Cell, Vol. 92, 83–92, January 9, 1998, Copyright ©1998 by Cell Press

# LAT: The ZAP-70 Tyrosine Kinase Substrate that Links T Cell Receptor to Cellular Activation

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## Summary

**Despite extensive study, several of the major components involved in T cell receptor–mediated signaling remain unidentified. Here we report the cloning of the cDNA for a highly tyrosine-phosphorylated 36–38 kDa protein, previously characterized by its association with Grb2, phospholipase C-γ1, and the p85 subunit of phosphoinositide 3-kinase. Deduced amino acid sequence identifies a novel integral membrane protein containing multiple potential tyrosine phosphorylation sites. We show that this protein is phosphorylated by ZAP-70/Syk protein tyrosine kinases leading to recruitment of multiple signaling molecules. Its function is demonstrated by inhibition of T cell activation following overexpression of a mutant form lacking critical tyrosine residues. Therefore, we propose to name the molecule LAT—linker for activation of T cells.**

## Introduction

T cell antigen receptor (TCR) engagement triggers complex cascades of biochemical events leading ultimately to enhanced gene transcription, cellular proliferation and differentiation (Weiss and Littman, 1994; Chan and Shaw, 1996; Wange and Samelson, 1996). Extensive investigation over the past ten years has revealed that the earliest signaling events coupled to the TCR are the activation of several protein tyrosine kinases (PTKs) and the subsequent tyrosine phosphorylation of multiple intracellular proteins. Activation of Lck or Fyn, two members of the Src family of PTK expressed in T cells, appears to be the proximal event in this pathway. Either one or both of these enzymes phosphorylate tyrosine residues found within multiple immunoreceptor tyrosine-based activation motifs (ITAMs) located in cytosolic domains of the TCRζ and CD3 chains. Each ITAM contains two tyrosine residues, which, upon phosphorylation, serve as binding sites for the tandem SH2 domains of ZAP-70, a Syk family PTK. Lck or Fyn then phosphorylate the bound ZAP-70, resulting in its activation. The engaged TCR is thus characterized by the presence of recruited and activated PTKs.

Characterization of many of the substrates of these activated PTKs has led to considerable understanding into the details of TCR-mediated signal transduction. Two direct consequences of protein tyrosine phosphorylation have been described in this system. First, tyrosine phosphorylation can activate enzymes. Examples are ZAP-70, which must be phosphorylated for full kinase activity (Chan et al., 1995; Wange et al., 1995), and phospholipase C-γ1 (PLC-γ1), which upon activation hydrolyzes phosphoinositides generating products that elevate intracellular calcium and activate protein kinase C (Rhee and Bae, 1997). Second, tyrosine phosphorylation creates sites for binding by proteins with SH2 domains (Pawson, 1995). One example is the interaction of Vav, SLP-76, and other proteins upon TCR activation. The Vav SH2 domain binds to phosphorylated tyrosine residues of SLP-76 (Wu et al., 1996). SLP-76 also contains an SH2 domain, which is capable of binding additional tyrosine-phosphorylated proteins (Motto et al., 1996). Though the function of these proteins, is not fully understood, their significance is demonstrated by the fact that overexpression of Vav and/or SLP-76 leads to enhanced T cell signaling (Wu et al., 1996).

Additional protein interactions, central to T cell activation, are indirectly regulated by tyrosine phosphorylation. For example, the Grb2 adaptor protein, consisting of an SH2 domain surrounded by two SH3 domains (Lowenstein et al., 1992), binds to proteins such as SLP-76, Cbl, and the SOS protein. All of these molecules bind Grb2 SH3 domains via proline-rich motifs (Buday et al., 1994; Donovan et al., 1994; Motto et al., 1994). Based on examples from growth factor receptor systems, cellular activation results in tyrosine phosphorylation of proteins that bind Grb2 via its SH2 domain thus recruiting its associated proteins. A related protein, Grap, is highly expressed in T cells and interacts with another set of molecules (Feng et al., 1996; Trüb et al., 1997). Upon TCR activation, the SH2 domains of both Grb2 and Grap are thought to interact with membrane proteins that have become tyrosine phosphorylated. In this manner, these signaling complexes can be recruited to the plasma membrane.

One of the remaining critical issues in the study of this PTK-directed signal transduction pathway is the identification of molecules that bridge the activated TCR with its associated PTKs to the tyrosine kinase substrates. In cellular systems in which growth factor receptor kinases are activated by their ligands, many signaling molecules directly bind phosphorylated tyrosine residues of the cytosolic domain of the receptor (Pawson and Schlessinger, 1993). However, this does not appear to be a feature of TCR activation. Attention, instead, has focused on a 36–38 kDa protein, one of the most prominently tyrosine-phosphorylated proteins detected following TCR engagement (June et al., 1990). Preliminary work has shown that this is a membrane-anchored protein, capable of binding SH2 domains of Grb2, Grap, PLC-γ1, and the p85 subunit of phosphoinositide 3-kinase (PI3K) (Gilliland et al., 1992; Buday et al., 1994; Sieh et al., 1994; Fukazawa et al., 1995; Trüb et al., 1997). This 36–38 kDa substrate has proven to be refractory to purification and characterization. The recently isolated Lnk protein was initially thought to be this 36–38 kDa protein (Huang et al., 1995). However, though Lnk is a PTK substrate and can bind Grb2, PLC-γ1, and the p85 subunit of PI3K, albeit through its own SH2 domain,

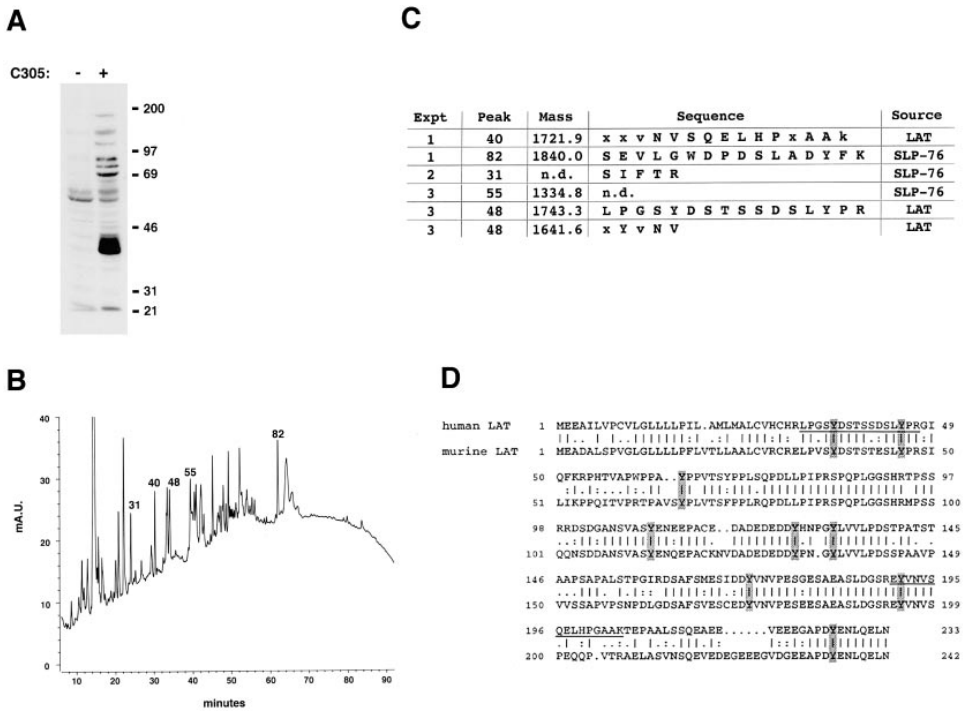


Figure 1. Purification, cDNA Cloning, and the Deduced Amino Acid Sequence of LAT

(A) Jurkat cells were either unstimulated (-) or stimulated (+) with C305 (1:50) for 2 min and lysed in 1% Brij97 lysis buffer. Lysates of $3 \times 10^5$ cell equivalent were loaded on SDS–PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody (PY20).

(B) Tryptic fragments from affinity-purified p36–38 protein isolated from stimulated Jurkat cells. The band corresponding to p36–38 was excised from the gel, digested with trypsin, and resolved by reverse-phase microbore HPLC. Five peptide peaks (fraction 31, 40, 48, 55, 82) were selected for mass spectrometry and microsequencing.

(C) Peptides from the indicated peaks were analyzed. The single amino acid code is used, with the following additions: X = no assignment, uppercase = full confidence assignment, lowercase = ambiguous residue, and n.d. = not determined.

(D) Deduced amino acid sequence of human and murine LAT. Two peptide sequences determined by microsequencing are underlined. The conserved tyrosines between human and murine LAT are shadowed.

it is unlikely that Lnk is the p36–38 substrate. It lacks any membrane targeting signal or any relevant SH2-binding sites. Moreover, Lnk overexpression has no effect on p36–38 tyrosine phosphorylation (Takaki et al., 1997). In view of the possible central role of the p36–38 protein as a molecule downstream of PTKs capable of binding critical PTK substrates and effector molecules, we have purified this protein and here report the cloning of its cDNA. We demonstrate that this protein, LAT (linker for activation of T cells), is a substrate of activated ZAP-70. Upon tyrosine phosphorylation, LAT binds Grb2, PLC-$\gamma$1, the p85 subunit of PI3K, and other critical signaling molecules. We provide evidence that LAT plays an important role in linking the TCR to cellular activation.

## Results

### Purification of LAT

Stimulation of the TCR on the Jurkat human T cell line by cross-linking with either C305 (anti-TCR$\beta$, Figure 1A) or OKT3 (anti-CD3$\epsilon$, not shown) monoclonal antibodies induces tyrosine phosphorylation of multiple proteins, most prominently, p36–38. To define the role of p36–38 in T cell signaling, we purified this protein from activated Jurkat cells. Since previous studies showed that p36–38 is membrane-associated, membrane fractions were prepared from OKT3-stimulated Jurkat cells and extracted with Brij97 detergent. The extracted membrane proteins were heat-treated, which induced precipitation of about 2/3 of the protein, though not p36–38. Anti-phosphotyrosine antibodies were used for affinity purification of phosphotyrosine-containing proteins. These were then specifically eluted with phenyl phosphate, concentrated, and resolved on SDS–PAGE. The p36–38 band was excised and digested in gel with trypsin, and the resultant tryptic peptides were resolved by microbore reverse-phase HPLC (Figure 1B).

### Peptide Sequencing and cDNA Cloning

Peptides from five HPLC fractions were either microsequenced or subjected to mass spectrometry. The peptide from fraction 40 had a molecular weight of 1721.9 daltons (Figure 1C). The residues at positions 1, 2, 3, 11, and 15 could not be assigned unambiguously. The available sequence was used to search the GenBank database with the BLAST algorithm, and an EST clone from human fetal heart was found to encode this peptide fragment. Review of the Edman sequence data at the ambiguous positions was consistent with the EST sequence. The predicted molecular weight of the putative tryptic fragment predicted from this EST sequence

is 1641.8 daltons. The 80.1 daltons difference suggested that a phosphate moiety of this molecular weight could be present at the Tyr residue. Two peptides from peaks 82 and 31 were shown by sequence analysis to be derived from the known tyrosine kinase substrate SLP-76. It is not known whether these fragments of this 76 kDa substrate were cleaved physiologically or during protein purification. To avoid sequencing other peptides derived from SLP-76, the masses of subsequent peptides were determined. With this strategy, we concluded that the peptide in fraction 55 was likely to originate from SLP-76, but the masses of two peptides in fraction 48 could not be of SLP-76 origin. The sequence of the larger of these two peptides was not found in the EST clone or GenBank. Interestingly, fraction 48 contained a small amount of a second peptide with a molecular weight of 1641.6, 80.3 daltons less than the mass of the peptide isolated and sequenced from peak 40. A partial amino acid sequence revealed that this peptide is the nonphosphorylated form of the peak 40 peptide.

A cDNA library from YT cells was probed with the EcoRI/PstI fragment of the EST clone. Thirty positive clones were isolated, of which the longest was 1.6 kb. Determination of the nucleotide sequences of these clones predicted a protein that contains the two tryptic peptide sequences found in the isolated peptides (underlined in Figure 1D). Through comparison of the EST and cDNA sequences, we found that the first 380 nucleotides in the EST clone are not present in the cDNA. This unknown sequence might be derived from intronic sequence. Probes from the human cDNA were used to screen a murine adult thymus library. The murine amino acid sequence we obtained is 66% identical to the human (Figure 1D).

The coding sequence of human p36–38 begins at nucleotide 58. The first methionine, not found within a consensus Kozak sequence, is followed by an open reading frame predicting a 233 amino acid polypeptide with a calculated molecular mass of 24,985 daltons. One cDNA clone has an 87-nucleotide insertion at position 396, encoding an additional 29 amino acids. Of the 233 amino acids found in the majority of clones, there are a total of 39 negatively charged residues (16 Asp and 23 Glu), but only 11 positively charged residues (2 Lys and 9 Arg). The high relative negative charge might result in retarded migration on SDS–PAGE leading to an apparent molecular weight of 36–38 kDa, in excess of the predicted mass. The deduced amino acid sequence contains no domain homologous to known tyrosine, serine/threonine, or lipid kinases, nor does it contain SH2, SH3, or PTB domains. A single region of hydrophobic amino acids extends from residues 5 to 27, and might form an $\alpha$-helical transmembrane domain. Two of the four residues N-terminal to this region are negatively charged in both human (two Glu) and murine (one Glu and one Asp). Immediately after this hydrophobic domain are positively charged residues (one Arg in the human and two Arg in the murine sequence). The relative charge difference across this putative transmembrane domain likely determines the orientation of the molecule in the membrane, resulting in an extremely short extracellular amino terminus and a long cytosolic tail.

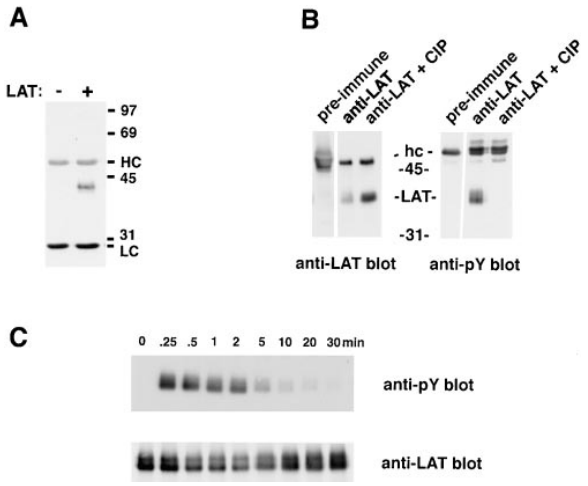


Figure 2. Characterization of the LAT Protein

(A) 293T cells were transfected with empty vector (pcDNA3) or with myc-tagged LAT in pcDNA3 (+). Thirty-six hours after transfection, 293T cells were lysed, and postnuclear lysates were immunoprecipitated with anti-myc antibody (9E10). Samples were resolved on SDS–PAGE and immunoblotted with anti-myc antibody.

(B) Jurkat T cells were stimulated with C305 and lysates immunoprecipitated with rabbit anti-LAT antibody. Proteins immunoprecipitated with anti-LAT antibody were treated with calf intestine alkaline phosphatase for 1 hr at 37°C where indicated.

(C) Kinetics of LAT phosphorylation in Jurkat cells. Cells ($1 \times 10^7$) in RPMI ($10^8$ cells/ml) were stimulated with OKT3 for the indicated times, lysed, and immunoprecipitated with anti-LAT antibody. Immunoprecipitated proteins were resolved on SDS–PAGE and blotted with anti-phosphotyrosine (pY) and anti-LAT antibodies.

Consistent with the prominent tyrosine phosphorylation of the protein in activated cells, the predicted cytosolic domain of human p36–38 contains ten tyrosines, of which nine are conserved between the human and murine proteins. Based on studies by Songyang et al. (1994), it is known that after phosphorylation there are five potential binding sites for Grb2 SH2 domains (YxN) at tyrosines 110, 127, 171, 191, and 226. Tyr171 and Tyr191 have an identical Grb2-binding motif (YVNV). A binding motif for the SH2 domain of the p85 subunit of PI3K is not observed. A sequence related to the consensus binding motif for N-terminal and C-terminal PLC-$\gamma$1 SH2 domains (YLVV, Tyr132) is present. Since the p36–38 protein most likely functions as a docking protein capable of recruiting signaling molecules, and in view of the observed features of its sequence, we propose that its name be LAT, linker for activation of T cells.

To confirm that the cDNA clone encodes the full-length LAT protein, and to resolve the discrepancy between the predicted and apparent molecular weight, the human *LAT* cDNA was subjected to in vitro transcription and translation. The reaction product contained a 38 kDa band not present when control DNA was used, confirming that the complete *LAT* coding sequence was present (not shown). The *LAT* cDNA was modified to include an epitopic tag (myc) at the C terminus, cloned into the pcDNA3 expression vector and transfected into 293T cells (Figure 2A). Immunoprecipitation and blotting demonstrated a 40 kDa protein in the transfected, but not nontransfected cells. These results also indicated

that the *LAT* cDNA clone contained the entire coding region of the protein.

To demonstrate the LAT protein in T cells, rabbit polyclonal antibodies were raised against the cytosolic portion of LAT fused to GST. The resulting antiserum was used to detect LAT in lysates of activated Jurkat cells (Figure 2B). The LAT antiserum precipitated and detected a doublet of 36 and 38 kDa, which comigrated with bands detected with antiphosphotyrosine antibodies. Repetitive immunoprecipitation with this antiserum depleted most of p36–38 proteins from stimulated Jurkat lysate (data not shown). Phosphorylation of LAT partially interfered with the detection of this protein as demonstrated by enhanced blotting following dephosphorylation of the immunoprecipitate with calf intestinal phosphatase (CIP). This antiserum enabled us to determine that LAT was rapidly tyrosine phosphorylated upon stimulation with OKT3 (Figure 2C). Maximal phosphorylation of LAT was seen after stimulation for 15 s, and after 2 min LAT was rapidly dephosphorylated. These phosphorylation kinetics were also seen in whole cell lysates (not shown). The results demonstrate that the *LAT* cDNA encodes a protein that migrates on SDS–PAGE with an apparent molecular weight of 36–38 kDa and that is tyrosine phosphorylated, as expected, following TCR activation.

### Tissue and Cellular Distribution of LAT

Northern blot analysis was performed with poly(A)+ RNAs isolated from different adult human tissues. Two transcripts (2.0 and 1.6 kb) were seen predominantly in thymus, peripheral blood, and at a low level of expression in spleen. There were no transcripts of *LAT* found in other tissues (Figure 3A and data not shown). *LAT* expression was assayed in human and rat cell lines of hematopoietic origin. *LAT* mRNA was only found in Jurkat, YT (NK-like cells), and RBL (a rat mast cell line) (Figure 3B). From these data, we conclude that LAT is expressed in T cells, NK cells, and mast cells, but not in B cells (Raji and Jiyoye) or monocytes (THP1).

The cellular localization of LAT was analyzed by immunofluorescence in COS cells, which were transiently transfected with *LAT* cDNA, fixed, permeabilized, and incubated sequentially with anti-LAT and TRITC-conjugated goat anti-rabbit antibodies. Visualization with confocal microscopy showed that LAT was localized to the plasma membrane, and a juxtanuclear intracellular compartment (Figure 4A). No staining was detected in untransfected COS cells. Plasma membrane localization of endogenous LAT was also specifically detected in Jurkat cells (Figures 4B and 4C). When cells were simultaneously stained with anti-LAT and anti-Lck antibodies, LAT and Lck were seen to colocalize at the plasma membrane and in an intracellular compartment (Figure 4D).

### LAT Is a Substrate of ZAP-70 and Syk Protein Tyrosine Kinases

Src family and Syk family kinases are directly involved in TCR-mediated signaling. To examine which tyrosine kinases can phosphorylate LAT, FLAG-tagged LAT was transiently coexpressed with Lck, Fyn, ZAP-70, or Syk, alone or in certain combinations in 293T cells. Phosphorylation of LAT was detected by anti-phosphotyrosine

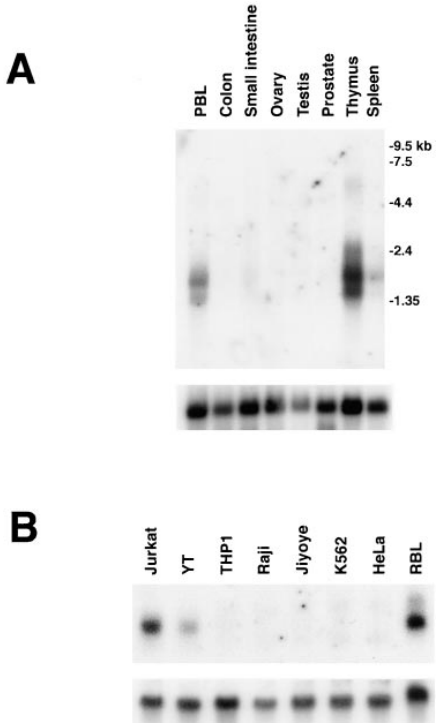


Figure 3. Tissue Distribution of LAT

(A) Northern blot analysis of poly(A)+ RNA from different human tissues. A prepared membrane (Clontech) was hybridized with randomly primed probes from *LAT* cDNA. The same membrane was also stripped and subsequently hybridized with a β-actin probe to normalize for the amount of RNA.

(B) Northern blot analysis of total RNA from different cell lines: Jurkat (T cell), YT (NK-like cell), THP1 (monocyte), Raji (B lymphoma), Jiyoye (B lymphoma), K562 (myelomonocytic cells), HeLa (fibroblastoid), and RBL (mast cell). The same membrane was also hybridized with a β-actin probe.

antibody blotting. There was no tyrosine phosphorylation of LAT when it was expressed alone in 293T cells (Figure 5A). When LAT was coexpressed with Lck, constitutively activated Lck (Y505F) or Fyn, only coexpression of activated Lck resulted in minimal tyrosine phosphorylation of LAT. Cotransfection with Syk resulted in marked LAT tyrosine phosphorylation. However, ZAP-70 alone failed to phosphorylate LAT. ZAP-70, unlike Syk, is known to require activation by a Src kinase family member. Consequently, when LAT was expressed with ZAP-70 and Lck or ZAP-70 and Fyn, LAT was tyrosine phosphorylated to a level comparable to that induced by Syk. At least two unidentified tyrosine-phosphorylated proteins of 90 and 60 kDa coprecipitated with LAT under these conditions. Likewise, the association of LAT with endogenous Grb2 linker protein required LAT tyrosine phosphorylation induced by either Syk or the combination of ZAP-70 with either Lck or Fyn. These results indicate that LAT is a substrate of ZAP-70 and Syk, and that tyrosines in LAT involved in binding to the Grb2 SH2 domain are phosphorylated by these PTKs.

### Association of LAT with Grb2, Grap, PI3K, and PLC-γ1 in 293T Cells

To investigate the association of LAT with critical signaling molecules, FLAG-tagged LAT in the pcDNA3 vector

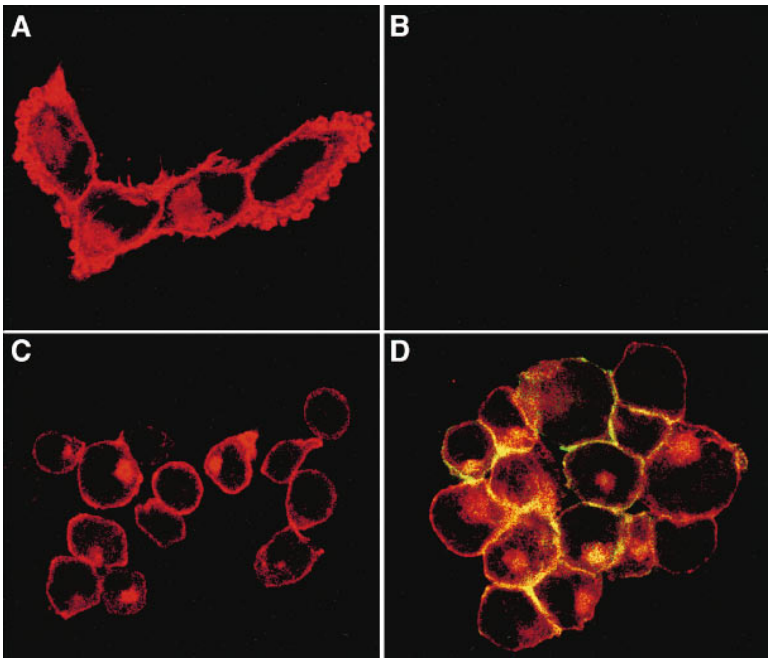


Figure 4. Cellular Localization of LAT

(A) COS cells were transfected with pcDNA3/LAT or pcDNA3 (not shown). After 24 hr, the transfected cells were fixed, permeabilized, labeled with anti-LAT antibody, and then stained with Texas red–conjugated goat anti-rabbit antibody. Cells were examined with confocal microscopy.

(B) Jurkat cells stained with preimmune serum using above procedure.

(C) Jurkat cells stained with anti-LAT serum.

(D) Jurkat cells were simultaneously stained with monoclonal anti-Lck antibody followed by FITC-goat anti-mouse Ig and rabbit anti-LAT antibody followed by TRITC-goat anti-rabbit Ig. LAT is stained in red. Lck is stained in green. The place where LAT and Lck colocalized is stained in yellow.

was cotransfected in 293T cells with plasmids expressing HA-tagged Grb2, myc-tagged Grap, HA-tagged p85 subunit of PI3K or PLC-γ1, in the presence or absence of both ZAP-70 and Lck. LAT was immunoprecipitated with anti-FLAG antibody, and associated proteins were detected by blotting with specific antibodies. As shown in Figure 5B, LAT tyrosine phosphorylation and association with endogenous Grb2 was induced by cotransfection with ZAP-70 and Lck as in Figure 5A. When LAT and the two PTKs were coexpressed with HA-tagged Grb2, both endogenous Grb2 and HA-Grb2 bound to phosphorylated LAT (HA-Grb2 migrates more slowly than endogenous Grb2 on SDS–PAGE). myc-tagged Grap, the Grb2-like protein, when coexpressed with LAT in the presence of the two PTKs, also associated with phosphorylated LAT. However, in this immunoprecipitation no endogenous Grb2 was bound to phosphorylated LAT, suggesting that Grap competes for Grb2-binding sites. HA-tagged p85 or PLC-γ1 also associated with phosphorylated LAT. Some endogenous Grb2 bound to LAT, but less than when LAT was cotransfected only with ZAP and Lck. Surprisingly, we found some association of LAT with PLC-γ1 without cotransfection of the PTKs, suggesting either binding to undetectable tyrosine phosphorylation sites or binding via an SH2-independent mechanism.

The above experiments were designed to demonstrate LAT associations by isolation of LAT followed by detection of associated molecules. The reciprocal approach was also performed to confirm these associations. 293T cells were cotransfected with FLAG-tagged LAT, ZAP-70 and Lck, and either HA-tagged Grb2 or myc-tagged Grap (Figure 5C). As above, immunoprecipitation of LAT allowed detection of that fraction of Grb2 or Grap associated with LAT. In addition, immunoprecipitation of either Grb2-HA or Grap-myc allowed detection of the fraction of LAT bound to either adaptor protein. Similar experiments were performed in cells cotransfected with HA-p85 or PLC-γ1. Immunoprecipitation of these proteins revealed associated LAT (not shown). Additional experiments were performed to determine if the PTKs ZAP-70 or Lck could bind to LAT. No associations between LAT and either PTK were detected (not shown).

### Association of LAT with Grb2, PLC-γ1, and PI3K in Jurkat T Cells

The ability of LAT to associate with signaling proteins in T cells was next evaluated. The interaction of Grb2 with p36–38 is thought to be mediated by the Grb2 SH2 domain. To test this, lysates prepared from resting and activated Jurkat cells stimulated with C305 were incubated with the immobilized fusion proteins, GST-Grb2, GST-Grb2 with an SH2 domain mutation (R86K), and GST-Grb2 with mutations at both SH3 domains (P49L/G203R). Only GST-Grb2 and GST-Grb2 with SH3 mutations bound a 36–38 kDa tyrosine-phosphorylated protein from C305-stimulated Jurkat lysate (Figure 6A). Mutation at the SH2 domain of Grb2 abolished the interaction between this 36–38 kDa protein and GST-Grb2. Subsequently the same membrane was probed with anti-LAT antibodies, which confirmed that this p36–38 is LAT. LAT was not detected in material subjected to purification with GST-Grb2 from unstimulated cells or with GST-Grb2 containing the SH2 mutation.

To demonstrate the interactions in T cells directly, lysates from unstimulated and stimulated Jurkat T cells were subjected to immunoprecipitation with anti-Grb2 and anti-PLC-γ1 antibodies followed by blotting with anti-LAT. As shown in Figure 6B, equal amounts of Grb2 or PLC-γ1 were immunoprecipitated from unstimulated or stimulated lysates. When the membrane was blotted with anti-phosphotyrosine antibody, a 36–38 kDa tyrosine–phosphorylated protein was detected in association with Grb2 and PLC-γ1 only in activated T cells. This protein was identified as LAT by immunoblotting with anti-LAT antibody. CIP treatment of the immunoprecipitate enhanced LAT detection. We also detected p85 in

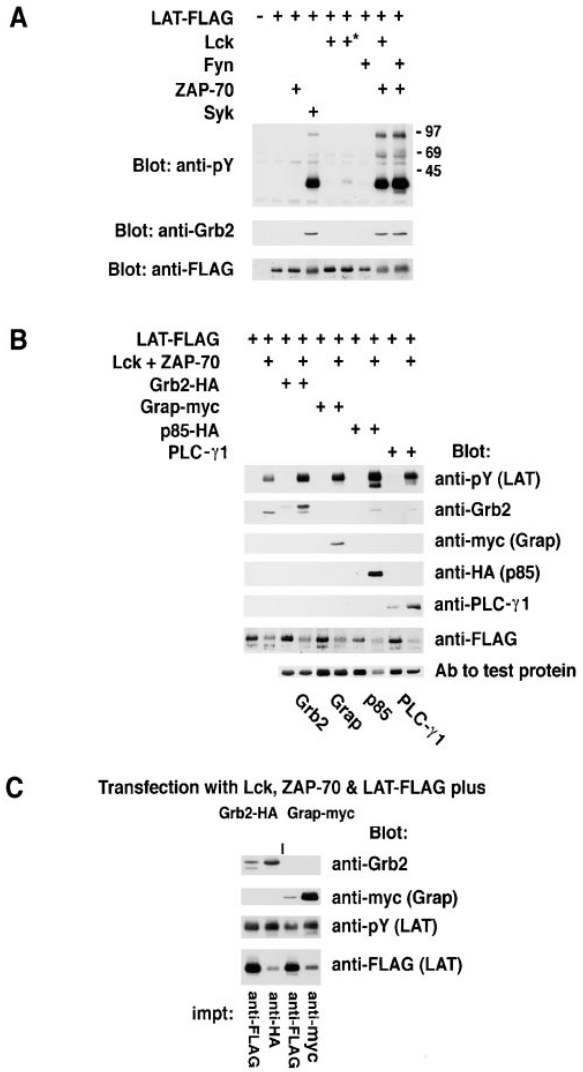


Figure 5. LAT Is Phosphorylated by PTKs and Associates with Grb2, Grap, the p85 Subunit of PI3K, and PLC-γ1 in 293T Cells

(A) Tyrosine phosphorylation of LAT by Syk and ZAP-70 PTKs. FLAG-tagged LAT in pcDNA3 was transfected into 293T cells either alone or cotransfected as indicated. Lck* is a constitutively activated mutant of Lck (Y505F). At 36 hr after transfection, the 293T cells were harvested and lysed in Brij lysis buffer. Lysates were subjected to anti-FLAG immunoprecipitation.

(B) Association of LAT with signaling molecules in 293T cells. FLAG-tagged LAT was cotransfected with pCEFL/HA-Grb2, pSRα/Grap-myc, pcDNA3/HA-p85, and pcDNA3/PLC-γ1 in the absence or presence of ZAP-70 and Lck PTKs. Brij lysates of transfected 293T cells were immunoprecipitated with anti-FLAG. The association of LAT with tested proteins was determined by blotting with specific antibodies.

(C) Determination of LAT association with Grb2 or Grap by reciprocal immunoprecipitation. Lck, ZAP-70, and LAT were cotransfected with either Grb2-HA or Grap-myc as indicated. At 36 hr after transfection, lysates of the 293T cells were immunoprecipitated with anti-FLAG and anti-HA antibodies if cotransfected with Grb2-HA, or anti-FLAG and anti-myc antibodies if cotransfected with Grap-myc.

anti-Grb2, but not in anti-PLC-γ1 immunoprecipitates in lysates of activated Jurkat cells. There was also some low level of association between Grb2 and PLC-γ1 probably through LAT (data not shown).

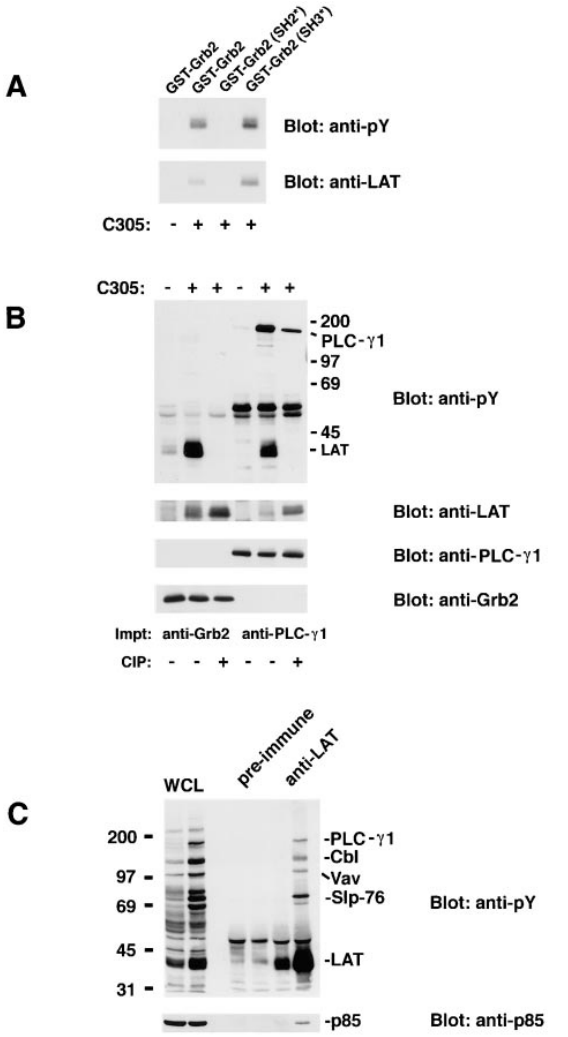


Figure 6. LAT Associates with Grb2, p85, and PLC-γ1 in Jurkat T Cells

(A) GST-Grb2 associates with LAT through its SH2 domain. GST fusion proteins cross-linked to glutathione-agarose were used to precipitate proteins from unstimulated or stimulated with C305 Jurkat lysates. GST-Grb2 (SH2*) has a mutation in the SH2 domain (R86K). GST-Grb2 (SH3*) has mutations at both N-terminal and C-terminal SH3 domains (P49L/G203R).

(B) Lysates from Jurkat T cell unstimulated or stimulated with C305 were subjected to anti-Grb2 or anti-PLC-γ1 immunoprecipitation. CIP was used to remove phosphate groups from LAT to enhance blotting by the anti-LAT antibody.

(C) Lysates from Jurkat cells were immunoprecipitated with preimmune serum or anti-LAT antibody. Samples were resolved on SDS–PAGE and immunoblotted with anti-phosphotyrosine antibody (4G10). The same membrane was stripped and blotted with anti-Grb2, p85, Vav, Cbl, PLC-γ1 to identify specific proteins. Only anti-p85 blotting is shown in this figure.

TCR-mediated activation results in tyrosine phosphorylation of many substrates (Figures 1A and 5C). To identify additional proteins capable of binding LAT following T cell activation, we used anti-LAT antisera to immunoprecipitate LAT from lysates of resting and activated Jurkat cells. As shown in Figure 6C, several tyrosine-phosphorylated proteins with molecular weights of about 70, 76, 100, 120, and 135 kDa were specifically

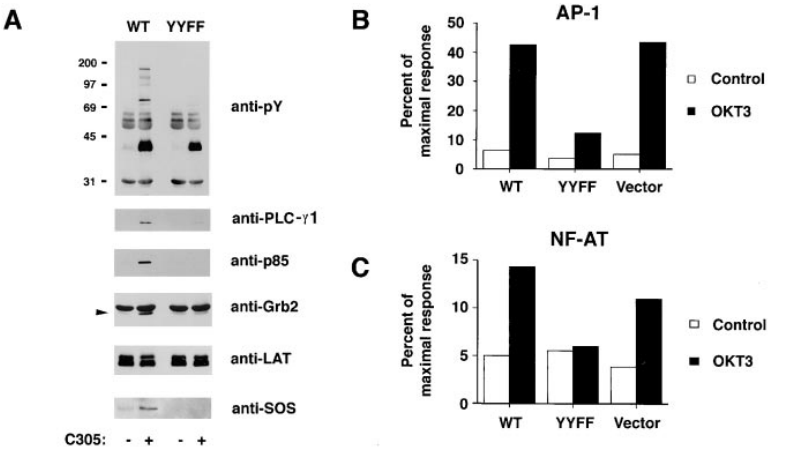


Figure 7. Overexpression of a Mutant Form of LAT (Y171/191F) Blocks Complex Formation and TCR-Mediated AP-1 and NFAT Transcriptional Activation

(A) Jurkat cells were stably transfected with wild-type (WT) or mutant (Y171/191F) LAT. Exogenous LAT was immunoprecipitated with anti-myc antibody from cells either left unstimulated or stimulated with C305 (1:50). Samples were analyzed on SDS–PAGE and blotted with 4G10. The same membrane was blotted with anti-PLC-γ1, anti-p85, anti-Grb2, and anti-LAT after stripping. Note that the arrowhead indicates the migration of Grb2 below the immunoglobulin light chain. An additional membrane prepared in the same way was probed with anti-SOS antibodies.

(B) Jurkat TAg cells were cotransfected with 20 μg pSX-AP-1/SEAP with 20 μg empty vector, pEF/LAT(WT), or pEF/LAT(Y171/191F). Twenty-four hours after transfection, cells were either unstimulated, stimulated with OKT3 (1:1000), or stimulated with PMA and ionomycin. The SEAP activity was assayed in triplicate. The data are representative of three independent experiments.

(C) Jurkat TAg cells were cotransfected with 20 μg pSX-NFAT/SEAP with 20 μg empty vector, pEF/LAT(Wt), or pEF/LAT(Y171/191F). The same procedure was used for stimulation and SEAP assay as in (B). The data are representative of three independent experiments.

coprecipitated with anti-LAT only from lysates of stimulated Jurkat cells. By using a panel of antibodies to immunoblot known tyrosine kinase substrates, we identified some of these proteins as PLC-γ1 (135 kDa), Cbl (120 kDa), and Vav (100 kDa) (not shown). The 76 kDa protein was most likely SLP-76. The associations of SLP-76 and Cbl with LAT were probably mediated indirectly through Grb2, because both bind the Grb2 SH3 domains. Vav is likely detected because of its association with SLP-76. The 70 kDa protein was not identified and was not Sam68 or ZAP-70. Anti-LAT immunoprecipitates from stimulated Jurkat also contained the p85 subunit of PI3K and Grb2 (not shown). These results demonstrate that LAT is present in signaling complexes containing multiple critical molecules.

### Function of LAT in TCR Signal Transduction Pathway

The hypothesis from the above studies is that the association of tyrosine-phosphorylated LAT with the various signaling molecules is required for the TCR signal transduction process. To directly address the role of LAT in signaling through the TCR, we overexpressed wild-type (WT) LAT or a mutant form of LAT, and examined the effect of this overexpression on TCR-mediated activation. The mutant form of LAT employed in these experiments contains Phe for Tyr substitutions at both positions Y171 and Y191 (Y171/191F). These two tyrosine residues were chosen for mutagenesis because they are within identical YVNV motifs, a motif which has previously been shown to mediate the binding of Shc to Grb2 (see Songyang et al., 1994). Moreover, phosphorylation at Tyr191 was detected during microsequencing and mass spectrometry analysis of peptides from p36–38 (see above).

To investigate the effect of mutant LAT on protein–protein interactions, we established stable cell lines overexpressing myc-tagged WT or Y171/191F LAT. myc-tagged LAT was immunoprecipitated with anti-myc antibody from unstimulated or C305-stimulated transfectants. As shown in Figure 7A, blotting with anti-LAT antibodies demonstrated that the amount of LAT immunoprecipitated with anti-myc was comparable in the two cell lines. The myc-tagged LAT appears as a doublet as does endogenous LAT. WT and Y171/191F LAT were both tyrosine phosphorylated upon stimulation, though the level of Y171/191F tyrosine phosphorylation was less than that of WT LAT, suggesting that tyrosine residues besides Y171 and Y191 are phosphorylated. Though p85, Grb2, and PLC-γ1 were observed to bind the WT form of LAT, mutations at Y171 and Y191 abolished the binding of Grb2 and p85, and greatly reduced the binding of PLC-γ1 upon activation. In addition, mutations at Y171 and Y191 prevented the association of SLP-76, Vav, and Cbl, but the 70 kDa tyrosine-phosphorylated protein remained associated with LAT. Additionally, we observed that the Ras activator protein SOS coprecipitated with LAT, likely due to its interaction with Grb2. The association with SOS was also disrupted by the double Y-to-F mutations.

The functional effect of mutant LAT overexpression was assayed by determining the effect on transcriptional activation of AP-1 and NF-AT, both involved in TCR-mediated transcriptional events. Jurkat TAg cells were transiently transfected with WT LAT, mutant LAT(Y171/191F), or vector only, together with a reporter construct for secreted alkaline phosphatase (SEAP) driven by the AP-1 or NF-AT response elements. Figure 7B demonstrates that overexpression of WT LAT did not result in any significant effect on AP-1 transcriptional activity and slightly increased NF-AT transcriptional activity compared with transfection of the control vector. However, overexpression of the mutant form of LAT (Y171/191F) blocked TCR-mediated AP-1 and NF-AT transcriptional activity. These results suggest that LAT plays an important role in activation of transcription mediated by AP-1 and NF-AT following TCR stimulation.

## Discussion

In this report, we describe the cloning of the cDNA encoding the prominent T cell PTK substrate, p36–38,

which has been observed and extensively studied for several years. The identification of p36–38 as LAT has now enabled us to demonstrate directly the interactions of this protein with critical signaling molecules in the TCR-mediated signaling pathway. Our results indicate that LAT is a substrate of ZAP-70/Syk tyrosine kinases. It associates with Grb2, PLC-γ1, and the p85 subunit of PI3K upon T cell activation, consistent with observation made previously for p36–38. Moreover, it also binds Cbl, SOS, Vav, and SLP-76, probably indirectly via Grb2. Two tyrosine residues, Y171 and Y191 of LAT, appear to be required for association with these signaling molecules. Significantly, overexpression of LAT with mutations at these tyrosines blocks the transcriptional activation of AP-1 and NF-AT. These data indicate that LAT is an important linker molecule coupling the TCR to downstream signaling events.

The amino acid sequence of LAT, deduced from the cDNA clones, reveals a unique hydrophobic region near the amino terminus, which is a potential transmembrane domain. This explains our biochemical data and that in the literature indicating that p36–38 is tightly associated with membranes. It is less likely that LAT is a peripheral membrane protein because a high concentration of salt failed to dissociate p36–38 from membranes (not shown). In the sequences of both human and murine LAT, this hydrophobic region is preceded by negatively charged and followed by positively charged amino acids. These features, in addition to the presence of multiple tyrosine phosphorylation sites distal to this region, indicate that the C terminus is cytosolic. This orientation and the absence of a signal peptide sequence allow this protein to be characterized as a type III membrane protein (von Heijne, 1994). The membrane localization of LAT suggests that its tyrosine phosphorylation could be readily accomplished by the activated TCR-PTK complex. The rapid kinetics of LAT tyrosine phosphorylation support this view. However, it is not clear whether LAT directly interacts with the TCR or with other signaling molecules. Our initial studies have not revealed any stable interactions with either TCR or PTK components. The limitations of coprecipitation studies are obvious, and additional work will address how LAT is recruited to the activated TCR.

LAT is expressed in Jurkat T cells as two forms at apparent molecular weights of 36 and 38 kDa, and the 36 kDa form is more tyrosine phosphorylated than the 38 kDa form. Although the cDNA cloning reveals two splice variants of both human and murine *LAT*, the doublet that is detected on SDS–PAGE appears not to be due to alternate splicing. Supporting this conclusion is the observation that when LAT is expressed from one cDNA clone, we still detect both forms of the protein with antibodies generated against the LAT cytosolic domain. Instead, these two forms are likely due to posttranslational modifications. We note the presence of two cysteine residues following the putative transmembrane domain. Studies are underway to test whether LAT is acylated at these sites. Whatever the nature of a possible posttranslational modification, its effect on LAT function remains to be determined.

Determining which PTKs phosphorylate substrates in the T cell system has been difficult. The TCR components are examples of proteins tyrosine phosphorylated by the Src kinases, Lck and Fyn. Substrates of ZAP-70 have been more difficult to identify. Tubulin and SLP-76 are examples of proteins demonstrated to be ZAP-70 substrates (Isakov et al., 1996; Wardenburg et al., 1996). Our analysis of LAT tyrosine phosphorylation in 293T cells indicates that overexpression of Syk alone or ZAP-70 with either Lck or Fyn results in sufficient tyrosine phosphorylation of LAT to bind endogenous Grb2. The fact that the conditions for Syk- and ZAP-70-induced tyrosine phosphorylation differ in this situation is compatible with the different requirements for their activation. Though Syk activity may be enhanced through its phosphorylation by a Src family kinase, Src kinases do not appear to be required (Chu et al., 1996; Pao and Cambier, 1997; Zoller et al., 1997). In contrast, there appears to be a requirement for ZAP-70 to be tyrosine phosphorylated by a Src family PTK for its activation (Chan et al., 1995; Wange et al., 1995). Our studies indicate that at least some critical tyrosines in LAT are phosphorylated by activated ZAP-70 and not by Src family kinases. The conclusion that LAT is a substrate of ZAP-70 is also supported by our unpublished data that in a ZAP-70-deficient cell line LAT is not tyrosine phosphorylated. Nonetheless, it is possible that some of the multiple LAT tyrosine residues can be tyrosine phosphorylated by Src family kinases, as suggested by the low level of phosphorylation induced by activated Lck.

That LAT functions as a central linker protein in T cell activation was postulated by previous investigators and directly confirmed in our studies. Grb2, PLC-γ1, p85 of PI3K, and other signaling molecules were found to associate with LAT upon activation. In the cytosolic domain of LAT, there are consensus motifs for SH2 domains of Grb2 and PLC-γ1, but not for p85. Recruitment of Grb2 to LAT brings its associated SLP-76, Cbl, and SOS molecules to the activated TCR-PTK complex. Vav is most likely associated with LAT through SLP-76. It is possible that p85 associates with LAT by binding to Grb2 or tyrosine phosphorylated Cbl. It is also likely that p85 interacts directly with LAT, as shown by Fukazawa et al. (1995), through a motif other than the one defined by Songyang et al. (1994). Recruitment to the membrane allows all these critical signaling molecules to be concentrated for the tyrosine phosphorylation needed for enzymatic activation or additional complex formation. Recruitment to the membrane in this manner also brings PLC-γ1, p85, and other enzymes in proximity to relevant protein and lipid substrates, resulting in such signaling events as calcium elevation and Ras activation.

Our overexpression experiments demonstrate the importance of LAT in the signaling process. Overexpression of WT LAT had no effect on the functions we measured, suggesting that LAT is not limiting in these processes. This result contrasts with the stimulatory effects of Vav and SLP-76 overexpression (Motto et al., 1996; Wu et al., 1996). However, expression of the double tyrosine to phenylalanine mutations (Y171/191F) was sufficient to markedly inhibit critical signaling pathways. Mutant LAT might saturate sites of interaction with the TCR or relevant signaling molecules, preventing access of endogenous LAT. Tyrosine phosphorylation of the mutant was observed, but those sites were evidently not competent to bind critical signaling molecules. The

mutated sites are clearly responsible for binding these signaling molecules. It is not clear how the two mutations in the Grb2 consensus binding sites completely abolished binding of PLC-γ1 and p85 of PI3K as well as Grb2. Perhaps all of these molecules share these same binding sites, or alternatively, these two sites may have a regulatory role in which Grb2 binding causes conformational changes that expose other binding sites. Further mapping of tyrosine phosphorylation sites and analysis of the stoichiometry of phosphorylation should define the function of all LAT tyrosine phosphorylation events.

We do not know how activation of AP-1 or NFAT is blocked by overexpression of mutant LAT. Inhibition of Grb2 and SOS binding would be expected to affect pathways downstream of Ras. Our preliminary results, obtained through coexpression of WT or mutant LAT with the Erk protein kinase in Jurkat, showed that the mutant did not block Erk activation. Erk activation may not solely be dependent on LAT. It is possible that LAT plays an important role in regulation of AP-1 activation by recruiting SLP-76 and/or Vav. Vav has been shown to be involved in Rac activation, which also affects AP-1 activation (Genot et al., 1996; Crespo et al., 1997). Extensive studies are underway to understand the interaction of LAT with multiple biochemical pathways in T cell activation through the TCR.

Generation of multiprotein complexes is now a central theme in the study of signal transduction (Pawson, 1995). Multiple linker molecules whose functions are to facilitate these interactions have been identified. LAT appears to be the critical linker in TCR-mediated activation, as evidenced by the identity of the signaling molecules it binds and the inhibition of function revealed by overexpression of a dominant negative LAT mutant.

## Experimental Procedures

### Antibodies, Immunoprecipitation, and Western Blotting

Antibodies used in the experiments were rabbit polyclonal anti-Grb2, anti-Grb2 cross-linked to agarose, anti-Cbl (C-15), and anti-Sam68 from Santa Cruz Biotechnology. Monoclonal anti-PLC-γ1 and anti-Vav, rabbit anti-p85 polyclonal were from Upstate Biotechnology. Others were anti-FLAG M2 (Kodak), and anti-phosphotyrosine antibody (PY20, Transduction Laboratory). PY20 was chemically cross-linked to protein A–sepharose using dimethyl pimelimidate (DMP). GST fusion proteins were cross-linked to glutathione-sepharose with 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDC, Pierce). Anti-LAT antibodies were generated against a GST fusion protein containing the cytosolic portion of LAT (aa 31–233).

Jurkat T cells were removed from culture, washed, and resuspended at $10^8$ cells/ml in RPMI. Cells were either stimulated with OKT3 ascites (1:100) or C305 (1:50) for 2 min or left untreated and lysed in 1% Brij lysis buffer (Wange et al., 1995). For treatment with calf intestine alkaline phosphatase (CIP), immunoprecipitates from $10^7$ cells were incubated with 10 units of CIP (Boehringer) in 40 μl CIP reaction buffer for 1 hr.

### Purification of LAT

Jurkat E6.1 cells ($10^{11}$) were stimulated for 1.5 min at $10^8$/ml in RPMI-1640 with OKT3 ascites (1:100) at 37°C. Immediately after stimulation, cells were washed with cold RPMI quickly and membrane fractions were prepared by Dounce homogenization and centrifugation. Membrane fractions were then extracted with 1% Brij lysis buffer, and insoluble material was removed by centrifugation. Membrane protein extracts were heated for 10 min at 75°C, followed by centrifugation to remove insoluble material. This supernatant was loaded onto a PY20 column, which was washed sequentially with lysis buffer containing 1% Brij97, 1% CHAPS, and 0.1% CHAPS. Phosphotyrosine-containing proteins were eluted with 0.1% CHAPS buffer containing 10 mM phenyl phosphate. Eluted proteins were concentrated and subjected to SDS–PAGE. The band for p36–38 was excised and subjected to in gel S-carboxyamidomethylation followed by tryptic digestion. The resulting peptide mixture was separated by microbore HPLC using a Zorbax C18 1.0 mm by 150 mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions were chosen based on differential UV absorbance at 205, 277, and 292 nm peak symmetry and resolution; fractions were then further screened for length and homogeneity by matrix-assisted laser desorption time-of-flight spectrometry (MALDI-MS) on a Finnigan Lasermat 2000 (Hemel, England). Tryptic peptides were submitted to automated Edman degradation on an Applied Biosystems Procise 494 or 477A protein sequencer (Foster City, CA).

### cDNA Cloning and Northern Blotting

A YT λZAP cDNA library ($10^6$ plaques) was screened with a random primed probe made from the EcoRI/PstI fragment of an EST clone (GenBank #w74254). Thirty overlapping phage clones were isolated. The cDNAs were excised from λ phages with the rapid excision kit from Stratagene. For Northern analysis, blots with poly(A)+ RNA from different human tissues (Clontech) were probed with a radiolabeled fragment of *LAT* cDNA or human β-*actin* cDNA under high-stringency conditions according to the manufacturer's instruction.

### Construction of LAT Expression Vector and Mutagenesis

The myc tag was fused to the C terminus of LAT by replacing the PstI/HindIII fragment of *LAT* in pBluescript (SK-) with a double-stranded oligonucleotide fragment encoding the myc tag sequence (SMEQKLISEEDLN). FLAG-tagged LAT was constructed by replacing the PstI/ClaI fragment of *LAT* in pBluescript with a double-stranded oligonucleotide fragment encoding the FLAG sequence (DYKDDDDK). The tagged *LAT* cDNAs were cloned into the pcDNA3 or pEF/BOS vector. Mutagenesis of Y171 to F and Y191 to F was done by PCR.

### Stable and Transient Transfections

Transfection of 293T cells was by the calcium phosphate method. Cells were harvested 36 hr after transfection. For transient transfection of Jurkat/Tag cells, $2 \times 10^7$ cells in 0.4 ml RPMI-1640, 25 mM HEPES, 2 mM Glutamine were incubated with pSX-NFAT/SEAP or pSX-AP-1/SEAP with 20 μg of pEF/LAT(wt), pEF/LAT(Y171/191F), or pEF/BOS vector DNA as control. Twenty-four hours after transfection, transfected cells were stimulated with OKT3 ascites (1:1000) or PMA (10 ng/ml) plus ionomycin (1.5 μM). SEAP assay was done as described (Spencer et al., 1993). For stable transfection of Jurkat E6.1, $10^7$ cells in 0.4 ml RPMI were electroporated using the same conditions as above. Stable transfectants were selected in the presence of 1.5 mg/ml G418.

### Immunofluorescence Staining and Confocal Microscopy

HeLa cells transfected with the *LAT* cDNA were grown on sterile glass coverslips overnight prior to antibody staining. Jurkat T cells were stained in suspension, and the cells were mounted onto coverslips immediately prior to analysis. Immunofluorescence staining was performed as described (Sloan-Lancaster et al., 1997) with anti-LAT at 1:500 dilution for T cells or 1:1000 dilution for HeLa cells, or monoclonal anti-Lck at 1:500 dilution.

### Acknowledgments

We thank W. S. Lane and his colleagues at the Harvard Microchemistry facility for their outstanding service; W. Leonard for many valuable suggestions and for providing the YT λZAP phage library; D. Wassarman with assistance during automated DNA sequencing; G. Crabtree, S. Gutkind, G. Koretzky, P. Love, S. G. Rhee, S. Shoelson, A. Weiss, and R. Williams for reagents; and J. Bonifacino, R. Klausner, J. van Leeuwen, and R. Wange for review of the manuscript. W. Z. is a Fellow of the Leukemia Society of America. J. S. L.

is a Fellow of the Damon Runyon-Walter Winchell Cancer Research Fund.

Received September 30, 1997; revised November 26, 1997.

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**GenBank Accession Number**

The GenBank accession numbers for the LAT sequences reported here are AF036905, AF036906, and AF036907.