Mapping the Zap-70 phosphorylation sites on LAT (linker for activation of T cells) required for recruitment and activation of signalling proteins in T cells

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T-cell-receptor (TCR)-mediated LAT (linker for activation of T cells) phosphorylation is critical for the membrane recruitment of signalling complexes required for T-cell activation. Although tyrosine phosphorylation of LAT is required for recruitment and activation of signalling proteins, the molecular mechanism associated with this event is unclear. In the present study we reconstituted the LAT signalling pathway by demonstrating that a direct tyrosine phosphorylation of LAT with activated protein-tyrosine kinase Zap70 is necessary and sufficient for the association and activation of signalling proteins. Zap-70 efficiently phosphorylates LAT on tyrosine residues at positions 226, 191, 171, 132 and 127. By substituting these tyrosine residues in LAT with phenylalanine and by utilizing phosphorylated peptides derived from these sites, we mapped the tyrosine residues in LAT required for the direct interaction and activation of Vav, p85/p110α and phospholipase Cγ1 (PLCγ1). Our results indicate that Tyr^{226} and Tyr^{191} are required for Vav binding, whereas Tyr^{171} and Tyr^{132} are necessary for association and activation of phosphoinositide 3-kinase activity and PLCγ1 respectively. Furthermore, by expression of LAT mutants in LAT-deficient T cells, we demonstrate that Tyr^{191} and Tyr^{171} are required for T-cell activation and Tyr^{132} is required for the activation of PLCγ1 and Ras signalling pathways.

Key words: phospholipase Cγ1, tyrosine phosphorylation, Vav and Ras signalling pathways.

INTRODUCTION

Stimulation of the T-cell receptor (TCR) triggers signalling cascades required for T-cell differentiation and proliferation. Signal transduction from the TCR is initiated by sequential activation of protein tyrosine kinases (PTKs) Lck/Fyn and Zap-70/Syk [1–3]. Upon TCR engagement, the CD8/CD4 co-receptors deliver Lck to the ζ-chain–CD3 complex to phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylated ITAMs bind the SH2 (Src homology 2) domains of the PTK Zap-70, bringing it in close proximity to Lck, which then phosphorylates and activates Zap-70 [4]. Activation of these kinases is coupled to downstream signalling cascades, including the mitogen-activated protein kinase (MAP kinase), phospholipase C-γ1 (PLCγ1) and Ras (a small GTPase)-related GTPase pathways, which regulate the transcriptional activation of critical immunoregulatory genes (for reviews, see [5,6]).

The molecular mechanism linking the PTKs to these signalling pathways has been under much scrutiny. The recently identified protein LAT (linker for activation of T cells) was shown to play a key role in linking Lck/Zap-70 to downstream signalling pathways [7,8]. LAT is a 36 kDa protein that was initially detected as a major PTK substrate during TCR activation [9]. The LAT protein contains a short extracellular domain, a transmembrane sequence, and an extended cytoplasmic domain with nine tyrosine residues conserved in the mouse and human sequences. Some of these tyrosine residues have been shown to be docking sites for mediators of TCR signalling. Mutation of two putative Grb2 (growth-factor receptor bound-2, an adapter protein) binding sites at Tyr^{171} and Tyr^{191} blocked the association of Grb2 and other signalling molecules. Furthermore, overexpression of the [Y171F/Y191F]LATdouble Tyr → Phe mutant in Jurkat cells prevented transcriptional activation of transcription factor AP-1 (activator protein-1) and nuclear factor in activated T cells (NFAT), exerting perhaps a dominant-negative influence on TCR signalling [7]. These findings suggest that Tyr^{171} and Tyr^{191} are involved in more than merely creating SH2-binding sites and may affect the native conformation, localization, stability and/or sequential phosphorylation of LAT during TCR stimulation. It was recently shown that Tyr^{226}, Tyr^{191}, Tyr^{171} are responsible for Gad binding while Tyr^{132} was required for binding and activation of PLCγ1 [10]. To date, the exact molecular interactions that govern the specific recruitment of signalling proteins and subsequent activation of downstream signalling pathways remain unresolved.

In LAT-deficient J.Cam2 [11] and ANJ3 cells [12], PLCγ1, Vav (a signalling protein with guanine-nucleotide-exchange activity) and Slp76 (an adapter protein) phosphorylation are not detected and TCR-mediated signalling events are severely

Abbreviations used: AP-1, activator protein-1 (a transcription factor); TCR, T-cell receptor; PTK, protein tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; MAP kinase, mitogen-activated protein kinase; PLCγ1, phospholipase Cγ1; LAT, linker for activation of T cells; NFAT, nuclear factor in activated T cells; SH2, Src homology 2; GEM, glycolipid-enriched domains; GST, glutathione S-transferase; PTyr or ^{P}Y, phosphotyrosine; PI 3-kinase, phosphoinositol 3-kinase; PDGF, platelet-derived growth factor; ERK, extracellular-signal-regulated kinase; [Y132F]LAT (etc.), LAT mutant in which Tyr^{132} has been mutated to Phe (etc.); FLAG, the epitope DYKDDDDK; *Sf*9 cells, *Spodoptera frugiperda* (fall armyworm) cells; PIP_2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; NP40, Nonidet P40; GEF, guanine nucleotide exchange factor; WT, wild-type; Vav, a signalling protein with guanine-nucleotide-exchange activity; p85/p110α, the α-isoform of PI 3-kinase; Grb2, growth-factor receptor bound-2, an adapter protein; Ras, a small GTPase; Cbl-b and Slp76, adapter proteins; Sos, a guanine nucleotide exchange factor; p42 and p44, ERK kinases; GAP, GTPase activating protein for Ras; Lck/Fyn and Zap-70/Syk, protein-tyrosine kinases; OKT3, an anti-CD3 monoclonal antibody.

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impaired. LAT was shown to be palmitoylated, and palmitoylation was required for LAT tyrosine phosphorylation and consequently for the recruitment of signalling proteins to glycolipid-enriched domains (GEM) [13,14]. These findings are consistent with the model that activation of TCR causes rapid activation of PTKs to phosphorylate LAT, followed by the recruitment of signalling proteins required for T cell activation.

In LAT-negative mice, a severe defect in TCR signalling is manifested by a block in early T-cell development similar to that observed in mice deleted in either Lck and Fyn, or Zap-70 and Syk [15]. This developmental phenotype indicates that LAT is an obligatory step downstream of both PTKs in the TCR signalling pathway and provides a crucial link to the Ras/MAP kinase and PLCγ1 pathways.

Although LAT phosphorylation was detected in cells co-expressing LAT and Lck-activated Zap-70 [7], it is not clear whether Zap-70 can directly phosphorylate LAT. Here we show that five of the ten tyrosine residues in LAT are directly phosphorylated by Zap-70 and Syk, but not by Lck, to create docking sites for signalling complexes. To further investigate the role of tyrosine phosphorylation in LAT signalling, we developed an experimental system that allows us to define the specific LAT phosphotyrosine residues responsible for direct recruitment of T-cell-signalling mediators. By utilizing LAT tyrosine mutants and phosphorylated peptides derived from LAT we demonstrate that distinct LAT tyrosine residues are critical for direct activation and association with Vav, p85/p110α (the α-isoform of PI 3-kinase) and PLCγ1.

EXPERIMENTAL

Cells and reagents

ANJ3 cells kindly provided by Dr R. T. Abraham (Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, U.S.A.) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum and 4 mM L-glutamine at densities $< 5.0 \times 10^5$ cells/ml. ANJ3 clones stably transfected with pCDNAneo vector (Invitrogen), FLAG-tagged wild-type LAT ([WT]LAT), [Y132F]LAT or [Y171F/L191F]LAT were maintained in RPMI media with 0.5 mg/ml G418 (Life Technologies) (FLAG is the epitope DYKDDDDK). OKT3 (an anti-CD3 monoclonal) antibody was provided by Dr A. Weiss (Department of Medicine, University of California at San Francisco, San Francisco, CA, U.S.A.) and was used for TCR stimulation. Monoclonal antibody to phosphotyrosine (4G10) and PLCγ1, and rabbit antibodies to Grb2, Zap70, p85 and LAT, were obtained from Upstate Biotechnology Inc. Antibody to Slp76 was a gift from Dr G. A. Koretzky (Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IN, U.S.A.) and anti-Cbl-b antibody was provided by Dr Olivier Rosnet (Laboratoire d'Oncologie Moleculaire, INSERM U119, Marseille, France) (Cbl-b is an adapter protein). The pan-Ras antibody was purchased from Calbiochem, anti-phospho-ERK from New England Biolabs (ERK is extracellular-signal-regulated kinase), anti-Sos from Santa Cruz Biotechnology and horseradish peroxidase-conjugated secondary antibodies for immunoblots were from Bio-Rad (Sos is a guanine nucleotide exchange factor). Anti-FLAG (M2) agarose beads used for LAT immunoprecipitations came from Sigma. The following synthetic phosphopeptides (SynPep Corp., Dublin, CA, U.S.A.) were used in the *in vitro* binding and kinase assays: PTyr^{226}, EGAPD^{p}YENLQE, PTyr^{191}, DGSRE^{p}YVNVSQ, PTyr^{171}, ESIDD^{p}YVNVPE, PTyr^{127}, EDEDD^{p}YPHNPG, PTyr^{132}, HNPG^{p}YLLVLP (PTyr and ^{p}Y are phosphotyrosine).

Plasmids and fusion proteins for biochemical assays

Glu-tagged Zap-70, glutathione S-transferase (GST)–Lck and GST–CD3ζ–ITAM cDNAs were cloned into pFAST-Bac and expressed in *Spodoptera frugiperda* (fall armyworm) (*Sf*9) cells. Selected clones were analysed for protein expression with Glu-Glu or anti-GST antibodies. Infected cell pastes were lysed, and recombinant proteins were purified by affinity chromatography as previously described [32]. Protein concentrations were determined using Bio-Rad Bradford reagent. The pGEX-[WT]LAT and pGEX-[Y171F/Y191F]LATconstructs expressing GST–LAT fusion proteins and pCDNA-[WT]LAT and pCDNA-[Y171F/Y191F] LATconstructs expressing FLAG-tagged LAT were kindly provided by Dr R. Wange and Dr L. Samelson (Laboratory of Cellular and Molecular Biology, Division of Basic Science, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.). The Y132F mutation was generated with the Quick-Change Mutagenesis Kit from Stratagene using the primers CACAACCCAGGCTTCCTGGTGGTGCTTC and GGAAGCACCACCAGGAAGCCTGGGTTG. A similar strategy was used to generate the remaining LAT mutants and mutations were confirmed by sequencing. GST–LAT proteins were purified from DH5α cells transformed with the pGEX constructs and induced for 4 h with 100 μM isopropyl β-D-thiogalactoside ('IPTG'). The bacteria were lysed in ice cold PBS with 0.1% Triton X-100 and protease-inhibitor cocktail (Boehringer-Mannheim) by sonication. GST–LAT proteins were precipitated with GSH–agarose beads. The PLCγ1 used in the overlay assay was purified from a GST–PLCγ1 construct generously given by Dr G. Carpenter (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN, U.S.A.). The recombinant PLCγ1 protein composed of the N-SH2 and C-SH2 domains was cleaved from the GST moiety using thrombin digestion. The NFAT and AP-1–luciferase reporter plasmids used in transcription activation assays were generously provided by Dr G. Crabtree (Developmental Biology and Pathology, Stanford University Medical School, Stanford, CA, U.S.A.).

Mapping the Zap-70 phosphorylation sites on LAT

LAT was phosphorylated by Zap-70 as mentioned below. Phosphorylated LAT was separated by SDS/PAGE, and the radioactive band excised and digested with trypsin or endoprotease Glu-C in 50 mM ammonium bicarbonate (more details are available from A. A. on request). Released peptides were separated by reverse-phase HPLC on a capillary C_{18} column using an ABI microblotter (Applied Biosystems). Radioactive peptides were coupled by their carboxylate groups to a Sequelon arylamine PVDF membrane. The discs were placed in the reaction chamber of a Procise 492 gas-phase sequencer (Applied Biosystems), using 100% trifluoroacetic acid to extract the released anilinothiazolinone amino acid and any released P_i, the mixture from each cycle collected in a fraction collector, dried to 5 μl in a Speedvac and each fraction spotted on to 3MM paper. The radioactivity associated with each fraction was quantified by PhosphorImager (Molecular Dynamics) analysis.

Gel overlay assay with recombinant PLCγ1 and Vav

PVDF membranes of resolved phosphorylated GST–LAT proteins were blocked overnight at 4 °C with 5% non-fat dry milk/PBS/0.05% Tween 20. The membrane was washed with PBS/Tween solution and incubated with 2 μg/ml purified PLCγ1 or Vav in binding buffer [25 mM sodium phosphate (pH 7.2)/

150 mM NaCl/0.1 % Tween 20/2.5 mM EDTA/20 mM NaF/1 % non-fat milk/1 mM dithiothreitol/10 μg/ml leupeptin and 10 μg/ml aprotinin] for 2 h at room temperature. Bound PLCγ1 was detected by an anti-PLCγ1 or anti-Vav Western blot.

In vitro kinase assays

Enzymically active recombinant Zap-70 was purified to homogeneity from *Sf*9 cells [32] co-infected with Lck and GST–ζ-ITAM baculovirus. Active Lck and Syk kinases were purified individually to homogeniety. Lck, Zap-70 or Syk (80 ng) were incubated for 30 min at 30 °C with 2 μg of GST–LAT or gastrin peptide in 40 μl of kinase buffer {50 mM Tris (pH 7.5)/50 mM NaCl/5 mM $MgCl_2$, containing 10 μM ATP and 5 μCi of [^{32}P]ATP}. Kinase reactions were terminated by the addition of sample buffer and boiling for 5 min. Samples were resolved by SDS/PAGE, transferred on nitrocellulose membranes and exposed to film. To quantify phosphorylation levels, bands corresponding to LAT were excised from the membranes and the amount of ^{32}P incorporated into LAT was measured by liquid-scintillation counting.

The p85/p110α complex was purified from *Sf*9 cells using a Glu antibody. The lipids phosphatidylethanolamine (PE) and phosphatidylinositol 4,5-bisphosphate (PIP_2; Avanti Polar Lipids) were dried under a stream of Ar, resuspended in buffer A [30 mM Hepes (pH 7.3)/1 mM EGTA/100 mM NaCl and 0.1 % sodium cholate] followed by 30 min of sonication in a water bath. P85/p110α complex (0.5 μg) was pre-incubated in 50 μl of buffer A (lacking sodium cholate) with the desired peptide concentrations. The reaction was initiated by adding 300 μM PE, 50 μM PIP_2, 2.5 mM $MgCl_2$, 20 μM ATP and 0.2 μCi of [^{33}P]ATP. The reaction mixture was incubated for 30 min at room temperature, terminated by precipitatation in 75 mM H_3PO_4 and the ^{33}P radioactivity incorporated into PIP_2 counted. The following peptide sequence derived from the platelet-derived-growth-factor (PDGF) receptor was used as a control: CGG^{p}YMDMSKDESVD^{p}YVPMLDM [16].

Cell stimulation, preparation of cell lysates, immunoprecipitations and *in vitro* binding

ANJ3 cells were harvested, resuspended in PBS at a density of 1.0×10^8 cells/ml and pre-warmed for 15 min at 37 °C before stimulation with OKT3 antibody for 2 min. TCR stimulation was terminated by adding an equal volume of 2 × detergent solution [2 % Brij97 (Sigma), 4 μM vanadate and 2 × protease inhibitor cocktail (Boehringer-Mannheim)]. Cells were lysed on ice for 15 min and cleared of debris by centrifugation at 5000 ***g*** for 30 min at 4 °C. LAT was immunoprecipitated from lysates with M2 beads (anti-FLAG) by incubating from 1 h at 4 °C. PLCγ1 was directly precipitated from the lysates using anti-PLCγ1 monoclonal antibody bound to Protein G–Sepharose beads. Precipitates were washed three times with 1 × lysis buffer, resuspended in SDS sample buffer, boiled for 5 min prior to separation by SDS/PAGE and analysed by Western blotting. Lysates were also analysed directly for phosphoproteins and phospho-ERK levels by Western blotting. For GST–LAT binding experiments, lysates from unstimulated Jurkat cells (5.0×10^7 cells/ml) were prepared using 0.5 % Nonidet P40 (NP40) detergent in PBS with vanadate and protease-inhibitor cocktail. Binding was performed at 4 °C for 30–60 min and GSH–agarose beads were washed three times with NP40 lysis buffer prior to analysis by Western blotting.

For peptide competition experiments, phosphorylated GST–LAT (2 μg) was preincubated with the indicated concentrations of phospho-specifc peptides followed by the addition of either purified recombinant Vav or p85/p110α proteins or cell lysate prepared from *Sf*9 cells expressing either Vav or p85/p110α proteins (2 μg/ml). The mixture was incubated for 45 min and LAT was precipitated with GSH–agarose beads. The beads were washed three times with lysis buffer, and Vav and p85/p110α were detected by Western blotting.

Intracellular Ca^{2+} measurement

Cells were loaded with the fluorescent calcium indicator dye Indo-AM (Molecular Probes) in Hanks Balanced Salt solution, pH 7.4, with 5 mM dextrose, washed, then stimulated with purified OKT3. The TCR-induced increase of intracellular Ca^{2+} was monitored by fluorescence (emission at 390 nm wavelength, excitation at 335 nm) using Perkin–Elmer Model L550B luminescence spectrometer.

Transfections and luciferase reporter assays

ANJ3 LAT cells (1×10^7) in serum-free media were transfected with 20 μg of NFAT–luciferase or AP-1 reporter plasmids by electroporation with a Bio-Rad Gene Pulser electroporator at 250 V and 960 μF settings. At 24–30 h after transfection, cells were equally divided into three portions and either unstimulated, or stimulated with OKT3 or 25 ng/ml PMA/1 μM ionomycin combination. Approx. 4–6 h later, cells were collected, lysed, and analysed for luciferase activity using Promega Luciferase Assay System with a Dynatech Laboratories ML2250 luminometer.

Ras activation

To measure levels of GTP-loaded Ras, 3.0×10^6 cells were lysed for 20 min in Ras lysis buffer [20 mM Hepes/150 mM NaCl/1 mM EDTA/25 mM NaF/10 mM $MgCl_2$ (pH 7.5), containing 1 % NP40, 0.25 % deoxycholate, 10 % (v/v) glycerol, 1 mM vanadate, and protease-inhibitor cocktail] after mock, OKT3 stimulation for 2 min or PMA (25 ng/ml) stimulation for 15 min. Lysates were cleared by centrifugation at 14000 rev./min for 30 min. Cleared extracts were mixed with 20 μl of GSH–agarose beads with bound GST–Raf fusion protein containing the Ras-binding domain. The samples were incubated for 30 min at 4 °C and precipitates were washed once with lysis buffer. Captured GTP–Ras protein was assayed by Western blotting with pan-Ras antibody.

RESULTS

Zap70/Syk PTK, but not Lck, phosphorylates LAT tyrosine *in vitro*

To understand better the functional relationship between Zap-70 and LAT, we analysed the ability of Zap-70 to phosphorylate LAT *in vitro*. Activated Zap-70, purified from insect cells co-infected with Lck- and GST–ζ-ITAM-expressing baculovirus, phosphorylated LAT in a dose-dependent manner (Figure 1B). In contrast, equal amounts of Lck failed to phosphorylate LAT, but did phosphorylate the gastrin peptide substrate effectively. Phosphorylation of gastrin by Zap-70 was considerably lower ($\approx$ 5 fold less) compared with Lck and Syk kinases, perhaps reflecting Zap70's high degree of substrate specificity (Figure 1A). These data are consistent with the results of an earlier study using a COS-cell expression system [7], and indicate that LAT is a substrate for Zap-70 and Syk kinases.

LAT phosphorylation by Zap70 creates SH2 binding sites for T-cell signalling molecules *in vitro*

LAT phosphotyrosine residues mediate the binding of signalling molecules such as PLCγ1 and Grb2 through interactions with

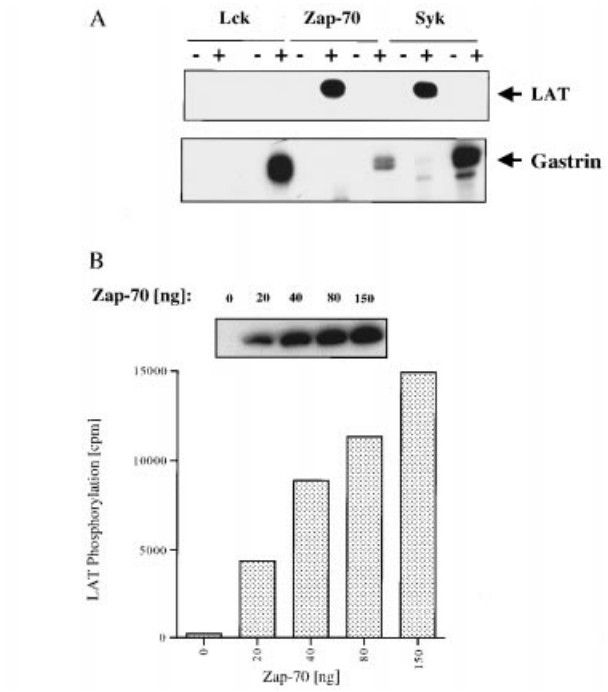

Figure 1 Direct phosphorylation of LAT by Zap-70 and Syk kinases

(**A**) Recombinant Lck, Zap-70 or Syk (80 ng) were incubated for 30 min at 30 °C with 2 μg of GST–LAT or gastrin peptide in kinase buffer containing either 5 μCi of [^{32}P]ATP (+) or no ATP (−). To activate Zap-70 kinase, we purified Zap-70 from *Sf*9 cells co-infected with Lck and GST–ζ-ITAM. (**B**) Increasing amounts of Zap-70 were incubated with GST–LAT and analysed in an *in vitro* kinase assay. Samples were resolved by SDS/PAGE and the LAT bands were excised from the gel and the amount of ^{32}P incorporated into LAT was measured by liquid-scintillation counting.

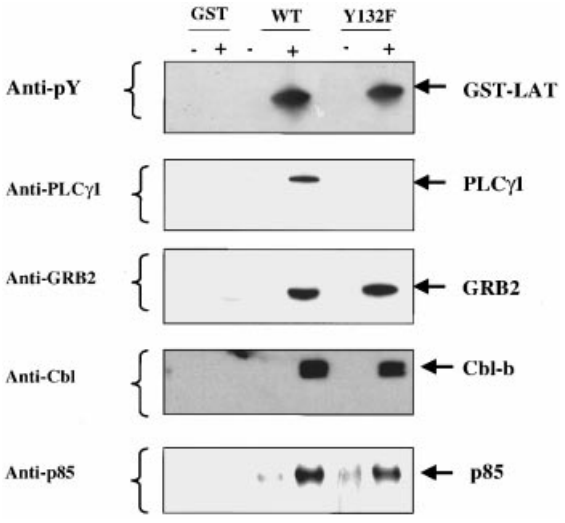

Figure 2 Association of T-cell signalling molecules *in vitro* with phosphorylated GST–LAT

Purified GST–LAT proteins immobilized on GSH–agarose beads were incubated for 30 min at 30 °C with activated Zap-70 in the presence (+) or the absence of (−) ATP. The GST–LAT beads were washed to remove Zap-70 and were incubated with 0.5% NP40 lysate of resting Jurkat cells. Precipitated samples were resolved by SDS/PAGE and immunoblotted with the indicated antibodies. {Compare the binding to LAT of signalling molecules between phosphorylated [WT]LAT and a mutant of LAT ([Y132F]LAT) mutated in the putative PLCγ1 site.}

SH2 domains. The anti-phosphotyrosine (anti-PTyr) Western blot in Figure 2 shows that Zap-70 phosphorylation of LAT occurred on tyrosine residues. To determine whether phosphorylation of LAT by Zap-70 is necessary and sufficient to recruit signalling proteins in T cells, we incubated GST–LAT previously phosphorylated by Zap-70 with lysate prepared from resting Jurkat cells. In this lysate-binding assay, recombinant GST-LAT immobilized on GSH–agarose beads were analysed for association of signalling proteins by immunoblotting. As shown in Figure 2, PLCγ1, Grb2, Cbl-b, and the p85 subunit of PI 3-kinase associated with GST–LAT only when LAT is phosphorylated by Zap-70 ('WT'+'ATP' lanes). To further validate this experimental approach, we also used a [Y132F]LAT mutant in which the putative tyrosine residue required for PLCγ1 binding was replaced with phenylalanine. As shown in Figure 2, PLCγ1 binding to LAT was impaired without affecting the association of other signalling proteins. These results demonstrate that the LAT phosphorylated by Zap-70 *in vitro* mimicked the TCR-induced LAT phosphorylations required for recruitment of these signalling molecules. This system thus provides a simple method for mapping the specific LAT PTyr–SH2 interactions involved in the recruitment of critical T-cell signalling proteins.

Zap-70 phosphorylates five different tyrosine residues on LAT

Although our results clearly show that tyrosine phosphorylation of LAT is required for association with multiple signalling proteins, it is not known which tyrosine residues are directly phosphorylated by Zap-70 and are subsequently responsible for interaction. Therefore we next mapped the phosphorylation residues on LAT. LAT was first phosphorylated by incubating it with active Zap-70 and radioactive [^{32}P]ATP, followed by trypsin digestion and peptide separation. Three radioactive peptides were identified, and Edman degradation analysis of these peptides showed a release of radioactivity following cycles 12, 2 and 22 (Figure 3). Tyr^{171}, Tyr^{191} and Tyr^{226} are the only tyrosine residues that lie 12, 2 and 22 residues from an arginine or lysine residue, strongly suggesting that these are the phosphorylated residues in these peptides (Figure 3). In support of this, endoprotease Glu-C digestion also generated three peptides, one of which released radioactivity after the 5th cycle of Edman degradation. Both Tyr^{171} and Tyr^{226} lie five residues following a glutamic acid residue, suggesting that this peptide represented at least one of these residues. The other two phosphorylated peptides, following digestion with endoprotease Glu-C, showed release of radioactivity at two positions, 16 and 21, and 9 and 14. These peptides probably represent partial digestions of the highly acidic region preceding Tyr^{127} and Tyr^{132}. These tyrosine residues would be unlikely to be detected following a trypsin digestion, as the peptide generated would be extremely large (60 amino acids), and would probably remain bound to the C_{18} column used in this analysis. We are unsure of the significance of the peak of radioactivity seen in cycle 8 of the peptide containing Tyr^{226}. This was not seen in all experiments, but could represent low-level phosphorylation of Ser^{212}, perhaps by a contaminating serine kinase present in either the LAT or ZAP-70 preparations. We therefore propose that ZAP-70 phosphorylates LAT on at least five tyrosine residues *in vitro*, namely Tyr^{127}, Tyr^{132}, Tyr^{171}, Tyr^{191} and Tyr^{226}.

Direct binding of Vav, p85/p110α with phosphorylated LAT

The human LAT protein contains ten tyrosine residues that could be potentially phosphorylated to create SH2 docking sites. While several signalling proteins were shown to co-immunoprecipitate with LAT, it is unclear whether the interaction is

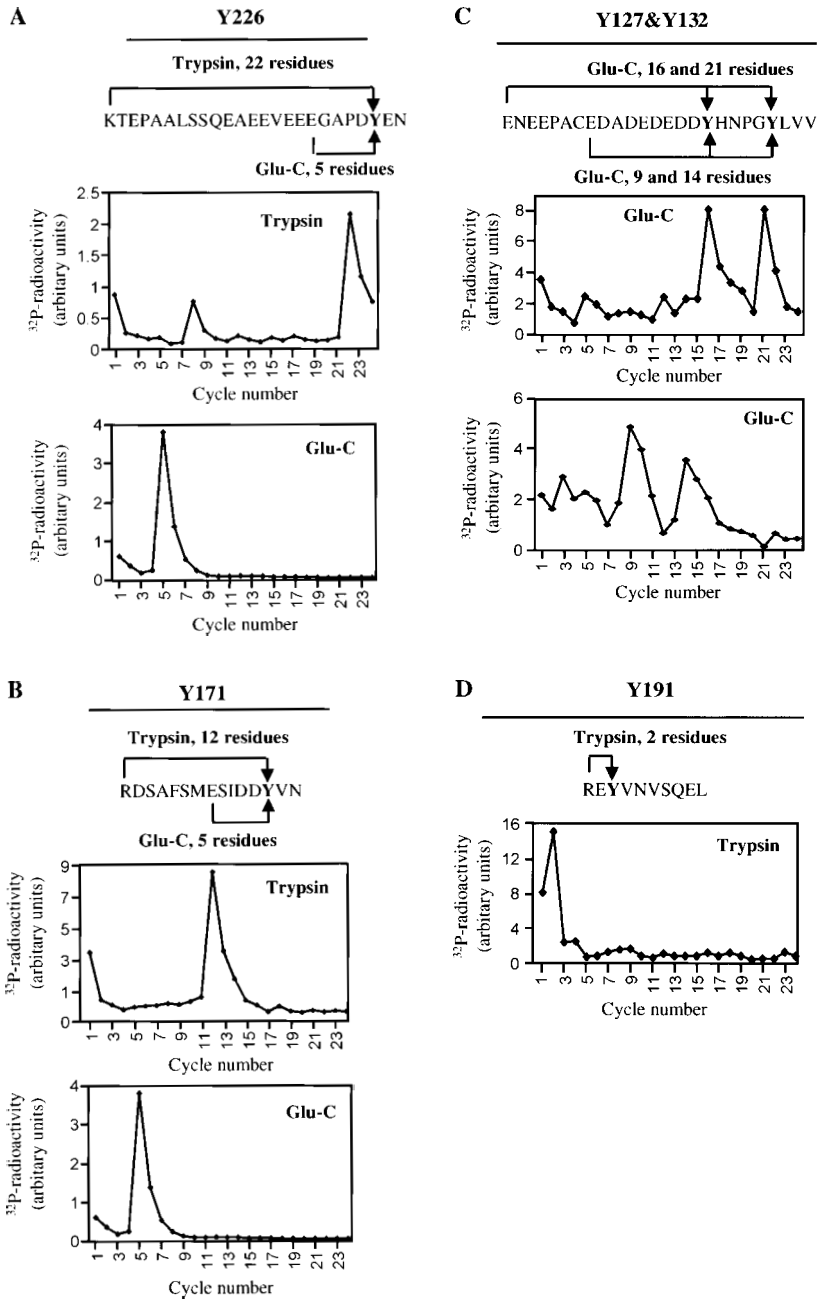

Figure 3 Identification of the sites on LAT phosphorylated by Zap-70 *in vitro*

LAT was phosphorylated by Zap-70 and digested with trypsin or endoprotease Glu-C as described in the Experimental section. Peptides were separated by reverse-phase HPLC, and the radioactive peptides coupled to an arylamine PVDF membrane. Following Edman degradation, fractions were collected, and radioactivity released after each was detected and plotted as shown. Phosphorylated tyrosine ('Y') residues could be unambiguously identified by differential release of phosphate following trypsin or endoprotease Glu-C digestion.

direct or whether it is mediated by an adaptor protein. We decided to examine the interactions of LAT with three important signalling proteins: Vav, p85/p110α and PLCγ1. As shown in Figures 4(A) and 4(B), recombinant Vav associated with phosphorylated [WT]LAT in both overlay and GST pull-down assays. To identify the tyrosine residues that are responsible for binding to Vav, we analysed a series of LAT tyrosine mutants by replacing Tyr^{132}, Tyr^{191}, Tyr^{226} and $Tyr^{171/191}$ with phenylalanine. While equal levels of tyrosine phosphorylation were detected among the various LAT mutants, a significant reduction in Vav binding to [Y191F]LAT, [Y226F]LAT and [Y171F/Y191F]LAT was observed (Figures 4A and 4B). These data suggest that multiple tyrosine residues participate in LAT and Vav interactions, including residues Tyr^{191}, Tyr^{226} and possibly Tyr^{171}. Comparable binding between [WT]LAT and [Y132F]LAT was observed, indicating that Tyr^{132} is not required for interaction with Vav (Figures 4A and 4B). To test whether Vav guanine-nucleotide-exchange-factor (GEF) activity toward the GTPases Rac and Cdc42 could be stimulated by phosphorylated LAT, we incubated phosphorylated LAT with Vav (previously

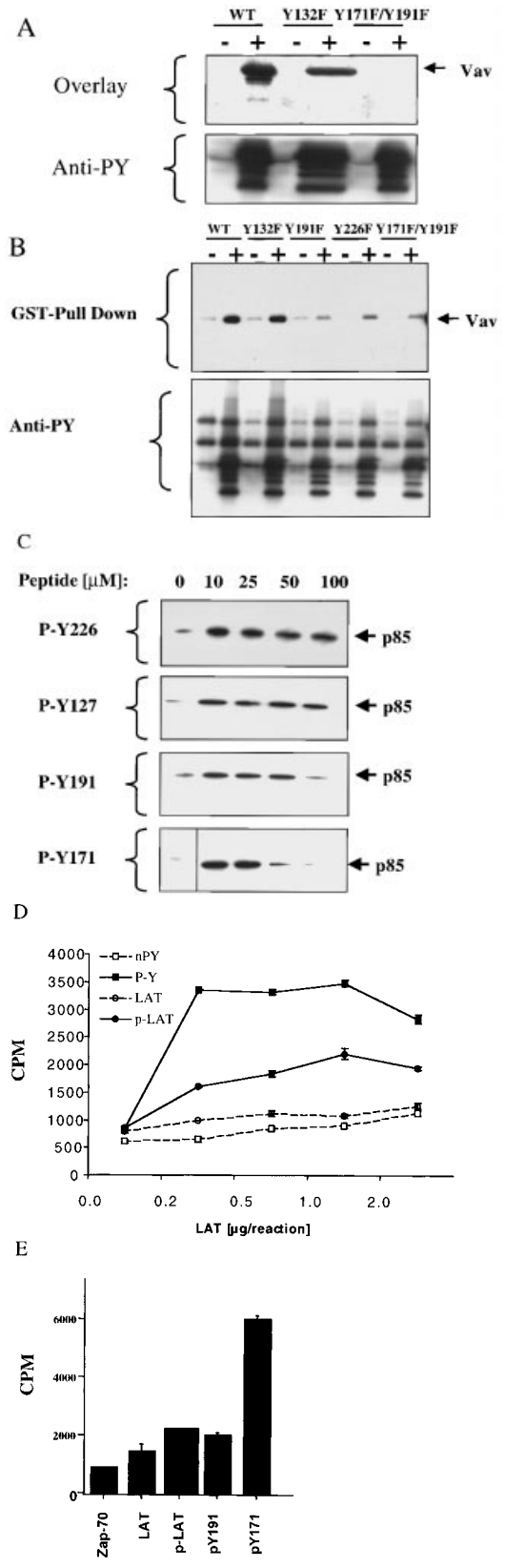

Figure 4 Vav and p85/p110α bind directly to phosphorylated LAT

(**A**) GST–[WT]LAT, GST–[Y132F]LAT and GST–[Y171F/Y191F]LAT phosphorylated by Zap70 were resolved by PAGE and transferred on to PVDF membrane. The membrane was incubated with 2 μg/ml recombinant Vav protein. Vav binding to phospho-LAT on the membrane was detected with anti-Vav antibody. The anti-phosphotyrosine antibody ('Anti-PY') blot shows equivalent LAT phosphorylation. (**B**) GST–LAT fusion proteins with various single Tyr → Phe substitutions were incubated with *Sf*9-cell lysates expressing Vav, after phosphorylation with activated Zap70 kinase with or without ATP (as described in Figure 2) and monitored for Vav binding by Western blotting. (**C**) GST–[WT]LAT was phosphorylated with active Zap-70 and was pre-incubated for 10 min with the indicated synthetic phosphopeptides prior to the addition of purified p85/p110α recombinant protein. The mixture was precipitated by GSH–agarose beads and the association of p85 was detected by Western blotting. A control for no peptide and non-phosphorylated LAT is indicated by '0' μM peptide. (**D**) Increasing amounts of either phosphorylated LAT (p-LAT), non-phosphorylated LAT (LAT), phosphorylated (P-Y) or non-phosphorylated (nPY) synthetic peptides derived from the PDGF receptor were incubated with recombinant p85/p110α complex and the PI 3-kinase activity was measured. (**E**) Recombinant p85/p110α complex was incubated either with Zap-70, LAT, phosphorylated LAT (p-LAT), synthetic peptides (100 μM) derived from LAT $PTyr^{191}$ and $PTyr^{171}$ and the PI 3-kinase activity was measured.

phosphorylated by Lck) in the presence of recombinant Rac or Cdc42 and measured the GEF activity. Although Vav interacts with phosphorylated LAT, further stimulation of GEF activity of either phosphorylated or non-phosphorylated Vav was not detected (results not shown). These results suggests that a direct association of Vav with LAT is not sufficient for the stimulation of Vav GEF activity.

PI 3-kinase was previously shown to co-immunopreciptate with LAT during T-cell activation [7]. To test whether the association of PI 3-kinase with LAT is direct, we incubated phosphorylated LAT with the PI 3-kinase subunits p85/p110α. As shown in Figure 4(C), recombinant p85 directly interacts with phosphorylated LAT. To identify the tyrosine residues on LAT that are responsible for the association with p85, we incubated phosphorylated LAT with increasing amounts of a series of synthetic phosphopeptides derived from LAT. The P85/p110α complex interacts only with phosphorylated LAT (indicated as '0' for no phosphorylation or peptide), and, at 10 μM, none of the tested peptides interfered with p85 binding to LAT. At 50 μM, the phosphopeptide $PTyr^{171}$ competed with p85/p110α for binding to LAT, whereas no competition was observed at 100 μM with phosphopeptides derived from $PTyr^{226}$ or $PTyr^{127}$ (Figure 4C). Phosphopeptide $PTyr^{191}$ was able to compete with p85/p110α binding at 100 μM (Figure 4C). While the data suggest that Tyr^{171} can bind to p85/p110α with the highest affinity, it is also possible that Tyr^{191} may serve as a docking site for p85/p110α.

It was previously demonstrated that the tyrosine kinase PDGF receptor can recruit and activate PI 3-kinase activity [16]. Therefore, we tested whether a direct binding of p85/p110α to LAT can also stimulate PI 3-kinase activity. Phosphorylated LAT was incubated with recombinant p85/p110α and the phosphorylation of PIP_2 was measured. A phosphopeptide derived from the PDGF receptor containing two phosphorylated tyrosine residues stimulates the activation of PI 3-kinase activity in a dose-dependent manner (Figure 4D). Similarly, phosphorylated LAT and phosphopeptide $PTyr^{171}$ also stimulate PI 3-kinase activity (Figures 4D and 4E). These findings suggest that, like the PDGF receptor, binding of PI 3-kinase to phosphorylated Tyr^{171} and possibly Tyr^{191} serves to activate PI 3-kinase activity.

Direct association of PLCγ1 with Tyr^{132} on LAT

The Tyr^{132} sequence, YLVV, is related to the consensus PLCγ1 SH2-binding motif previously defined by Songyang and colleagues [16]. We therefore analysed a series of LAT tyrosine mutants in a lysate binding assay and found that Tyr^{132} is the main docking site for PLCγ1. The Tyr^{64} mutant exhibits a reduction in PLCγ1 association, suggesting this residue may be

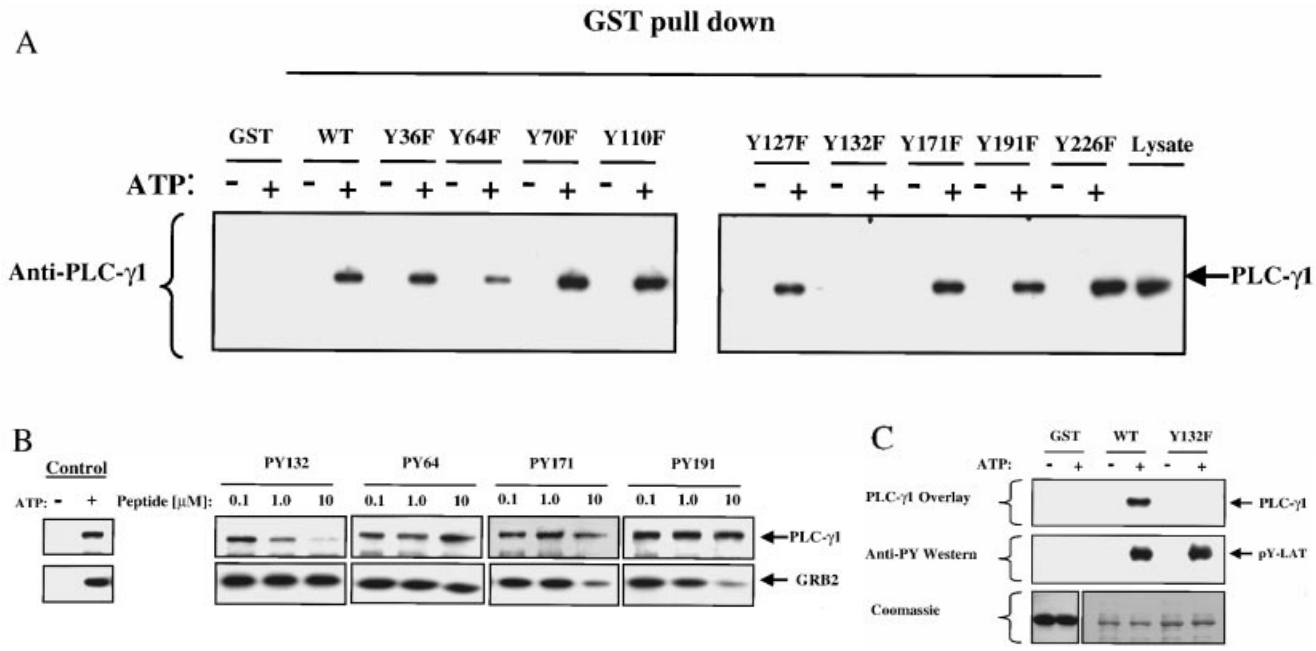

Figure 5 Direct association of PLCγ1 with Tyr^{132} on LAT

(**A**) GST–LAT fusion proteins with various single Tyr → Phe substitutions were incubated with Jurkat-cell lysates, after phosphorylation with activated Zap70 kinase with or without ATP (as described in Figure 2) and monitored for PLCγ1 binding by Western blotting. (**B**) Jurkat-cell lysates were pre-incubated with 0.1, 1.0 or 10.0 μM synthetic phosphopeptides prior to addition to phosphorylated GST–LAT. PLCγ1 and Grb2 recovered from treated lysates were revealed by Western blotting. (**C**) GST alone, GST–[WT]LAT and GST–[Y132F]LAT phosphorylated by Zap70 were resolved by PAGE and transferred on to PVDF membrane. The membrane was incubated with 2 μg/ml recombinant PLCγ1 protein containing both N-terminal and C-terminal SH2 domains. PLCγ1 binding to phospho-[WT]LAT on the membrane was detected with anti-PLCγ1 antibody. The anti-phosphotyrosine antibody ('Anti-PY') blot shows equivalent LAT phosphorylation, and staining with Coomassie Brilliant Blue indicates the presence of GST–LAT fusion protein on the membrane.

important in stabilizing the interaction (Figure 5A, 'Y64F'+'ATP' lane). To determine whether the PTyr^{132} sequence is directly involved in PLCγ1 binding, a synthetic phosphopeptide containing the Y^{132} sequence (PTyr^{132}) was tested for the inhibition of PLCγ1 association with phosphorylated LAT in our lysate binding assay. Phosphopeptides encompassing Tyr^{64}, Tyr^{171} and Tyr^{191} were also tested for comparison. As shown in Figure 5(B), only PTyr^{132} reduced PLCγ1 binding to LAT in a dose-dependent manner. The inhibitory effect of PTyr^{132} is specific for PLCγ1, since Grb2 (Figure 5B, lower panels) and Cbl-b (result not shown) associations with LAT were unaffected. PTyr^{64} peptide did not inhibit PLCγ1 binding, suggesting that PTyr^{64} is not directly involved in PLCγ1–LAT association. PTyr^{171} and PTyr^{191} phosphopeptides also had no effect on PLCγ1 binding, whereas Grb2 association was reduced, in agreement with previous reports that Tyr^{171} and Tyr^{191} are binding sites for the Grb2 SH2 domains [7].

To determine whether the interaction between PLCγ1 and LAT is direct, we tested the ability of a recombinant PLCγ1 to bind phosphorylated LAT. To address this issue, a membrane-overlay assay using purified PLCγ1 as a probe was performed. The truncated form of PLCγ1 used in this assay (PLCγ1N$^+$C$^+$) contains both N-terminal and C-terminal SH2 domains and has been shown to bind directly to phosphotyrosine peptides from PDGF and epidermal-growth-factor receptors [17,18]. As shown in Figure 5(C), the PLCγ1 N$^+$C$^+$ probe bound exclusively to phosphorylated [WT]LAT in the absence of any other proteins. In the same overlay, PLCγ1 N$^+$C$^+$ binding was not detected in GST alone nor phosphorylated [Y132]LAT. In summary, these results suggest LAT Tyr^{132} is directly involved in the recruitment of PLCγ1.

Restoration of LAT function in LAT-deficient ANJ3 cells

To extend our *in vitro* observations, we used the LAT-deficient ANJ3 Jurkat cell line to analyse the function of [WT]LAT, [Y132F]LAT and [Y171F/Y191F]LAT. FLAG-tagged LAT constructs were stably transfected in the ANJ3 cell line [12]. LAT expression was not detected in ANJ3 cells stably transfected with vector control, while equal levels of [WT]LAT, [Y132F]LAT and [Y171F/Y191F]LAT expression were observed (Figure 6A). To examine the ability of LAT to restore TCR-mediated tyrosine phosphorylation in the ANJ3 cell line, we looked at LAT tyrosine phosphorylation induced by OKT3. Phosphorylation of [WT]LAT and [Y132]LAT were similar, while little or no phosphorylation was detected on [Y171F/Y191F]LAT (Figure 6A, bottom panel).

To assess the functional properties of LAT mutants expressed in ANJ3 cells, we immunoprecipitated the FLAG-tagged LAT from resting and OKT3-stimulated cells followed by immunoblot analysis with a panel of antibodies against signalling proteins known to associate with LAT [7]. PLCγ1, Cbl-b, Slp76, and Grb2/SOS were detected in [WT]LAT immunoprecipitated from activated cells (Figure 6B, 'WT'+'OKT3' lane), while association of PLCγ1, Cbl-b, Slp76, and Grb2/SOS were either absent or severely reduced in the double tyrosine mutant [Y171F/Y191F]LAT (Figure 6B, 'Y171/191F'+'OKT3' lane). Consistent with the *in vitro* experiments, PLCγ1 did not co-precipitate

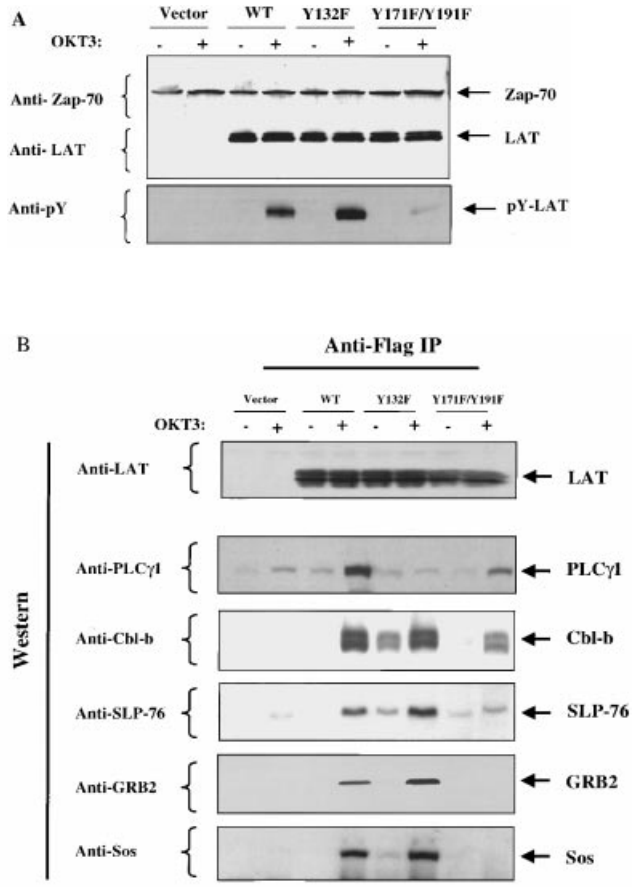

Figure 6 Reconstitution of LAT expression and association of signalling molecules in ANJ3 Jurkat cell lines

(**A**) Equivalent expression of endogenous Zap-70 and FLAG-tagged LAT was detected in the cell lines generated. Expression of FLAG-tagged LAT constructs stably transfected in LAT-deficient ANJ3 cells was confirmed using anti-LAT antiserum. LAT phosphorylation following OKT3 stimulation (2 min) was detected on LAT-Flag precipitated from Brij97 lysates of $\approx 1.0 \times 10^7$ cells using an anti-phosphotyrosine monoclonal antibody (4G10). (**B**) LAT was immunoprecipitated (IP) with FLAG antibody from lysates of resting or OKT3-stimulated cells (1 : 5 for 2 min at 37 °C) at 5×10^7 cells/ml in 1% Brij97 detergent and analysed by Western blotting with the indicated antibodies.

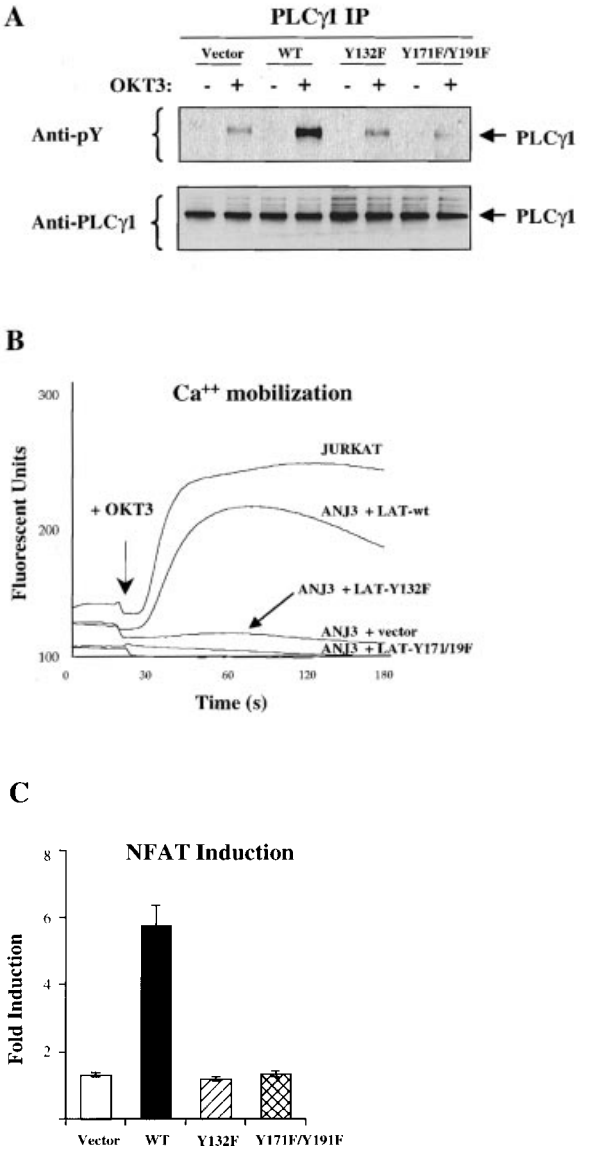

Figure 7 Defect in PLCγ1 activation in [Y132F]LAT-expressing cells results in failure to mobilize Ca^{2+} and induce NFAT activation

(**A**) PLCγ1 was immunoprecipitated from resting and OKT3-stimulated Anj3 cells expressing either vector control, [WT]LAT, [Y132F]LAT or [Y171F/Y191F]LAT. The state of tyrosine phosphorylation on PLCγ1 was measured by immunoblot using anti-phosphotyrosine antibody. (**B**) TCR-induced intracellular Ca^{2+} flux was measured in ANJ3 cells stably expressing the indicated LAT construct. Cells were loaded with the Ca^{2+} indicator Indo1-AM and stimulated with purified OKT3 and the Indo-1 fluorescence emission at 435 nm was measured over 180 s. (**C**) Quantification of TCR-mediated NFAT activity in ANJ3 cells expressing various LAT constructs. ANJ3 cells were transfected with NFAT luciferase reporter and cells were either left untreated or stimulated with OKT3 (1 : 20) or PMA/ionomycin (25 ng/ml, 1.0 μM) at 24–30 h post transfection. The level of luciferase activity was measured in cell lysates as described in the Experimental section.

with the [Y132F]LAT mutant, whereas Cbl-b, Slp76 and Grb2–Sos were detected at levels comparable with those observed with [WT]LAT (Figure 6B, 'Y132F' + 'OKT3' lane). The specific defect in PLCγ1 association clearly demonstrates that LAT recruitment of PLCγ1 during TCR stimulation is dependent on the TCR-induced phosphorylation of Tyr^{132}.

Our *in vitro* data suggest that multiple tyrosine residues are required for Vav and p85/p110α binding to LAT. Although Tyr^{171} and Tyr^{191} are required for docking to LAT, we could not identify a single tyrosine residue that is responsible for binding either Vav or p85/p110α *in vivo* (results not shown). Therefore we further characterized the ANJ3-[Y132F]LAT cells.

ANJ3-[Y132F]LAT cells are defective in the PLCγ1 pathway

PLCγ1 activation triggered by TCR stimulation subsequently leads to generation of diacyglycerol and inositol trisphosphate by-products and the rapid cytoplasmic influx of Ca^{2+} ions [20,21]. PLCγ1 phosphorylation by an unidentified tyrosine kinase has been shown to be an indicator of PLCγ1 activation [22,23]. Thus we also compared the extent of TCR-induced tyrosine phosphorylation of PLCγ1 in ANJ3 vector control, [WT]LAT, [Y132F]LAT and [Y171F/Y191F]LAT cell lines. TCR-mediated PLCγ1 phosphorylation was detected in ANJ3 cells expressing [WT]LAT, but only suboptimal levels were observed in vector only, [Y132F]LAT and [Y171F/Y191F]LAT ANJ3 cells (Figure 7A). Furthermore, TCR-induced Ca^{2+} mobilization (Figure 7B)

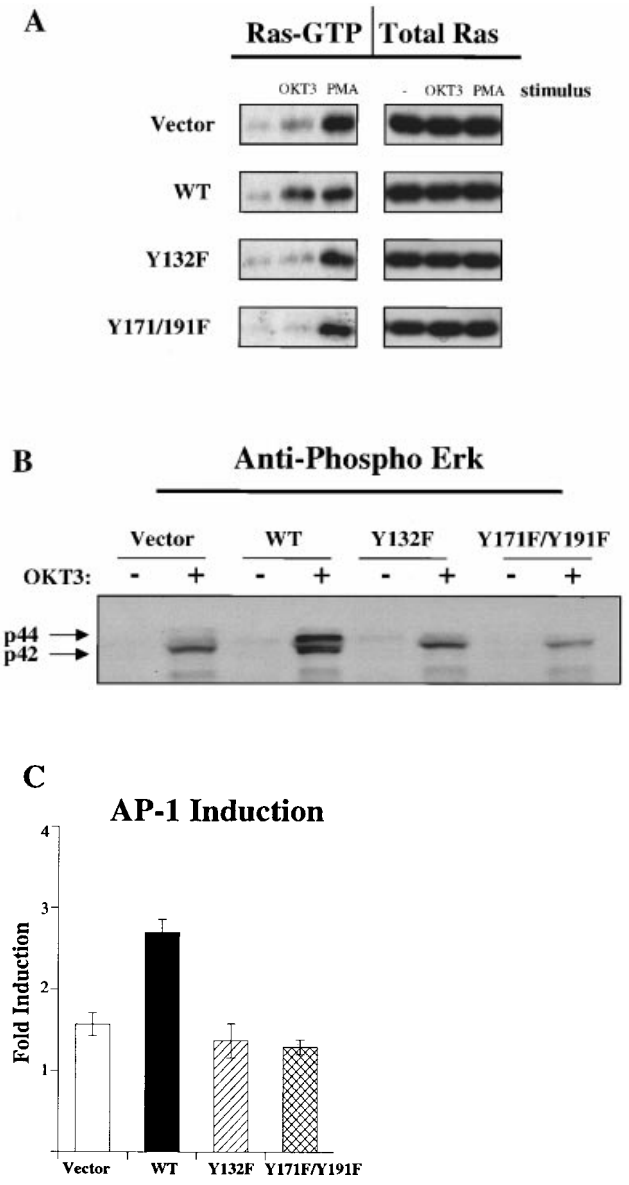

Figure 8 Ras signalling pathway in ANJ3 cells expressing different LAT constructs

(**A**) TCR-mediated Ras activation in ANJ3 cells. GST–Raf–agarose beads containing the Ras binding domain were incubated with lysates prepared from the indicated ANJ3 cells either untreated or treated with PMA (15 min) or OKT3 (3 min). The GST–Raf beads were washed and resolved by SDS/PAGE, followed by immunoblot with pan-Ras antibody. (**B**) The indicated cell lines were stimulated with OKT3 for 2 min and cell lysates were prepared and immunoblotted using phospho-ERK antibody. (**C**) TCR-mediated AP-1 activity in ANJ3 cells expressing various LAT constructs. ANJ3 cells were transfected with AP-1 luciferase reporter and cells were either left untreated or stimulated as described above. The level of luciferase activity was measured in cell lysates as described in the Experimental section.

and transcriptional activation of the Ca^{2+}-dependent NFAT reporter construct (Figure 7C) were observed only in ANJ3 cells expressing [WT]LAT. These experiments prove that phosphorylation of Tyr^{132} is critical for both the recruitment and subsequent activation of the PLCγ1 during TCR stimulation. The inability of [Y171F/Y191F]LAT to associate with PLCγ1 and other signalling proteins suggests this double mutant undergoes conformational changes severely affecting its native structure, stability and/or function. Alternatively, it is possible that phosphorylation of Tyr^{132} and other tyrosine residues that directly bind signalling proteins do not proceed in the absence of phosphorylation on Tyr^{171} and Tyr^{191}, suggesting a sequentially regulated phosphorylation process for LAT.

Recruitment of PLCγ1 to LAT is essential for activation of the Ras pathway

It was previously shown that LAT is required for the activation of the Ras signalling pathway [7]. In stimulated T cells, the Grb2–Sos complex is recruited by LAT to the plasma membrane to initiate GEF activity on Ras. To determine whether normal Ras activation follows TCR stimulation, we looked at Ras/GTP levels in the different LAT-expressing cell lines. Ras/GTP was captured from cell lysates of unstimulated, OKT3-stimulated or PMA-activated cells with a GST–Raf fusion protein containing the Ras-binding domain [24]. Ras–GTP complexes bound on GST-Raf were revealed by Western blotting with a pan-Ras antibody. As shown in Figure 8(A), formation of Ras–GTP complexes during OKT3 stimulation was seen only in [WT]LAT cells. PMA treatment generated equivalent Ras/GTP in all four cell lines, indicating that Ras signalling downstream of LAT was intact (Figure 8A, right panels). The phospho-ERK blot showed both [Y132F]LAT and [Y171/191F]LAT stimulated cells with undetectable ERK kinase p44 and severely reduced ERK kinase p42 levels, further indicating a defect in the Ras-dependent activation of the MAP kinase pathway (Figure 8B). In addition, activation of the transcription factor AP-1, an event that is Ras-dependent, was restored in the [WT]LAT cells and not in the LAT mutant cell lines (Figure 8C). Our results clearly demonstrate that PLCγ1 activation is linked to activation of the Ras signalling pathway.

DISCUSSION

TCR engagement induces tyrosine phosphorylations on LAT essential for the assembly of signalling complexes required for T-cell activation. LAT contains five potential Grb2 SH2 binding sites and a consensus sequence-binding motif for PLCγ1 SH2 domain. It was previously shown that co-expression of activated Zap-70 in 293 and COS cells is sufficient to induce LAT tyrosine phosphorylation [7]. However, the exact molecular events regulating recruitment of specific signalling proteins to LAT are not well understood. Here we show that Zap-70 can directly phosphorylate LAT on five tyrosine residues to create docking sites for a number of signalling proteins. Several tyrosine residues are implicated in a direct interaction with Vav and p85/p110α. However, LAT recruitment of PLCγ1 is mediated exclusively through the Zap-70-dependent phosphorylation on Tyr^{132}. Moreover, LAT recruitment of PLCγ1 is essential for its phosphorylation and activation of both the PLCγ1 and Ras signalling pathways.

We reconstituted the LAT signalling pathway by using enzymically active Zap-70 purified from *Sf*9 cells co-infected with Lck and GST–ITAM. Zap-70 kinase activity was enhanced severalfold when Zap-70 was co-expressed with both Lck and GST–ITAM, and maximum phosphorylation of LAT occurred when Zap-70 and ITAM were phosphorylated by Lck. We demonstrated that the direct phosphorylation of LAT by Zap-70/Syk was sufficient to create docking sites for signalling complexes, indicating that the sequence of events during T-cell activation was reconstituted in our *in vitro* system. Only five of the ten tyrosine residues in LAT were directly phosphorylated by

Zap-70. Nevertheless, it is possible that the remaining five tyrosines in LAT could be phosphorylated by a tyrosine kinase other than Zap-70/Syk kinases [25].

Our *in vitro* binding experiments with Zap70-phosphorylated LAT and Jurkat lysates allowed us to directly test the capacity of LAT to recruit signalling molecules. First we demonstrated that LAT requires Zap-70 phosphorylation for binding a number of signalling proteins containing SH2 domains. Secondly, by using lysate from unstimulated Jurkat cells we showed that 'pre-activation' of those signalling proteins was not required for their recruitment to LAT. This indicates that the recruitment of signalling proteins by LAT is the initial switch that connects the PTKs to downstream signalling pathways. Finally we have shown that Vav, p85/p110α, and PLCγ1 directly interact with phosphorylated LAT. Although multiple tyrosine residues were implicated in Vav binding, Tyr1[32] and Tyr[171] were the primary residues responsible for PLCγ1 and p85/p110α binding. By utilizing synthetic phosphopeptides derived from LAT, we mapped the docking site on LAT required for the association and activation of p85/p110α kinase activity. Although we have shown that PTyr[171] can stimulate PI 3-kinase activity, it is possible that, like the situation in the PDGF receptor, multiple tyrosine residues will contribute to PI 3-kinase activation [19]. In contrast with PI 3-kinase, the direct enzymic activation of Vav by LAT was not seen. While Vav was directly associated with phosphorylated LAT, no further activation of Vav GEF activity towards Rac and Cdc42 was detected. Our data support the model that LAT functions to recruit Vav to Lck, and subsequently result in phosphorylation of Vav and stimulation of its GEF activity. Consistent with this, it was recently shown that TCR-mediated Rac activation requires LAT and Vav but is independent of Slp76 [33]. These data support the notion that a direct link between LAT and Vav may be essential for its recruitment to Lck and susequently for Rac activation.

With respect to PLCγ1 recruitment and activation, our results agree with those of a recent report [10] and clearly suggest that Tyr[132] is the LAT docking site for PLCγ1. The replacement of Tyr[132] by phenylalanine completely abrogated the interaction between LAT and PLCγ1, but had no detectable effect on the binding of other signalling proteins tested, including Grb2/Sos, Cbl-b, p85 and Slp76. Using synthetic phosphopeptide 132, we were able to compete for PLCγ1 binding with LAT. Furthermore, gel-overlay assays using purified recombinant PLCγ1 indicated a direct interaction between PLCγ1 SH2 domains and PTyr[132].

To study the role of LAT phosphotyrosine residues in T-cell signalling, we used a mutant variant of Jurkat cells, ANJ3, shown to cause a dramatic reduction in the LAT level and a defect in TCR-mediated Ca^{2+} flux. Recently it was shown that normal T-cell function was restored when [WT]LAT was re-introduced into those cells [11]. We confirmed this finding and restored normal T-cell function by stably expressing [WT]LAT in the ANJ3 cell line. Expression of [Y132F]LAT, however, failed to rescue the signalling defects in ANJ3. We validated our *in vitro* observations by showing that PLCγ1 binding cannot take place in the absence of PTyr[132], although [Y132F]LAT phosphorylation and association with Grb2/Sos, Cbl and Slp76 were comparable with [WT]LAT. The inability of [Y132F]LAT to recruit PLCγ1 resulted in the failure to phosphorylate, and consequently to activate, the PLCγ1 pathway. As was shown with Vav and p85/p110α, these results suggest that activation and phosphorylation of PLCγ1 follow the recruitment of PLCγ1 to LAT. Results from the [Y171/191F]LAT mutant confirmed previous findings and indicate that this mutant can no longer interact with any identified signalling protein including Vav and p85α. It is possible that phosphorylation of these residues is required for conformational changes on LAT that expose other tyrosine residues for further phosphorylation. Recently, Samelson's group reported that the three distal tyrosine residues on LAT are involved in Grb2 and Gads recruitment and Tyr[132] is important for PLCγ1 binding [10]. We extend this observation and show both direct and exclusive PLCγ1 and p85/p110α binding to the PTyr[132] and PTyr[171] residues respectively. Interestingly, as described with Gad2 and Grb2 [10], Vav interaction with LAT required multiple tyrosine residues.

In agreement with our data, it was recently shown that Slp76-deficient T cells exhibit a reduction in TCR-induced tyrosine phosphorylation and activation of PLCγ1 and Ras signalling pathways, despite normal recruitment of PLCγ1 and Grb2/SOS to LAT. It was proposed that Slp76 participates in the formation of complexes that link PLCγ1 to the Ras signalling pathway [23]. It is likely that the role of Slp76 in PLCγ1 activation involves the recruitment of a tyrosine kinase to the LAT complex to phosphorylate PLCγ1. A good candidate is the Tec family of tyrosine kinases Itk/Rlk, which was recently shown to be involved in PLCγ1 phosphorylation and activation during T cell activation [26].

Although it was shown that TCR-mediated Ras activation required LAT phosphorylation, it is unclear if docking of Grb2/Sos by LAT is sufficient for maximal Ras activation. We detected normal recruitment of the Grb2–Sos complex by [Y132F]LAT, but activation of the Ras/MAP kinase pathway was clearly impaired, indicating that normal levels of the Ras GEF Sos on the plasma membrane are insufficient to activate the Ras signalling pathway. Our findings show that Ras signalling pathways require an intact PLCγ1 pathway. It was previously shown in T cells that Ras is activated efficiently with the PKC activator PMA through a mechanism that inhibits the activity of Ras GAP (GTPase activating protein for Ras) [27,28]. According to this model, activation of PLCγ1 results in the generation of inositol trisphosphate and diacylglycerol, which directly activates PKC and subsequently inhibits Ras GAP to obtain prolonged Ras activation. A possible mediator of this event is PKCφ, which was recently shown to play a key role in the activation of the Ras pathway in T cells [29]. Alternatively, the PLCγ1 pathway may be involved in stimulating an unknown Ras exchange factor.

It is also possible that the negative regulator Cbl influences T-cell functions by complexing with different proteins regulated by PLCγ1. It has been shown that TCR activation dissociates Cbl from Grb2 to form a complex with the adapter protein CrkL. Crkl is constitutively associated with C3G, a GEF for the Ras-related protein Rap-1 [30]. Rap1 antagonizes Ras activation and has been implicated in the induction of energy in T cells [31]. The combination of Grb2/SOS recruitment by LAT and PKCθ inhibition of Ras GAP may act in synergy to fully activate Ras during TCR stimulation. Thus PLCγ1 may act as a critical modulator in T cells to control the Ras signalling pathway by either regulating the combinations of signalling complexes associated with LAT or by directly coupling PKC with the Ras signalling pathway. Further studies are required to determine the molecular link between PLCγ1 and Ras signalling pathways.

In summary, we reconstituted the LAT signalling pathway by demonstrating that Lck activated Zap-70 can directly phosphorylate five tyrosine residues in LAT required for association with Vav, p85/p110α and PLCγ1. Our analysis suggests that LAT is involved in both the docking and activation of signalling proteins during T-cell activation.

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