptors. Although these proteins lack intrinsic enzymatic activity, they function to promote intermolecular interactions utilizing multiple protein-protein interaction domains or motifs (4). Examples of interaction domains include Src homology (SH) 2 domains, which bind phosphotyrosine residues, and SH3 domains, which bind proline-rich regions.

One adaptor protein essential for T cell activation is the transmembrane adaptor protein, linker for activation of T cells (LAT) (5–7). Following TCR engagement, LAT becomes phosphorylated on multiple tyrosine residues thereby allowing other proteins important for T cell activation to be recruited by SH2-phosphotyrosine interactions. Multiple proteins have been demonstrated by coimmunoprecipitation experiments to be recruited to LAT, either directly or indirectly, such as phospholipase C-γ1 (PLC-γ1), Grb2, Sos, Gads, SLP-76, Vav, Cbl, Itk, and the p85 subunit of phosphatidylinositol 3-kinase (5, 8–11). In addition to the many tyrosine residues, LAT also contains two cysteine residues proximal to the transmembrane domain. These two cysteine residues are palmitoylated resulting in localization of LAT into glycolipid-enriched microdomains (GEMs) within the plasma membrane (12). Without correct localization into GEMs, LAT cannot mediate downstream signaling events induced by TCR stimulation (13, 14).

The essential role of LAT in T cell development has been demonstrated using targeted gene disruption in mice. LAT-deficient mice have a block in thymocyte development at the immature CD25\(^+\)CD4\(^+\), CD4\(^+\)CD8\(^−\) stage and a complete lack of mature peripheral T cells (6). Blocks at this early stage of thymocyte development are similar to those observed in other mice lacking proteins, such as the Src kinases Lck and Fyn and the Syk kinases ZAP-70 and Syk, that mediate pre-TCR signaling events (15).

The LAT-deficient Jurkat derivative, J.CaM2, has provided considerable insight into the mechanism by which LAT mediates TCR-signaling events. This cell line is deficient in many pathways activated by TCR stimulation such as Ca\(^{2+}\) mobilization and Ras/MAP kinase activation (7, 16). These defects are, in part, due to an inability to recruit PLC-γ1 to the membrane where it is phosphorylated and becomes activated. PLC-γ1 activation is essential for the generation of the second messengers inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate. IP\(_3\) binds receptors that regulate the release of stored Ca\(^{2+}\), whereas DAG is required to activate proteins such as protein kinase C and Ras guanyl nucleotide releasing protein (Ras-GRP) (17–19). The absence of LAT may also result in the loss of Ras activation through the inability to recruit the Ras exchange factor Sos to the membrane via the adaptor Grb2.

Analysis of the various coimmunoprecipitation studies involving LAT has led to a model where a complex of adaptors involving LAT, Grb2, Gads, and SLP-76 are localized to GEMs within the plasma membrane in order to recruit other signaling proteins (5, 8, 20). Grb2 is an adaptor protein with a central SH2 domain flanked on both sides with SH3 domains, which bind Sos. Human LAT contains four tyrosine residues that fit...
the Grb2-SH2 binding motif of XYN that are conserved between human, mouse, and rat (Tyr<sup>110</sup>, Tyr<sup>171</sup>, Tyr<sup>191</sup>, and Tyr<sup>226</sup>). Three of these tyrosines (Tyr<sup>171</sup>, Tyr<sup>191</sup>, and Tyr<sup>226</sup>) when mutated have been shown to disrupt Grb2 recruitment to LAT. A Grb2-like molecule, known as Gads, also binds directly to LAT at only two of these sites (Tyr<sup>171</sup> and Tyr<sup>191</sup>). Like Grb2, Gads contains two SH3 domains flanking a single SH2 domain. However, Gads also contains a proline-rich region between the SH2 domain and the C-terminal SH3 domain. Studies by Liu et al. (8) have demonstrated that Gads is responsible for mediating the recruitment of SLP-76 to LAT.

The SLP-76 adaptor protein contains proline-rich regions, a C-terminal SH2 domain, and three N-terminal phosphoryrosine residues. All have been demonstrated to recruit other signaling proteins. The C-terminal SH3 domain of Gads binds to proline-rich sequences within SLP-76 for its recruitment to LAT (8). SLAP-130/Fyb and HPK1 both contain phosphoryrosine residues that can bind the SH2 domain of SLP-76 (21).<sup>2</sup> Proteins such as Itk, Rlk, and Vav are thought to bind directly to phosphoryrosine residues at the N terminus of SLP-76, explaining why they are detected in LAT immunoprecipitations (22–24).

Mice with targeted disruption of SLP-76 have a phenotype very similar to that of LAT-deficient mice, a profound block in thymic development with the absence of peripheral T cells (25, 26). Not surprisingly, the recently described Gads-deficient mice also have a similar, although less severe, phenotype (27). This is most likely due to a partial compensation by Grb2 in the absence of Gads. A Jurkat mutant cell line lacking SLP-76 displays a phenotype similar to J.CaM2 cells, yet somewhat less drastic. These cells, named J14, have decreases in Ca<sup>2+</sup> flux and Ras activation in response to TCR stimulation (28).

These findings suggest that SLP-76 plays a critical role in LAT-dependent signal transduction. Indeed, overexpression of a LAT-MLP chimera was recently shown to substitute for LAT function (29).

The multiple protein interactions that have been demonstrated with LAT led us to examine further the role of different tyrosine residues and the activities that they mediate. Human LAT has 10 total tyrosines, and 9 are conserved between human, mouse, and rat. Previous studies have shown the importance of some of these residues for recruitment of PLC-γ1, Grb2, and Gads (8, 20). In this study, we expand on the analysis of these and other tyrosine residues by identifying the minimal tyrosine residues required for LAT function. By utilizing the LAT-deficient J.CaM2 cell line, multiple stable lines expressing various tyrosine mutants of LAT were generated, and their abilities to mediate activation of downstream pathways were studied. Ca<sup>2+</sup> mobilization is dependent on three of the tyrosines (Tyr<sup>132</sup>, Tyr<sup>191</sup>, and Tyr<sup>191</sup>), whereas full Ras pathway activation requires two additional tyrosine residues (Tyr<sup>171</sup> and Tyr<sup>226</sup>). These two additional tyrosines contribute to the recruitment of Grb2 to the LAT complex. In addition, the critical tyrosine residues must also be on the same molecule of LAT for reconstitution of TCR-mediated signaling events. Therefore, these data provide evidence for the important function of an assembly of proteins on a single LAT molecule. Moreover, these studies show that the tyrosine residues have different roles in mediating events downstream of LAT.

### EXPERIMENTAL PROCEDURES

#### Cell Lines, Transfections, and Plasmids

The LAT-deficient Jurkat mutant J.CaM2 (7, 16) and subsequent stable lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mm glutamine, penicillin, and streptomycin. For stable and transient transfections, 2 × 10<sup>6</sup> J.CaM2 cells were resuspended in 400 μl of RPMI 1640 and electroporated at 250 V, 960 microfarads using a Gene Pulser electroporator (Bio-Rad). For generation of stable lines, transfected cells were plated 48 h after electroporation in media containing 2 μg/ml G418. For transient assays, cells were utilized 24 h after transfection. Myc-tagged Erk2 was expressed using the pEF-BOS expression plasmid, which also encodes a neomycin resistance gene for production of stable lines. Point mutants of LAT were generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Clones present in this report are as follows: wt LAT (clone 70), Y-F6 (clone 107), Y-F7,8 (clone 73), Y-F6–8 (clone 55), F-Y6 (clone 35), F-Y7,8 (clone 15), F-Y6–8 (clone 40.8).

#### Antibodies—TCR stimulation was performed with the anti-Jurkat TCR β-chain monoclonal antibody (mAb) C305 (30). The polyclonal anti-LAT, anti-PLC-γ1, mixed mAb, and anti-phosphotyrosine 4G10 mAb were from Upstate Biotechnology, Inc. (Lake Placid, NY), whereas the polyclonal anti-Grb2 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-phospho-Erk was from Cell Signaling Technology, Inc. (Beverly, MA), and the anti-Myc mAb was derived from the 9E10 hybridoma.

#### Measurement of Intracellular Ca<sup>2+</sup> Mobilization—To analyze intracellular Ca<sup>2+</sup> mobilization, 3 × 10<sup>6</sup> cells were resuspended in 1 ml of RPMI 1640 with 10% fetal bovine serum and labeled in 3 μM of the fluorescent Ca<sup>2+</sup> indicator dye Indo-1 AM (Molecular Probes, Eugene, OR) for 1 h at 37 °C. Cells were washed 3 times in Ca<sup>2+</sup> buffer (0.1% glucose w/v, 25 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM Na<sub>HPO<sub>4</sub></sub>, 0.1% bovine serum albumin, w/v, 50 mM MgCl<sub>2</sub>, 100 mM CaCl<sub>2</sub>, pH 7.4), and the cell pellet was kept on ice. Prior to the assay, cells were resuspended in Ca<sup>2+</sup> buffer and warmed to 37 °C. 10<sup>6</sup> cells were stimulated with 300 ng/ml ionomycin (1 μM). The fluorescence emission at 400- and 500-nm wavelengths was measured with an excitation at 355 nm using a Hitachi F-4500 fluorescence spectrophotometer, and the intracellular Ca<sup>2+</sup> concentration was calculated based on the ratio of the fluorescence at 400 and 500 nm.

#### TCR Stimulation, Preparation of Lysates, Immunoprecipitations, and Western Blotting—Cells 2.5 × 10<sup>6</sup> cells/ml were first resuspended at 37 °C for 20 min and then stimulated with 1:250 dilution of purified anti-TCR (1 mg/ml stock solution, C305) in phosphate-buffered saline. Cells were lysed at 10<sup>6</sup> cells/ml in lysis buffer (1% Triton X-100, 50 mM NaCl, 10 mM Tris-H<sub>3</sub>C<sub>O</sub> <sub>4</sub>, pH 7.6). For coimmunoprecipitation assays, 1% Brij in 150 mM NaCl and 10 mM Tris, pH 7.6, was used instead. Both lysis buffers contained 2 mM EDTA and a combination of proteinase and phosphatase inhibitors as described previously (31). For immunoprecipitations, lysates were incubated with primary antibody for 45 min, followed by protein G-Sepharose beads for 45 min, and were then washed 3 times with lysis buffer. Samples were separated by SDS-PAGE, and proteins were analyzed by Western blotting. Membranes were incubated with the indicated primary antibodies followed by the appropriate secondary antibody conjugated to horse-radish peroxidase. Reactive proteins were visualized by Renaissance a chemiluminescence reagent (PerkinElmer Life Sciences). For quantitation of bands, chemiluminescence was assessed on a Kodak one-dimensional image analysis software version 3.5 (Rochester, NY).

#### NF-AT Luciferase Assays—J.CaM2 cells were transfected as before with 20 μg of a 3 × NF-AT-luciferase reporter construct and 1 μg of the indicated LAT construct in pcDEF3. The TCR was stimulated with immobilized C305 for 8 h. Cells were harvested, lysed, and assayed for luciferase activity (32). Lysates were also blotted for LAT expression.

### RESULTS

#### Generation of Stable LAT Mutant Lines from LAT-deficient J.CaM2 Cells—The LAT-deficient Jurkat-derived cell line J.CaM2 was used to produce cell lines stably expressing various tyrosine mutants of LAT. To aid in the description of the various tyrosine mutants of LAT, each of the 10 tyrosines was given a number. Fig. 1A depicts the location of the tyrosine residues in human LAT by amino acid numbers. The fourth tyrosine in human LAT was designated site 3.5 since it is not conserved between species. Throughout this paper, Y-F denotes mutant forms of LAT where tyrosine residues were changed to phenylalanine, whereas F-Y denotes mutants in which all tyrosines were initially changed to phenylalanine and then the specified residue mutated back to tyrosine. For example, F-Y6

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2 K. Bauer, J. Liu, S. B. Singh, D. Yablonski, A. Weiss, and R. M. Perlmutter, submitted for publication.
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The Y-F6 cell line had a markedly decreased Ca\(^{2+}\) flux, whereas Y-F7,8 had a normal response when compared with wt LAT (Fig. 2A). However, Y-F6–8 had a complete loss in its ability to flux Ca\(^{2+}\) in response to TCR stimulation. These results implicate sites 6–8 in mediating the Ca\(^{2+}\) response. To extend further this analysis, cells expressing forms of LAT containing only certain tyrosine residues on an all phenylalanine background were tested in the same manner. The F-Y6 version of LAT did not reconstitute Ca\(^{2+}\) signaling, and the F-Y7,8 version of LAT only restored a slight, almost undetectable flux. However, the F-Y6–8 form of LAT reconstituted the Ca\(^{2+}\) flux following TCR stimulation to the same extent as wt LAT (Fig. 2A). For these experiments, multiple clones of all mutants gave similar results. These data demonstrate that sites 6–8 are necessary and sufficient for Ca\(^{2+}\) mobilization in the LAT-deficient J.CaM2 cell line. A potential explanation could come from previous studies that have shown that site 6 binds to PLC\(^{\gamma}\) and sites 7 and 8 bind Gads (5, 8, 20), thereby indirectly recruiting SLP-76 to LAT. This suggests that a LAT-PLC\(^{\gamma}\)-Gads-SLP-76 complex may be necessary and sufficient for Ca\(^{2+}\) mobilization.

PLC\(^{\gamma}\) Phosphorylation and Recruitment to LAT—Since defective Ca\(^{2+}\) mobilization was observed with Y-F6, F-Y6, and F-Y7,8, we decided to investigate PLC\(^{\gamma}\) phosphorylation in these cell lines, given that PLC\(^{\gamma}\) is responsible for generating the IP\(_3\) required for Ca\(^{2+}\) mobilization. Cells were stimulated with anti-TCR, PLC\(^{\gamma}\) immunoprecipitations were isolated from cellular lysates, and the samples were separated by SDS-PAGE. Western blotting with an anti-phosphotyrosine antibody indicated that PLC\(^{\gamma}\) was phosphorylated in J.CaM2 cells reconstituted with wt LAT, Y-F6, Y-F7,8, F-Y7,8, and F-Y6–8 (Fig. 3A, middle panel). Thus, the phosphorylation state of PLC\(^{\gamma}\) does not exactly correlate with the mobilization of intracellular Ca\(^{2+}\). In support of this observation, phosphorylation-independent mechanisms of PLC\(^{\gamma}\) activation have been noted previously in the literature (33). Moreover, our analysis fails to distinguish among the various reported sites of PLC\(^{\gamma}\) tyrosine phosphorylation (34, 35). When the association of PLC\(^{\gamma}\) with phospho-LAT was examined, a direct correlation was observed between association and Ca\(^{2+}\) flux in response to TCR stimulation (Fig. 3A, lower panel). Due to the poor ability of the LAT antibody to recognize phosphorylated LAT, we were not able to show inducible association of LAT with PLC\(^{\gamma}\). However, a phosphorylated band that comigrates with LAT can be detected by phosphotyrosine blotting. Samples were also blotted with an anti-PLC\(^{\gamma}\) antibody to show equal protein levels in the immunoprecipitations (Fig. 3A, upper panel). To ensure that the lack of a phospho-LAT band in the PLC\(^{\gamma}\) immunoprecipitates is not due to an inability of these mutants of LAT to become phosphorylated, an additional study was done. Anti-Myc immunoprecipitates were performed on the LAT mutant lines in which an interaction of PLC\(^{\gamma}\) with phospho-LAT could not be detected but still exhibited PLC\(^{\gamma}\) phosphorylation upon stimulation. Immunoprecipitates from unstimulated or TCR-stimulated cells reconstituted with wt LAT, Y-F6, and F-Y7,8 were separated by SDS-PAGE followed by blotting with an anti-phosphotyrosine antibody. Fig. 3B shows that the phosphorylation state of the Y-F6 mutant is very similar to that of wt LAT and that the F-Y7,8 mutant is still phosphorylated, although at a reduced level. These data suggest that phosphorylation of PLC\(^{\gamma}\) alone is not sufficient to elicit a Ca\(^{2+}\) flux. Mobilization of intracellular Ca\(^{2+}\) following TCR stimulation also requires PLC\(^{\gamma}\) recruitment to LAT.

Tyrosines in LAT Required for Reconstitution of Erk Phosphorylation—Since the F-Y6–8 form of LAT was sufficient to reconstitute the mobilization of intracellular Ca\(^{2+}\) following...
stabilization through the TCR, we were interested to determine if any or all of these three sites could reconstitute other signaling pathways in J.CaM2. Previous studies performed with J.CaM2 have demonstrated a deficiency in the activation of the Ras pathway. One downstream indicator of this pathway is J.CaM2 have demonstrated a deficiency in the activation of the Ras pathway. Previous studies performed with J.CaM2 have demonstrated a deficiency in the activation of the Ras pathway. One downstream indicator of this pathway is phosphorylation of Erk on Thr202 and Tyr204, which can be followed if any or all of these three sites could reconstitute other signal-

When J.CaM2 cells were transfected with Erk and a vector control or the all-F LAT, Erk did not become activated following TCR stimulation (Fig. 4A). If the cells were cotransfected with a wt LAT construct, Erk was phosphorylated following TCR stimulation. Neither the F-Y6 mutant nor the F-Y7,8 mutant form of LAT could reconstitute Erk phosphorylation in J.CaM2 cells. However, an F-Y6–8 form of LAT could partially reconstitute the defective signaling pathway, demonstrating the importance of all three of these sites (Fig. 4A). For reasons unknown, mutants of LAT that have most or all of the tyrosine residues changed to phenylalanine reproducibly have a slightly faster mobility. One possible explanation is that the loss of hydroxyl groups on the tyrosine residues creates a protein that is now more hydrophobic resulting in a change in mobility.

A time course of Erk activation was then performed to determine if the partial response at 3 min is due to a shift in kinetics or a decrease in the maximum response. J.CaM2 cells cotransfected with Erk2 and either wt LAT or F-Y6–8 were stimulated with anti-TCR for 0, 1, 3, 10, or 30 min. The phosphorylation states of Erk at the different time points were compared. The results show that the partial reconstitution of the Erk pathway is due to a decreased magnitude in the response rather than a shift in kinetics (Fig. 4B).

To identify further tyrosine residues that may play a role in Erk pathway activation, other forms of LAT that contained additional tyrosines added back were tested. From the amino acid sequence, there are 2 more potential Grb2 binding motifs, sites 4 and 9, that are fully conserved between human, mouse, and rat. The LAT/Grb2 interaction could function to recruit Sos to the membrane and therefore also contribute to Ras activation, which is upstream of the MAP kinases. Sites 4 and 9 were added alone or together with sites 6–8 to determine if they contribute to activation of the Erk pathway. Addition of either site 4 or 9 to F-Y6–8 only slightly increased the phosphorylation of Erk over sites 6–8 following TCR stimulation (Fig. 4C). However, F-Y4,6–9 could fully reconstitute the Erk deficiency found in J.CaM2 demonstrating that the additional potential Grb2-binding sites are important for the activation of Erk.

Phosphorylation and Association of LAT Mutants with Grb2—To investigate further the significance of sites 4 and 9, the phosphorylation state of the F-Y6–8 mutant was compared with the F-Y4,6–9 mutant to determine if the sites are phosphorylated in response to TCR stimulation. J.CaM2 cells were transfected with either empty vector as a control, wt LAT, F-Y6–8, or F-Y4,6–9. Cells were then stimulated with anti-TCR, and the transfected proteins were immunoprecipitated with anti-Myc. As demonstrated in Fig. 5A, the F-Y4,6–9 mutant is phosphorylated to a greater extent when compared with the F-Y6–8 mutant, indicating that either sites 4 or 9 or both are indeed phosphorylated upon TCR stimulation.

To explore the possible mechanism by which sites 4 and 9 function to increase Erk phosphorylation, immunoprecipitation studies were carried out. Since both sites 4 and 9 are Grb2 consensus binding motifs (YXXN), Grb2 immunoprecipitates...
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were performed to determine if the addition of sites 4 and 9 could enhance phospho-LAT recruitment to Grb2. Grb2 immunoprecipitates from cells transfected with wt LAT resulted in phospho-LAT interaction with Grb2, whereas cells transfected with the F-Y6–8 mutant resulted in a reduced interaction (Fig. 5B). It is, however, important to keep in mind that the phosphorylation state of the F-Y6–8 mutant is lower than that of wt LAT. When sites 4 and 9 are added to the F-Y6–8 mutant, a greater amount of phospho-LAT is seen coimmunoprecipitating with Grb2. These data demonstrate that sites 4 and 9 function to enhance the recruitment of Grb2 to LAT.

Sites 4 and 6–9 Reconstitute NF-AT Activation to wt LAT Levels—As a functional readout for TCR stimulation, transcriptional reporter studies were performed with a reporter that contains composite binding sites for AP-1 and the nuclear factor of activated T cells (NF-AT). A 3× NF-AT-luciferase reporter construct was co-transfected with the various LAT mutants into J.CaM2 cells. Reconstitution of J.CaM2 with wt LAT resulted in approximately a 4-fold activation of NF-AT over unstimulated cells, whereas the Y-F6–8 mutant was equivalent to that of a vector control (Fig. 6). The F-Y6–8 only resulted in a partial reconstitution, whereas the Y-F6–8 mutant restored the response comparably to that of wt LAT. Since the reporter construct is composed of both AP-1 and NF-AT sites, a deficiency in the function of either transcription factor would lead to failure to induce luciferase expression. Thus, the NF-AT results are similar to those seen in Erk activation where the F-Y6–8 provides only partial rescue, whereas the Y-F6–8 essentially reconstitutes wt LAT function.

Reconstitution of the Tyrosine Residues in Trans Does Not Rescue Erk Signaling—Since LAT can recruit several molecules to GEMs within the plasma membrane, an interesting question is whether this recruitment simply localizes the proteins to a discrete location within the plasma membrane or whether LAT is involved in the formation and coordination of a multiprotein complex on a single LAT molecule at the plasma membrane (13, 14). One possible interpretation of the data concerning site 6 and sites 7 and 8 is the formation of a 4-part complex including the interaction of PLC-γ1 with site 6 and a Gads/SLP-76 interaction with sites 7 or 8. Therefore, deletion of either site 6 or sites 7 and 8 could result in less efficient recruitment of PLC-γ1 and Gads/SLP-76 or less stable complex formation. To test this hypothesis, we transfected J.CaM2 cells with either wt LAT or an equal mix of LAT constructs that each have only one of the 10 tyrosine residues. The wt LAT construct reconstituted the Erk phosphorylation seen following TCR stimulation, whereas the mix of LAT constructs was similar to vector alone (Fig. 7). To investigate further the potential coop-
The critical role of the transmembrane adaptor protein LAT in mediating signals generated by TCR engagement has been demonstrated by many approaches. Experiments involving targeted gene disruption of LAT and characterization of Jurkat T cell mutants, which lack expression of LAT, have shed considerable light on LAT’s function in signaling (5–7). Following TCR stimulation, LAT rapidly becomes tyrosine-phosphorylated at multiple residues, allowing for the recruitment of multiple signaling proteins to the plasma membrane. Localization of LAT to cholesterol-rich lipid microdomains within the plasma membrane, otherwise known as GEMs, has been demonstrated to be critical for function (13, 14). However, the minimal tyrosine residues required for LAT function was unclear. Moreover, it was not clear whether a single LAT molecule can form a discrete signaling complex or whether many LAT molecules interacting with distinct complexes can function in trans.

To address such issues, we performed a structure/function analysis of LAT, with a focus on the different tyrosine residues. Our analysis of the tyrosine residues in LAT has led to some interesting findings. In the Y-F6- and Y-F7,8-reconstituted cells, PLC-γ1 phosphorylation following TCR stimulation was restored, yet a defect in Ca2+ flux was still detected. It is believed that Syk and Tec family kinases Itk and Rlk are involved in the phosphorylation of PLC-γ1 in T cells. Since Itk has been demonstrated previously to associate with the LAT complex, a simple model of activation of PLC-γ1 activity would involve recruitment of PLC-γ1 to LAT where Itk and Rlk are also recruited, possibly via a Gads/SLP-76 interaction (9, 11, 24). It may also be possible that Tec family members could bind LAT directly since they contain SH2 domains. Alternatively, since Itk and PLC-γ1 both contain PH domains, which are important in membrane localization, PLC-γ1 could potentially become phosphorylated without recruitment to LAT. However, to activate PLC-γ1 enzymatic activity, perhaps localization to GEMs via an association to the LAT complex is required. Data presented in this study support this model since mutation of site 6 only partially reduced the association of PLC-γ1 with LAT, and only a reduction of Ca2+ mobilization was observed. Our data differ with previously published data (20) that showed that mutation at site 6 resulted in the loss of PLC-γ1 phosphorylation and an increased magnitude but more transient Ca2+ mobilization following TCR stimulation. Although it is difficult to reconcile these differences, in both cases multiple clones of the same mutation were tested. One potential difference is the method of stimulation. In the studies reported here, the TCR was stimulated with relatively high concentrations of purified C305, whereas previous studies used relatively low concentrations of C305 for biochemical studies and the anti-CD3 mAb OKT3 for Ca2+ flux studies. The decreased Ca2+ flux in response to C305 presented in this paper was also observed on a FACS Calibur with cells loaded with the Ca2+ indicator Fluo-3 (data not shown).

Our studies suggest a link but not a complete overlap for the requirements for PLC-γ1 and Erk activation. Erk activation is likely to reflect Ras activation in T cells (36). Previous studies from chicken B cells have suggested that PLC-γ2 plays a role in activating the Ras pathway. Loss of PLC-γ2 expression or mutations that abrogate PLC-γ2 activity prevent Ras activation (37). Recent studies have demonstrated that overexpression of another exchange factor for Ras, RasGRP, can enhance TCR-Ras-Erk signaling and increase interleukin-2 production in Jurkat cells (18). Some of the most convincing data come from targeted gene disruption of RasGRP in mice. Thymocytes from these mice fail to activate the Ras pathway following CD3 stimulation (19). Since RasGRP has both Ca2+-binding EF hands and a DAG-binding C1 domain, it is believed that Ras-GRP is dependent on PLC-γ2 activity, since PLC-γ2 is responsible for Ca2+ release and DAG production. Indeed, pharmacologic inhibitors of PLC-γ2 diminished activation of Ras following TCR stimulation (18). Another possible mechanism for PLC-γ1 regulation of Ras activation is for PLC-γ1 to recruit directly Sos to the LAT complex independent of Grb2. Such an interaction has been reported to be constitutive and dependent on the SH3 domain of PLC-γ1 (38, 39). Kim and colleagues (38) also showed that expression of a lipase-inactive PLC-γ1 could potentiate Ras activation; however, these studies were carried out in non-lymphoid cells.

**DISCUSSION**

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Our studies suggest a link but not a complete overlap for the requirements for PLC-γ1 and Erk activation. Erk activation is likely to reflect Ras activation in T cells (36). Previous studies from chicken B cells have suggested that PLC-γ2 plays a role in activating the Ras pathway. Loss of PLC-γ2 expression or mutations that abrogate PLC-γ2 activity prevent Ras activation (37). Recent studies have demonstrated that overexpression of another exchange factor for Ras, RasGRP, can enhance TCR-Ras-Erk signaling and increase interleukin-2 production in Jurkat cells (18). Some of the most convincing data come from targeted gene disruption of RasGRP in mice. Thymocytes from these mice fail to activate the Ras pathway following CD3 stimulation (19). Since RasGRP has both Ca2+-binding EF hands and a DAG-binding C1 domain, it is believed that Ras-GRP is dependent on PLC-γ2 activity, since PLC-γ2 is responsible for Ca2+ release and DAG production. Indeed, pharmacologic inhibitors of PLC-γ2 diminished activation of Ras following TCR stimulation (18). Another possible mechanism for PLC-γ1 regulation of Ras activation is for PLC-γ1 to recruit directly Sos to the LAT complex independent of Grb2. Such an interaction has been reported to be constitutive and dependent on the SH3 domain of PLC-γ1 (38, 39). Kim and colleagues (38) also showed that expression of a lipase-inactive PLC-γ1 could potentiate Ras activation; however, these studies were carried out in non-lymphoid cells.

**FIG. 6.** NF-AT activation in cells expressing mutants of LAT. 20 μg of NF-AT-luciferase reporter construct was cotransfected with 1 μg of the various LAT mutants into J.CaM2 cells. 1 × 106 cells were either unstimulated or stimulated with plate-bound C305, 8 h later, cells were lysed and assayed for luciferase activity. The graph represents the fold activation induced by TCR stimulation over unstimulated. Data are derived from three independent experiments, each done in duplicate.
Full reconstitution of Erk is dependent on sites 4 and 6–9 within LAT. Only partial reconstitution of the Erk activation was seen with F-Y6–8. Our calcium mobilization data suggest that sites 6–8 are required and sufficient for full PLC-\(\gamma\)1 activation. The addition of sites 4 and 9 functions to recruit more molecules of Grb2 to the membrane. Since Grb2 is known to be coupled with the exchange factor Sos, this recruitment to the membrane may result in further activation of Ras. Sites 7 and 8 have been shown to not only bind Gads but Grb2 as well. This could explain the partial Erk activation seen in the F-Y6–8. These data demonstrate that sites 6–8, which are sufficient for PLC-\(\gamma\)1 activation, are not sufficient for full Erk activation. Partial, an additive effect is needed requiring all of the conserved Grb2 consensus sites for full Erk activation.

The importance of having sites 6–8 on the same molecule of LAT supports the idea that LAT is involved in nucleating a multimolecular signaling complex (Fig. 7). Potential interactions among multiple domains may increase the stability of the complex. Data supporting a direct PLC-\(\gamma\)1 interaction with SLP-76 has recently been obtained by Yablonski and colleagues (43). In that study, mutants of SLP-76 were used to reconstitute the SLP-76-deficient Jurkat derivative J14. A proline-rich region of SLP-76 is required for reconstitution of NF-AT activation and was shown to bind to the SH3 domain of PLC-\(\gamma\)1. Furthermore, a GST-SH3 domain of PLC-\(\gamma\)1 could directly interact with SLP-76. Interestingly, tyrosine to phenylalanine mutations at sites 7 and 8 were seen to decrease PLC-\(\gamma\)1 association in the original LAT cloning paper even though subsequent studies have suggested that site 6 is primarily responsible for PLC-\(\gamma\)1 association (5, 20).

Other recent evidence also supports the notion that there is a direct interaction between PLC-\(\gamma\)1 and SLP-76. Boerth et al. (29) demonstrated that targeting of SLP-76 to the plasma membrane was sufficient to reconstitute LAT-deficient J.CaM2 cells. In this report, a chimeric molecule was constructed containing the transmembrane domain of LAT attached to SLP-76 in order to localize it to the GEMs. When this chimeric protein was expressed in J.CaM2 cells, PLC-\(\gamma\)1 became phosphorylated following TCR stimulation. Although the phosphorylation state does not necessarily demonstrate activation of enzymatic activity of PLC-\(\gamma\)1, this chimeric molecule was able to rescue NF-AT activation implying that mobilization of intracellular...
Ca$^{2+}$ took place. One possible interpretation of this observation is that SLP-76 recruits PLC-γ$_1$ to GEMS independent of the tyrosine residues of LAT in this chimeric molecular system. Collectively, the data here and those of other studies (20, 43) suggest that PLC-γ$_1$ simultaneously interacts with SLP-76 and LAT, which stabilizes a LAT-Gads-SLP-76-PLC-γ$_1$ signaling complex (Fig. 8).

The adaptor protein Gads, which links SLP-76 to LAT, is also important in generating a Ca$^{2+}$ flux in response to receptor stimulation. Ishiai et al. (40) examined the role of T cell-specific adaptor proteins in a B cell system. The adaptor protein B cell linker (BLNK) is thought to perform the functions of both adaptor proteins in a B cell system. The adaptor protein B cell of these two sites (Tyr110 and Tyr 226) results in increased both of which have the Grb2-binding motif YN. The addition X these studies involve PLC-γ$_2$, previous data from DeBell et al. (42) had demonstrated that PLC-γ$_2$ could functionally reconstitute a PLC-γ$_2$-deficient DT40 B cell.

The data presented here show that distinct tyrosine residues in LAT are involved in different pathways. We have shown that three specific tyrosine residues (Tyr$^{132}$, Tyr$^{171}$, and Tyr$^{191}$) are necessary and sufficient to achieve a Ca$^{2+}$ flux following TCR stimulation. However, these same tyrosines can only partially reconstitute Erk activation. Complete reconstitution of Erk requires two additional tyrosine residues (Tyr$^{110}$ and Tyr$^{226}$), both of which have the Grb2-binding motif YNX. The addition of these two sites (Tyr$^{110}$ and Tyr$^{226}$) results in increased association of phospho-LAT with Grb2. Our study also suggests that LAT plays a key role in coordinating the interaction of multiple molecules within a single complex. Evidence in support of a LAT-Gads-SLP-76-PLC-γ$_1$ signaling complex is suggested by the data demonstrating that sites 6–8 must be on the same molecule of LAT. However, formal demonstration of this multiprotein complex awaits further investigation.

Acknowledgments—We thank the members of the Weiss lab for their helpful advice and emotional support. We are also grateful to Mike Tomlinson and Larry Kane for critically reading the manuscript.

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J. Biol. Chem. 2001, 276:29588-29595.
doi: 10.1074/jbc.M102221200 originally published online June 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102221200

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