# Phosphorylation of the Linker for Activation of T-Cells by Itk Promotes Recruitment of Vav

Juan J. Perez-Villar,‡ Gena S. Whitney,‡ Mitchell T. Sitnick, Robert J. Dunn,§ Srividhya Venkatesan, Kathleen O'Day, Gary L. Schieven, Tai-An Lin, and Steven B. Kanner*

*Department of Immunology, Inflammation, and Pulmonology Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543*

*Received January 15, 2002; Revised Manuscript Received May 30, 2002*

ABSTRACT: The linker for activation of T-cells (LAT) is a palmitoylated integral membrane adaptor protein that resides in lipid membrane rafts and contains nine consensus putative tyrosine phosphorylation sites, several of which have been shown to serve as SH2 binding sites. Upon T-cell antigen receptor (TCR/CD3) engagement, LAT is phosphorylated by protein tyrosine kinases (PTK) and binds to the adaptors Gads and Grb2, as well as to phospholipase C$\gamma$1 (PLC$\gamma$1), thereby facilitating the recruitment of key signal transduction components to drive T-cell activation. The LAT tyrosine residues $Y^{132}$, $Y^{171}$, $Y^{191}$, and $Y^{226}$ have been shown previously to be critical for binding to Gads, Grb2, and PLC$\gamma$1. In this report, we show by generation of LAT truncation mutants that the Syk-family kinase ZAP-70 and the Tec-family kinase Itk favor phosphorylation of carboxy-terminal tyrosines in LAT. By direct binding studies using purified recombinant proteins or phosphopeptides and by mutagenesis of individual tyrosines in LAT to phenylalanine residues, we demonstrate that $Y^{171}$ and potentially $Y^{226}$ are docking sites for the Vav guanine nucleotide exchange factor. Further, overexpression of a kinase-deficient mutant of Itk in T-cells reduced both the tyrosine phosphorylation of endogenous LAT and the recruitment of Vav to LAT complexes. These data indicate that kinases from distinct PTK families are likely responsible for LAT phosphorylation following T-cell activation and that Itk kinase activity promotes recruitment of Vav to LAT.

The activation of T-cells through the antigen receptor (TCR/CD3)[1] results in the stimulation of select members from several protein tyrosine kinase (PTK) families that phosphorylate proteins involved in downstream signal transduction events. These kinase families include the Src family, where at least one key member (Lck) is required for phosphorylation of TCR/CD3 $\zeta$-chain (CD247) ITAMs leading to the recruitment of the Syk-family tyrosine kinase ZAP-70 (*1*−*5*). In addition, the Tec-family kinase Itk appears to be essential for activation of the phospholipase C/calcium cascade and IL-2 production (*1*−*5*). These activated tyrosine kinases phosphorylate enzymes, cytoskeletal components, receptors, and adaptor proteins, which promote the interaction between many signaling molecules through both SH2 and PTB domains to induce formation of protein modules that serve to regulate downstream T-cell responses (*1*−*5*).

One of the earliest described tyrosine kinase substrates identified in T-cells was a 36 kDa protein (*6*, *7*). Subsequent study indicated that this protein formed complexes with phospholipase C$\gamma$1 (PLC$\gamma$1), Grb2, and the PI-3K p85 subunit (*8*−*12*). Cloning of the 36 kDa protein revealed a palmitoylated integral membrane adaptor molecule with nine consensus tyrosine phosphorylation sites that would allow other molecules to bind through their SH2 and PTB domains (*13*, *14*). The protein was termed LAT (linker for activation of T-cells) since it was demonstrated that this protein facilitated the recruitment of many signaling proteins in T-cells to the plasma membrane where it resides in glycolipid/cholesterol-enriched microdomains (GEMs or lipid rafts) and links receptors, tyrosine kinases and their substrates, and other effector molecules together (*13*, *15*, *16*).

The essential role of LAT in both T-cell development and signal transduction has been demonstrated in a variety of independent studies. Targeted disruption of the *LAT* gene in mice resulted in an intrathymic arrest of thymocyte development at the $CD25^+CD44^-$ DN stage and an absence of all mature peripheral T-cells including $\alpha\beta$, $\gamma\delta$, and IEL subsets (*17*). Further, Jurkat T-cell lines lacking the expression of LAT were defective in several TCR/CD3-mediated signaling events, including calcium mobilization, hyperphosphorylation of PLC$\gamma$1, SLP-76, and the guanine nucleotide exchange factor (GEF) Vav, expression of CD69, Erk activation, and AP-1/NFAT-directed gene transcription (*18*, *19*). These TCR/CD3 responses were restored upon expression of wild-type LAT (*18*, *19*). Expression of mutant LAT molecules with distinct substitutions of tyrosine residues for phenylalanine failed to establish functional signaling activities in the LAT-negative Jurkat line (*20*, *21*).

* To whom correspondence should be addressed at Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543. Fax: (609) 252-6058. E-mail: steven.kanner@bms.com.

‡ These authors have contributed equally to this work.

§ Present address: IDEC Pharmaceuticals, San Diego, CA 92121.

[1] Abbreviations: TCR/CD3, T-cell antigen receptor; LAT, linker for activation of T-cells; PTK, protein tyrosine kinase; SH2, Src homology 2 domain; PLC$\gamma$1, phospholipase C$\gamma$1; GEF, guanine nucleotide exchange factor; PTB, phosphotyrosine binding.

10.1021/bi025554o CCC: $22.00 
Published on Web 08/03/2002

Specific mutation of LAT at the tyrosine residue $Y^{132}$ ablated PLC$\gamma$1 binding and subsequent Erk and NFAT activation in LAT-Y132F reconstituted LAT-negative Jurkat T-cells, whereas expression of individual mutants with phenylalanine substitutions of the carboxy-terminal LAT tyrosines $Y^{171}$, $Y^{191}$, or $Y^{226}$ did not affect binding of Grb2, Gads, or PLC$\gamma$1 or interfere with other downstream events (*20*). In contrast, combinations of tyrosine substitutions at the three carboxy-terminal tyrosines resulted in blockade of Grb2, Gads, and PLC$\gamma$1 binding as well as defects in Erk and NFAT activation, suggesting that multiple protein interactions are required at those residues for function (*20*). Of interest, despite the disruption of PLC$\gamma$1 binding in the Y132F mutant, calcium fluxing was only partially impaired. However, in the triple mutant Y171F/Y191F/Y226F, PLC$\gamma$1 binding also was eliminated but not the calcium fluxing capacity, and only when all four tyrosines (Y132F/Y171F/Y191F/Y226F) were mutated was calcium mobilization ablated, suggesting again that multiple sites of regulatory protein binding on LAT converge to control effector functions (*20*).

Itk (Emt, Tsk) is a PTK expressed mainly in T-cells and mast cells (*22*). Upon T-cell activation, Itk is rapidly phosphorylated on tyrosine residues and transiently associates with phospholipids, intracellular signaling proteins, and different cell surface antigens (*22*−*27*). Itk-deficient mice have reduced numbers of peripheral T-cells which exhibit diminished TCR/CD3-induced responses including $Ca^{2+}$ influx, NFAT translocation, apoptosis, and antiviral cytolytic activity, while the B-cell compartment appears relatively normal (*28*, *29*). These data suggest that Itk plays a central role in many TCR/CD3-mediated cellular effector functions.

In this report, we demonstrate that, both in vitro and in vivo, Itk and ZAP-70 phosphorylated LAT, where both kinases favored phosphorylation of carboxy-terminal LAT tyrosines. Further, a soluble SH2 domain from the Vav GEF bound to LAT following phosphorylation by Itk or ZAP-70, preferentially at $Y^{171}$ and $Y^{226}$. Monoclonal antibodies (mAbs) to LAT were generated and used to detect LAT by immunoblotting, where LAT was isolated from T-cells or from LAT-transfected COS cells using a soluble Vav-SH2 domain fusion protein. Expression of a dominant negative Itk mutant reduced TCR/CD3-induced LAT phosphorylation and Vav recruitment to LAT. Finally, coexpression of either wild-type LAT or point mutant versions of LAT with various tyrosine kinases in COS cells showed that Vav specifically bound to the LAT tyrosine residue $Y^{171}$.

## EXPERIMENTAL PROCEDURES

*Cell Lines, Antibodies, and Reagents*. The human leukemic T-cell line Jurkat was cultured in RPMI 1640 containing 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin. COS cells were maintained in DMEM supplemented with 10% heat-inactivated FCS. Anti-CD3 mAb G19-4 was described previously (*30*). Secondary antibodies (HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG) were purchased from Biosource International (Camarillo, CA). Phosphopeptides were purchased from Yale University (New Haven, CT), including the following: ZAP-70, MPMDTSVpYESPYSDPEELK; SLP-76, SSFEEDDpYESPNDDQDGED; SLAP-130, QLDMGDEVpYDDVDTSDFPVSS; CD3-$\zeta$, NQLpYNELNLGREEpYDVLD; LAT-pTyr171, AFSMESIDDpYVNVPESGESA; and LAT-pTyr226, EVEEEGAPDpYENLQELN. For the ZAP-70 and SLP-76 peptides, nonphosphorylated versions were also prepared.

*Generation of LAT Constructs.* The LAT full-length cDNA was amplified from a Jurkat cDNA library and cloned into the bacterial expression vector pGEX-4T-3 (Amersham Biotech, Little Chalfont, Buckinghamshire, England) using engineered *Eco*RI and *Xho*I sites. GST protein production and purification were performed as specified by the manufacturer. Truncated forms $LAT_{28-126}$, $LAT_{28-109}$, $LAT_{28-63}$, $LAT_{126-233}$, $LAT_{165-233}$, $LAT_{111-170}$, $LAT1_{28-190}$, $LAT1_{28-225}$, and $LAT_{172-225}$ were generated by PCR including the amino acid residues as indicated in each name (reference LAT protein sequence accession number AAC39636). In addition, Y → F mutants were generated at positions 110, 127, 132, 171, and 226 individually and 127/132 in combination, all by PCR. The desired mutations of the tyrosine residues were introduced by overlapping extension PCR using the $LAT_{28-126}$ plasmid DNA as the template.

*Generation of SH2 Domain Constructs*. SH2-Ig constructs were generated for Grap, Grb2, SLP-76, and Vav using previously described procedures (*9*), introduced into the expression vector pGEX-4T-3 using the *Eco*RI and *Xho*I restriction sites, and transformed into DH5$\alpha$ *Escherichia coli* cells. GST protein production and purification were performed as specified by the manufacturer.

*Generation of Kinase Fusion Constructs*. GST-Itk/Emt was produced using the Baculo-Gold baculovirus expression system (PharMingen). The PCR-amplified coding region for residues 352 through 620 of human Itk/Emt was inserted into the pAcGHLT baculovirus transfer vector with its GST, 6×His, and thrombin cleavage upstream sequences. The resultant recombinant vector was cotransfected with the linearized Baculo-Gold baculovirus DNA into Sf9 insect cells. His-ZAP-70 was produced using the Bac-to-Bac baculovirus expression system (Invitrogen). Briefly, two restriction sites, *Eco*RI and *Sal*I, were introduced to the 5′ and 3′ ends of the ZAP-70 coding region, respectively, using PCR. The full-length coding region was then inserted to the pFastBac HTa baculovirus donor vector. The procedures for the generation of recombinant baculovirus and expression of His-ZAP-70 were performed according to the manufacturer's instruction (Invitrogen). His-ZAP-70 was purified using TALON metal-affinity resin (Clontech). His-Lck was produced similarly as described above for His-ZAP-70.

*Generation of the Kinase-Inactive Itk Mutant*. The kinase-inactive mutant of Itk (K391E) was described previously (*31*). Briefly, Itk-K391E was generated by overlapping extension PCR using the pcDNA3.1 Itk DNA as a template. The sequences of the oligonucleotide primers were as follows: forward Itk-wt primer (5′-GCGGCCGAATTCATGAACAACTTTATCCTCCTG-3′), reverse Itk-wt primer (5′-GCGATCGCGGCCGCCTAAAGTCCTGATTCTGCAAT-3′), forward Itk-K391E primer (5′-AAGGACAAGGTGGCTATCGAAACCATTCGGGAAGGGGCT-3′), and reverse Itk-K391E primer (5′-AGCCCCTTCCCGAATGGTTTCGATAGCCACCTTGTCCTT-3′). The mutation within Itk was confirmed by sequencing. Itk wild-type and mutant constructs were transfected into Jurkat T-cells using previously described methods (*27*).

*Transfection of COS-7 Cells.* COS-7 cells were maintained in DMEM + 10% FBS and split prior to transfection. Fresh medium was added containing 10% NU serum, 10 $\mu$g of maxi-prep DNA, and 500 $\mu$L of 25× DEAE-Dextran. After 4−5 h at 37 °C, the medium was aspirated, and the cells were rinsed in 10 mL of PBS + 10% DMSO for 2 min. The cells were then incubated in DMEM + 10% FBS ON at 37 °C. For tyrosine phosphorylation induction, the medium was aspirated, the cells were incubated with 10 mL of trypsin−EDTA for 10 min at 37 °C, and the unattached cells were harvested for study. For pervanadate induction (*32*), the cells were incubated with sodium pervanadate (1 mM sodium orthovanadate, 0.3% $H_2O_2$) at 37 °C for the indicated time periods.

*Generation of Monoclonal Antibodies.* The full-length GST-LAT fusion protein was used to immunize Balb/c mice (100 $\mu$g/mouse) in RIBI adjuvant, followed by three boosts at 50 $\mu$g every 3 weeks. Once titers were established, mice were boosted with 50 $\mu$g of antigen in PBS by intravenous administration 3 days prior to cell harvesting. Splenocytes were fused with P3x myeloma cells (ATCC), and positive cultures were detected by ELISA. Stable hybridomas were prepared by cloning, and selected mAbs were purified from culture supernatants using protein A columns.

*Immunoprecipitation, "Pull-Down" Assays, and Western Immunoblotting.* Jurkat T-cells were washed and incubated at 4 °C. For TCR/CD3 stimulation, anti-CD3 mAb G19-4 was added (2 $\mu$g/mL) at 37 °C for the indicated time points. For pervanadate induction (*32*), the cells were incubated with sodium pervanadate (1 mM sodium orthovanadate, 0.3% $H_2O_2$) at 37 °C for the indicated time periods. After stimulation, the cells were lysed in 1 mL of lysis buffer containing 50 mM Tris, pH 7.5, 1% NP-40, 150 mM NaCl, 2 mM EGTA, and 1 mM sodium orthovanadate, plus Complete Protease Inhibitor Mixture (Boehringer Mannheim). Samples were centrifuged at 14000 rpm for 2 min; lysates were precleared twice with 100 $\mu$L of protein A−Sepharose beads (Pharmacia) for 60 min at 4 °C and subjected to immunoprecipitation with the indicated antibodies. For the GST pull-down experiments, equivalent amounts of the indicated GST fusion proteins were incubated with unstimulated or TCR/CD3-stimulated Jurkat cell lysates for 60 min at 4 °C, followed by the addition of glutathione−Sepharose beads (Pharmacia) for 60 min at 4 °C. Immunoprecipitates were subjected under reducing conditions to SDS−PAGE in 4−20% gels and subsequently transferred to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore, Marlborough, MA). For LAT detection, membranes were blocked in 5% BSA and treated with an anti-LAT mAb mixture as the primary reagent at 1 $\mu$g/mL and anti-mouse IgG-HRP as the secondary reagent. For phosphotyrosine analysis, blots were treated with anti-phosphotyrosine antibody 4G10-HRP (Upstate Biotechnology) at 0.2 $\mu$g/mL. The binding of HRP was detected by ECL (Amersham, Buckinghamshire, England) and exposure to film. Blot stripping was carried out by membrane incubation in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 50 mM $\beta$-mercaptoethanol at room temperature for 90 min.

*ELISA.* GST-LAT constructs at 4 $\mu$g/mL were loaded onto standard 96-well ELISA plates in 50 mM $NaHCO_3$, pH 9, overnight at 4 °C. Wells were blocked for 1 h with 5% nonfat milk in PBS + 0.05% Tween 20. Wells were washed three times with PBS-T, once with PBS, and once with 25 mM Hepes, pH 7. Kinase reactions were performed by adding to wells titrated amounts of the indicated enzyme in kinase buffer (25 mM Hepes, pH 7.0, 0.1 mg/mL BSA, 100 $\mu$M ATP, 5 mM $MgCl_2$, 1 mM DTT, 1 mM $Na_3VO_4$) for the indicated time points. The reaction mix was removed, and wells were washed with PBS-T. HRP-conjugated anti-phosphotyrosine antibodies PY99 (Transduction Laboratories) and 5H1 (*33*) diluted 1:1000 in PBS-T + 1% BSA were added to the wells for 1 h. Wells were then washed three times with PBS-T and twice with PBS. Reactions were developed using the Kirkegaard & Perry Laboratory TMB ELISA kit (100 $\mu$L/well of 50:50 TMB substrate and $H_2O_2$ solutions). Color reactions were stopped with 100 $\mu$L/well 1 N $H_2SO_4$, and the absorbency at 450−650 nm was measured.

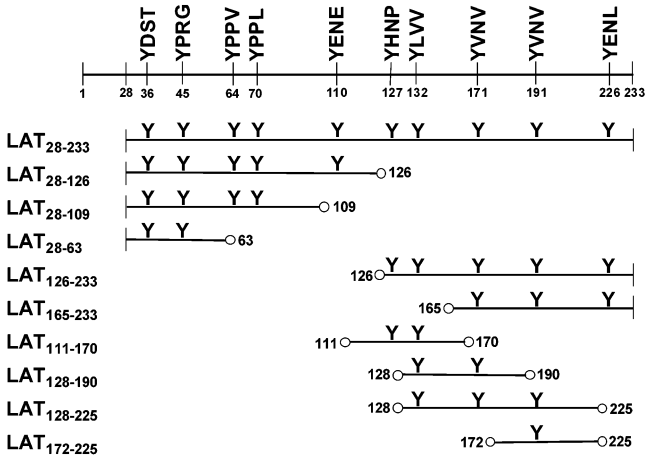


FIGURE 1: LAT truncation constructs. Full-length LAT was defined as residues 28−233, lacking the first 27 amino acids, which include the transmembrane domain. All constructs were fused with GST at their amino terminus (not shown), and baculovirus-produced fusion proteins were purified on glutathione−Sepharose. The circles at the ends of constructs indicate the starting or ending residue and show the absence of individual tyrosines in proximity.

## RESULTS

*Generation of LAT Truncation Mutant Constructs*. In an effort to determine the substrate recognition sites in LAT for the nonreceptor tyrosine kinases Itk and ZAP-70, we generated a series of truncation mutants of human LAT as GST fusion proteins for in vitro phosphorylation. The full-length GST-LAT construct ($LAT_{28-233}$) contained at its amino terminus GST, followed by LAT starting at residue 28 (which excludes the transmembrane region), and has the carboxy-terminal asparagine residue at position 233 (Figure 1). Nine additional GST fusion constructs were generated that contained at least one and up to five potential tyrosine phosphorylation sites, including both amino-terminal and carboxy-terminal tyrosine residues. Expression and purification of each GST fusion construct yielded the expected size protein detected by SDS−PAGE (data not shown).

*In Vitro Phosphorylation of LAT Truncation Constructs*. Fusion proteins containing the tyrosine kinases from human Itk and human ZAP-70 were generated, expressed, and purified for use in kinase reactions in vitro with the GST-LAT truncation constructs. Incubation of the individual

kinase fusions with either GST alone or full-length GST-LAT ($LAT_{28-233}$) showed that only $LAT_{28-233}$ served as a substrate for the kinases as detected by an anti-phosphotyrosine mAb in an ELISA format (Figure 2A). For comparison, we also generated and tested the ability of the human Lck kinase (His fusion) to phosphorylate LAT, and we observed a degree of reactivity similar to that of the other two kinases (Figure 2A). Further, the extent of phosphorylation was similar for all three kinases, and each exhibited biphasic kinetics. Although full-length versions of these kinases were also generated, expression levels were limited and thus were not useful for the present studies.

To determine which tyrosines in LAT were substrates of these kinases, each GST-LAT truncation shown in Figure 1 was incubated individually with 12 ng of recombinant kinase fusions. The pattern of phosphorylation revealed a degree of substrate specificity, where constructs containing carboxy-terminal tyrosines were phosphorylated preferentially by ZAP-70 and Itk (Figure 2B). By deduction, Itk appeared to phosphorylate the LAT tyrosines $Y^{171}$ and $Y^{191}$ (the tyrosine residues common to the LAT constructs $LAT_{111-170}$, $LAT_{128-190}$, $LAT_{128-225}$, and $LAT_{172-225}$) but not $Y^{127}$ or $Y^{132}$. Again, as a control, we tested recombinant Lck kinase against the LAT truncation constructs and observed that Lck phosphorylated amino-terminal tyrosine residues in the LAT truncation fusion proteins in vitro (Figure 2B).

Previous work by Zhang et al. (*20*) demonstrated that binding of LAT to the adaptor proteins Gads and Grb2 required the LAT tyrosine residues $Y^{171}$, $Y^{191}$, and $Y^{226}$. Tyrosines mutated to phenylalanine did not interfere individually with binding to the adaptors, whereas combinations of tyrosine mutations appeared to be required for the protein interactions (*20*). This result prompted us to investigate the binding of the SH2 domains from Grb2, the Grb2/Gads-related family member Grap (*34*), and Vav to the phosphorylated truncation mutants of LAT. To perform this experiment, ZAP-70 was mixed with Lck to phosphorylate both amino- and carboxy-terminal tyrosines on the LAT constructs in vitro, followed by binding to recombinant SH2-Ig fusion constructs (*9*) generated from Grap, Grb2, and Vav. Binding of the SH2-Ig to the LAT constructs was detected with anti-Ig.

*The Vav-SH2 Domain Binds to LAT in Vitro*. The pattern of binding of the Grap-SH2 and Grb2-SH2 domains to the LAT truncation mutants was similar (Figure 3A), although the Grb2-SH2 bound to the carboxy-terminal constructs of LAT to a lesser degree than the Grap-SH2 domain. The binding of Grap-SH2 and Grb2-SH2 domains in vitro correlated with previous observations using the point mutants of LAT expressed in cells (*20*). In contrast, in attempts to address the binding of the Vav-SH2 domain to the LAT truncation fusions, the Vav-SH2 domain did not bind to the amino-terminal construct $LAT_{28-126}$ as did the Grap-SH2 and Grb2-SH2 domains. The data showed that Vav associated with carboxy-terminal tyrosines in LAT and, by deduction, suggested that Vav may bind $Y^{171}$ and $Y^{226}$ due to its interaction with $LAT_{165-233}$ but not $LAT_{172-225}$ (Figure 3A).

To control for nonspecific binding of SH2-Ig fusions to the LAT constructs, we compared the binding of the Vav-SH2 domain and the SLP-76-SH2 domain to either phosphorylated full-length LAT or a phosphopeptide from SLAP-130 (a known binding partner for the SLP-76-SH2 domain)

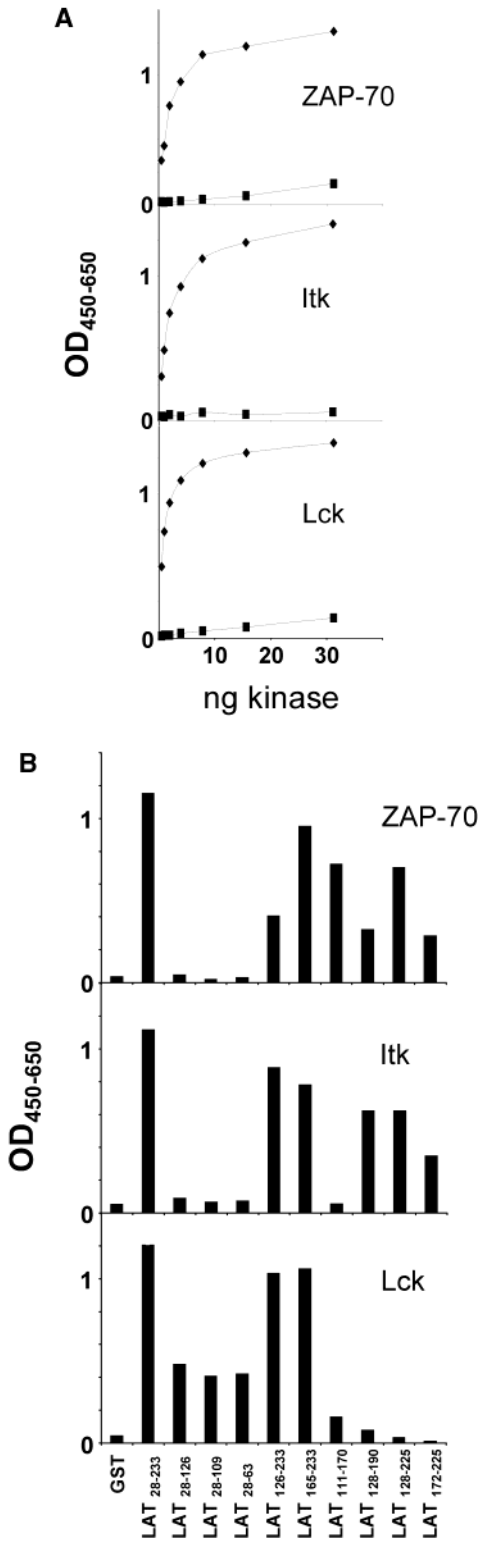


FIGURE 2: ZAP-70 and Itk phosphorylate distinct regions of LAT in vitro. (A) Full-length GST-$LAT_{28-233}$ (◆) or GST alone (■) at 4 $\mu$g/mL was used to coat plates overnight. Washed plates were incubated with the indicated amounts of purified recombinant kinase fusions for 60 min. Phosphorylation reactions were detected by the addition of anti-phosphotyrosine, and the optical density was measured at 450 and 650 nm. All reactions were performed in triplicate. (B) Individual LAT truncation constructs were immobilized on plastic at 4 $\mu$g/mL prior to incubation with the indicated soluble recombinant kinases (12 ng/reaction) for 60 min. Detection of phosphorylation was as described in panel A. All reactions were performed in triplicate.

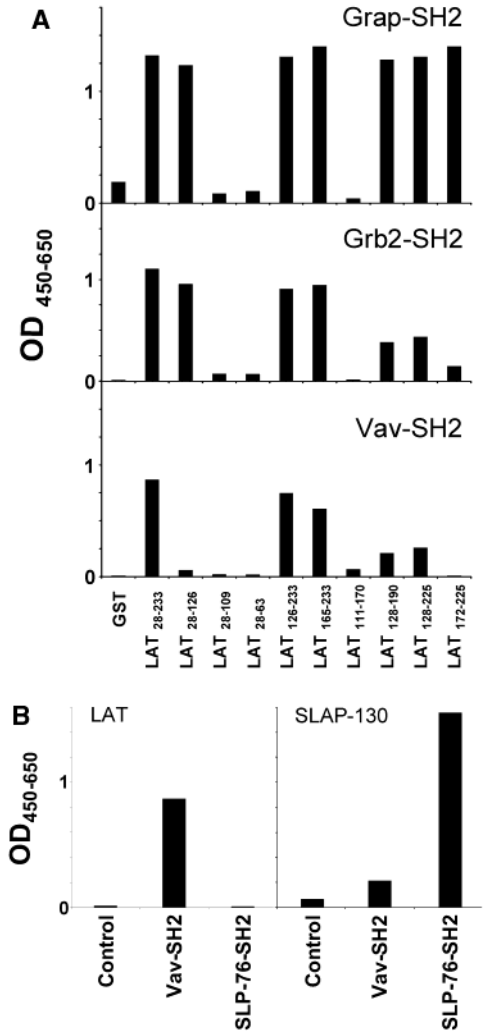


FIGURE 3: SH2 domains of Grap, Grb2, and Vav bind to distinct regions of LAT in vitro. (A) GST alone (GST) or individual LAT truncation mutants were immobilized on plastic at 4 $\mu$g/mL prior to incubation with ZAP-70 (12 ng/reaction activated by Lck at 12 ng/reaction) for 60 min. After removal of kinases by washing, individual soluble recombinant SH2-Ig fusion proteins as indicated were added to the wells. Binding of SH2-Ig fusions was detected with anti-Ig in an ELISA format. All reactions were performed in triplicate. (B) Full-length $LAT_{28-233}$ or a phosphopeptide of SLAP-130 (QLDMGDEVpYDDVDTSDFPVSS) at 4 $\mu$g/mL was immobilized on plastic. For full-length $LAT_{28-233}$, ZAP-70 (12 ng/reaction for 60 min) was added to the wells and then removed by washing. Control Ig or SH2-Ig from SLP-76 or Vav was then incubated as indicated. Color development was performed as described in panel A. All reactions were performed in triplicate.

(*35*). Clearly, Vav-SH2 bound only to phosphorylated LAT while the SLP-76-SH2 only bound to the SLAP-130 phosphopeptide, indicating that the SH2-Ig constructs maintain a measurable and differential selectivity in these in vitro reactions (Figure 3B).

*Generation of Monoclonal Antibodies to LAT*. As a means of analyzing endogenous cell-expressed LAT or transfected LAT constructs, we generated a panel of monoclonal antibodies (mAbs) by immunizing mice with the full-length LAT construct (GST-$LAT_{28-233}$). Five mAbs were identified that were able to both immunoprecipitate and immunoblot LAT (LAT1, LAT7, LAT13, LAT15, and LAT18). Interestingly, LAT1, LAT7, and LAT13 mAbs detected TCR/CD3-induced tyrosine-phosphorylated LAT, while LAT15 and

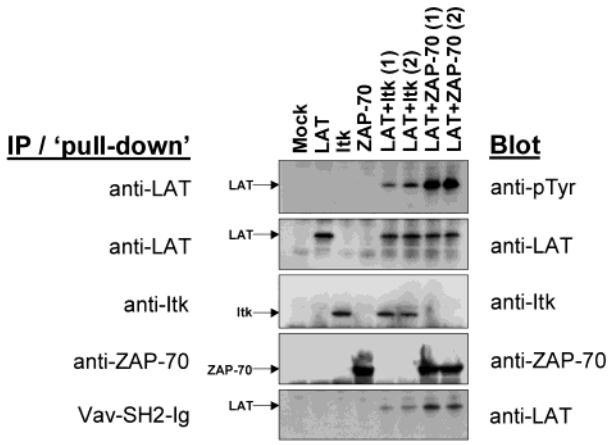


FIGURE 4: Cotransfection of LAT and Itk (or ZAP-70) promotes interaction with Vav-SH2. COS cells were transfected with empty vector (mock), $LAT_{28-233}$ (LAT), Itk, ZAP-70, or combinations of LAT and Itk or LAT and ZAP-70. The combinations indicated as (1) and (2) delineate two separate transfection plates for comparison. Immunoprecipitates of LAT (mAb LAT1) were immunoblotted with anti-phosphotyrosine (top panel) or anti-LAT (second panel). Immunoprecipitates of Itk were immunoblotted with anti-Itk (third panel), and immunoprecipitates of ZAP-70 were immunoblotted with anti-ZAP-70 (fourth panel). Recombinant Vav-SH2 was used in a pull-down assay, and LAT binding was detected by immunoblotting with anti-LAT mAb LAT1 (bottom panel).

LAT18 were strong immunoprecipitating mAbs but did not detect phosphorylated LAT (data not shown).

*Phosphorylation of LAT by Itk and Binding to Vav-SH2 in COS Cell Transfectants*. In an effort to demonstrate that the phosphorylation of LAT by Itk or ZAP-70 observed in vitro also occurs in intact cells and that the Vav-SH2 interacts with cell-expressed LAT, we cotransfected full-length LAT with Itk or ZAP-70 into COS cells. In Figure 4, we observed that both Itk and ZAP-70 individually phosphorylated LAT on tyrosine (top panel). The expression levels of LAT (second panel), Itk (third panel), and ZAP-70 (fourth panel) were confirmed using specific mAbs. Importantly, LAT was isolated from COS cell transfectants using recombinant Vav-SH2 in pull-down assays only in cotransfectants with LAT and Itk or ZAP-70 (Figure 4, bottom panel), demonstrating the requirement of LAT phosphorylation for binding Vav-SH2. We also tested the ability of Lck to phosphorylate LAT in this system and did not detect LAT phosphorylation (data not shown), corroborating previous observations (*13*).

*Interaction between LAT and Vav in T-Cells*. To confirm our observations described in COS cells, we investigated the interaction in T-cells with endogenous LAT and tyrosine kinases, wherein we stimulated Jurkat T-cells with either anti-TCR/CD3 or pervanadate and examined the association between Vav and LAT. In Figure 5A, we observed the interaction between Vav-SH2 and endogenous LAT only following T-cell stimulation through the TCR/CD3 or by treatment with pervanadate. Further, the association between LAT and Vav-SH2 correlated with the tyrosine phosphorylation of LAT. The complexity of the pattern of tyrosine-phosphorylated proteins associated with the Vav-SH2 domain was evident in comparison with that of anti-LAT, indicating that the Vav-SH2 fusion protein has a binding capacity to a wider array of phosphotyrosine-containing proteins in activated T-cells. Of interest, endogenous Vav was also detected

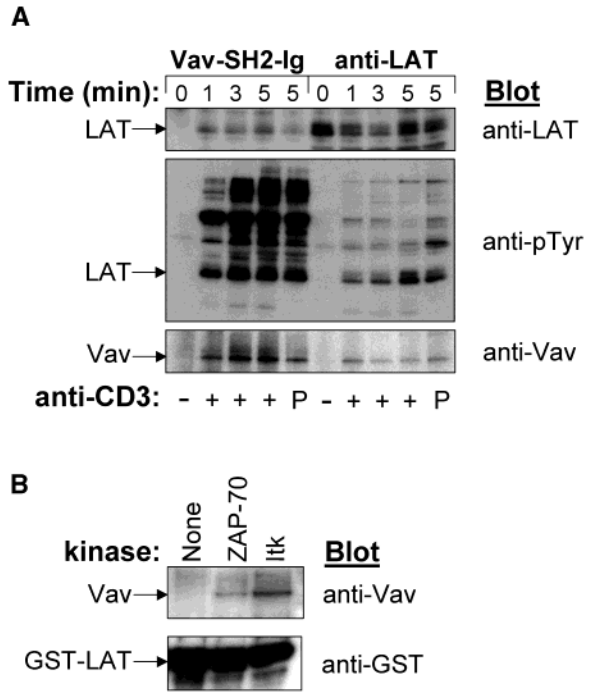


FIGURE 5: Interaction of Vav and LAT in T-cells. (A) Jurkat T-cells were left unstimulated (−), were stimulated with anti-TCR/CD3 (+) for 1, 3, or 5 min, or were treated with pervanadate (P) for 5 min and then lysed. Recombinant Vav-SH2 was used in pull-down assays, and anti-LAT (mAb LAT1) was used in immunoprecipitation reactions from the cell lysates. Proteins were immunoblotted with anti-LAT (upper panel), anti-pTyr (middle panel), or anti-Vav (bottom panel). (B) Full-length GST-$LAT_{28-233}$ was incubated alone (none) or with recombinant ZAP-70 or Itk kinase fusions (12 ng/reaction) for 60 min. The LAT fusions were used in pull-down assays using lysates from unstimulated Jurkat T-cells. The reactants were immunoblotted with anti-Vav (upper panel) or anti-GST to control for $LAT_{28-233}$ input (lower panel).

in the Vav-SH2 pull-down assay and by coimmunoprecipitation with LAT (Figure 5A), suggesting that endogenous Vav interacts in a regulated manner with LAT and that likely not all LAT molecules are associated with Vav. In a converse experiment, $LAT_{28-233}$ was phosphorylated in vitro with either ZAP-70 or Itk and then used as a pull-down reagent to isolate endogenous Vav from unstimulated Jurkat T-cells. In Figure 5B, we observed the association of Vav with phosphorylated $LAT_{28-233}$ following phosphorylation with either ZAP-70 or Itk.

*Generation of Site-Directed Mutants of LAT*. To explore the observation that Vav-SH2 bound to truncation fusions of LAT containing $Y^{171}$ and $Y^{226}$ in vitro, individual tyrosine residues in LAT were mutated to phenylalanine by site-directed mutagenesis. Six LAT constructs containing either single or double Tyr → Phe (Y → F) substitutions were generated (Figure 6A). All of these constructs were generated as full-length GST fusions spanning LAT residues 28−233 and were purified on glutathione−Sepharose.

*The Vav-SH2 Domain Binds to LAT* $Y^{171}$. To further define the putative tyrosine residues that were binding sites for Vav, we used the Y → F substitution mutants in binding assays in vitro. Only Y171F showed reduced binding of the Vav-SH2 domain, while Y226F appeared relatively unaffected following ZAP-70 phosphorylation and only partially reduced after phosphorylation by Itk (Figure 6B). The data in Figure 6B are calculated as a function of the phosphorylation level of each construct to reflect the minor differences among them we observed. To follow this observation further, we analyzed the binding of the Vav-SH2 domain to a series of phosphopeptides, including ones from critical or putative SH2 binding sites: ZAP-70, MPMDTSVpYESPYSDPEELK (Vav-SH2 binding motif); SLP-76, SSFEEDDpYESPNDDQDGED (Vav-SH2 binding motif); SLAP-130, QLDMGDEVpYDDVDTSDFPVSS (SLP-76 SH2 binding motif); CD3-ζ, NQLpYNELNLGRREEpYDVLD (ZAP-70 SH2 binding motif); LAT-pTyr171, AFSMESIDDpYVNVPESGESA (putative SH2 binding motif); and LAT-pTyr226, EVEEEGAPDpYENLQELN (putative SH2 binding motif). The lower case p in each peptide refers to the phosphorylated tyrosine residue (Y) in the peptide. For the ZAP-70 and SLP-76 peptides, nonphosphorylated versions were also prepared. As shown in Figure 6C, while Vav-SH2 was able to bind to previously defined phosphotyrosine binding sites in ZAP-70 and SLP-76, it also bound strongly to a phosphopeptide spanning residues containing LAT $Y^{171}$ but weakly to a phosphopeptide containing $Y^{226}$.

*Vav Binds to Itk-Phosphorylated LAT in COS Cell Transfectants*. To confirm the in vitro results involving the Vav-SH2 and LAT $Y^{171}$ interaction, we coexpressed the LAT Y171F mutant in COS cells with either Itk or ZAP-70 and tested the ability of the Vav-SH2 domain to interact with the mutant LAT. The level of overall tyrosine phosphorylation of LAT Y171F by either Itk or ZAP-70 was similar to that of wild-type LAT (Figure 7). Although the level of phosphorylation of LAT Y171F did not diminish significantly when phosphorylated by either ZAP-70 or Itk (in comparison to wild-type LAT), we suggest that the level of phosphorylation on this molecule at one or more of the other eight potential phosphorylation sites precluded our ability to distinguish this mutation using the anti-pTyr blot method. In contrast, the binding of the Vav-SH2 domain to the LAT Y171F mutant was considerably diminished compared to wild-type LAT (Figure 7), correlating with the inhibition in binding we observed in vitro (Figure 6B). Overall, these data suggest that LAT $Y^{171}$ serves as a major phosphorylation site for Itk and a docking site for Vav in cells.

*Disruption of Vav/LAT Interaction by Dominant Negative Itk*. In an effort to investigate the role of Itk in T-cells regarding the association of Vav and LAT, we transfected a kinase-deficient mutant of Itk-K391E (*31*) into Jurkat T-cells and studied the effects of the mutant on LAT phosphorylation and Vav recruitment to LAT. In Figure 8, we show that expression of the kinase-inactive Itk mutant K391E resulted in a reduction in the tyrosine phosphorylation of LAT and associated phosphoproteins in comparison with Itk-wt expression. This was corroborated by a diminished level of tyrosine phosphorylation of PLCγ1, a putative substrate of Itk (*26*). Further, the level of LAT phosphorylation was upregulated by overexpression of Itk-wt kinase. The association between LAT and Vav, measured by using either anti-Vav (Figure 8A) or the Vav-SH2 fusion protein (Figure 8B), was reduced significantly by the dominant negative Itk mutant. For control purposes, we measured the expression of Itk in the cells after transfection and observed approximately 2-fold overexpression of the Itk-wt and Itk-K391E mutant kinases relative to mock transfection (Figure 8C). Overall, these data further demonstrate a key role for

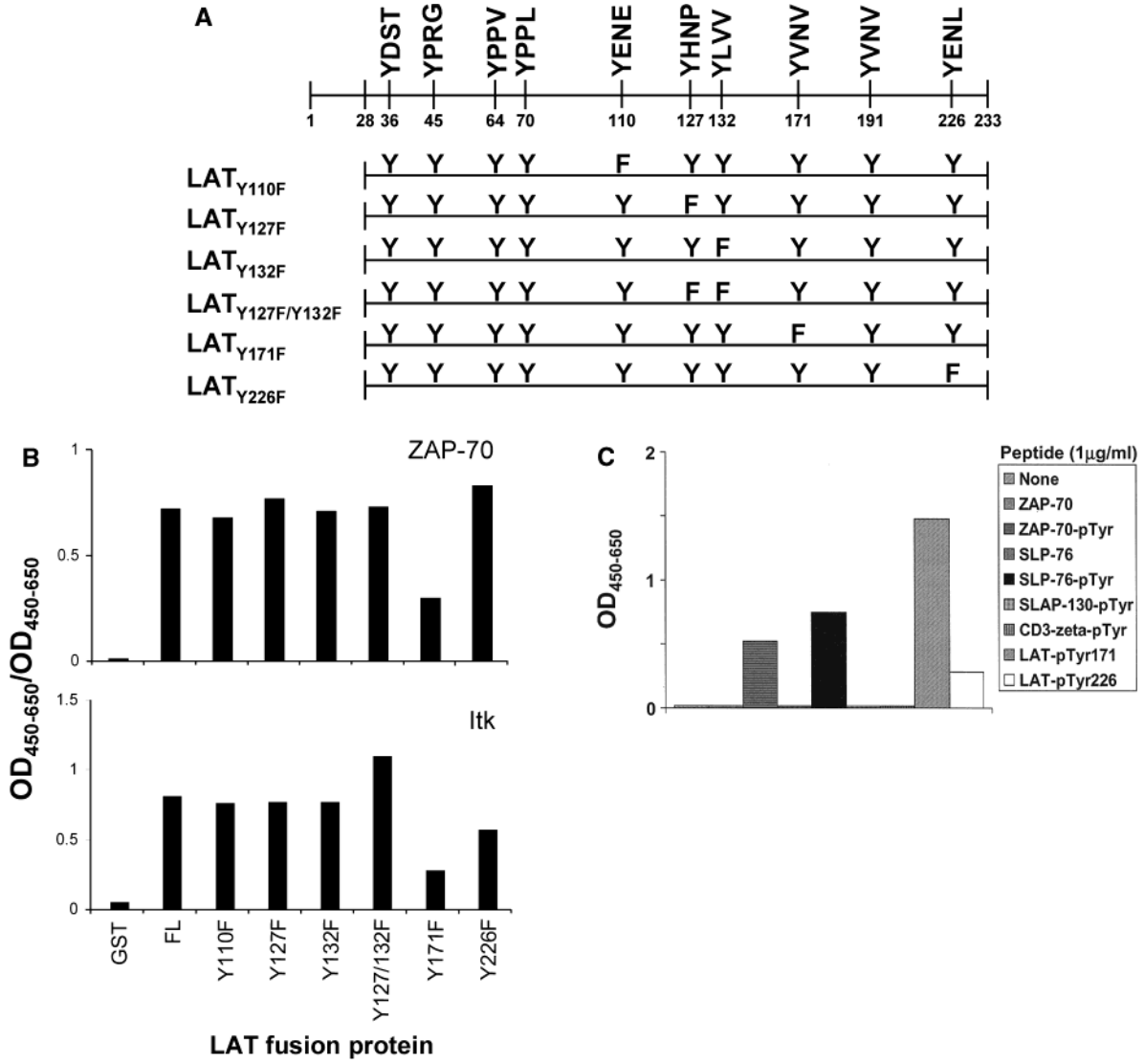


FIGURE 6: Mapping of LAT tyrosine residues that affect binding of Vav. (A) LAT Tyr → Phe substitution mutants. Full-length LAT was defined as residues 28−233. All constructs were fused with GST at their amino terminus, and baculovirus-produced fusion proteins were purified on glutathione−Sepharose. Key: Tyr (Y); Phe (F). (B) Association of Vav-SH2 with Tyr → Phe substitution mutants of $LAT_{28-233}$. GST alone (GST), wild-type $LAT_{28-233}$ (FL), or Tyr → Phe substitution mutants of $LAT_{28-233}$ were immobilized on plastic at 4 $\mu$g/mL overnight. The constructs were phosphorylated with the indicated kinase at 12 ng/reaction for 60 min. Phosphorylation of the bound proteins was measured using anti-phosphotyrosine by ELISA. In parallel wells, recombinant Vav-SH2 was incubated and assayed for binding with anti-Ig. All reactions were performed in triplicate. The data are expressed as the $OD_{450-650}$ from the Vav-SH2 binding assay divided by the $OD_{450-650}$ from the anti-phosphotyrosine assay as a measure of the binding of Vav-SH2 to the LAT fusions relative to their phosphorylation status. (C) Phosphopeptide binding to recombinant Vav-SH2. A panel of synthetic peptides [ZAP-70, MPMDTSVpYESPYSDPEELK (Vav-SH2 binding motif); SLP-76, SSFEEDDpYESPNDDQDGED (Vav-SH2 binding motif); SLAP-130, QLDMGDEVpYDDVDTSDFPVSS (SLP-76 SH2 binding motif); CD3-$\zeta$, NQLpYNELNLGRREEpYDVLD (ZAP-70 SH2 binding motif); LAT-pTyr171, AFSMESIDDpYVN-VPESGESA; LAT-pTyr226, EVEEEGAPDpYENLQELN] were coated at 4 $\mu$g/mL onto plastic. The lower case p in each peptide refers to the phosphorylated tyrosine residue Y in the peptide. For the ZAP-70 and SLP-76 peptides, nonphosphorylated versions were also prepared and bound to plastic exactly as the phosphopeptides. Binding of Vav-SH2 was measured by ELISA, and all reactions were performed in triplicate.

Itk in the phosphorylation of LAT and the recruitment of Vav to LAT in T-cells.

## DISCUSSION

Following the molecular characterization of the LAT adaptor protein, numerous studies have shown its importance in T-cell development and activation and its role in other hematopoietic cell types (*17*, *20*, *21*, *36*, *37*). Clearly, LAT couples early signaling events in T-cells including the stimulation of tyrosine kinases to downstream responses including Erk and NF-AT activation (*36*, *37*). Many of the intermediate signaling components that LAT links together to drive TCR/CD3 signals have been identified, including Grb2, Gads, PLC$\gamma$1, Itk, SLP-76, Vav, and PI3K (*34*, *35*). The orientation of these molecules into specific protein complexes is partially understood, although their spatial and temporal dynamics have not been fully explored.

In this report we addressed the specific phosphorylation of LAT by the Itk tyrosine kinase and the subsequent interaction between LAT and the Vav guanine nucleotide exchange factor. Our observations indicate that LAT serves as a substrate for Itk both in vitro and in cells. Previous

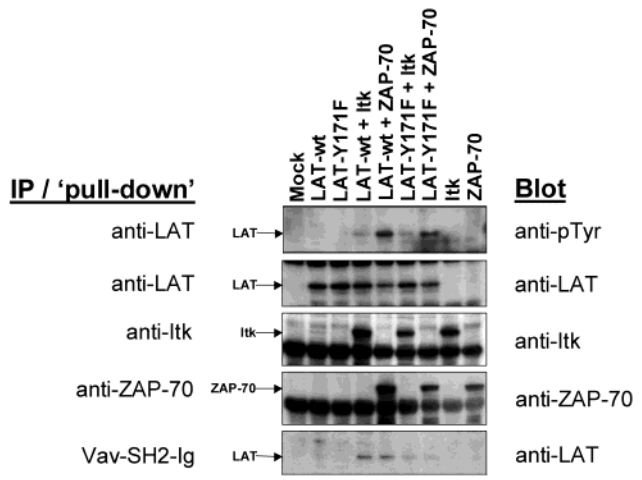


FIGURE 7: Association of LAT and Vav is dependent upon LAT $Y^{171}$. COS cells were transfected with empty vector (mock), full-length LAT (LAT-wt), a mutant LAT (Y171F), Itk, ZAP-70, or combinations of LAT-wt (or LAT-Y171F) with Itk or ZAP-70. Immunoprecipitates of LAT (mAb LAT1) were immunoblotted with anti-phosphotyrosine (top panel) or anti-LAT (second panel). Immunoprecipitates of Itk were immunoblotted with anti-Itk (third panel), and immunoprecipitates of ZAP-70 were immunoblotted with anti-ZAP-70 (fourth panel). Recombinant Vav-SH2 was used in a pull-down assay, and LAT binding was detected by immunoblotting with anti-LAT mAb LAT1 (bottom panel).

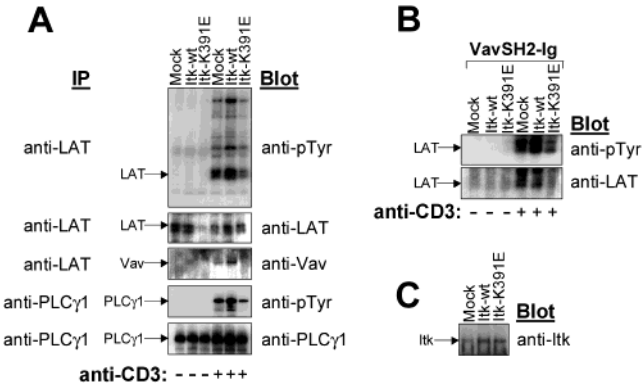


FIGURE 8: Dominant negative Itk mutant (K391E) inhibits LAT recruitment of Vav in T-cells. Jurkat T-cells were transfected with an empty vector (mock) or a vector containing wild-type Itk or a kinase-inactive Itk mutant K391E. The cells were collected 48 h after transfection and either were left unstimulated (−) or were stimulated with anti-TCR/CD3 (+) for 5 min. (A) Anti-LAT (mAb LAT1) was used in immunoprecipitation reactions from the cell lysates. Proteins were immunoblotted with anti-pTyr (upper panel), anti-LAT (second panel), or anti-Vav (third panel). Alternatively, anti-PLC$\gamma$1 was used in immunoprecipitation reactions from the cell lysates, and the proteins were immunoblotted with anti-pTyr (fourth panel) or anti-PLC$\gamma$1 (bottom panel). (B) Recombinant Vav-SH2 was used in pull-down assays from the cell lysates, and the proteins were immunoblotted with anti-pTyr (upper panel) or anti-LAT (lower panel). (C) Itk was immunoprecipitated from the cell lysates of the transfectants, and the proteins were directly immunoblotted with anti-Itk.

reports have indicated an association between LAT and Itk (*23*, *38*), suggesting that LAT may serve as a substrate for Itk. Despite the observation that Lck was required for the activation of ZAP-70 to phosphorylate LAT when transfected into cells (*13*), we observed phosphorylation of LAT with coexpression of full-length Itk in COS cells. Previous studies suggested that the expression of ZAP-70 was critical for the interaction between LAT and Itk (*23*). In our studies, the Itk kinase domain alone was sufficient for phosphorylation of recombinant LAT fusion proteins in vitro, further implicating Itk in the pathway for LAT coupling to signaling components.

The nature of the interaction between LAT and Vav has been reported previously, but with varying observations. An initial hypothesis suggested that the interaction was indirect due to the association of Vav with SLP-76 (*39*, *40*) and the subsequent identification of SLP-76/LAT complexes (*36*, *37*). Further, Vav is constitutively bound to the carboxy-terminal Grb2 SH3 domain (*41*), suggesting an alternative indirect association. A recent study has shown that purified recombinant Vav binds directly to denatured LAT on Far Western immunoblots (*42*), thus suggesting a direct association. Our studies would support the conclusion that Vav interacts with LAT directly as shown by specific LAT phosphopeptide interaction with the Vav-SH2 domain, as well as the association between the Vav-SH2 domain and phosphorylated LAT expressed in cells or prepared as a soluble recombinant protein. Further, our observations suggest that Vav interacts with LAT mainly via $Y^{171}$ and that $Y^{226}$ may contribute to the interaction. Although we did not observe a fundamental change in binding of Vav to LAT-Y226F when phosphorylated by ZAP-70, we did observe a reduction following Itk phosphorylation. A conformational change in LAT-Y226F induced by Itk phosphorylation at other tyrosine residues may account for this difference. Our results in part corroborate those of Paz et al. (*42*), who reported that a LAT Y171F/Y191F double mutant showed absent or decreased association between Vav and LAT, while Y191F and Y226F mutants showed diminished association between Vav and LAT in pull-down assays. Considering that both $Y^{171}$ and $Y^{191}$ are canonically identical Grb2 binding sites (YVNV), Vav may bind to both sites. However, our data do not implicate $Y^{191}$ in association with Vav. On the basis of these data and our observations with the kinase-inactive Itk mutant K391E, we conclude that Itk plays a key role in the phosphorylation of LAT in T-cells that generates at least one and likely two docking sites for Vav.

The importance of an association between Vav and LAT may be to sequester a significant amount of Rho/Rac-family GTPase activity into TCR/CD3 signaling complexes. Such clustering may enhance the ability of Vav to stimulate GTP/GDP exchange during T-cell activation and for Vav to serve as an additional docking site for other signaling proteins. Our observation that the recombinant Vav-SH2 domain could isolate endogenous Vav from T-cell lysates suggests that the nature of the multicomponent complexes is dynamic. For example, previous reports have demonstrated the interaction between Vav and SLP-76 (*39*, *40*), as well as between Vav and ZAP-70 (*43*, *44*), suggesting that the Vav-SH2 domain we used could bind to phosphotyrosine residues on these other molecules and thus form a bridge between the recombinant Vav-SH2 and endogenous Vav. Our observations strongly suggest, but do not prove, that Vav and LAT form a direct link in T-cells, thereby adding to the list of putative interaction partners that Vav may bind to during a T-cell response.

Previous studies have shown that ablation of Vav in mice leads to impaired T-cell development, where *Vav*$^{-/-}$ T-cells

have reduced capacity to generate PLC$\gamma$1 second messengers ($IP_3$ and $Ca^{2+}$), diminished signaling through the transcription factors NF-AT and NF$\kappa$B, and limited organization and polymerization of F-actin (*45*). In contrast, early signaling events including the activation of tyrosine kinases and the phosphorylation of LAT or other molecules are normal in *Vav*$^{-/-}$ T-cells (*45*). Collectively, these data suggest that Vav plays a dominant role in downstream activation pathways and that the potential for bridging LAT and Vav molecules together into an early active complex likely drives subsequent cascades, particularly interactions between pathway intermediates that require LAT and Vav for subcellular relocalization and recruitment. Additionally, the LAT/Vav complex likely promotes increased tyrosine phosphorylation of Vav, thereby increasing its enzymatic activity to exchange guanine nucleotides on Rac/Rho molecules (*46*). Finally, disruption of the LAT/Vav complex by chemical and/or genetic approaches may lead to therapeutic intervention for both immunological and inflammatory diseases.

## ACKNOWLEDGMENT

We thank Dr. Alejandro Aruffo for support and encouragement.

## REFERENCES

1. Chan, A. C., and Shaw, A. S. (1996) *Curr. Opin. Immunol. 8*, 394−401.
2. Clements, J. L., and Koretzky, G. A. (1999) *J. Clin. Invest. 103*, 925−929.
3. Cantrell, D. (1996) *Annu. Rev. Immunol. 14*, 259−274.
4. Qian, D., and Weiss, A. (1997) *Curr. Opin. Cell Biol. 9*, 205−212.
5. Wange, R. L., and Samelson, L. E. (1996) *Immunity 5*, 197−205.
6. June, C. H., Fletcher, M. C., Ledbetter, J. A., and Samelson, L. E. (1990) *J. Immunol. 144*, 1591−1599.
7. Garcia-Morales, P., Minami, Y., Luong, E., Klausnre, R. D., and Samelson, L. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9255−9259.
8. Kanner, S. B., Kavanagh, T. J., Grossmann, A., Hu, S. L., Bolen, J. B., Rabinovitch, P. S., and Ledbetter, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 300−304.
9. Gilliland, L. K., Schieven, G. L., Norris, N. A., Kanner, S. B., Aruffo, A., and Ledbetter, J. A. (1992) *J. Biol. Chem. 267*, 13610−13616.
10. Sieh, M., Batzer, A., Schlessinger, J., and Weiss, A. (1994) *Mol. Cell. Biol. 14*, 4435−4442.
11. Buday, L., Egan, S. E., Rodriguez, V. P., Cantrell, D. A., and Downward, J. (1994) *J. Biol. Chem. 269*, 9019−9023.
12. Fukazawa, T., Reedquist, K. A., Panchamoorthy, G., Soltoff, S., Trub, T., Druker, B., Cantley, L., Shoelson, S. E., and Band, H. (1995) *J. Biol. Chem. 270*, 20177−20182.
13. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998) *Cell 92*, 83−92.
14. Weber, J. R., Ørstavik, S., Torgersen, K. M., Danbolt, N. C., Berg, S. F., Ryan, J. C., Taskén, K., Imboden, J. B., and Vaage, J. T. (1998) *J. Exp. Med. 187*, 1157−1161.
15. Zhang, W., Trible, R. P., and Samelson, L. E. (1998) *Immunity 9*, 239−246.
16. Lin, J., Weiss, A., and Finco, T. S. (1999) *J. Biol. Chem. 274*, 28861−28864.
17. 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.
18. Zhang, W., Irvin, B. J., Trible, R. P., Abraham, R. T., and Samelson, L. E. (1999) *Int. Immunol. 11*, 943−950.
19. Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E., and Weiss, A. (1998) *Immunity 9*, 617−626.
20. Zhang, W., Trible, R. P., Zhu, M., Liu, S. K., McGlade, C. J., and Samelson, L. E. (2000) *J. Biol. Chem. 275*, 23355−23361.
21. Lin, J., and Weiss, A. (2001) *J. Biol. Chem. 276*, 29588−29595.
22. Yang, W. C., Collette, Y., Nunes, J. A., and Olive, D. (2000) *Immunity 12*, 373−382.
23. Shan, X., and Wange, R. L. (1999) *J. Biol. Chem. 274*, 29323−29330.
24. Bunnell, S. C., Diehn, M., Yaffe, M. B., Findell, P. R., Cantley, L. C., and Berg, L. J. (2000) *J. Biol. Chem. 275*, 2219−2230.
25. Ching, K. A., Kawakami, Y., Kawakami, T., and Tsoukas, C. D. (1999) *J. Immunol. 163*, 6006−6013.
26. Perez-Villar, J. J., and Kanner, S. B. (1999) *J. Immunol. 163*, 6435−6441.
27. Perez-Villar, J. J., O'Day, K., Hewgill, D. H., Nadler, S. G., and Kanner, S. B. (2001) *Int. Immunol. 13*, 1265−1274.
28. Schaeffer, E. M., Debnath, J., Yap, G., McVicar, G., Liao, X. C., Littman, D. R., Sher, A., Varmus, H. E., Lenardo, M. J., and Schwartzberg, P. L. (1999) *Science 284*, 638−641.
29. Liao, X. C., and Littman, D. R. (1995) *Immunity 3*, 757−769.
30. Ledbetter, J. A., June, C. H., Grosmaire, L. S., and Rabinoitch, P. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1384−1388.
31. Tanaka, N., Abe, H., Yagita, H., Okumura, K., Nakamura, M., and Sugamura, K. (1997) *Eur. J. Immunol. 27*, 834−841.
32. Phillips, J. H., Chang, C., Mattson, J., Gumperz, J. E., Parham, P., and Lanier, L. L. (1996) *Immunity 5*, 163−172.
33. Burkhardt, A. L., Costa, T., Misulovin, Z., Stealy, B., Bolen, J. B., and Nussenzweig, M. C. (1994) *Mol. Cell. Biol. 14*, 1095−1103.
34. Trub, T., Frantz, J. D., Miyazaki, M., Band, H., and Shoelson, S. E. (1997) *J. Biol. Chem. 272*, 894−902.
35. Musci, M. A., Hendricks-Taylor, L. R., Motto, D. G., Paskind, M., Kamens, J., Turck, C. W., and Koretzky, G. A. (1997) *J. Biol. Chem. 272*, 11674−11677.
36. Samelson, L. E., Bunnell, S. C., Trible, R. P., Yamazaki, T., and Zhang, W. (1999) *Cold Spring Harbor Symp. Quant. Biol. 64*, 259−263.
37. Zhang, W., and Samelson, L. E. (2000) *Semin. Immunol. 12*, 35−41.
38. Ching, K. A., Grasis, J. A., Tailor, P., Kawakami, Y., Kawakami, T., and Tsoukas, C. D. (2000) *J. Immunol. 165*, 256−262.
39. Wu, J., Motto, D. G., Koretzky, G. A., and Weiss, A. (1996) *Immunity 4*, 593−602.
40. Tuosto, L., Michel, F., and Acuto, O. (1996) *J. Exp. Med. 184*, 1161−1166.
41. Lahesmaa, R., Allsup, A., Soderberg, C., Jackman, J., Findell, P., and Peltz, G. (1995) *J. Immunol. 155*, 3815−3822.
42. Paz, P. E., Wang, S., Clarke, H., Lu, X., Stokoe, D., and Abo, A. (2001) *Biochem. J. 356*, 461−471.
43. Katzav, S., Sutherland, M., Packham, G., Yi, T., and Weiss, A. (1994) *J. Biol. Chem. 269*, 32579−32585.
44. Deckert, M., Tartare-Deckert, S., Couture, C., Mustelin, T., and Altman, A. (1996) *Immunity 5*, 591−604.
45. Bustelo, X. R. (2000) *Mol. Cell. Biol. 20*, 1461−1477.
46. Bustelo, X. R. (2001) *Oncogene 20*, 6372−6381.

BI025554O