THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 12, Issue of March 19, pp. 11229–11235, 2004
Printed in U.S.A.
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The Importance of Three Membrane-distal Tyrosines in the Adaptor Protein NTAL/LAB*

Received for publication, October 16, 2003, and in revised form, January 6, 2004
Published, JBC Papers in Press, January 13, 2004, DOI 10.1074/jbc.M311394200

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NTAL (non-T cell activation linker)/LAB (linker for activation of B cells) is a LAT (linker for activation of T cells)-like molecule that is expressed in B cells, mast cells, natural killer cells, and monocytes. Upon engagement of the B cell receptor or Fc receptors, it is phosphorylated and interacts with Grb2. LAB is capable of rescuing thymocyte development in LAT$^{-/-}$ mice. In this study, we utilized various LAB Tyr to Phe mutants to map the phosphorylation and Grb2-binding sites of LAB. We also examined the function of these mutants by investigating their ability to rescue signaling defects in LAT-deficient Jurkat cells and thymocyte development in LAT$^{-/-}$ mice. Our results indicated that human LAB was primarily phosphorylated on three membrane-distal tyrosines, Tyr^{136}, Tyr^{193}, and Tyr^{233}. Mutation of these three tyrosines abolished Grb2 binding and LAB function. Our data suggested that these tyrosines are the most important tyrosines for LAB function.

Adaptor proteins play an important role in the antigen receptor signaling pathways by facilitating formation of multiple protein complexes. These protein complexes are required for activation of enzymes in the signaling cascades (1–3). Adaptor proteins can be categorized into two classes: membrane-associated adaptors such as PAG (4), SIT (5), TRIM (6), LAX (7), and LAT[1] (Linker for activation of T cells) (8), and cytosolic adaptors such as Grb2, Gads (9) SLP-76 (10), and BLNK (11). The importance of LAT, Gads, SLP-76, and BLNK adaptor proteins in lymphocyte activation and development has been clearly demonstrated in cell lines or mice deficient in these proteins (12–19).

LAT is a membrane-associated adaptor protein that contains multiple tyrosine residues in its cytoplasmic domain (8, 20). Upon T cell activation, it is phosphorylated and binds several signaling proteins (8, 9, 21). LAT functions to recruit Sos to the membrane to activate Ras through binding Grb2. LAT also interacts with PLC-γ1, and this interaction is required for optimal phosphorylation and activation of PLC-γ1. In addition, LAT recruits the Gads-SLP-76 complex to the membrane, and SLP-76 further interacts with Vav and PLC-γ1 (22, 23). Jurkat cells deficient in LAT protein fail to signal from the TCR to activate the Ras-MAPK and Ca^{2+} pathways (12, 24), indicating that LAT is essential in TCR-mediated signaling. A LAT deficiency in mice also severely affects thymocyte development (13). Thymocyte development in LAT$^{-/-}$ mice is arrested at the CD4^{-}CD8^{-} stage, leading to the absence of mature T cells in the periphery. Among the nine conserved tyrosine residues in LAT, the four membrane-distal tyrosines (Tyr^{132}, Tyr^{171}, Tyr^{191}, and Tyr^{226} in human LAT) are crucial for LAT phosphorylation, its interaction with Grb2, Gads, and PLC-γ1, and LAT function in T cell activation and development (25–27). Interestingly, LAT knock-in mutant mice expressing a LAT mutant that does not bind PLC-γ1 (LATY136F) develop a severe autoimmune disease with lymphocyte infiltration in various organs, suggesting that the LAT interaction with PLC-γ1 is important in normal T cell function and homeostasis (28, 29).

During a search for a LAT-like molecule in B cells, a novel membrane-associated adaptor molecule, NTAL (non-T cell activation linker)/LAB (linker for activation of B cells), was identified (30, 31). Similar to LAT, this molecule is localized to lipid rafts. Upon B cell receptor (BCR) or Fc receptor cross-linking, LAB is phosphorylated and associates with the adaptor protein Grb2. The cytoplasmic domain of LAB contains nine tyrosines conserved between mouse and human. Five of these tyrosines are within a Grb2-binding motif (Tyr^{95}, Tyr^{118}, Tyr^{136}, Tyr^{193}, and Tyr^{233}); however, there is no apparent PLC-γ1 or -γ2-binding motif, and LAB does not bind PLC-γ1 or -γ2. The functional similarity between LAT and LAB was demonstrated by an adoptive transfer experiment in which LAB could rescue thymocyte development in LAT$^{-/-}$ mice (31).

In this study, we generated a series of LAB mutants with tyrosine to phenylalanine mutations and stably expressed them in a mouse B cell line to map the phosphorylation and Grb2-binding sites of LAB. We also examined the function of these mutant proteins by determining their ability to rescue signaling defects in LAT-deficient Jurkat cells and reconstitute thymocyte development in LAT$^{-/-}$ mice. Our results indicated that the three membrane-distal tyrosines are the most important tyrosines in LAB function.

EXPERIMENTAL PROCEDURES

Constructs—Site-directed mutagenesis was performed to mutate tyrosine residues in human LAB to phenylalanine by PCR. LAB mutants with combinations of different mutations were constructed by assembling cDNA fragments of the single Tyr to Phe mutants using restriction endonuclease sites present in the LAB cDNA or created during mutagenesis. Two retroviral vectors, pMSCV-IRES/*Bla* (blasticidin-resistant gene) and pMSCV-IRES/*GFP* (green fluorescent protein), were used in our experiments. In these two vectors, expression of LAT or LAB was driven by the 5′ long terminal repeat. The blasticidin resistance and *GFP* genes were placed under the control of an IRES (internal ribosomal entry site) element. LAB mutants used in this study are listed in Fig. 1. pMSCV-IRES/*Bla* viruses were used to transduce A20 cells (murine B cell line) to generate stable cell lines, whereas pMSCV-

* This work was supported by National Institutes of Heath Grants 1R01 AI048674 and AI056156. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LAT, linker for activation of T cells; LAB, linker for activation of B cells; NTAL, non-T cell activation linker; BCR, B cell receptor; TCR, T cell receptor; PLC, phospholipase C; IRES, internal ribosomal entry site; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; WT, wild type; EST, expressed sequence tag.

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IRES/*GFP* viruses were primarily used to transduce bone marrow cells from $LAT^{-/-}$ mice in the adoptive transfer experiment and for transient expression of LAB in the LAT-deficient Jurkat cells.

Accession Numbers—Pig, cow, and chicken LAB protein sequences were obtained by translating expressed sequence tag (EST) clones. The GenBank™ accession numbers of EST clones are BF078123 and BE231818 for pig, AW487463, BF890213, and BE755183 for cow, and BU242463 for chicken.

Cell Lines, Transfection, and Retroviral Transduction—A20 cells and LAT-deficient cells (J.CaM2.5) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, and Phoenix ecotropic virus-packaging cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. LAT-deficient Jurkat cells expressing the ecotropic receptor mCAT-1 were described previously (26). A total of 15 μg of each retroviral construct was used to transfect Phoenix ecotropic packaging cells using calcium phosphate. Forty-eight to seventy-two hours after transfection, the cell culture supernatant containing recombinant retroviruses was harvested either for immediate viral transduction or to be frozen at −80 °C. A20 and J.CaM2.5 cells expressing various LAB mutants were generated by transducing these cells with recombinant retroviruses followed by selection in the presence of blasticidin (10 μg/ml).

Antibodies, Immunoprecipitation, and Western Blotting—The following antibodies were used for immunoprecipitation and Western blotting: anti-mouse IgG $F(ab')_2$ from Zymed Laboratories Inc. (San Francisco, CA); anti-TCR (C305); anti-myc (9E10); rabbit anti-LAB antisera and mouse monoclonal anti-LAB (31); anti-phosphotyrosine (PY20) and anti-Grb2 monoclonal antibodies (Transduction Laboratories, Lexington, KY); anti-phosphotyrosine (4G10) and rabbit anti-LATpY191(Upstate Biotechnology, Lake Placid, NY); rabbit anti-Grb2 polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-phospho-Erk (Cell Signaling, Beverly, MA). For immunoprecipitation, A20 cells expressing different LAB mutants were either stimulated with anti-mouse IgG $F(ab')_2$ (20 μg/ml) for 1.5 min or left untreated. A total of 1×10^7 cells were lysed in 500 μl of ice-cold lysis buffer (1% Brij-97, 25 mM Tris-Cl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4) with protease inhibitors. Postnuclear lysates were immunoprecipitated with different antibodies as indicated in each figure. Immunoprecipitated proteins were resolved on the SDS-PAGE and blotted with different antibodies as indicated in each figure.

MAPK Activation and Ca^{2+} Flux—Intracellular free Ca^{2+} measurements were performed as described previously (32). Twelve to sixteen hours after retroviral transduction, J.CaM2.5 cells were loaded with Indo-1. Anti-CD3ε (OKT3) was used to activate these cells. Ca^{2+} flux in GFP^+ cells was analyzed using flow cytometry (FACStar, BD Biosciences) to monitor the fluorescence emission ratio at 405–495 nm.

Transduction of Bone Marrow Cells and Adoptive Transfer—Bone marrow cell transduction and adoptive transfer were performed as described previously (26). Briefly, bone marrow cells from $LAT^{-/-}$ mice were cultured in Iscove's modified Dulbecco's medium with 15% fetal bovine serum in the presence of interleukin (IL)-3, IL-6, and stem cell factor for 3–4 days. They were then mixed with retroviral supernatant in the presence of polybrene (8 μg/ml) and centrifuged at 1300 × *g* for 3 h at 22 °C. After culturing for 24 h, these cells were transduced again by retroviruses and cultured for 2 days before injection into mice. A total of $0.5–1 \times 10^6$ cells were injected via a tail vein into $LAT^{-/-}$ mice that were sublethally irradiated at 5Gy.

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

RESULTS

Transfection of LAB Mutants into Murine A20 B Cell Line—In the cytoplasmic domain of LAB, there are nine tyrosine residues conserved between mouse and human (30, 31). Five of these tyrosines are within a Grb2-binding motif (^{95}YQNF^{98}, ^{118}YYNW^{121}, ^{136}YENV^{139}, ^{193}YQNS^{196}, and ^{233}YVNG^{236} in human LAB) and can potentially bind Grb2 once phosphorylated. Although previous studies have shown that LAB is phosphorylated upon BCR ligation, it has not been determined which of these tyrosines are phosphorylated *in vivo* and which of them are required for the LAB-Grb2 association.

To answer these questions, we generated LAB mutants with mutations at each tyrosine and mutants with combinations of different Tyr to Phe mutations of the three membrane-distal tyrosines (Fig. 1). These mutants together with wild type (WT)-LAB were introduced into the A20 murine B cell line by retroviral transduction. Stable transductants were selected in the presence of blasticidin and were used for further analysis without subcloning to avoid differences among subclones. As shown in Fig. 2*A*, all the cell lines expressing WT and mutant LAB were well matched for LAB expression.

Establishment of these stable cell lines enabled us to examine whether BCR-mediated phosphorylation of LAB was affected by these mutations. The transduced A20 cells were stimulated with anti-mouse IgG $F(ab')_2$ for 1.5 min before lysis. LAB protein was immunoprecipitated from cell lysates with rabbit anti-LAB. Tyrosine phosphorylation of LAB was determined by Western blotting with anti-phosphotyrosine. As shown in Fig. 2*B*, WT-LAB was heavily phosphorylated after BCR stimulation. Mutation at Tyr^{58}, Tyr^{84}, Tyr^{110}, or Tyr^{118} did not affect phosphorylation of LAB, suggesting that these tyrosine residues might not be phosphorylated upon BCR stimulation. Although mutation at Tyr^{95} or Tyr^{136} only slightly reduced phosphorylation of LAB, mutation at Tyr^{193} or Tyr^{233} caused a significant decrease in phosphorylation when compared with WT-LAB. Among the mutants with a single Tyr to Phe mutation, the Y233F mutant was least phosphorylated. In addition, combinations of the Y233F mutation with mutation at other tyrosines, such as 2YF2 or 2YF4, also caused a dramatic reduction in tyrosine phosphorylation of LAB. Mutations of three membrane-distal tyrosines (Tyr^{136}, Tyr^{193}, and Tyr^{233}) or four tyrosines (Tyr^{95}, Tyr^{136}, Tyr^{193}, and Tyr^{233}) almost totally abolished LAB tyrosine phosphorylation. These data indicated that the three membrane-distal tyrosines, Tyr^{136}, Tyr^{193}, and Tyr^{233}, are the primary phosphorylation sites.

We also examined phosphorylation of LAB using rabbit antisera that recognize phosphorylated LAT $pTyr^{191}$. LAT Tyr^{191} is within the sequence context of YVNV similar to that of LAB Tyr^{233} (YVNG). Thus, it is possible that this phospho-LAT antibody could also recognize phosphorylated LAB. Postnuclear lysates of different stable cell lines were immunoblotted with this antisera. As shown in Fig. 2*C*, anti-LAT $pTyr^{191}$ sera did not detect LAB in unstimulated A20 transfectants but did recognize WT-LAB and mutants containing Tyr^{233} (Y136F, Y193F, 2YF1, and 2YF3) upon BCR stimulation. Furthermore, it failed to detect mutants that contained a mutated Tyr^{233} (Y233F, 2YF2, 2YF4, 3YF, and 4YF), suggesting that the antisera specifically recognized phospho-Tyr^{233} in LAB. This finding further supported the data from Fig. 2*B* and indicated that Tyr^{233} of LAB is phosphorylated upon BCR stimulation. Interestingly, mutation of Tyr^{136} in LAB as in Y136F and 2YF3 enhanced phosphorylation of LAB at Tyr^{233} upon BCR stimulation when compared with wild-type (Fig. 2*C*). It is possible that Tyr^{136} negatively regulates LAB phosphorylation at Tyr^{233}.

LAB Interaction with Grb2—LAT interacts with several signaling proteins such as Grb2, Gads, and PLC-γ1 (8, 21). Unlike LAT, LAB only binds Grb2, not PLC-γ1 or PLC-γ2, upon activation via the BCR (30, 31). Our data from Fig. 2 suggested that most of the LAB tyrosine phosphorylation occurred on three membrane-distal tyrosines, Tyr^{136}, Tyr^{193}, and Tyr^{233}. Therefore, we chose to focus on these three tyrosines to study whether they are required for the Grb2-LAB interaction. Transduced A20 cells were lysed after stimulation with anti-mouse IgG $F(ab')_2$. Postnuclear extracts were immunoprecipitated with rabbit anti-Grb2. The association of LAB with Grb2 was examined by anti-LAB and anti-pTyr Western blotting.

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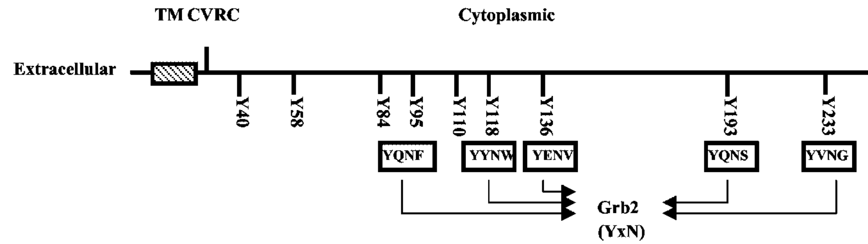
A



B

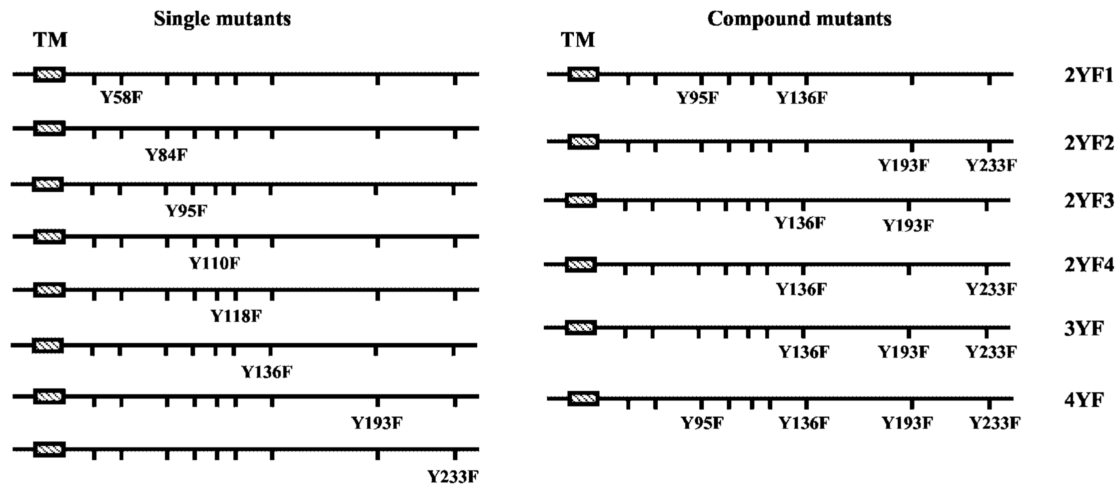
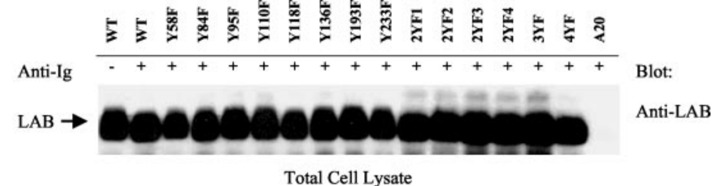


FIG. 1. **Tyrosine residues in LAB and a list of LAB mutants with Tyr to Phe substitutions.** *A*, the structural domains of LAB. The five tyrosine residues within Grb2-binding motif (YXN) are indicated by *arrows*. *B*, a list of the LAB mutants used. Only the mutated tyrosine residues in different mutants are indicated; tyrosine residues at other positions are still intact. *TM*, transmembrane domain.

A



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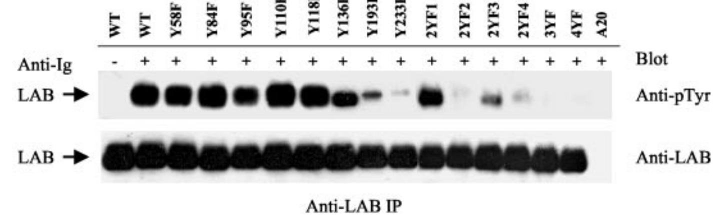

C

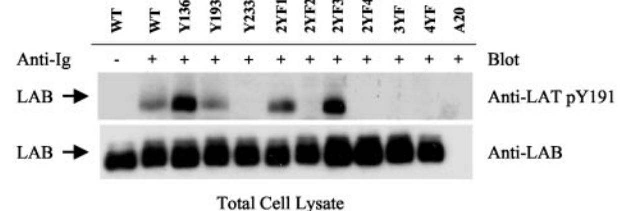

FIG. 2. **LAB expression and tyrosine phosphorylation upon BCR stimulation.** As shown in *A*, A20 cells transduced with different LAB mutants were lysed in 1% Brij lysis buffer. Postnuclear lysates were analyzed by an anti-LAB blot. As shown in *B*, lysates before and after stimulation were subjected to immunoprecipitation with rabbit anti-LAB sera. Phosphorylation of LAB was detected with an anti-phosphotyrosine blot. The amount of immunoprecipitated protein was determined by blotting with a LAB monoclonal antibody. As shown in *C*, total lysates were analyzed by rabbit LAT $pTyr^{191}$ specific antisera. Representative experiments are shown ($n = 5$).

As shown in Fig. 3, mutation at Tyr^{136}, Tyr^{193}, or Tyr^{233} alone only slightly reduced the LAB-Grb2 association, suggesting that Grb2 binds to LAB via multiple tyrosine residues. Interestingly, the apparent molecular weight of the LAB Y136F mutant associated with Grb2 appeared less than that of WT-LAB on the SDS-PAGE. Mutation of two tyrosines in LAB as in 2YF2, 2YF3, and 2YF4 and three tyrosines dramatically reduced the Grb2-LAB interaction. These results indicated that these membrane-distal tyrosines were the most important in the interaction of LAB with Grb2. In addition, comparison of LAB amino acid sequences deduced from the EST data base showed that these three tyrosines are well conserved in LAB

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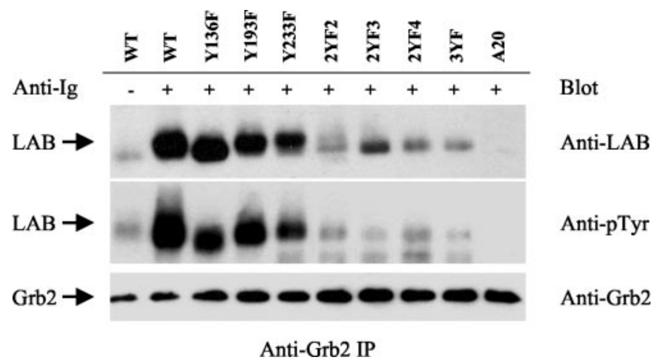

FIG. 3. **Association of LAB mutants with Grb2.** A20 cells reconstituted with WT or various LAB mutants were lysed in 1% Brij lysis buffer. Postnuclear lysates were subjected to immunoprecipitation with anti-Grb2 sera. The association of LAB with Grb2 was detected by blotting with anti-LAB and anti-pY. The amount of Grb2 immunoprecipitated was determined by an anti-Grb2 blot. A representative experiment is shown ($n = 5$).

from human, mouse, pig, cow, and chicken (Fig. 4), implying that they are important in LAB function.

Rescue of Ca^{2+} Flux in LAT-deficient Cells by LAB Mutants—Previous studies showed that ectopic expression of NTAL/LAB in LAT-deficient Jurkat T cells partially rescued TCR-mediated activation of Erk1/2 and calcium response (30). We decided to examine whether these LAB mutants could correct signaling defects in LAT-deficient cells. Recombinant retroviruses were used to transiently express different mutants in J.CaM2.5 cells. As shown in Fig. 5*A*, no TCR-mediated Ca^{2+} flux was seen in the parental cells. Introduction of LAT rescued the Ca^{2+} flux defect as reported previously (12). Transient expression of WT-LAB also restored Ca^{2+} flux in LAT-deficient cells, albeit at a reduced level when compared with WT-LAT. Mutation of any of the three membrane-distal tyrosines reduced the ability of LAB function in TCR-mediated Ca^{2+} flux. Mutation of any two or three distal tyrosines in LAB rendered LAB virtually nonfunctional in Ca^{2+} flux. Expression levels of various mutants and WT-LAB were similar (Fig. 5*B*). We also examined whether TCR-mediated Erk activation could be rescued by WT and mutant LAB. However, in contrast to previous reports (30), we failed to rescue Erk activation with expression of WT-LAB or any of these LAB mutants (data not shown). Our data suggested that the three membrane-distal tyrosines of LAB are important in coupling the receptor engagement to Ca^{2+} flux.

The Three Membrane-distal Tyrosines Are Important in Rescuing Thymocyte Development in $LAT^{-/-}$ Mice—In LAT-deficient mice, thymocytes are unable to differentiate past the $CD25^+CD44^-$ subset of the $CD4^-CD8^-$ stage. Previously, we showed that WT-LAB could compensate for a LAT deficiency when it was introduced into $LAT^{-/-}$ bone marrow cells (31). To study whether the three membrane-distal tyrosines are important in LAB function, we used recombinant retroviruses to introduce WT-LAB and various LAB mutants into the bone marrow cells from $LAT^{-/-}$ mice. After transduction, these bone marrow cells were allowed to expand and were then transferred into irradiated $LAT^{-/-}$ mice by injection via a tail vein. Because these recombinant viruses carried both *LAB* and *GFP* genes, lymphocytes derived from transduced bone marrow cells should be GFP^+ and express LAB. Five to six weeks after injection, thymocytes and splenocytes from the reconstituted mice were analyzed for expression of GFP, CD4, CD8, and TCRβ by flow cytometry.

As shown in Fig. 6, WT-LAB rescued thymocyte development in $LAT^{-/-}$ mice as expected. The majority of thymocytes in mice reconstituted with WT-LAB were GFP^+. Flow cytometric analysis of these thymocytes showed that these GFP^+ cells expressed CD4, CD8, or both. The low percentage of $CD4^+CD8^+$ (double positive) thymocytes was likely due to a limited number of T progenitor cells transferred and to the fact that most of the double positive thymocytes had developed into single positive thymocytes. These thymocytes further developed into mature single positive T cells, which were detected in the periphery. Interestingly, there was a preferential skewing toward the $CD4^+$ lineage. Similar results were seen in mice adoptively transferred with bone marrow cells expressing LAB Y193F (Fig. 6*A*). However, in mice reconstituted with Y136F and Y233F mutants, there were even more $CD4^+$ T cells. TCR expression in both thymocytes and splenic T cells from mice reconstituted with WT or single mutants was low when compared with that in T cells from B6 mice (Fig. 6*B*). The mutants with two or three of the membrane-distal tyrosine residues mutated failed to restore thymocyte development as neither single positive nor double positive T cells were detected in these mice (Fig. 6*A*). Because GFP^+ cells were present in the thymuses and spleens from these mice, failure of thymocyte development was not due to unsuccessful adoptive transfer or retroviral transduction. These data suggested that the three membrane-distal tyrosines were also important in correcting the thymocyte development defect in $LAT^{-/-}$ mice.

DISCUSSION

LAB is a membrane-associated adaptor molecule. Upon stimulation via the BCR, it interacts with the adaptor Grb2. In this study, we used different Tyr to Phe mutants to map the tyrosine phosphorylation sites in LAB and to study the effect of these mutations on LAB function. Our results showed that mutation of any of the three membrane-distal tyrosines, Tyr^{136}, Tyr^{193}, or Tyr^{233}, reduced LAB phosphorylation. Mutation of all three membrane-distal tyrosines virtually abolished LAB phosphorylation, indicating that LAB is primarily phosphorylated at these tyrosines. These tyrosines are all within a Grb2 SH2 domain-binding motif. Mutation of any of them alone reduced the Grb2-LAB interaction, whereas mutation of two or three abolished Grb2 binding. We also investigated the function of these mutants by examining their ability to reconstitute Ca^{2+} flux in a LAT-deficient cell line and to rescue thymocyte development in $LAT^{-/-}$ mice. Our data showed that the three membrane-distal tyrosines, which are well conserved among different species (Fig. 3), are the most important for LAB function.

Among the single Tyr to Phe mutants, mutation at Tyr^{233} caused a significant reduction in tyrosine phosphorylation and the LAB-Grb2 interaction. This tyrosine is contained within a YVNG sequence, which is very similar to the Tyr^{171} and Tyr^{191} in LAT (YVNV). These two tyrosines in LAT are required for proper binding of Grb2, Gads, and PLC-γ1. Thus, Tyr^{233} in LAB may be a functional equivalent of Tyr^{171} or Tyr^{191} in LAT. The dramatic reduction in phosphorylation of the LAB Y233F mutant suggested that Tyr^{233} is a primary target of the Syk family kinases. However, we could not rule out an alternative scenario in which Tyr^{233} is required for the recruitment of kinases to LAB. Interestingly, when Tyr^{136} was mutated, phosphorylation of Tyr^{233} was enhanced, although overall tyrosine phosphorylation of LAB was reduced (Fig. 2*C*). It is possible that, upon BCR engagement, Tyr^{136} is phosphorylated and recruits a phosphatase to dephosphorylate LAB. Alternatively, phosphorylation of Tyr^{136} may cause a conformational change in LAB so that Tyr^{233} is not accessible to tyrosine kinases.

The increase in intracellular free Ca^{2+} provides an important intermediary in lymphocyte activation. In comparison with LAT, LAB has no PLC-γ1-binding site and is incapable of binding PLC-γ1 or -γ2. Our data showed that introduction of WT-LAB or a single Tyr to Phe LAB mutant into the LAT-

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                              25  28               40                 58
MSSGTE LLWP-GAALLVLLGVAASL CVR CSRPGAKRSEKI YQQRSLREDQQSFTGSRTYS Human
MSAELE LLWPVSGLLLLLLGATAWL CVH CSRPGVKRNEKI YEQRNRQENAQSSAAAQTYS Murine
MGPETE LLWP-GAALLLLVGAAAGL CVR CSRPGAKRSEKI YEQRSL-ENQQSFAVARTYT Pig
MSADTE LLWP-GAALLLLLGAAASL CVR CSRPGAKKSEKM YEQRSLQENQQSFAVARTYT Cow
                 MLLGVAVSA CVR CQLYATKRG-KD GSQGSRLERPQRFEVIRSCS Chicken

                                   84         95              110
-LVGQAWPGPLADMAPT----RKDKLLQFYPSLEDPASSRYQNFSKGSRHGSEEAYIDPIA
-LARQVWPGPQMDTAPNKSFERKNKML--FSHLEGPESPRYQNFYKGSNQEPDAAYVDPIP
-LVREAWPEPLMDTASNVTSTRKDKLLQFSPSLEDPDSSR-----------------DPLAT
-LVREAWPG---DTASNMELTRKDKLLRFSPSLEDSZSPR-----------------DPTS
AVTRRPERIKEPENLARKAPEELSTSC--HVGFESSAEPRYQNFLTEDWLHEDAAYVEPVP

118/119          136
MEYYNWGRFSKPPEDDD-ANS Y ENVLICKQKTTETGAQQEGIGGLCRGKLSLSLALKTGPT
TNYYNWGCFQKPSEDDD-SNS Y ENVLVCKPSTPESGV------------------------
DDYYNWGQSQKPSEDDDDTNS Y ENVLICKPPKIESG-------------------------
TDYYNYDPFQKPVEDDDDTNS Y ENVLICKPKRMESA-------------------------
LDYYSHNRFFSPANDED-SHS Y QNVIIGDPCSSE---------------------------

               193                                         233
SGLCPSASPEEDEESED Y QNSASIHQWRESRKVMGQKQREASPGPVGSPDEEDGEPD Y VN
------------EDFED Y QNSVSIHQWRESKRTMG-----APMSLSGSPDE---EPD Y VN
-----------DEGSED Y QNSASIQQWRESKRVVEPVTREAPPSPGGS------EPD Y VN
-----------DEGSED Y QNSASIQQWRESKKVMEPAPREAPLSLAGSPDEDG-EPD Y VN
-----------LDDAED Y ENSTAIEVW----KVQQADA----MLYAESQDE---EPD Y VN

 GEVAATEA              243
 GDVAAAENI             203
 GDVAATKA              192
 GDVAAIEA              191
 TEPTIDAVVLSK          199
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FIG. 4. **Comparison of the amino acid sequences of LAB from human, mouse, pig, cow, and chicken.** The human LAB sequence was used to search the EST data base for EST clones encoding LAB. The nucleotide sequences from these clones were used to deduce LAB amino acid sequences. The last three membrane-distal tyrosine residues, Tyr^{136}, Tyr^{193} and Tyr^{233}, are within a Grb2-binding motif and highly conserved among these species.

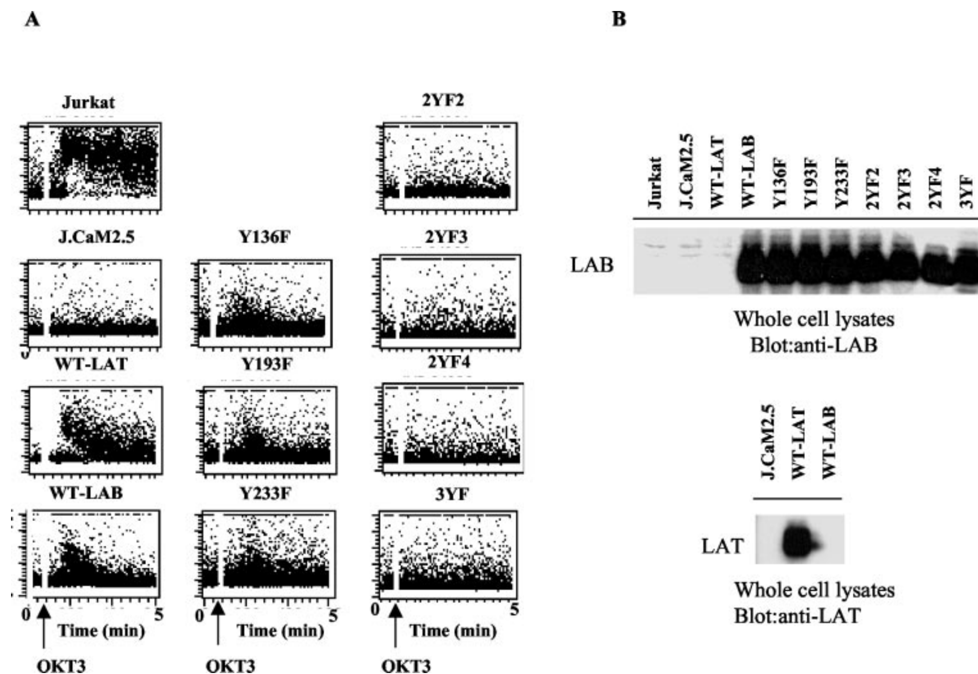

FIG. 5. **Calcium flux in LAT-deficient cells reconstituted with wild type LAT, LAB, and different LAB mutants.** As shown in *A*, 12–16 h after retroviral transduction, LAT-deficient cells were loaded with Indo-1. Ca^{2+} flux was induced with anti-CD3ε (*OKT3*), and the fluorescence emission ratio at 405/495 nm was measured by flow cytometry. Ca^{2+} flux in GFP^+ cells was analyzed. As shown in *B*, the expression of LAB and LAT was detected with antibodies against each protein. Representative experiments are shown (n = 4).

deficient cells partially rescued Ca^{2+} flux but not MAPK activation. Ca^{2+} mobilization restored by either WT-LAB or single mutants was similar to that observed in LAT Y132F transfectants (33) as a transient Ca^{2+} flux, but not a sustained phase, was attained. Binding of PLC-γ1 to LAT is essential for maximal PLC-γ1 phosphorylation and activation (12, 33). How LAB or the LAT Y132F mutant partially restored Ca^{2+} flux in the absence of PLC-γ binding is still not known. Because both LAB and LATY132F have Grb2- and Gads-binding motifs, it is possible that PLC-γ1 may interact indirectly with these two adaptor molecules via the Gads-SLP-76 or Grb2-SLP-76 complex. Mutation of these Grb2-binding sites would disrupt this indirect association of LAB with PLC-γ1. However, we could not exclude the possibility that LAB might contain an undefined PLCγ-binding motif that mediates the LAB-PLCγ association. This interaction may be too weak to detect by coimmunoprecipitation. Another possibility is that TCR-mediated calcium mobilization may not be completely dependent on PLC-γ1.

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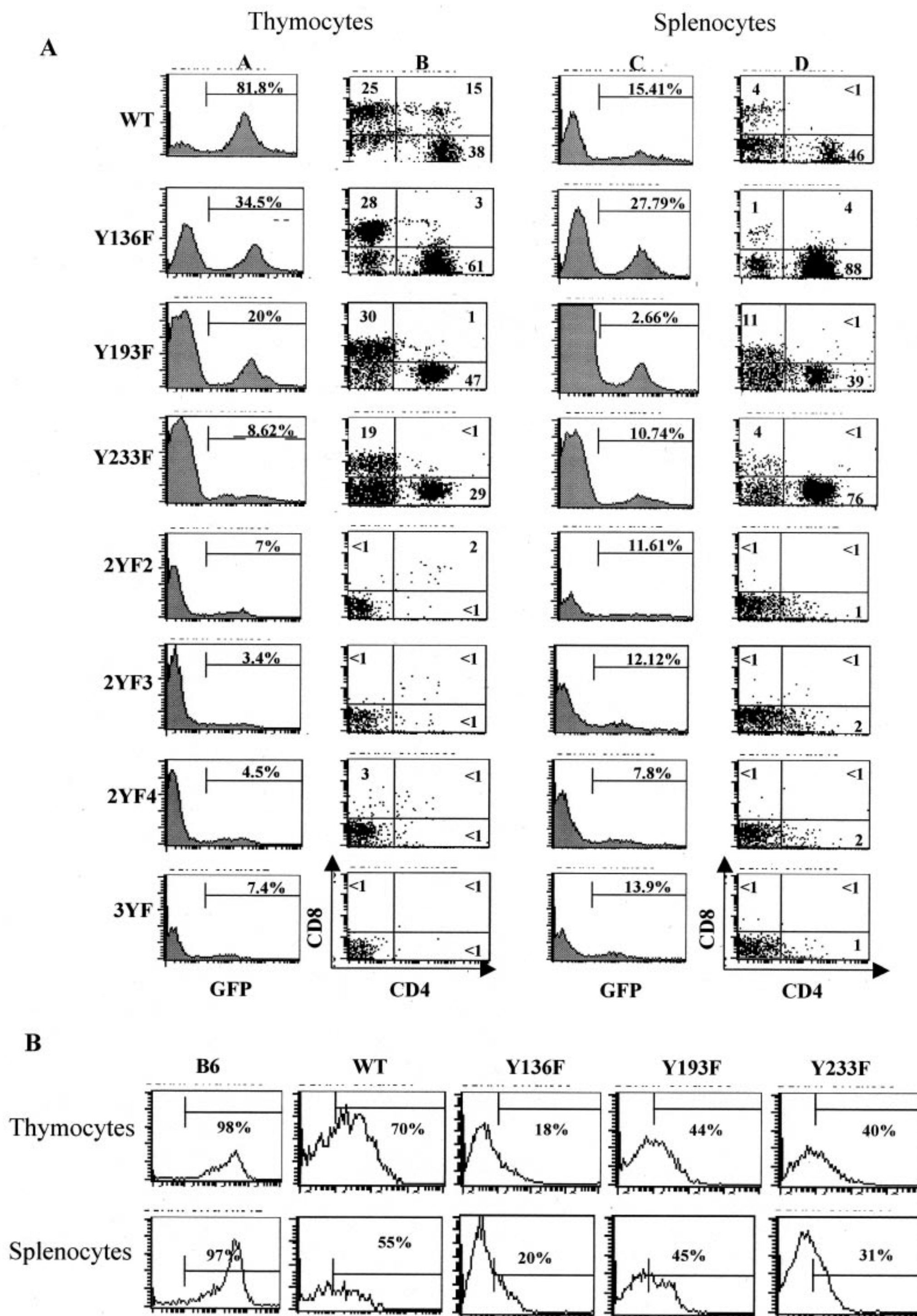

FIG. 6. **Rescue of thymocyte development by LAB.** Bone marrow cells from LAT$^{-/-}$ mice were transduced with retroviruses encoding WT-LAB and mutants and then transferred into irradiated LAT$^{-/-}$ mice. Five weeks after transfer, thymocytes and splenocytes from these mice were harvested and analyzed by fluorescence-activated cell sorter. As shown in *A*, thymocytes and splenocytes from adoptively transferred mice were analyzed for GFP, CD4, and CD8 expression. As shown in *B*, TCRβ expression on CD4$^+$ thymocytes and splenocytes reconstituted with WT and LAB mutants was also examined (n = 4).

Jurkat cells deficient in PLC-γ1 have a similar phenotype with regard to Ca^{2+} flux as LAT Y132F transfectants (34). In this case, other PLC isoforms, such as PLC-γ2, might be recruited to LAT or LAB.

Although LAB associated with Grb2, it failed to reconstitute Erk activation in LAT-deficient cells like the LAT Y132F mutant. This may also be attributed to the lack of the LAB-PLCγ association. Insufficient activation of PLC-γ1 could affect activation of PKC or RasGRP1, which acts upstream of Ras. Recruitment of the Grb2-Sos complex to LAT may be required, but not sufficient, for Erk activation in T cells. Our results differed from the published data in which LAB/NTAL successfully rescued MAPK activation in LAT-deficient cells (30). The reason for this discrepancy is unclear. It may be due to variations in the NTAL/LAB expression levels when different vectors were used.

WT-LAB and single Tyr to Phe mutants were capable of rescuing thymocyte development in LAT$^{-/-}$ mice. It is not clear why TCR expression in T cells reconstituted with LAB was low

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when compared with that in normal T cells. Similar TCR expression was also found in T cells from the Y136F knock-in mice (28, 29). It is possible that LAB functions like the LATY136F mutant. Although three LAB single tyrosine mutants, Y136F, Y193F, and Y233F, were all capable of rescuing thymocyte development like WT-LAB, $CD4^+$ T cell development was more prominently skewed in mice reconstituted with the Y136F and Y233F mutants. LAB-mediated reconstitution of $CD8^+$ T cell development could be affected by mutation at Tyr^{136} or Tyr^{233} more than at Tyr^{193}. The precise mechanism for this difference is not clear as current understanding of the differential signals for $CD4^+$ and $CD8^+$ T cell development is still limited. Although mutation of any one of the three membrane-distal tyrosines had no significant effect on the ability of LAB to function in thymocyte development, mutation of two or three of these tyrosines abolished LAB function; therefore, multiple Grb2-binding sites are required for LAB function. These findings were similar to results from studies of LAT mutants (26). In LAT, mutation of the four membrane-distal tyrosines abolished LAT tyrosine phosphorylation, its interaction with Grb2, Gads, and PLC-γ1, and LAT function in T cell activation, and thymocyte development. Our data showed that mutation of three membrane-distal tyrosines of LAB disrupted LAB tyrosine phosphorylation, its interaction with Grb2, and its ability to rescue a LAT deficiency. In summary, our data indicated that among nine tyrosine residues in human LAB, the three membrane-distal tyrosines are the most important in LAB function.

Acknowledgment—We thank the Duke University Cancer Center Flow Cytometry for fluorescence-activated cell sorter analysis.

REFERENCES

1. Wange, R. L., and Samelson, L. E. (1996) *Immunity* **5,** 197–205
2. Clements, J. L., Boerth, N. J., Lee, J. R., and Koretzky, G. A. (1999) *Annu. Rev. Immunol.* **17,** 89–108
3. Samelson, L. E. (2002) *Annu. Rev. Immunol.* **20,** 371–394
4. Brdicka, T., Pavlistova, D., Leo, A., Bruyns, E., Korinek, V., Angelisova, P., Scherer, J., Shevchenko, A., Hilgert, I., Cerny, J., Drbal, K., Kuramitsu, Y., Kornacker, B., Horejsi, V., and Schraven, B. (2000) *J. Exp. Med.* **191,** 1591–1604
5. Marie-Cardine, A., Kirchgessner, H., Bruyns, E., Shevchenko, A., Mann, M., Autschbach, F., Ratnofsky, S., Meuer, S., and Schraven, B. (1999) *J. Exp. Med.* **189,** 1181–1194
6. Bruyns, E., Marie-Cardine, A., Kirchgessner, H., Sagolla, K., Shevchenko, A., Mann, M., Autschbach, F., Bensussan, A., Meuer, S., and Schraven, B. (1998) *J. Exp. Med.* **188,** 561–575
7. Zhu, M., Janssen, E., Leung, K., and Zhang, W. (2002) *J. Biol. Chem.* **277,** 46151–46158
8. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998) *Cell* **92,** 83–92
9. Liu, S. K., and McGlade, C. J. (1998) *Oncogene* **17,** 3073–3082
10. Jackman, J. K., Motto, D. G., Sun, Q., Tanemoto, M., Turck, C. W., Peltz, G. A., Koretzky, G. A., and Findell, P. R. (1995) *J. Biol. Chem.* **270,** 7029–7032
11. Fu, C., Turck, C. W., Kurosaki, T., and Chan, A. C. (1998) *Immunity* **9,** 93–103
12. Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998) *Immunity* **9,** 617–626
13. Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., Long, E. O., Love, P. E., and Samelson, L. E. (1999) *Immunity* **10,** 323–332
14. Yoder, J., Pham, C., Iizuka, Y. M., Kanagawa, O., Liu, S. K., McGlade, J., and Cheng, A. M. (2001) *Science* **291,** 1987–1991
15. Yablonski, D., Kuhne, M. R., Kadlecek, T., and Weiss, A. (1998) *Science* **281,** 413–416
16. Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F. W., and Geha, R. S. (1998) *Cell* **94,** 229–238
17. Clements, J. L., Yang, B., Ross-Barta, S. E., Eliason, S. L., Hrstka, R. F., Williamson, R. A., and Koretzky, G. A. (1998) *Science* **281,** 416–419
18. Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A. C., and Kurosaki, T. (1999) *Immunity* **10,** 117–125
19. Pappu, R., Cheng, A. M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B. P., and Chan, A. C. (1999) *Science* **286,** 1949–1954
20. Weber, J. R., Orstavik, S., Torgersen, K. M., Danbolt, N. C., Berg, S. F., Ryan, J. C., Tasken, K., Imboden, J. B., and Vaage, J. T. (1998) *J. Exp. Med.* **187,** 1157–1161
21. Liu, S. K., Fang, N., Koretzky, G. A., and McGlade, C. J. (1999) *Curr. Biol.* **9,** 67–75
22. Wu, J., Motto, D. G., Koretzky, G. A., and Weiss, A. (1996) *Immunity* **4,** 593–602
23. Yablonski, D., Kadlecek, T., and Weiss, A. (2001) *Mol. Cell. Biol.* **21,** 4208–4218
24. Zhang, W., Irvin, B. J., Trible, R. P., Abraham, R. T., and Samelson, L. E. (1999) *Int. Immunol.* **11,** 943–950
25. Lin, J., and Weiss, A. (2001) *J. Biol. Chem.* **276,** 29588–29595
26. Zhu, M., Janssen, E., and Zhang, W. (2003) *J. Immunol.* **17,** 325–333
27. Sommers, C. L., Menon, R. K., Grinberg, A., Zhang, W., Samelson, L. E., and Love, P. E. (2001) *J. Exp. Med.* **194,** 135–142
28. Sommers, C. L., Park, C. S., Lee, J., Feng, C., Fuller, C. L., Grinberg, A., Hildebrand, J. A., Lacana, E., Menon, R. K., Shores, E. W., Samelson, L. E., and Love, P. E. (2002) *Science* **296,** 2040–2043
29. Aguado, E., Richelme, S., Nunez-Cruz, S., Miazek, A., Mura, A. M., Richelme, M., Guo, X. J., Sainty, D., He, H. T., Malissen, B., and Malissen, M. (2002) *Science* **296,** 2036–2040
30. Brdicka, T., Imrich, M., Angelisova, P., Brdickova, N., Horvath, O., Spicka, J., Hilgert, I., Luskova, P., Draber, P., Novak, P., Engels, N., Wienands, J., Simeoni, L., Osterreicher, J., Aguado, E., Malissen, M., Schraven, B., and Horejsi, V. (2002) *J. Exp. Med.* **196,** 1617–1626
31. Janssen, E., Zhu, M., Zhang, W., Koonpaew, S., and Zhang, W. (2003) *Nat. Immunol.* **4,** 117–123
32. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998) *Mol. Cell. Biol.* **18,** 1388–1399
33. Zhang, W., Trible, R. P., Zhu, M., Liu, S. K., McGlade, C. J., and Samelson, L. E. (2000) *J. Biol. Chem.* **275,** 23355–23361
34. Irvin, B. J., William, B. L., Nilson, A. E., Maynor, H. O., and Abraham, R. T. (2000) *Mol. Cell. Biol.* **20,** 9149–9161

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The Importance of Three Membrane-distal Tyrosines in the Adaptor Protein NTAL/LAB

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J. Biol. Chem. 2004, 279:11229-11235.
doi: 10.1074/jbc.M311394200 originally published online January 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311394200

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