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Minimal Requirement of Tyrosine Residues of Linker for Activation of T Cells in TCR Signaling and Thymocyte Development[1]

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Linker for activation of T cells (LAT) is a membrane-associated adaptor protein that is phosphorylated on multiple tyrosines upon TCR cross-linking. Previous studies show that LAT is essential for TCR-mediated signaling and thymocyte development. In this study, we expressed a series of LAT Tyr to Phe mutants in LAT-deficient J.CaM2.5 cells and examined their tyrosine phosphorylation; association with Grb2, Gads, and phospholipase C (PLC)-γ1; and function in T cell activation. Our results showed that the five membrane-distal tyrosines were phosphorylated upon T cell activation. Grb2, Gads, and PLC-γ1 associated with LAT preferentially via different sets of tyrosine residues; however, they failed to interact with LAT mutants containing only one tyrosine. We also determined the minimal requirement of LAT tyrosine residues in T cell activation and thymocyte development. Our results showed that a minimum of three tyrosines is required for LAT to function in T cell activation and thymocyte development. LAT mutants that were capable of binding Grb2 and PLC-γ1 could reconstitute T cell activation in LAT-deficient cells and thymocyte development in LAT-deficient mice. ***The Journal of Immunology*****, 2003, 170: 325–333.**

Ligation of the TCR triggers a cascade of biochemical events that lead to cytokine gene expression, cellular proliferation, and T cell effector functions. The biochemical events initiated via the TCR involve activation of tyrosine kinases, phosphorylation of cellular proteins, and recruitment of proteins to the plasma membrane (1–3). The recruitment of cytosolic signaling molecules to the membrane is mediated by membrane-associated adaptor proteins (4–6). One of these adaptor proteins is linker for activation of T cells (LAT).[3] LAT function in TCR signaling is clearly demonstrated in LAT-deficient Jurkat cells (7, 8). These mutant Jurkat cells are defective in TCR-mediated Ras-mitogen-activated protein kinase (MAPK) activation, Ca^{2+} flux, and NF-AT activation. Reconstitution of these cells with LAT restores the TCR signaling pathway, indicating that LAT is essential for TCR-mediated activation of Ras-MAPK and Ca^{2+} flux.

LAT has a short extracellular domain, a transmembrane domain, and a cytosolic tail with multiple tyrosines (9, 10). It is palmitoylated via two cysteine residues near its transmembrane domain (11). Palmitoylation of LAT is required for its localization in lipid rafts. Upon T cell activation, LAT is phosphorylated by ZAP-70 tyrosine kinase and associates with Grb2, Grb2-related adapter downstream of Shc (Gads), and phospholipase C (PLC)-γ1. Of the 10 tyrosines in human LAT, Y110, Y127, Y171, Y191, and Y226 are within a Grb2-binding motif, and Y132 is within a PLC-γ1-binding motif. These motifs allow LAT to interact with the Src homology 2 (SH2) domains of Grb2 and PLC-γ1 directly. Mutation of three Grb2 binding sites (Y171, Y191, and Y226) simultaneously abolishes the interaction of LAT with Grb2 and Gads, and mutation of Y132 in LAT abolishes PLC-γ1 binding, leading to defective Ras-MAPK activation and Ca^{2+} flux (12). These data indicate that recruitment of Grb2 and PLC-γ1 is essential for LAT function.

In addition to its role in the TCR signaling pathway, LAT is also essential during thymocyte development. Disruption of LAT in mice has a profound effect on thymocyte development (13). LAT-deficient mice lack mature T cells in the periphery, while they have normal B cells. Even though thymocytes from these mice have a normal TCRβ chain rearrangement, they are arrested at the $CD25^+CD44^-$ subset of the $CD4^-CD8^-$ stage. These cells cannot be rescued by injection of anti-CD3 Abs, suggesting that LAT is required in the pre-TCR signaling pathway. Recent data show that mice expressing LAT with mutations in the four membrane-distal tyrosines also exhibit defective thymocyte development, indicating that these tyrosines are essential for LAT function in thymocyte development (14).

In this study, we made a series of LAT mutants with Y to F mutations to define the minimal tyrosines that are required for LAT binding to Grb2, Gads, and PLC-γ1. We also examined the function of these mutants in TCR-mediated Ras-MAPK activation, Ca^{2+} flux, NF-AT activation, and thymocyte development. Our results showed that LAT binding of Grb2, Gads, and PLC-γ1 requires multiple tyrosines. LAT binding to Grb2 and PLC-γ1 is necessary for T cell activation and thymocyte development. LAT with a minimal three of four membrane-distal tyrosines can function in T cell activation and thymocyte development.

Materials and Methods

Constructs

Site-directed mutagenesis was performed to mutate tyrosine residues in LAT using the Quick-Exchange kit from Stratagene (La Jolla, CA). LAT mutants with combinations of different tyrosine residues were constructed by using restriction endonuclease sites present in *LAT* cDNA or created

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[3] Abbreviations used in this paper: LAT, linker for activation of T cells; DN, double negative; DP, double positive; Gads, Grb2-related adapter downstream of Shc; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; PY, phosphotyrosine; SH2, Src homology 2; SP, single positive; WT, wild type.

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during mutagenesis. These LAT mutants were cloned into two retroviral vectors, pLXIN (Clontech, Palo Alto, CA) and pMSCV-IRES (internal ribosomal entry site)/GFP (green fluorescent protein). LAT expression is driven by the 5′ LTR. The Neo^R and GFP genes were placed after LAT under the control of an internal ribosomal entry site element (Fig. 1*A*). The LAT mutants used in this study were listed in Fig. 1*B*. The pLXIN constructs were used to generate stable cell lines expressing LAT mutants in the J.CaM2.5 cell line, while the pMSCV constructs were mainly used to transduce bone marrow cells from $LAT^{-/-}$ mice in the adoptive transfer experiments.

Cell lines, transfection, and retroviral transduction in LAT-deficient cells

LAT-deficient cells (J.CaM2.5) were maintained in RPMI 1640 medium supplemented with 10% FBS. Phoenix-ecotropic virus-packaging cells were maintained in DMEM medium supplemented with 10% FBS. A total of 10 μg of each retroviral construct was used to transfect Pheonix-ecotropic packaging cells using calcium phosphate. Forty-eight to seventy-two hours after transfection, the cell culture supernatant containing recombinant retroviruses was harvested for immediate viral transduction or frozen at −80°C.

To make J.CaM2.5 cells transducible by ecotropic viruses, the ecotropic receptor MCAT-1 was introduced. A cDNA fragment from *MCAT*-1 was cloned into pMSCVpuro (Clontech). The resulting construct was then used to pack pantropic retroviruses using VSV-G as the envelope glycoprotein (Clontech). Retroviruses containing the *MCAT*-1 gene were subsequently used to transduce J.CaM2.5 cells. These transduced J.CaM2.5 cells were selected in the presence of puromycin and further subcloned. Each clone was tested for the expression of the ecotropic receptor by its ability to be transduced by a retrovirus expressing GFP. One designated as J.CaM2.5-MCAT was chosen for further studies. J.CaM2.5 cells expressing various LAT mutants were generated by transducing J.CaM2.5-MCAT cells with recombinant retroviruses containing different LAT mutants in pLXIN. Stable transductants were selected for in the presence of G418 (1.2 mg/ml).

Abs, immunoprecipitation, and Western blotting

The following Abs were used for immunoprecipitation and Western blotting: anti-TCR (C305), anti-*myc* (9E10), and rabbit polyclonal anti-LAT Abs (10); anti-phosphotyrosine (PY20) and anti-Grb2 mAbs were from Transduction Laboratories (Lexington, KY); monoclonal anti-PY (4G10), anti-PLC-γ1, and rabbit anti-Gads Abs were from Upstate Biotechnology (Lake Placid, NY); rabbit anti-Grb2 polyclonal Ab was from Santa Cruz Biotechnology (Santa Cruz, CA); an anti-active MAPK mAb was purchased from New England Biolabs (Beverly, MA).

For immunoprecipitation, J.CaM2.5 cells expressing different LAT mutants were either stimulated with C305 (1:50 tissue culture supernatant) for 1 min and 30 s or left untreated. A total of 1×10^7 cells were lysed in 500 μl of ice-cold lysis buffer (1% Brij97, 25 mM Tris-Cl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4) with protease inhibitors. Cell lysates were subjected to immunoprecipitation with different Abs, as indicated in each figure. Immunoprecipitated proteins were resolved on SDS-PAGE, transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA), and blotted with different Abs, as indicated in each figure. Immunoreactive proteins were detected with an HRP-coupled secondary Ab (Amersham, Arlington Heights, IL), followed by detection with SuperSignal West Pico Luminol/Enhancer Solution (Pierce, Rockford, IL).

MAPK activation, Ca^{2+} flux, and luciferase assay

Twelve to sixteen hours after transduction with retroviruses containing LAT mutants and GFP, GFP^+ cells were sorted by FACS and cultured for another 24 h before harvest. These sorted cells were either left untreated or stimulated with C305 for 5 min. Cell lysates were resolved on SDS-PAGE and further analyzed by an anti-active MAPK Western blot.

Intracellular free Ca^{2+} measurement was performed as described (15). Twelve to sixteen hours after retrovirus transduction, J.CaM2.5 cells were loaded with Indo-1. Anti-CD3ϵ Ab (OKT3) was used to activate those cells. Ca^{2+} flux in GFP^+ cells was analyzed using a flow cytometer (FACScan; BD Biosciences, San Jose, CA) by monitoring the fluorescence emission ratio at 405–495 nm.

For luciferase assays, 1.6×10^6 J.CaM2.5 cells were transfected with 1.5 μg pNFAT-luciferase plasmid and 0.5 μg of different LAT plasmids or an empty vector using Superfect transfection reagent (Qiagen, Valencia, CA). Twenty to twenty-four hours after transfection, J.CaM2.5 cells were stimulated with OKT3 (1:500 ascites) coated on tissue culture plates, PMA (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, Madison, WI).

Transduction of bone marrow cells, and adoptive transfer

Bone marrow cells were taken from the femurs of LAT knockout mice that were treated with 5-fluorouracil (150 mg/kg) for 48 h. After removal of RBCs, they were cultured in IMDM with 15% FBS in the presence of IL-3 (20 ng/ml), IL-6 (20 ng/ml), and stem cell factor (50 ng/ml) for 3–4 days. For retroviral transduction, bone marrow cells were then mixed with retroviral supernatant in the presence of polybrene (8 μg/ml) and centrifugated at $1300 \times g$ for 3 h at 22°C. These cells were cultured in the presence of IL-3, IL-6, and stem cell factor for an additional 24 h and then transduced with retroviruses again. The efficiency of retroviral transduction of bone marrow cells was examined by monitoring GFP expression using fluorescence microscopy. Before adoptive transfer, transduced bone marrow cells were harvested and washed once with IMDM without FBS. A total of $0.5–1 \times 10^6$ cells were injected i.v. via a tail vein into LAT knockout mice that were irradiated at 5 Gy.

Flow cytometric analysis

Five to six weeks after adoptive transfer, thymocytes and splenocytes were prepared from adoptively transferred mice. After removal of RBCs, these cells were stained with the following Abs: biotin-conjugated anti-TCRβ and B220; PE-conjugated anti-CD4, CD25, and IgM; APC-conjugated anti-CD4 and CD8; and PE-Cy5-conjugated anti-CD44. Each sample was further analyzed on the FACStar cytometer.

Results

Five membrane-distal tyrosines of LAT are phosphorylated upon T cell activation

The LAT-deficient Jurkat cell line, J.CaM2.5, is defective in Ca^{2+} flux, Ras-MAPK activation, and AP-1- and NF-AT-mediated transcriptional activation in response to anti-TCR or anti-CD3 stimulation. Reconstitution of this cell line with wild-type (WT) LAT corrects these signaling defects (7). We chose to use this cell line to analyze LAT function in TCR signaling pathways by reconstituting it with different LAT mutants containing Y to F substitutions. In the cytoplasmic domain of human LAT, there are nine conserved tyrosines. Five of these tyrosines, Y110, Y127, Y171, Y191, and Y226, are within a Grb2-binding motif and are likely to bind Grb2 upon phosphorylation. Y171 and Y191 also probably interact with Gads. Y132 is crucial for interaction with PLC-γ1, as demonstrated in our previous studies (12). Although previous studies had shown that Y132, Y171, Y191, and Y226 are critical for TCR-mediated signaling (12, 14), it has not been formally demonstrated which of these tyrosines are phosphorylated in vivo and which of these are required for the formation of LAT-mediated signaling complexes.

To answer these questions, we generated a LAT mutant with all tyrosines mutated (10YF) and then made a series of additional mutants in which each of these tyrosines was singly added back. We also made a mutant with the four membrane-distal tyrosines (LAT-4Y), mutants with either two or three of four membrane-distal tyrosines, and a mutant with mutations at five membrane-distal tyrosines (Fig. 1*B*). We used recombinant ecotropic retroviruses encoding LAT mutants to transduce LAT-deficient cells. Stable transductants were selected in the presence of G418 and were used for further analysis without subcloning to avoid differences among each subclone, such as levels of LAT and TCR expression.

For biochemical analysis of LAT mutants, J.CaM2.5 cells reconstituted with different LAT mutants were stimulated with an anti-TCR Ab, C305. Because all LAT mutants had a Myc tag, LAT was immunoprecipitated from cell lysates with an anti-*myc* Ab. Tyrosine phosphorylation of LAT was determined by Western blotting with a combination of two anti-PY mAbs, 4G10 and PY20. As shown in Fig. 2*A*, different LAT mutants were expressed

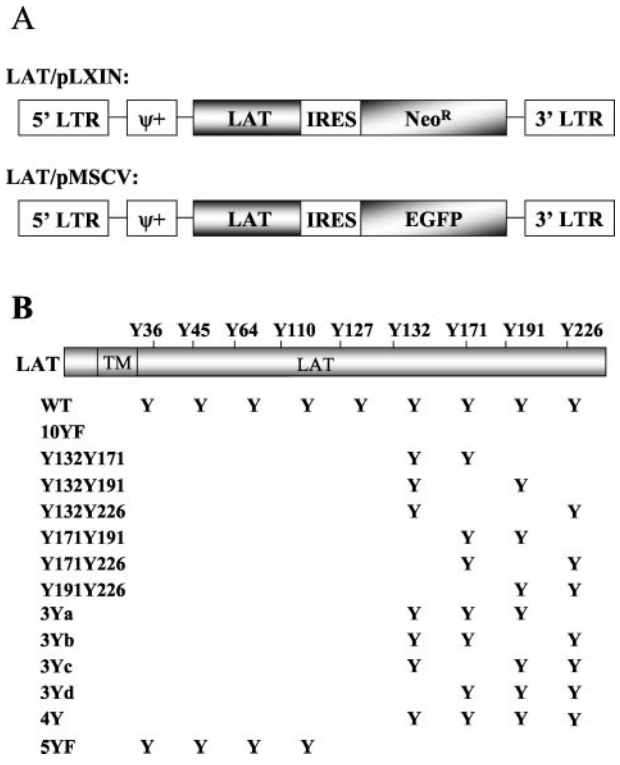

FIGURE 1. Retroviral vectors and a list of LAT mutants with Tyr to Phe substitutions. *A*, Two versions of retroviral vectors used to express LAT and neoR or GFP simultaneously. *B*, List of different LAT mutants used. Only the tyrosine residues present in different mutants were indicated. Tyrosine residues at other positions were replaced with phenylalanines.

at similar levels in J.CaM2.5 cells. LAT-WT was heavily phosphorylated after C305 stimulation. Among those mutants with one tyrosine, only the mutants with a tyrosine at 127, 171, 191, or 226 were phosphorylated upon TCR cross-linking (Fig. 2*B*). There was no tyrosine phosphorylation of LAT mutants with any of the four membrane-proximal tyrosines, Y36, Y45, Y64, and Y110 (data not shown), and the LAT mutant with a tyrosine at 132. Lack of phosphorylation of four membrane-proximal tyrosines was supported by analysis of another mutant, 5YF, which had mutations at five membrane-distal tyrosines. Although this mutant still had either four membrane-proximal tyrosines, it was not phosphorylated upon stimulation (Fig. 2*B*).

Surprisingly, the LAT mutant with a single tyrosine at Y132 was not phosphorylated because our previous results showed that Y132 is required for PLC-γ1-binding and MAPK activation (12). It is likely that phosphorylation of LAT Y132 is dependent on prior phosphorylation of four membrane-distal tyrosines, although we could not rule out the possibility that phosphorylated Y132 could not be recognized by these two anti-PY Abs. These data, together with our previous data, indicate that five membrane-distal tyrosines, Y127, Y132, Y171, Y191, and Y226, are phosphorylated upon T cell activation.

Interaction of LAT with Grb2, Gads, and PLC-γ1 requires multiple tyrosines

The results from Fig. 2*B* indicated that the LAT mutants with a tyrosine at 127, 171, 191, or 226 could be phosphorylated. Next we examined the association of these mutants with Grb2, Gads, and PLC-γ1. After stimulation with C305, reconstituted J.CaM2.5 cells were lysed, and detergent extracts were immunoprecipitated with anti-Grb2, Gads, or PLC-γ1 Abs, respectively. Due to the presence of nonspecific proteins comigrated with LAT on SDS-PAGE in anti-LAT Western blotting, association of LAT with Grb2, Gads, or PLC-γ1 could only be determined by anti-PY Western blotting. As shown in Fig. 2*C*, none of the LAT mutants with one tyrosine was capable of interacting with Grb2, Gads, or PLC-γ1, despite the fact that four of these mutants were phosphorylated. A weak band detected by anti-PY Western blotting in anti-Gads or PLC-γ1 immunoprecipitation was not LAT, because it migrated slightly faster than LAT-*myc* on SDS-PAGE. These data suggest that LAT interactions with these signaling proteins require the phosphorylation of more than one tyrosine. However, it is also possible that phosphorylation of these single tyrosine mutants was too weak to detect in anti-Grb2, Gads, and PLC-γ1 immunoprecipitation. However, this conclusion was supported by the results in Fig. 3, as discussed below.

Grb2, Gads, and PLC-γ1 bind LAT via different sets of tyrosines

To further determine which tyrosines in LAT are required for its interaction with Grb2, Gads, and PLC-γ1, we made another series of LAT mutants with combinations of two or three of the four membrane-distal tyrosines (Fig. 1*B*). We concentrated on these four tyrosines because the LAT mutant with only these four tyrosines (LAT-4Y) was well phosphorylated, capable of interacting with Grb2, Gads, and PLC-γ1 (Fig. 3, *lane 12*), and functional in TCR signaling, as discussed below (Fig. 4). In addition, a LAT mutant with mutations of these four tyrosines failed to function in thymocyte development (14), suggesting that these four tyrosines are critical in LAT function. These mutants were introduced into J.CaM2.5 with retroviruses and expressed at similar levels (Fig. 3). The interactions of these mutants with Grb2, Gads, and PLC-γ1 were examined by immunoprecipitation of each protein, followed by anti-PY blotting.

As shown in Fig. 3, these mutants were all phosphorylated after C305 stimulation. Of the six mutants with two tyrosines (*lanes 2-7*), only LATY171Y191, Y171Y226, and Y191Y226 mutants were able to associate with Grb2 (Fig. 3, *lanes 5–7*). Tyrosines 171, 191, and 226 are all within a consensus Grb2-binding motif (YXN). Because LAT mutants with only one Grb2-binding motif were not able to bind Grb2, it is possible that two Grb2 molecules bind two phosphorylated tyrosines on LAT cooperatively. In contrast to Grb2, Gads only interacted with the LAT mutant Y171Y191, two tyrosines that are both within the YVNV context (Fig. 3, *lane 5*). Other two-tyrosine mutants were well phosphorylated similarly to the Y171Y191 mutant, with an exception of the Y132Y191 mutant, but they failed to interact with Gads. Different from Grb2 and Gads, PLC-γ1 could only associate with the Y132Y191 mutant (Fig. 3, *lane 3*). This mutant was least phosphorylated compared with other two-tyrosine mutants. These results indicated that failure of those two-tyrosine mutants to interact with Grb2, Gads, or PLC-γ1 was not due to weak phosphorylation and further supported the conclusion from Fig. 2 that Grb2, Gads, and PLC-γ1 interaction with LAT requires multiple tyrosines. These data suggest that Grb2, Gads, and PLC-γ1 interact with LAT via different sets of tyrosines.

Because Y127 was also phosphorylated in our single mutants, we wanted to determine whether it was involved in the interaction of LAT with Grb2, Gads, and PLC-γ1. We made mutants with a tyrosine at 127 in combinations with one of the four membrane-distal tyrosines. None of these mutants was able to interact with Grb2, Gads, or PLC-γ1 (data not shown), suggesting that Y127 is less likely involved in binding these signaling molecules.

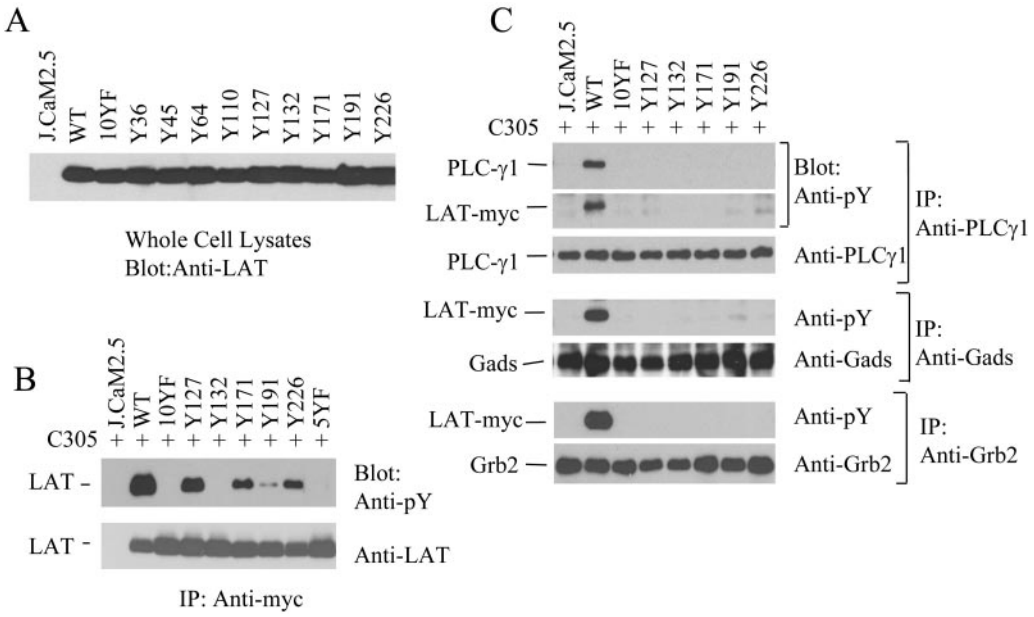

FIGURE 2. Phosphorylation and association of LAT mutants with one tyrosine with Grb2, Gads, and PLC-γ1. J.CaM2.5 cells reconstituted with different LAT mutants were lysed in 1% Brij lysis buffer. Cell lysates were immunoprecipitated with Abs against Myc, Grb2, Gads, and PLC-γ1, respectively. The association of LAT with these signaling proteins was detected with an anti-PY Western blot. The amount of immunoprecipitated protein was determined by Western blotting using Abs against each individual protein.

Grb2 stabilizes the interaction between LAT and Gads

To ascertain whether an additional phosphorylated tyrosine could recruit another protein, perhaps involved in stabilization of the LAT signaling complex, we examined the interaction of Grb2, Gads, and PLC-γ1 using LAT mutants with combinations of three of four membrane-distal tyrosines (LAT-3Ya, b, c, and d; Fig. 1*B*).

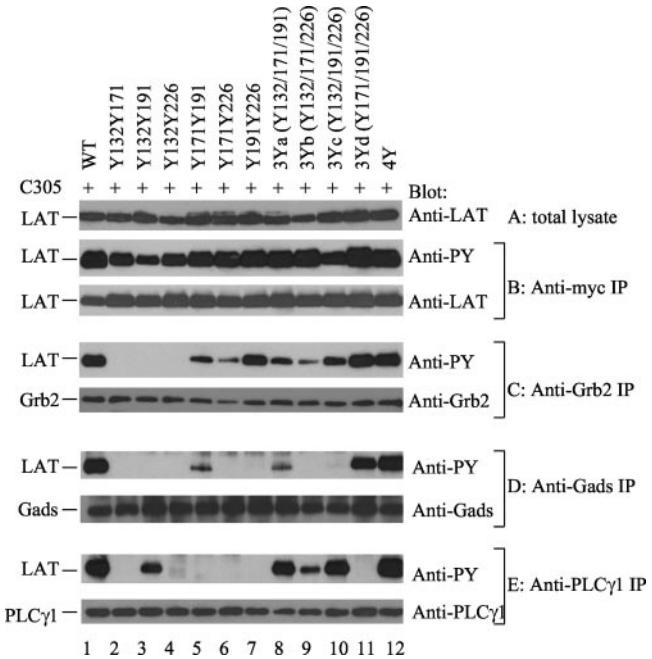

FIGURE 3. Association of LAT-4Y and LAT-3Y mutants with Grb2, Gads, and PLC-γ1. J.CaM2.5 cells reconstituted with WT or various LAT mutants were lysed in 1% Brij lysis buffer. Cell lysates were immunoprecipitated with Abs against Myc, Grb2, Gads, and PLC-γ1, respectively. The association of LAT with these signaling proteins was detected with an anti-PY Western blot. The amount of immunoprecipitated protein was determined by Western blotting using Abs against each individual protein.

The addition of Y132 to any of the two-tyrosine mutants did not enhance the interaction of LAT with Grb2 and Gads (*lane 8* vs *5*, *lane 9* vs *6*, and *lane 10* vs *7*). This result was expected because Y132 is most likely involved in PLC-γ1 binding. As discussed previously, of the six two-tyrosine mutants, Gads only associated very weakly with LAT-Y171Y191 (*lane 5*) compared with LAT-WT (*lane 1*) and LAT-4Y (*lane 12*). Strikingly, addition of a Grb2 binding site at 226 (LAT-3Yd) dramatically increased the interaction between Gads and LAT (*lane 11* vs *5*). This increased association was not due to increased phosphorylation of LAT-3Yd, because this mutant was phosphorylated to a similar extent as LAT-Y132/171/191 (3Ya) and LAT-Y171Y191 (*lane 11* vs *5* and *8*). This result suggests that binding of Grb2 to phosphorylated Y226 could stabilize a Gads-LAT interaction.

Compared with the LAT mutant with two tyrosines at 132 and 191 (Fig. 3, *lane 3*), the addition of a tyrosine at 171 (*lane 8*) or 226 (*lane 10*) did not significantly increase the LAT-PLC-γ1 interaction. The apparent increased association with phosphorylated LAT (*lanes 8* and *10* vs *3*) was most likely due to increased tyrosine phosphorylation of these mutants (*lanes 8* and *10* vs *3*). It is interesting that in the absence of Y191, the LAT mutant with tyrosines at 132, 171, and 226 (3Yb) could also interact with PLC-γ1 to some extent (*lane 9*). This result is in agreement with our previous finding that mutation of Y191 alone reduced PLC-γ1 binding (12).

Interaction of LAT with both Grb2 and PLC-γ1 is essential for T cell activation

We also examined whether these two- and three-tyrosine mutants could correct signaling defects in LAT-deficient cells. Recombinant retroviruses were used to deliver different mutants into J.CaM2.5 cells transiently. None of the two-tyrosine mutants was able to restore Ca^{2+} mobilization in those cells (data not shown). Cells reconstituted with 3Yb and 3Yc were capable of fluxing some Ca^{2+} in response to receptor stimulation (Fig. 4*A*), although not as well as LAT-WT and LAT-4Y (Fig. 4*A*). Cells with 3Ya and

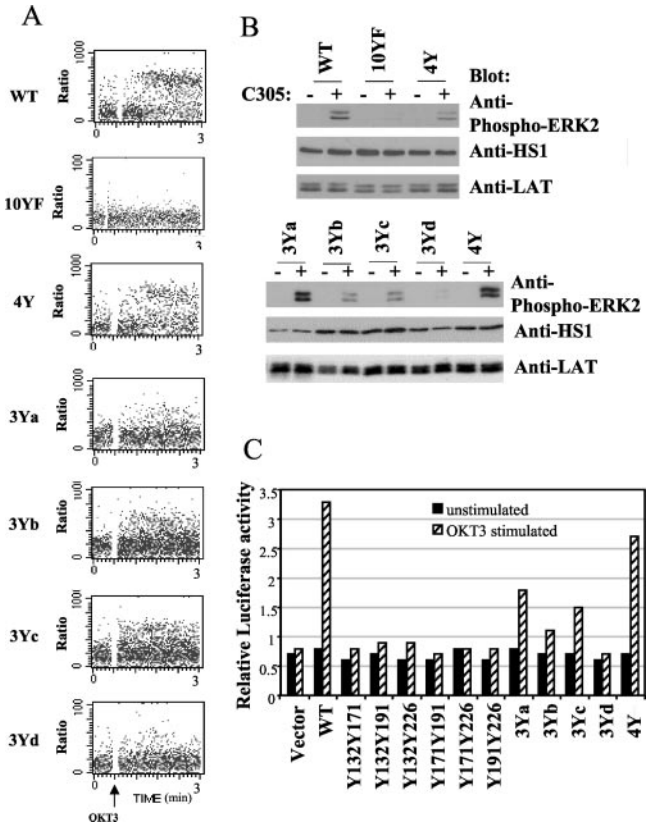

FIGURE 4. Four membrane-distal tyrosines are sufficient for LAT association with Grb2, Gads, and PLC-γ1, and function in T cell activation. *A*, Ca^{2+} flux in LAT-deficient cells reconstituted with different LAT mutants. Twelve to sixteen hours after retrovirus transduction, J.CaM2.5 cells were loaded with Indo-1. Ca^{2+} flux was induced with anti-CD3ϵ Ab (OKT3), and the fluorescence emission ratio at 405–495 nm was measured by flow cytometry. Ca^{2+} flux in GFP^+ cells was analyzed. *B*, MAPK activation. GFP^+ cells after retroviral transduction were sorted by FACS and cultured for an additional 24 h. These cells were stimulated with C305 for 5 min or left untreated. The cell lysates were separated by SDS-PAGE and blotted with an anti-active MAPK Ab. The expression of LAT was detected with an anti-LAT blot, and an equal amount of cell lysate was loaded on SDS-PAGE, as determined by an anti-HS1 blot. *C*, NF-AT-dependent transcription in LAT-deficient cells reconstituted with different mutants. The NF-AT-luciferase plasmid and different LAT plasmids were transfected into J.CaM2.5 cells using Superfect transfection reagent. Thirty-six hours after transfection, these cells were stimulated with OKT3 coated on 24-well tissue culture plates, PMA and ionomycin, or left untreated. Six hours later, those cells were lysed, and cell lysates were used to determine luciferase activity.

3Yd had weak Ca^{2+} flux. We also determined whether these mutants could restore MAPK activation. LAT-3Ya could reconstitute MAPK activation similar to LAT-4Y, although this mutant only weakly reconstituted Ca^{2+} flux in J.CaM2.5 cells. LAT-3Yb and LAT-3Yc could partially reconstitute MAPK activation, while LAT-3Yd failed completely (Fig. 4*B*). We also transiently transfected these LAT mutants together with an NF-AT-luciferase reporter construct to determine whether NF-AT activation in J.CaM2.5 cells could be restored. As shown in Fig. 4*C*, LAT-WT and LAT-4Y restored NF-AT activation in J.CaM2.5 cells. LAT mutants with only two tyrosines were unable to restore NF-AT activation when introduced into J.CaM2.5 cells (Fig. 4*C*). LAT-3Ya, 3Yb, and 3Yc could partially restore NF-AT activation. LAT-3Yd was unable to correct the defective NF-AT activation in J.CaM2.5 cells. LAT-3Ya, 3Yb, and 3Yc, which are all capable of binding Grb2 and PLC-γ1, could partially restore signaling defects in LAT-deficient cells, suggesting that Grb2 and PLC-γ1 binding to LAT is sufficient for its function in NF-AT activation. However, Gads binding to LAT is required for full activation of T cells.

Rescue of thymocyte development with LAT-WT

To study LAT function in thymocyte development, we used recombinant retroviruses to introduce LAT-WT and various LAT mutants into bone marrow cells from $LAT^{-/-}$ mice in vitro. These transduced cells were then transferred into irradiated $LAT^{-/-}$ mice by injection via a tail vein. Five to six weeks after injection, thymocytes and splenocytes from these reconstituted mice were analyzed by FACS. Because these recombinant viruses carried both LAT and GFP genes, all lymphocytes derived from transduced bone marrow cells should be GFP^+ and express LAT.

We first tested whether introduction of LAT-WT into bone marrow cells from $LAT^{-/-}$ mice could rescue thymocyte development. Thymocytes from LAT knockout and B6 mice were used as controls. As shown in Fig. 5*A*, GFP^+ thymocytes were observed in LAT knockout mice reconstituted with $LAT^{-/-}$ bone marrow cells transduced with viruses containing LAT-WT. Thymocytes from these reconstituted mice were further analyzed for CD4, CD8, TCRβ, CD44, and CD25 expression. As shown in previous studies

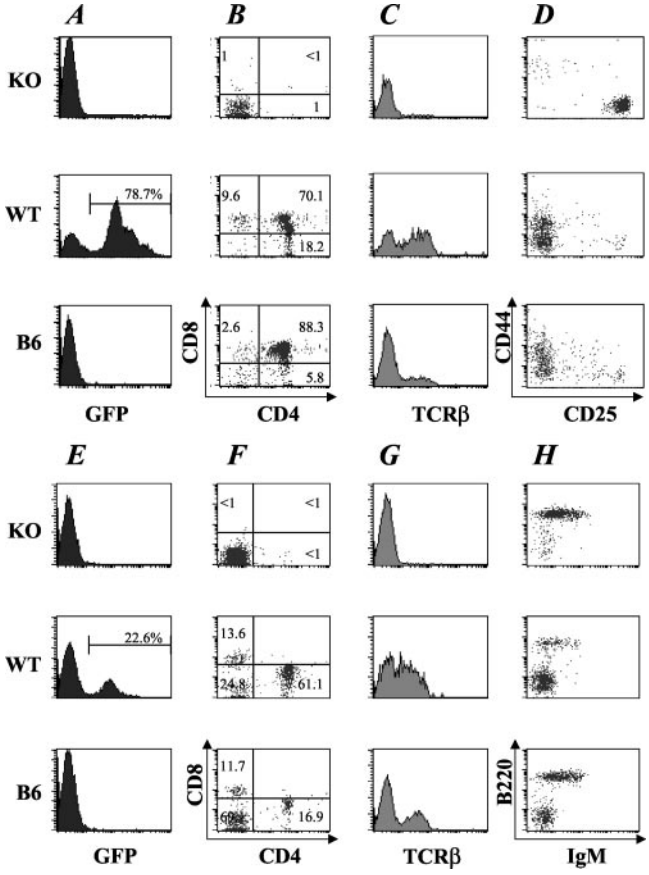

FIGURE 5. Rescue of thymocyte development by adoptive transfer of LAT-WT into $LAT^{-/-}$ mice. Bone marrow cells taken from $LAT^{-/-}$ mice were cultured in vitro and transduced with retroviruses containing WT-LAT. These bone marrow cells were then injected into $LAT^{-/-}$ mice. Five to six weeks after adoptive transfer, thymocytes (*A–D*) and splenocytes (*E–H*) from these mice were stained for CD4, CD8, TCRβ, CD25, CD44, B220, and IgM. GFP^+ cells from mice reconstituted with WT-LAT were analyzed for expression of different surface markers. Thymocytes and splenocytes from B6 and $LAT^{-/-}$ mice were used as controls in FACS analysis.

(13), LAT knockout mice had no CD4 and CD8 double-positive (DP) and single-positive (SP) thymocytes, and thymocyte development was blocked at the $CD25^+CD44^-$ subset of the double-negative (DN) stage (Fig. 5, *B* and *D*). In mice reconstituted with $LAT^{-/-}$ bone marrow cells transduced with viruses containing WT-LAT, the majority of GFP^+ thymocytes, which should be derived from transduced bone marrow cells, were $CD4^+CD8^+$ DP, similar to thymocytes from B6 mice (Fig. 5*B*). Some GFP^+ cells were either $CD4^+$ or $CD8^+$ SP. These thymocytes expressed the TCRβ chain on their cell surface (Fig. 5*C*). To determine whether thymocyte development at the DN stage was normal in these reconstituted mice, $GFP^+CD4^-CD8^-$ thymocytes were analyzed for CD44 and CD25 expression. As shown in Fig. 5*D*, introduction of LAT into $LAT^{-/-}$ thymocyte could drive thymocyte development beyond the $CD25^+CD44^-$ stage. CD44 and CD25 expression in thymocytes from reconstituted mice was very similar to that in B6 thymocytes.

Next we examined whether there were mature SP T cells in the periphery after reconstitution. Splenocytes from $LAT^{-/-}$ mice, WT-LAT reconstituted knockout mice, and B6 mice were stained with anti-CD4, CD8, TCRβ, B220, or IgM Abs. SP T cells were absent in LAT knockout mice, as previously described (Fig. 5*F*). Approximately 22.6% of splenocytes from the LAT-WT-reconstituted knockout mouse were GFP^+ (Fig. 5*E*). Among GFP^+ cells, 61.1% were $CD4^+$ and 13.6% were $CD8^+$. These SP T cells expressed the TCRβ chain, as indicated by anti-TCRβ chain staining (Fig. 5*G*). A small percentage of GFP^+ cells were B cells, as revealed by staining with anti-B220 and IgM Abs (Fig. 5*H*), suggesting that transduced bone marrow cells could also develop into B cells. These data indicated that thymocyte development in $LAT^{-/-}$ mice was successfully rescued by introduction of LAT-WT into $LAT^{-/-}$ bone marrow cells. We also performed the same experiment with the LAT-10YF mutant (with mutations at all tyrosines). This mutant failed to rescue thymocyte development of bone marrow cells from $LAT^{-/-}$ mice (data not shown).

Binding of LAT to both Grb2 and PLC-γ1 is important during thymocyte development

Because LAT-4Y was able to associate with Grb2, Gads, and PLC-γ1 and rescue Ras-MAPK activation as well as Ca^{2+} flux in LAT-deficient cells, we determined whether this mutant was also able to rescue thymocyte development in LAT knockout mice. As shown in Fig. 6, in the mice reconstituted with LAT-4Y, a large percentage of thymocytes were GFP^+, and most of these GFP^+ cells were DP or SP (Fig. 6*B*). Although it appeared that there were less DP cells in thymuses from mice reconstituted with LAT-4Y than in those in thymuses from mice reconstituted with LAT-WT in this experiment, this subtle difference was also seen in different mice reconstituted with LAT-WT most likely due to variation in viral transduction and adoptive transfer. These GFP^+ thymocytes expressed the TCRβ chain (Fig. 6*C*). We also stained splenocytes from mice reconstituted with LAT-4Y with anti-CD4, CD8, and

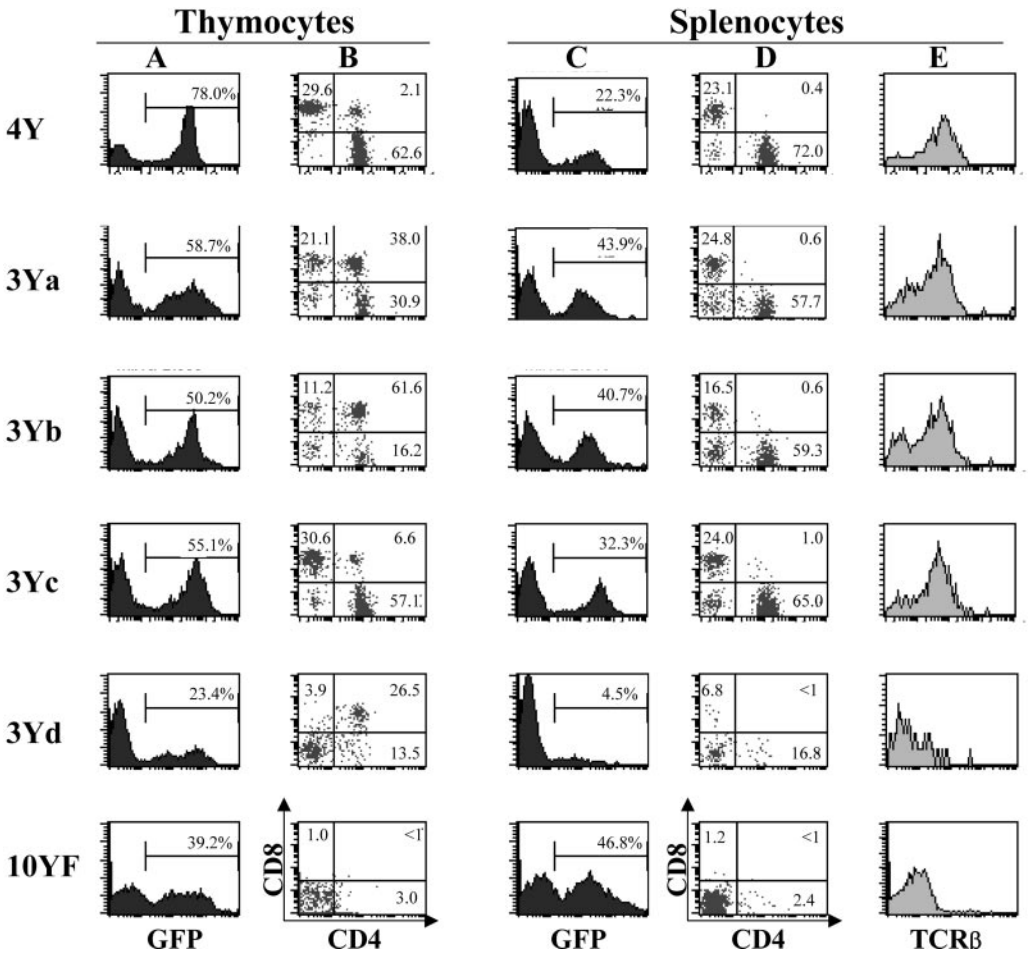

FIGURE 6. Rescue of thymocyte development of bone marrow cells from $LAT^{-/-}$ by LAT-4Y and LAT-3Y mutants. Bone marrow cells from $LAT^{-/-}$ mice were transduced with the LAT-4Y, LAT-3Ya, 3Yb, 3Yc, 3Yd, and LAT-10YF mutants and then transferred back into irradiated $LAT^{-/-}$ mice. FACS analysis was performed as in Fig. 5.

TCRβ Abs. $CD4^+$ and $CD8^+$ SP cells were present in mice reconstituted with LAT-4Y (Fig. 6*E*), and these cells expressed the TCRβ chain. These data indicated that the four membrane-distal tyrosines were sufficient in TCR-mediated signaling and thymocyte development.

We next tested whether LAT mutants with combinations of two tyrosines could rescue thymocyte development. As shown in Fig. 3, some of these two-tyrosine mutants could bind either Grb2 or PLC-γ1, but not both Grb2 and PLC-γ1. We were not able to detect any GFP^+ DP or SP thymocytes in mice reconstituted with any of the two-tyrosine mutants (data not shown). We also transduced $LAT^{-/-}$ bone marrow cells with LAT mutants with combinations of three of the four membrane-distal tyrosines to determine the minimal LAT tyrosine residues required for thymocyte development. As shown in Fig. 6, LAT-3Ya, 3Yb, and 3Yc, which could bind both Grb2 and PLC-γ1 (Fig. 2), were capable of efficiently rescuing thymocyte development of bone marrow cells from $LAT^{-/-}$ mice. Mice reconstituted with $LAT^{-/-}$ bone marrow cells transduced with LAT-3Ya, 3Yb, and 3Yc retroviruses had a high percentage of GFP^+ thymocytes (Fig. 6*A*). Most GFP^+ thymocytes were either DP or SP. SP thymocytes from these mice expressed the TCRβ chain on their surface (data not shown). When GFP^+ splenocytes from those mice were examined, $CD4^+$ and $CD8^+$ cells were present in spleens from the mice reconstituted with three-tyrosine mutants (Fig. 6*D*). Anti-TCRβ staining of these splenocytes showed that they expressed the TCRβ chain (Fig. 5*E*). In mice reconstituted with LAT-3Yd, which only associated with Grb2 and Gads, but not PLC-γ1, there were fewer GFP^+ thymocytes, and most of these thymocytes were DN. In the spleens from these mice, 5% of splenocytes were GFP^+. Although these GFP^+ cells were either $CD4^+$ or $CD8^+$, they expressed a very low level of TCR on their surface. We also transduced bone marrow cells from $LAT^{-/-}$ mice with the LAT mutant-10YF and transferred these cells back into $LAT^{-/-}$ mice. As shown in Fig. 5, there was no significant number of DP or SP T cells in the thymuses and spleens from these mice. The differences in the percentage of DP cells in thymuses reconstituted with LAT-4Y, 3Ya, 3Yb, and 3Yc could be due to either variation in viral transduction and efficiency of adoptive transfer or differences in signal strength of various LAT mutants. Future studies with transgenic mice or knockin mice will be done to examine these possibilities.

	3Ya (Y132/171/191)	3Yb (Y132/171/226)	3Yc (Y132/191/226)	3Yd (Y171/191/226)	4Y (Y132/171/191/226)	WT
Grb2 association	++	++	++	++	+++	+++
Gads association	+	-	-	++	+++	+++
PLCγ1 association	+++	+	+++	-	+++	+++
Ca^{++} flux	+/-	+	+	+/-	+++	+++
Erk activation	+++	+	+	+/-	+++	+++
NFAT activation	+	+	+	-	++	+++
Thymocyte development	+++	+++	+++	+	+++	+++

FIGURE 7. Summary of critical tyrosine residues of LAT in association with other signaling molecules, T cell activation, and thymocyte development.

Results from biochemical analysis and thymocyte reconstitution were summarized in Fig. 7. Three LAT mutants that were competent to efficiently rescue thymocyte development were all capable of binding both Grb2 and PLC-γ1, suggesting that LAT binding to both Grb2 and PLC-γ1, not Grb2 or PLC-γ1 alone, is important for LAT function. Of these three mutants, LAT-3Yb and 3Yc failed to associate with Gads, but were able to rescue thymocyte development, indicating that Gads binding to LAT is dispensable for thymocyte development. This is in agreement with the data from Gads-deficient mice (16). In summary, our data indicated that LAT interaction with both Grb2 and PLC-γ1 is important in thymocyte development.

Discussion

LAT is a membrane-associated adaptor molecule. Upon T cell activation, it is phosphorylated on multiple tyrosines and interacts with several important signaling proteins. Among LAT-interacting proteins, Grb2 and Gads each contain an SH2 domain, while PLC-γ1 possesses two SH2 domains. These three proteins most likely interact with phosphorylated LAT directly via their SH2 domains. Previous studies showed that mutation of the four membrane-distal tyrosines, Y132, Y171, Y191, and Y226, in human LAT affects LAT binding to these molecules (12). In this study, we constructed a series of LAT mutants with Tyr to Phe substitutions. We showed that in addition to Y132, Y171, Y191, and Y226, Y127 was also phosphorylated upon stimulation via the TCR. Interestingly, none of the mutants with one phosphorylated tyrosine associated with Grb2, Gads, and PLC-γ1. Instead, binding of LAT to these signaling proteins required phosphorylation on multiple tyrosines of LAT. Grb2, Gads, and PLC-γ1 interacted with LAT preferentially via different sets of tyrosine residues. We also examined the function of these LAT mutants in the TCR signaling pathway by using LAT-deficient cells, and in thymocyte development by reconstituting bone marrow cells from $LAT^{-/-}$ mice. Our results indicated that LAT function in T cell activation and thymocyte development requires a minimum of three membrane-distal tyrosines.

Human LAT has 10 tyrosine residues in its cytoplasmic domain, 9 of which are conserved between murine and human LAT. Our results obtained using LAT mutants with one tyrosine showed that Y127, Y171, Y191, and Y226 were phosphorylated upon T cell activation. Surprisingly, the mutant LAT with one tyrosine at 132 was not phosphorylated. Y132 was demonstrated to be required for PLC-γ1 binding and activation of Ras-MAPK (12), implying that this residue must be phosphorylated after TCR engagement. Based on published data and our data in this study, it is likely that phosphorylation of Y132 is dependent on the phosphorylation of other tyrosines. Phosphorylation of those tyrosines could change the conformation of LAT so that Y132 could be further phosphorylated. It is also possible that other phosphorylated tyrosines might recruit tyrosine kinases, such as IL-2-inducible T cell kinase to LAT to phosphorylate Y132. Because the LAT mutant with tyrosines at 132 and 191 could associate with PLC-γ1, Y191 might be one of the tyrosines required for phosphorylation of Y132. We could not exclude the possibility that any of the four membrane-proximal tyrosines could be phosphorylated in a way similar to Y132. Y110 might be phosphorylated during T cell activation, as suggested by recent data (17). In addition, studies described in this work mainly focused on TCR-mediated tyrosine phosphorylation of LAT. It is possible that costimulation via CD28 might induce phosphorylation of these membrane-proximal tyrosines. Because LAT-deficient cells are also deficient in CD28 and other surface molecules (7), future studies need to be done to address this possibility after reconstitution of LAT-deficiency cells with CD28.

Our data showed that Y127 was phosphorylated, possibly more than any other tyrosine in LAT. This tyrosine was also found to be phosphorylated in vitro by ZAP-70 (18). Although this tyrosine is within a Grb2-binding motif (Y^{127}HNP), this tyrosine after phosphorylation was not involved in binding of Grb2, Gads, or PLC-γ1. LAT mutants with Y127 in combination with any one of these tyrosines, Y132, Y171, Y191, or Y226, failed to associate with Grb2, Gads, and PLC-γ1 (data not shown). When we examined the sequence between Y127 and Y132 in human LAT (Y^{127}HNPGY^{132}), we found that it contains a binding motif (NPGY^{132}) for proteins containing a PTB domain. However, this motif is not present in murine LAT. It is not clear whether this motif in LAT binds any PTB-containing proteins. It is possible that phosphorylation of Y127 might change the local structure of LAT and affect the binding of PLC-γ1 to Y132. It is also possible that this tyrosine after phosphorylation is involved in association with other signaling proteins.

One surprising finding from our studies is that the LAT mutants with one single tyrosine at 127, 171, 191, or 226 failed to associate with Grb2, although these tyrosines are all in the YXN motif recognized by Grb2 and were phosphorylated upon T cell activation. However, the LAT mutants with any two of these tyrosines (Y171, Y191, or Y226) could associate with Grb2. This result suggests that two Grb2 molecules might bind to LAT cooperatively to form a stable complex. In the solved three-dimensional structure of Grb2, two Grb2 molecules in one asymmetric unit form a large contact area over 4100 $Å^2$ (19), suggesting that Grb2 could potentially form a dimer on LAT.

Our studies also revealed that PLC-γ1 preferentially binds to LAT with tyrosines at Y132 and Y191. The LAT mutant with only these two tyrosines could associate with PLC-γ1, although weakly compared with LAT with four membrane-distal tyrosines. This mutant failed to associate with Grb2 or Gads, suggesting that PLC-γ1 could bind LAT directly as proposed in other studies (20, 21). Because PLC-γ1 has two SH2 domains, it is possible that each SH2 domain binds one phosphorylated tyrosine on LAT. The N-terminal SH2 domain of PLC-γ1 might bind phosphorylated Y132, while the C-terminal SH2 domain binds Y191. Y191 might not be directly involved in interaction with PLC-γ1. Phosphorylation of Y191 is simply required for phosphorylation of Y132. Phosphorylation of Y191 might change LAT conformation or recruit other tyrosine kinases so Y132 could be phosphorylated.

The LAT mutant with four membrane-distal tyrosines could associate with Grb2, Gads, and PLC-γ similar to LAT-WT. It could reconstitute TCR-mediated Ras-MAPK and Ca^{2+} flux in LAT-deficient cells, although not as well as LAT-WT. This result suggests that these four tyrosines play a major role in LAT-mediated signaling events, while other tyrosines, such as Y110 and Y127, might also contribute to LAT function to some extent. The LAT mutants with one or two of these four membrane-distal tyrosines failed to reconstitute Ras-MAPK activation, Ca^{2+} flux, and NF-AT activation. None of these two-tyrosine mutants could associate with both PLC-γ1 and Grb2. However, three three-tyrosine LAT mutants, LAT-3Ya, 3Yb, and 3Yc, were capable of correcting these signaling defects to some extent. LAT-3Yb and -3Yc, which did not associate with Gads, could partially reconstitute Ras-MAPK, Ca^{2+} flux, and NF-AT activation in LAT-deficient cells. This result is different from the data from Gads knockout mice. Peripheral T cells from Gads knockout mice are defective in TCR-induced Ca^{2+} mobilization (16). It is possible that Gads might play some role in TCR-mediated signaling independent of its interaction with LAT.

We used retroviruses to deliver various LAT mutants into $LAT^{-/-}$ bone marrow cells, followed by adoptive transfer to study the importance of LAT binding to Grb2, Gads, and PLC-γ1 in thymocyte development. Compared with the procedure used to generate transgenic mice and knockin mice, this method is expedient and simple. The disadvantage of this retroviral system is the variations in retroviral transduction and adoptive transduction. Due to these variations, it is difficult to address whether LAT mutants have quantitative differences in TCR signaling during thymocyte development. Our experiments showing successful reconstitution of $LAT^{-/-}$ bone marrow cells with WT-LAT demonstrated that this system could be used to study LAT function in thymocyte development. Our results showed that the LAT mutant with four membrane-distal tyrosines was capable of binding to Grb2, Gads, and PLC-γ1 and correcting the defective TCR signaling events, such as the Ras-MAPK activation, Ca^{2+} mobilization, and NF-AT activation in LAT-deficient cells. This mutant was also sufficient for rescuing thymocyte development. This is in agreement with results from a study of knockin mice with mutation of these four tyrosines. Mutation of these four tyrosines abolishes LAT function in thymocyte development (14). Among these four tyrosines, Y132 in LAT has been shown to be responsible for binding of PLC-γ1. Y171, Y191, and 226 are the binding sites for Grb2. Y171 and Y191 are also the binding sites for Gads (12, 17). When we examined the function of LAT mutants with combinations of three of these four membrane-distal tyrosines, we found that only LAT-3Ya (Y132/171/191), LAT-3Yb (Y132/171/226), and LAT-3Yc (Y132/191/226) efficiently rescued thymocyte development. Of these three LAT mutants, LAT-Y132/171/226 and Y132/191/226 could not associate with Gads, suggesting that LAT binding to Gads is not required for thymocyte development. This conclusion is in agreement with the data from Gads knockout mice. In Gads knockout mice, there is a severe block in proliferation of $CD4^{-}CD8^{-}$ thymocytes, but those thymocytes can still differentiate into mature T cells (16). Because recruitment of SLP-76 to the membrane might be important for SLP-76 function (22), it is possible that SLP-76 might be recruited to LAT complexes via adaptor proteins other than Gads, such as Grb2. SLP-76 was originally identified as a protein that interacts with Grb2. It is possible that Grb2 recruits SLP-76 to LAT in the absence of Gads or the Gads-LAT interaction.

It was unexpected that LAT-3Yd with tyrosines at 171, 191, and 226 could rescue thymocyte development, although it did so less efficiently. This mutant could only bind to Grb2 and Gads, not PLC-γ1. This mutant failed to reconstitute MAPK and NF-AT activation like the LAT-Y132F mutant. T cells from mice reconstituted with this mutant have no or low TCR expression on their cell surface similar to those from mice reconstituted with the LAT Y132F mutant (data not shown) and mice with a knockin mutation at this tyrosine (Y136F in mouse LAT). Interestingly, these mutant mice developed a lymphoproliferative disorder and autoimmune disorder (23, 24). Our results showed that LAT with four membrane-distal tyrosines, Y132, Y171, Y191, and Y226, could function in thymocyte development like LAT-WT, while the mutant with three tyrosines, Y171, Y191, and Y226, functioned similarly to the Y132F mutant, suggesting that Y171, Y191, and Y226 are the ones that cause lymphoproliferation in mice by interacting with Grb2, Gads, or other unidentified proteins. How these tyrosines cause lymphoproliferative disorder and further autoimmune disease in the absence of PLC-γ1 interaction remains to be determined in the future.

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