Roles of the C-terminal tyrosine residues of LAT in GPVI-induced platelet activation; insights in the mechanism of PLCγ2 activation.

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Abstract

Linker for activation of T cells (LAT) is an adaptor protein required for organisation of the signalling machinery downstream of the platelet collagen receptor, the glycoprotein VI (GPVI). Here, we investigated the effect of LAT mutations on specific signalling pathways and on platelet functions in response to GPVI triggering by convulxin (Cvx). Using mice containing tyrosine to phenylalanine mutations of the adaptor, we show the crucial role played by the tyrosine residues at position 175, 195 and 235 in the phosphorylation of LAT and in the whole pattern of protein tyrosine phosphorylation in response to Cvx. These three C-terminal tyrosine residues are important to recruit the tyrosine kinase Fyn which may be involved in LAT phosphorylation. Efficient phosphoinositide 3-kinase (PI3K) activation requires the three C-terminal tyrosine residues of LAT but not its tyrosine 136. Interestingly, single mutation of the tyrosine 136 results in the loss of phospholipase C \(\gamma_2\) (PLC\(\gamma_2\)) activation without affecting its PI3K-dependent membrane association, and is sufficient to impair platelet responses to Cvx. Thus, activation of PLC\(\gamma_2\) via GPVI is dependent on two complementary events; its interaction with the tyrosine 136 of LAT and its membrane location which itself requires events mediated by the three C-terminal tyrosines of LAT.
Introduction

Subendothelial collagen fibers play an important role in platelet adhesion and activation at sites of vessel damage.\(^1\) Besides α2β1, a major receptor supporting platelet adhesion to collagen, the glycoprotein VI (GPVI) is considered as the receptor mediating collagen-induced platelet activation.\(^2\) GPVI is a member of the immunoglobulin superfamily of type I transmembrane proteins and is noncovalently associated with the Fc receptor (FcR) γ-chain. It stimulates platelets through a tyrosine kinase-based signalling pathway.\(^2\) The initial step of tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the FcR γ-chain by Src family kinases leads to the recruitment and the activation of the tyrosine kinase Syk and in turn to the phosphorylation of a number of signalling proteins. Several adaptor molecules, including linker for activation of T cell (LAT) and Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) coordinate the assembly of a multiprotein signalling complex essential for GPVI-induced activation of key enzymes such as phospholipase C\(γ\)2 (PLC\(γ\)2) and phosphoinositide 3-kinase (PI3K).\(^3,4,5,6,7\)

LAT is a membrane-associated adaptor molecule containing a short extracellular domain, a single transmembrane-spanning region and a long cytosolic tail with nine tyrosines conserved in mouse and human sequences. It is thought that the palmitoylation of cysteine residues near the plasma membrane anchors LAT to lipid rafts. This hematopoietic-specific adaptor protein is expressed in early B cells, T cells, mast cells, natural killer cells, megakaryocytes and platelets. It plays a crucial role in T cell activation and thymocyte development.\(^8\) LAT-deficient mice (\(Lat^{-}\)) have revealed a role of this adaptor protein during T cell maturation.\(^9\) LAT is also involved in FceRI activating pathway in mast cells\(^10,11\) and might facilitate early B-cell differentiation.\(^12,13\) Studies in T lymphocytes have shown the importance of the four C-terminal tyrosine residues (Y132, Y171, Y191 and Y226 in humans, and their homologues in mice Y136, Y175, Y195 and Y235) for most of the signalling
activity of LAT. The three membrane-distal tyrosine residues Y171, Y191 and Y226 bind Gads and Grb2 while Y132 is required for PLC γ1 interaction. Mice expressing LAT with mutations in the four distal tyrosines exhibit defective thymocyte development. LAT knock-in harbouring point mutations in the Y175, 195 and 235 results in the selective development and expansion of γδ T cells. Mice bearing a single Y136F mutation have an aberrant αβ T cell proliferation characterized by an exaggerated polyclonal differentiation into CD4 cells that secreted abnormally high levels of Th2 cytokines. It is thus becoming clear that subtle mutations in LAT, leading to a defective LAT signalosome, have important effects on the differentiation and function of multiple immune cell types.

Platelets from \textit{Lat}−/− mice exhibit a defective response to GPVI-agonists such as the collagen related peptide (CRP) and the C-type lectin present in the venom of the rattlesnake \textit{Crotalus durissus terrificus} convulxin (Cvx). \textit{Lat}−/− platelets are still able to aggregate in response to high concentrations of GPVI agonists suggesting that this adaptor may be dispensable for some platelet functions in certain conditions. However, LAT appears essential for the platelet procoagulant response and the full surface expression of P-selectin. As GPVI null mice, \textit{Lat}−/− mice show no signs of haemorrhage suggesting that other agonists or adhesive proteins using different signalling pathway can compensate for these deficiencies. Recent evidence suggests that, \textit{in vivo}, the type of initiating event appears to determine the relative importance of GPVI-mediated platelet activation in thrombus formation. It is also important to note that GPVI is involved in thrombotic disorders. Besides its important role in signal transduction upon GPVI triggering, LAT is also involved downstream of FcγRIIa and GpIb. It plays an essential role in platelet stimulation by the C-terminal peptide of thrombospondin-1. Most tyrosine kinase-dependent platelet activation pathways actually use LAT to efficiently orchestrate the spatio-temporal assembly of signalling proteins to obtain a complete and rapid platelet response.
There are similarities of LAT functions in immune cells and in platelets but several important differences in the signalling mechanisms involving SLP-76 and LAT have also been observed between both systems. Here, we investigated the effects of LAT mutations on the disruption of specific signalling pathways downstream of GPVI and analysed their functional consequences on platelet responses. We demonstrate the crucial role of the three C-terminal tyrosines (Y175, Y195 and Y235) in the phosphorylation of the adaptor itself, in the whole pattern of protein tyrosine phosphorylation and in platelet responses to GPVI triggering. PI3K activation is strongly inhibited upon mutation of the distal three tyrosine residues of LAT but not of the Y136 residue. Conversely, a single mutation of the Y136 residue of LAT results in the loss of PLCγ2 activation but spares its PI3K-dependent association with the membrane.
Methods

Materials. The anti-Fyn monoclonal antibody and the anti-PLCγ2 polyclonal antibody were from Santa-Cruz Biotechnology. The mouse anti-phosphotyrosine 4G10 was from UPSTATE Biotechnology, Inc. 5-hydroxy[14C]tryptamine (56.0 mCi/mmol) and SuperSignal West Pico Chemiluminescent Substrate were from Amersham International. [γ-32P] ATP (3000 Ci/mmol) was from New England Nuclear. GST-LAT cDNA was generated by inserting full-length cytosolic domain of LAT obtained by RT-PCR from megakaryocytic DAMI cells into pGEX-KG vector (Pharmacia). Polyclonal LAT antibody was produced by immunizing rabbit with this fusion protein. Cvx was purified from the venom of Crotalus durissus terrificus as previously described.32 Horm collagen from equine tendon was from Nycomed, Germany. Thin layer chromatography (TLC) plates were from Merck. Wortmannin, LY294002 and other reagents were from Sigma.

Mice. Mice deficient in recombination activation gene 1 (Rag1-/- mice) were originally obtained from E. Spanopoulou (Mount Sinai Scholl of Medecine, New York).33 Mice deficient in LAT (Lat-/-), and mice homozygous for a mutation that replaced with phenylalanine tyrosine 136 (LatY136F) or the three last carboxy-terminal tyrosines (Lat3YF) were previously described.17,18 To prevent the development of noxious T cells, mice deficient for the Rag1 gene and homozygous for the LatY136F or Lat3YF mutations were used. Mice were housed under specific pathogen-free conditions in accordance with institutional guidelines approved by French laws and were used between 6 and 10 weeks of age.

Platelet preparation and in vitro aggregation studies. Whole blood was collected by puncturing the inferior vena cava with syringes containing acid citrate dextrose (1/9 volume) from anesthetized mice. Pooled blood samples were diluted in one volume of modified Heps
Tyrode’s buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO₃, 0.3 mM NaH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, 5 mM Hepes, pH 6.7) containing 0.35% human serum albumin, and platelet-rich plasma was obtained by centrifugation (4 minutes, 250 g, 37°C). Thereafter, prostaglandin I₂ at a final concentration of 500 nM was added, and platelets were pelleted once by centrifugation (4 minutes, 1,000 g, 37 °C). The platelet pellet was finally resuspended in modified Hepes-Tyrode’s buffer pH 7.38, containing 2 mM CaCl₂, at a density of 5x10⁸ platelets per milliliter in the presence of 0.02 unit/ml of the ADP scavenger apyrase (adenosine-5'-triphosphate diphosphohydrolase), and incubated for 45 minutes at 37 °C before stimulating with Cvx. Optical aggregation experiments were monitored by a turbidimetric method using a dual-channel Payton aggregometer (Payton Assoc, Scarborough, ON) with continuous stirring (900 rev/min) at 37 °C.

**Dense granule secretion.** Dense granule secretion was investigated by platelet 5-hydroxy[¹⁴C]tryptamine release in response to Cvx stimulation under stirring conditions, as described previously.³⁴

**In Vitro Flow-based Adhesion studie.** Glass microcapillaries were coated with 500 µg/ml Horm collagen from equine tendon for 1 hour at 37°C. The flow chamber, mounted on an epifluorescence microscope (Axiovert 200; Carl Zeiss, Inc.), allowed direct visualization of the platelet adhesion and aggregation process, which was recorded with a CCD camera (Cool Snap HQ, Roper Scientific). Mouse blood was drawn into lepirudin (200 IU/ml) and DiOC₆ (2 µM, 30 minutes at 37 °C) was used to label platelets in whole blood. Labelled blood was then perfused through collagen-coated glass microcapillaries for 2 minutes at a wall shear rate of 1,500 s⁻¹ (15 dyn/cm²), followed by washing for 2 minutes at the same shear rate with phosphate-buffered saline. Thrombus formation was visualized with a 40X long working
distance objective in real time (acquisition rate: 1 frame every 5 seconds) for both fluorescent and transmitted light microscopy. Image sequences of the time lapse recording and analysis of surface coverage were performed off-line on single frame using the Metamorph software (Universal Imaging Corp.). After deconvolution, a lower intensity threshold was applied to distinguish platelets from the background and a similar threshold was then used for analyzing all Z-stack collected for a given experiment. Thrombus volume was calculated as the summation of partial volumes measured from the area occupied by platelets in each plane of Z-stacks as described previously.35

**Lipid extraction and analysis.** Platelets were labelled with 0.6 mCi/ml $[^{32}\text{P}]$orthophosphate during 45 minutes in a phosphate-free Hepes-Tyrode’s buffer (pH 6.5) at 37°C. $^{32}$P-Labelled platelets were then washed once in the same buffer and finally suspended at a final concentration of $1\times10^9$ platelets/ml in modified Hepes-Tyrode’s buffer (pH 7.38). After stimulation, reactions were stopped by addition of chloroform/methanol (1/1, v/v) containing 0.4 N HCl, and lipids were immediately extracted as described previously. For phosphatidylinositol 3,4- bisphosphate (PtdIns(3,4)P$_2$), phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) quantification, lipids were first resolved by TLC using chloroform/acetone/methanol/acetic acid/water (80/30/26/24/14, v/v). The spots corresponding to PtdIns(3,4)P$_2$, PtdIns(3,4,5)P$_3$ and PtdIns(4,5)P$_2$ were then scraped off, immediately deacylated and analyzed by high performance liquid chromatography on a Whatman Partisphere 5 SAX column (Whatman International Ltd., UK) as described. For phosphatidic acid (PtdOH) quantification, lipids were resolved by TLC using chloroform/methanol/10N HCl (87/13/0.5, v/v) as described previously. The spots corresponding to PtdOH were scrapped off and directly quantified by liquid-scintillation counting.
Immunoprecipitations and immunoblotting. 500 µl of 7.5x10^8/ml resting or stimulated platelets were lysed in RIPA buffer at final concentrations of 150 mM NaCl, 20 mM Tris-HCl, pH 7.7, 4 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin. The lysates were submitted to standard immunoprecipitation and immunoblotting protocols. For re-immunoprecipitation, the immunoprecipitated complexes were dissociated by addition of 60 µl of a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 1% SDS and boiled for 5 minutes. The final volume was then brought to 1.5 ml by RIPA buffer containing 0.5% Triton X-100 and the second immunoprecipitation was performed with the appropriate antibody.

Biotinylated Cvx (2 µg/mL), prepared as described previously, was used to analyse GPVI expression and was probed with HRPO-streptavidin.

In vitro kinase assay. Samples were immunoprecipitated as described above, washed twice with RIPA buffer, and once with kinase buffer (50 mM Tris-HCl, 2.5 mM MnCl2, 5 mM MgCl2, pH 7.4). The beads were resuspended in 40 µl of kinase buffer containing 10 µCi of [γ-32P] ATP with 10 µM cold ATP and incubated at 30°C for 30 minutes under shaking. Reactions were terminated by addition of 10 µl of 5x Laemli buffer and submitted to a 10% SDS-PAGE. To perform re-immunoprecipitation, the immune complexes from the in vitro kinase assay (IVK) were submitted to the protocol described above using LAT antibody.

Cytosol depletion. After stimulation, platelets were centrifuged (3,000 g for 30 seconds) and suspended in 20 mM Pipes buffer (pH 6.8) containing 150 mM KCl, 2 mM EDTA, and 30 µg/ml saponin. After 5 minutes at room temperature under shaking, supernatant and pellet
fractions were separated by centrifugation (12,000 g for 40 seconds). The pellet, containing membranes and cytoskeleton, was suspended in Laemli’s sample buffer, and PLCγ2 was probed by immunoblotting using anti-PLCγ2 antibody.
Results

The C-terminal three tyrosine residues of LAT and tyrosine 136 are critical for GPVI-induced platelet activation. To address the role of the C-terminal tyrosine residues of LAT, platelets from wild-type or knock-in mice expressing LAT with combinations of tyrosine mutations were stimulated with the potent GPVI agonist Cvx. The platelet counts of the mice with LAT mutated either on the three last C-terminal tyrosine residues 175, 195 and 235 (Lat3YF) or on the tyrosine residue 136 only (LatY136F) were normal (492,000 ± 36,000/mm³ for WT versus 470,000 ± 47,000/mm³ for LatY136F and 447,000 ± 97,000/mm³ for WT(Rag-/-) versus 541,000 ± 37,000/mm³ for Lat3YF, n=5) and the platelet expression level of GPVI, LAT and PLCγ2 were fairly comparable (supplemental Figure 1).

As expected, stimulation of wild-type platelets with increasing concentrations of Cvx resulted in an irreversible platelet aggregation, in a dose dependent manner (Figure 1A-B, left panels). Conversely, platelets from Lat3YF (Figure 1A) or from LatY136F (Figure 1B) mice did not aggregate in response to low doses of Cvx (<5 nM) and slightly aggregated at the highest concentrations of Cvx tested (up to 20 nM). Lat+/− platelets did not aggregate upon 5 nM Cvx but, as reported previously, 23 a significant aggregation response was observed upon high concentrations of Cvx (>20 nM, not shown). To preserve the selectivity of the Cvx/GPVI activation pathway we used a dose of 5 nM of Cvx in our biochemical studies, since higher concentrations may induce GPVI independent events. It is noteworthy that platelets from LatY136F and Lat3YF mice aggregated as wild-type platelets in response to 0.2 IU/ml thrombin (Supplemental Figure 2C-D). Platelet dense granule secretion was nearly maximal at 10 nM Cvx in wild-type platelets but was hardly detectable in Lat3YF (Figure 1C) and LatY136F (Figure 1D) platelets upon stimulation with Cvx concentrations up to 10 nM.

To further investigate the functional role of the C-terminal tyrosine residues of LAT on platelets adhesion and aggregation, we have examined platelet under physiological flow...
conditions using an *in vitro* flow-based platelet aggregation assay. Fluorescently labelled platelets in whole blood were perfused over a matrix of collagen at a shear rate of 1500 s⁻¹ (15 dyn / cm²). Blood from control mice exhibited robust formation of densely packed platelet thrombi on collagen. In marked contrast, *Lat*3YF platelets attached along the length of the collagen fibers, but the formation of platelet thrombi was strongly deficient (Figure 2A-B). These results are consistent with the notion that these platelets formed a single layer on the collagen fibers in contrast to the multilayer platelet thrombi observed in control blood as shown with the differential interference contrast pictures and the total fluorescence intensity. As shown in figure 2C and D, the surface covered by platelet thrombi (12 ± 0.6 % for *Lat*3YF versus 20.9 ± 1.6 % for wild-type platelets) and the thrombus volume (0.6 ± 0.01 x 10⁵ µm³ for *Lat* 3YF versus 1.6 ± 0.1 x 10⁵ µm³ for wild-type platelets) were significantly reduced. Similar results were obtained with *Lat*Y136F platelets (not shown).

All together, these results strongly suggest that the docking properties of the distal four tyrosine residues of LAT link GPVI to intracellular signalling pathways essential for platelet functions.

**The C-terminal three tyrosine residues of LAT are critical for most GPVI-induced tyrosine phosphorylations.** To analyze the signalling pathways linked to the three distal tyrosine residues of LAT, we first investigated the tyrosine phosphorylation events occurring in platelets from the different mice strains stimulated by Cvx. Stimulation with 5 nM Cvx led to a rapid and dramatic increase in the level of tyrosine phosphorylation of several proteins in wild-type platelets (Figure 3A). Particularly, a marked increase in the phosphorylation of 70 kDa proteins (matching Syk and SLP76) was observed. An increase in the tyrosine phosphorylation of proteins of about 120 kDa and 145 kDa (matching PLCγ2) was also clearly detected. Interestingly, combination of Y175F, Y195F, and Y235F mutations of LAT
dramatically decreased the phosphorylation of the 70 and the 145 kDa proteins in response to Cvx (Figure 3A). Conversely, the tyrosine phosphorylation pattern of platelets from knock-in mice presenting a single mutation (Y136F) was comparable to that of wild-type platelets in response to Cvx (Figure 3B) except a reduction in the degree of phosphorylation of the 145 kDa protein (matching PLCγ2). As suggested in Figure 3A, the heavy tyrosine-phosphorylation of LAT observed in response to Cvx in wild-type mice platelets was abolished by mutation of its three C-terminal tyrosine residues (Figure 3C). Thus, although LAT has 9 tyrosine residues, mutations of its three last C-terminal tyrosines are sufficient to abolish its own phosphorylation in response to GPVI triggering. The single mutation Y136F did not significantly affect the degree of tyrosine phosphorylation of LAT in response to Cvx (Figure 3D). This Y136 residue of LAT is the major binding site for PLCγ1 in T lymphocytes.15 Two forms of PLCγ have been identified and PLCγ2 is the form predominantly expressed in platelets. GPVI-induced PLCγ2 tyrosine phosphorylation was fully inhibited in Lat3YF platelets (Figure 3E). Interestingly, although strongly affected, a tyrosine phosphorylation of PLCγ2 was still detectable in LatY136F platelets (Figure 3F). These results suggest that besides an important role of the Y136 residue, the distal three tyrosine residues of LAT (Y175, Y195 and Y235) are required for PLCγ2 phosphorylation.

**Fyn interacts with LAT in response to GPVI triggering: a role for the three C-terminal tyrosines of LAT.** To check whether a kinase that would phosphorylate LAT interact with this adaptor through its distal three tyrosine residues, we performed an *in vitro* kinase assay in LAT immunoprecipitates. As shown in Figure 4A, a strong *in vitro* LAT phosphorylation was observed after 30 seconds Cvx stimulation of wild-type platelets. This phosphorylation was only very weak in Lat3YF platelets. These results suggest that the three last tyrosine residues of LAT are important for the binding of a protein kinase involved in its phosphorylation. The
Src family kinase member Fyn is known to form part of the GPVI signalling cascade. Interestingly, Fyn interacted with LAT upon Cvx activation in wild-type mice platelets (Figure 4B). This interaction was no longer observed when LAT was mutated on its three last C-terminal tyrosine residues. These results were confirmed by reverse experiment. Indeed, phosphorylated LAT was detected in Fyn immunoprecipitates obtained from wild-type mice platelets while only a weak signal was detected when the immunoprecipitated was performed from Lat3YF mice platelets (Figure 4C).

**Both PLCγ2 and PI3K activations are abolished by mutations of the three last C-terminal tyrosines of LAT, while the Y136F mutation selectively affect PLC and spares PI3K activation.** Activation of PLC and PI3K are recognized as critical processes in the stimulation of platelets by GPVI. To monitor PLC activity in [32P]-labelled platelets it is classical to followed the production of [32P]-PtdOH, a metabolite of 1,2-diacylglycerol. PI3K activation is usually measured by following the levels of the different [32P]-labelled phosphoinositides by an HPLC technique.

In contrast to wild-type platelets, which rapidly form PtdIns(3,4,5)P3 in response to Cvx, platelets from Lat3YF mice produced only very small amounts of this PI3K product (Figure 5A). Moreover, PtdIns(3,4)P2 known to accumulate in an integrin-engagement and a platelet aggregation-dependent manner was no longer produced in platelets from Lat3YF mice (Figure 5A). As shown by the lack of PtdOH production, the activation of PLC was fully inhibited in platelets from Lat3YF mice (Figure 5A). In agreement, the weak decrease in PtdIns(4,5)P2, the substrate of PLC, observed in wild-type platelets stimulated by Cvx was not observed in platelets from Lat3YF mice (Figure 5A). Similar results were obtained with Lat+/+ platelets (not shown). In contrast, PtdIns(3,4,5)P3 was still produced in platelets from LatY136F mice stimulated by Cvx, with only a slight decrease in the maximal synthesis of this second
messenger after 1 minute of activation (Figure 5B). The initial production of PtdIns(3,4)P$_2$ observed at 30 seconds of Cvx stimulation was preserved by the Y136F mutation of LAT but the accumulation of this lipid was abolished likely because aggregation was impaired (Figure 5B). Interestingly, mutation of the PLC$_\gamma$-binding site of LAT (Y136) fully inhibited the production of PtdOH and the decrease in PtdIns(4,5)P$_2$ (Figure 5B). It is noteworthy that this mutation only partly affected the phosphorylation of PLC$_\gamma$2 in response to Cvx (Figure 3F).

These results show that a full PI3K activation requires the distal three tyrosine residues of LAT (Y175, Y195 and Y235) but not its Y136 residue. Conversely, mutation of the Y136 residue of LAT results in the loss of PLC$_\gamma$2 activation but spares PI3K activation in response to Cvx.

**PLC$_\gamma$2 association with the platelet membrane/cytoskeleton is not affected by the Y136F mutation of LAT but is abolished by mutations of the distal three tyrosine residues.** The stable recruitment of activated PLC$_\gamma$2 to the membrane is critical for the enzyme to hydrolyse PtdIns(4,5)P$_2$ and to produce inositol-1,4,5 trisphosphate (InsP$_3$) and diacylglycerol. Since Y136F mutation of LAT abolishes PtdOH production we checked whether this mutation also affected the association of PLC$_\gamma$2 with the membrane/cytoskeleton. As shown in Figure 6A, Cvx stimulation induced a rapid interaction of PLC$_\gamma$2 with the membrane fraction of platelets from both wild-type and $Lat^{Y136F}$ mice. In both cases, this interaction was fully inhibited by the PI3K inhibitor wortmannin (Figure 6A) and the other unrelated PI3K inhibitor LY294002 (not shown). The fact that Y136F mutation of LAT did not affect the association of PLC$_\gamma$2 with the membrane/cytoskeleton clearly shows that this step is not sufficient to allow PLC activation. Interestingly, mutations of the distal three tyrosine residues of LAT which dramatically inhibited PI3K activation (Figure 5A) also strongly prevented the relocation of PLC$_\gamma$2 (Figure 6B). The association of PLC$_\gamma$2 with the membrane/cytoskeleton is essential
since in platelets from wild-type mice, PI3K inhibitors abolished PtdOH production (Figure 6C) while sparing PLC γ2 phosphorylation (Figure 6D).
Discussion

In this study, we show that tyrosine to phenylalanine mutations in the C-terminal part of LAT has profound effects on signalling and on platelet responses upon GPVI triggering by Cvx. Concomitant mutation in the three membrane distal tyrosine residues at position 175, 195 and 235 result in the inhibition of the phosphorylation of LAT itself and in a dramatic reduction of the whole pattern of protein tyrosine phosphorylation in response to Cvx. Accordingly, platelet secretion and aggregation are impaired under these conditions. In T cells, the four last tyrosine residues of LAT have also a critical role as the mutant mice with the last four tyrosines mutated to phenylalanine have a similar phenotype as LAT-deficient mice with an early block in T cell differentiation.\(^{16,40}\) ZAP-70, a kinase activated after T cell receptor stimulation, is involved in the direct phosphorylation of LAT in T cells.\(^{41,42}\) However, other kinases such as Itk and Lck, have been suggested to participate in its phosphorylation.\(^{20}\) It has been reported that in human T cells, the phosphorylation of tyrosine residues at position 191 is required for the phosphorylation of the tyrosine residues at position 132 suggesting that LAT phosphorylation occurs in different waves involving different tyrosine kinases.\(^{43}\) In platelets, little is known about the kinase(s) responsible for the direct phosphorylation of LAT. This adaptor becomes phosphorylated downstream of Syk activation but whether this kinase is responsible for the direct phosphorylation of LAT is unknown. Asazuma et al.\(^{22}\) previously observed a co-immunoprecipitation of LAT and the tyrosine kinase Lyn. Here we show that another member the Src-family, Fyn, co-precipitates with LAT upon Cvx stimulation in mice platelets. Interestingly, the three C-terminal tyrosine residues of LAT are required for the rapid recruitment of Fyn. Moreover, \textit{in vitro} kinase assays performed on LAT immunoprecipitates indicate that a kinase associated to these three C-terminal tyrosines, possibly Fyn, is able to phosphorylate LAT itself. Lyn and Fyn are known to play a crucial role in the very early phase of GPVI pathway since they
phosphorylate the ITAM of the FcR γ-chain enabling the binding of the tyrosine kinase Syk. 39,44 Our results suggest that these kinases also participate in the direct phosphorylation of LAT which is likely a sequential event upon GPVI-triggering. Thus, in addition to Syk and Lyn, Fyn may directly participate in the maximal phosphorylation of LAT.

Our results clearly show the importance of the three C-terminal tyrosine residues of LAT in platelet activation via GPVI. They are essential for the tyrosine phosphorylation of most proteins in response to GPVI, efficient PI3K activation and PLC activation. These mutations strongly affect platelet secretion and aggregation in washed platelet activation assays and thrombus growth in whole blood under flow conditions. The tyrosine residue at position 136, known to interact with PLCγ1 in T lymphocytes, is also critical. Mutation of this particular tyrosine residue of LAT results in the loss of PLCγ2 activation in response to Cvx and in platelet responses. Interestingly, this point mutation does not affect significantly PI3K activation and spares the tyrosine phosphorylation of most proteins in response to GPVI triggering. The tyrosine phosphorylation of PLCγ2, which is impaired by mutations of the three last tyrosine of LAT, is strongly reduced but still detectable by mutation of tyrosine 136. These results suggest that other proteins depending on the phosphorylation of the last three tyrosine residues of LAT regulate some phosphorylation sites of PLCγ2 in the absence of its docking site on the adaptor. However, this phosphorylation appears insufficient to activate the enzyme or more likely concerns tyrosine residues not involved in its activation. Recently, it was shown in stimulated Jurkat T cells that in addition to LAT, several other phosphotyrosyl-proteins including SLP76, c-Cbl and Vav1, co-precipitate with PLCγ1, the PLCγ isoform playing a major role in these cells.45 Particularly, the interaction of PLCγ1 with SLP-76 coincides with the activating tyrosine phosphorylation of the enzyme (i.e. tyrosine 783) possibly via a Tec-family kinase. Our result would fit with this model as SLP-76 is essential in GPVI-induced PLCγ2 phosphorylation and activation in platelets.46
Although the recruitment of PLCγ2 and its phosphorylation are critical steps, they do not necessarily result in production of diacylglycerol and InsP₃. Indeed, a stable and adequate interaction of the enzyme with its substrate in the membrane is mandatory for an efficient production of second messengers. In platelets, the PI3K product PtdIns(3,4,5)P₃ is required for PLCγ2 activation but not for its tyrosine phosphorylation.³⁴,⁴⁷,⁴⁸ Bobe et al. have shown that in megakaryocytes, the translocation of PLCγ2 to the plasma membrane in response to GPVI activation is dependent on PI3K.⁴⁹ Here, we show that the translocation of PLCγ2 to platelet membranes in response to GPVI-triggering is fully dependent on PI3K and is not affected by mutation of the tyrosine 136 residue of LAT. This result indicates that interaction of PLCγ2 with LAT, through tyrosine 136, is not required for the location of the phospholipase to the membrane/cytoskeleton. However, this PI3K-dependent stabilization is not sufficient to activate the production of the second messengers diacylglycerol and InsP₃. Thus, both tyrosine 136 residue of LAT and PI3K activation through the last three C-terminal tyrosine residues of LAT are required for PLCγ2 activation in response to GPVI triggering (Figure 7). Thus, highly coordinated mechanisms, involving specific tyrosine residues of LAT, SLP-76 and PI3K, control PLCγ2 activation and in turn platelet functions downstream of GPVI.

Interestingly, consistent with these observations, mutation of tyrosine residue 136 or mutations of tyrosine residues 175, 195 and 235 of LAT strongly affect platelet aggregation in response to low concentrations of collagen (<5 µg/ml) (Supplemental Figure 2A,B). However, at concentrations higher than 6 µg/ml this agonist can induce platelet aggregation in the presence of the mutation of tyrosine residue 136 of LAT. Even higher concentrations are required to promote aggregation in the presence of the mutation of tyrosine residue 175, 195 and 235. These results and previous reports in which Lat⁻/⁻ platelets were analyzed indicate that, at high concentrations, collagen can induce platelet aggregation through
signalling pathways independent of LAT and strengthen the idea of multiple receptors and/or alternative signalling mechanisms to allow platelet activation by this multivalent agonist.

In conclusion, using LAT mutants we demonstrated the critical role of the three last tyrosine residues of LAT for most functions of the adaptor downstream of GPVI activation in platelets. These tyrosine residues are essential for the recruitment of Fyn which may in turn contribute to LAT phosphorylation. Mutation of tyrosine 136 of LAT has a more subtle effect as it does not affect the PI3K-dependent association of PLC\(\gamma\)2 with the membrane, partly inhibit the phosphorylation of the phospholipase but impairs its activation and platelet responses upon GPVI triggering. These results indicate that, as in immune cells, subtle mutation of LAT affecting specific signalling pathway may alter platelet reactivity.

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**Authorship contribution statement:** A.R., S.S. and M-P.G. have designed and performed most experiments and analysed data; E.A., B. and M. M. have produced transgenic mice, M. J-P. has purified Cvx, J.R. and B.P. supervised the work, analyzed data and wrote the paper.
References


Legends to the figures

Figure 1: Mutations of the three C-terminal tyrosines or tyrosine 136 of LAT inhibit platelet aggregation and secretion in response to Cvx. (A-B) Platelets from the different mouse strains were stimulated with increasing concentrations of Cvx and aggregation was assessed using a Chrono-log dual channel aggregometer under stirring at 900 rev/minutes. The profiles shown are representative of four independent experiments. (C-D) To measure dense granule secretion, 5-hydroxy[14C]tryptamine-labelled platelets were stimulated by different concentration of Cvx. Results are expressed as percentage of 5-hydroxy[14C]tryptamine (serotonin) secretion and are means ± SEM of five independent determinations.

Figure 2: Mutation of the three C-terminal tyrosines of LAT affect platelet thrombi formation on collagen under flow. DiOC6-labelled platelets in whole blood were perfused through a collagen-coated microcapillary at a shear rate of 1,500 s⁻¹ for 2 minutes. (A) Thrombus formation was visualized with a 40X long working distance objective in real time and then imaged using transmitted light microscopy. (B) After a washing step with phosphate-buffered saline for 2 minutes at the same shear rate to remove non adherent cells, slides were visualized using differential interference contrast microscopy. Representative images of platelet adhesion from wild-type and Lat3YF mice are shown. A representative time course for both wild-type (WT) (top panel) and Lat3YF (bottom panel) platelet accumulation on collagen is shown. (C) Area covered by platelet thrombi and (D) thrombus volume were measured at two surface locations in each of three different experiments (mean ± SEM). **, significant difference (p<0.005) versus wild-type, according to Student t test. Scale bar = 20 μm.
Figure 3: Effects of mutations of the C-terminal tyrosines of LAT on the tyrosine phosphorylations of PLCγ2 in response to Cvx. (A-B) Platelets from WT, Lat3YF and LatY136F mice were stimulated by 5 nM Cvx for the time indicated. Reactions were stopped by addition of RIPA buffer, protein separated by a 12.5% SDS-PAGE, transferred onto nitrocellulose and the tyrosine phosphorylated proteins were detected by immunoblotting with the anti-phosphotyrosine antibody 4G10. The membrane was stripped and reprobed for LAT, Syk, SLP76 and PLCγ2 which positions are indicated by the black arrow (from the bottom of the nitrocellulose, respectively). Platelets from WT (C-D, left panel), Lat3YF (C, right panel) and LatY136F (D, right panel) mice were stimulated by 5 nM Cvx for the time indicated. Reactions were stopped by addition of RIPA buffer and LAT was immunoprecipitated using a specific antibody. Tyrosine phosphorylation of LAT was assessed by immunoblotting using the anti-phosphotyrosine antibody 4G10 (upper panels). The membrane was stripped and reprobed for LAT with the anti-LAT antibody (lower panels). PLCγ2 was immunoprecipitated from the lysate of platelets from WT (E and F, left panel), Lat3YF (E, right panel) and LatY136F (F, right panel) mice stimulated by 5 nM Cvx for the indicated times. Tyrosine phosphorylation of PLCγ2 was assessed by immunoblotting using the anti-phosphotyrosine antibody 4G10 (upper panels). The membrane was stripped and reprobed for PLCγ2 with the anti-PLCγ2 antibody (lower panels). Results are representative of three independent experiments.

Figure 4: The interaction of Fyn with LAT is abolished by mutations in the three distal C-terminal tyrosines of LAT. (A) Platelets from WT (left panel) and Lat3YF (right panel) mice were stimulated by 5 nM Cvx for the time indicated and LAT was immunoprecipitated and submitted to an in vitro kinase assay (IVK) in the presence of [γ-32P]ATP. After separation by 10% SDS-PAGE, the radioactivity incorporated in LAT was assessed by
PhosphorImager analysis. (B) Alternatively, following immunoprecipitation with the anti-LAT antibody, immunoprecipitated complexes were dissociated and re-immunoprecipitated with anti-Src family antibody. After separation by 10% SDS-PAGE and transfer onto nitrocellulose the re-immunoprecipitate was immunoblotted with a specific anti-Fyn antibody. (C) Fyn was immunoprecipitated from lysate of WT (left panel) or Lat3YF (right panel) platelets stimulated by 5 nM Cvx for the indicated times. Immunoprecipitated complexes were submitted to an in vitro kinase assay in the presence of [γ-32P]ATP and re-immunoprecipitated with the anti-LAT antibody as described above. The radioactivity incorporated in LAT was assessed by PhosphorImager analysis. Results are representative of two independent experiments.

Figure 5: Impact of mutations in the C-terminal tyrosines of LAT on PI3K and PLC activation in response to GPVI triggering. 32P-labelled platelets from WT (●) and Lat3YF (▲) (A) or from WT (Rag-/-) (●) and LatY136F mice (●) (B) platelets were stimulated by 5 nM Cvx for the indicated times and the levels of 32P-PtdIns(3,4,5)P3, 32P-PtdIns(3,4)P2, 32P-PtdIns(4,5)P2 and 32P-PtdOH were analyzed as indicated in Materials and Methods. Results are mean ± SEM of 4 experiments.

Figure 6: Mutation of tyrosine 136 of LAT does not affect GPVI-dependent membranes/cytoskeleton recruitment of PLCγ2. WT, LatY136F (A) and Lat3YF (B) platelets treated or not with the PI3K inhibitor wortmannin (50 nM) were stimulated by 5 nM Cvx for the indicated times. Half of the cells were analysed for the total amount of PLC-γ2 (A-B lower panels) and the other half was immediately permeabilised by saponin for cytosol depletion. After centrifugation (12,000 g for 40 seconds), the pellet (corresponding to the membranes and cytoskeleton fraction, Mbr/Csk) was suspended in Laemli's sample buffer,
and the amount of PLC-γ2 was analysed by immunoblotting (A-B upper panels). The effect of PI3K inhibition by wortmannin on PtdOH formation (C) and PLCγ2 tyrosine phosphorylation (D) of platelets from wild-type mice was then analyzed.

Figure 7: A schematic model highlighting the essential role of the tyrosine residues of LAT involved in PLCγ2 activation upon GPVI triggering in platelets.
Figure 1

A  WT  Lat3YF

B  WT (Rag-/-)  LatY136F

C  WT  Lat3YF

D  WT (Rag-/-)  LatY136F

% of secretion

0 5 10 15 20

Cvx (nM)

0 5 10 15 20

Cvx (nM)

0 5 10 15 20

Cvx (nM)

0 5 10 15 20

Cvx (nM)
Figure 2
Figure 3 A-D
Figure 3 E-F
Figure 4
Figure 5A
Figure 5B
Figure 6
Figure 7

LAT

- essential for PLCγ2 activating tyrosine phosphorylation

- required for LAT phosphorylation and downstream events including PI3K activation as well as PLCγ2 membrane association and activation

PLCγ2 activation (IP3 and DAG production)

Platelet secretion and aggregation
Roles of the C-terminal tyrosine residues of LAT in GPVI-induced platelet activation; insights in the mechanism of PLCγ2 activation

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