The tyrosine phosphatase 1B regulates LAT phosphorylation and platelet aggregation upon FcγRIIa cross-linking.

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Running title: LAT regulation by PTP1B in human FcγRIIa activated platelets.
Summary

Human platelets express the receptor for immunoglobulin G, FcγRIIa, that triggers cell aggregation upon interaction with immune complexes. Here, we report that the rapid tyrosine phosphorylation of the Linker for Activation of T-cell (LAT) in human platelets stimulated by FcγRIIa cross-linking was followed by its complete dephosphorylation in an αIIb/β3 integrin-dependent manner. Concomitant to LAT dephosphorylation, the protein tyrosine phosphatase 1B (PTP1B) was activated through a mechanism involving its proteolysis by calpains downstream of integrins. Both PTP1B and LAT were associated with the actin cytoskeleton complex formed during platelet aggregation. Moreover, phosphoLAT appeared as a good substrate of activated PTP1B in vitro and these two proteins interacted upon platelet activation by FcγRIIa cross-linking. The permeant substrate trapping PTP1B (TAT-PTP1B D181A) partly inhibited LAT dephosphorylation in human platelets, strongly suggesting that this tyrosine phosphatase was involved in this regulatory pathway. Using a pharmacological inhibitor, we provide evidence that PTP1B activation and LAT dephosphorylation processes were required for irreversible platelet aggregation. Altogether, our results demonstrate that PTP1B plays an important role in the integrin-mediated dephosphorylation of LAT in human platelets and is involved in the control of irreversible aggregation upon FcγRIIa stimulation.
Introduction

Human platelets possess only one Fc γ receptor for immunoglobulin G (FcγRIIa) which is a 40 kDa single chain transmembrane glycoprotein also present in monocytes, neutrophils and B lymphocytes (1). In platelets, clustering of FcγRIIa induces shape change, secretion and aggregation, which are typical physiological responses required for efficient hemostatic function of these cells (2). This activating signal contributes to the rapid destruction of platelets during heparin-induced thrombocytopenia and in some autoimmune diseases (3,4). FcγRIIa-mediated signaling pathway implicates the cytoplasmic tail of the receptor which presents an amino acid sequence called Immunoreceptor Tyrosine-based Activation Motif (ITAM). Mice platelets lack this receptor but develop a similar signaling pathway downstream the glycoprotein VI (GPVI), also present in human platelets. Upon clustering, GPVI recruits and requires the Fc γ-chain sharing a strong homology with the human FcγRIIa receptor (5,6). In human platelets, upon FcγRIIa receptor cross-linking, the tyrosine residues within the ITAM motif are rapidly phosphorylated and become docking sites for proteins containing Src homology 2 (SH2) domains (7). Activated GPVI is coupled with the Fc-γ-chain protein which contains the similar ITAM motif. The phosphorylation of ITAM appears to be mediated by the Src related kinases p59Fyn and p56Lyn and allows the recruitment and the activation of the p72Syk kinase and subsequently the tyrosine phosphorylation of phospholipase C γ2 (PLCγ2) (8). However, the links between Src kinases, Syk and PLCγ2 are not clearly established in platelets and likely involve adaptor molecules.

Linker for Activation of T cells (LAT), a transmembrane 36-38 kDa adaptor protein essential for T cell receptor (TCR)-mediated activation, is also present in platelets (9). In T cells, LAT is tyrosine phosphorylated after TCR stimulation by the Syk related kinase ZAP 70 (10) and contains in its intracellular part, five optimal binding sequences for linking to SH2 domains containing proteins. In T lymphocyte, numerous signaling molecules have been
shown to associate with phosphorylated LAT including PLCγ1, the p85 subunit of PI 3-kinase, Grb2 and SLP76 (11). In platelets, LAT is strongly tyrosine phosphorylated downstream of GPVI clustering by collagen or convulxin stimulation. Several signaling proteins including the p85 subunit of PI 3-kinase and PLCγ2 have been shown to interact with phosphorylated LAT in platelets (9,12,13). In collagen stimulated platelets, the signaling complexes recruited by tyrosine phosphorylated LAT are essential for PLCγ2 activation (14).

The tyrosine phosphorylation level of proteins is the result of a controlled balance between protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The mechanism involved in protein dephosphorylation are still poorly known in platelets. Until now, no trans-membrane PTP has been described at the platelet surface but almost three cytosolic PTPs have already been identified in these cells (SHP-1, SHP-2 and PTP1B) (15-17). SHP-1, a PTP containing two SH2 domains in its N-terminus, is highly expressed in haematopoietic cells, where it is often implicated in the negative regulation of a number of membrane receptors (18). SHP-1 is rapidly phosphorylated and translocated to the platelet cytoskeleton upon thrombin stimulation (17,19,20). However, the role of SHP-1 in platelet functions is still unclear. SHP-2, another SH2 domain-containing PTP, is associated with the receptor PECAM 1 (CD31) in platelets and could be involved in the signaling pathway initiated by this adhesion molecule (21,22). The 50 kDa PTP1B has also been described in human platelets (16). In these cells, the amount of PTP1B is about 0.2 % of total detergent soluble proteins, a level comparable with that of pp60Sre kinase. In resting platelets, the full-length PTP1B tightly associates with the endoplasmic reticulum via its C-terminal 35 amino acids (23). When platelets are activated by thrombin, PTP1B undergoes a proteolytic cleavage in a region between its catalytic domain and its membrane anchoring C-terminal targeting sequence. This process is dependent on integrin engagement and platelet aggregation and leads to enzymatic activation of PTP1B (16). In other cells as fibroblasts, PTP1B plays an
important role in integrin-mediated cell adhesion and spreading (24,25). It has also been
demonstrated that PTP1B can dephosphorylate p130Cas, suggesting that it might have a
regulatory role in mitogen-mediated signal transduction pathway via integrin (26). The
tyrosine kinase pp60c-src has also been identified as a good substrate of PTP1B leading to an
activation of this kinase (27). Recently, a critical role for PTP1B in the negative regulation of
insulin signaling has been well documented (28). PTP1B appears as the major PTP
responsible of insulin receptor regulation (29,30) suggesting that PTP1B inhibitors may
become new drugs for type 2 diabetes treatment (31,32).

In platelets, the substrate and the role of PTP1B are still unknown. The aim of our
study was to investigate the mechanisms involved in the tight control of LAT adaptor protein
dephotphorylation in platelets activated by FcγRIIa clustering. We found that the adaptor
LAT, which phosphorylation appeared transient in this signaling cascade, was actually one of
the PTP1B substrates. Indeed, our results demonstrate that LAT dephosphorylation requires
PTP1B activation via calpains downstream of αIIb/β3 integrin engagement. Our data suggest
a role of PTP1B in the coordination of signaling process leading to irreversible platelet
aggregation through dephosphorylation of proteins such as LAT.

Experimental procedures
Reagents, antibodies and fusion proteins.

The anti-FcγRIIa monoclonal antibody (mAb IV.3), the monoclonal PTP1B antibody,
the polyclonal Src family kinase antibody (SRC-2) and the anti-phosphotyrosine 4G10
antibody were purchased from Upstate Biotechnology Inc. The specific F(ab’)2 fragment
were from Jackson Immunoresearch Laboratories. The monoclonal Anti-HA antibody was
from Eurogentec. The fluorescein-conjugated anti mouse Ig secondary antibody (ALEXA
488) and rhodamin-conjugated phalloidin (ALEXA 594) were from Molecular Probe.
Convulxin was purified from the venom of *Crotalus durissus terrificus* as previously described (33). Enhanced chemiluminescence (ECL) western blotting reagents were from Amersham International. Poly (Glu₄-Tyr₁)ₙ, calpains inh1, RGDS and other chemical products were from Sigma Chemical Co. The phosphatase inhibitor PTP InhI was from Calbiochem Co. [γ-³²P] ATP (3000 Ci/mmole) was from New England Nuclear. Ni-ProBond resin was from Invitrogen.

GST-LAT cDNA was generated by inserting cytosolic domain of LAT containing amino acid 18-255 obtained by RT-PCR from megacaryocytic DAMI cells into pGEX-KG vector. The fusion protein was purified using glutathion sepharose beads using Pharmacia instructions. Rabbit anti-LAT antibody was produced in our laboratory by immunizing rabbit with this GST-LAT fusion protein. The full length human cDNA (GST-PTP1B), containing a Cys-215 to Ser mutation (GST-PTP1B C215S) was a gift from Dr. J. Chernoff (Fox Chase Cancer Center, Philadelphia, USA). The full length cDNA construct PTP1B (D181A) obtained from Dr. N. Tonks (Cold Spring Harbor Laboratory, New York, USA) was subcloned in pTAT-HA-His vector (34) as a Ncol/EcoR1 fragment. Isolation of TAT-fusion protein was realised after sonication of bacteria in 8M urea HEPES (pH 7.2), 100mM NaCl buffer, 20mM imidazole and the clarified sonicate was applied to Ni-ProBond resin. TAT fusion protein elution was performed by the same buffer containing 100mM imidazole. Detection of the TAT-HA-His PTP1B (D181A) fusion protein was performed by western blotting using anti-HA or anti-PTP1B antibodies. Quantification of the purified protein was done by BIO-RAD protein assay system.

*Platelet preparation and stimulation.*

Human blood platelet concentrates were obtained from the local blood bank (Etablissement de transfusion Sanguine, Toulouse, France). Platelet preparation and FeγRIIa
cross-linking were performed as previously described (35). Platelet aggregation was measured at 37°C by a turbidimetric method in a dual-channel aggregometer (Payton Associates, Scarborough, Ontario, Canada). In some experiments, platelets were not shacked. To test the effect of inhibitors on LAT tyrosine phosphorylation, platelets suspension (1x10⁹ platelets/ml) were pre-incubated for 3 min at 37°C with 500 µM RGDS, or 10 µM calpains inh1. In some experiments, the phosphatase inhibitor (PTP InhI) was added at 12.5 µM final concentration on platelet suspensions after indicated time of stimulation. To test the effect of PTP1B substrate trapping, 10 µg of TAT-HA-His-PTP1B (D181A) fusion protein were added to 500 µl platelets and cells were incubated at 37°C for 20 min before stimulation.

Mice platelets were prepared according the following protocol: C57/Bl6 animals were anaesthetized with a mix of Imalgene (kétamine) Rompun and blood was collected by cardiac puncture using heparin (100U/ml) as anticoagulant. Pooled blood was centrifuged at 1500 rpm for 8 min at 22°C and platelet-rich plasma was removed and centrifuged at 3000 rpm for 8 min at 22°C. The platelet pellet was washed twice in tyrode’s buffer (134 mM NaCl, 2.9 mM KCl, 12 mM NaHCO₃, 0.34 mM NaH₂PO₄, 20 mM HEPES, pH 7.3, 1 mM MgCl₂, 5 mM glucose) and finally suspended in the same buffer at the density of 8x10⁸ platelets/ml. Stimulations of mice platelets were carried out with 5 nM convulxin at 37°C under stirring conditions.

Isolation of platelet cytoskeleton.

500 µl of human platelets (1x10⁹ cells/ml) were activated by FcγRIIA cross-linking for indicated times and stimulation was stopped by addition of cytoskeleton buffer (CSK) to give a final concentration of 50 mM tris-HCL, pH 7.4, 10 mM EGTA, 1% tritonX-100, 1 mM sodium orthovanadate, 1 mM phenyl-methylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin. The lysate was incubated for 5 min at room temperature and 10 min at 4°C under
shaking. Cytoskeletal material was collected by centrifugation (12,000 x g, 10 min., 4°C), washed three times with CSK buffer and cytoskeletal proteins were sonicated three times for 10 sec in sample Lemblii buffer then submitted to western blotting using appropriate antibodies.

**Immunoprecipitation and immunoblotting.**

500 µl of 1x10⁹/ml resting or stimulated platelets were lysed in RIPA buffer at final concentration: 150mM NaCl, 20 mM Tris-HCl, pH 7.7, 4mM EDTA, 0.5% triton X-100, 1mM sodium orthovanadate, 1mM phenyl-methylsulfonyl fluoride, 10µg/ml leupeptin, 10µg/ml aprotinin. 5 µg of the indicated antibody was added to the clarified lysate in a final volume of 750 µl and placed on a rocking platform for 1 h at 4°C. The immune complexes were collected by adding 35 µl of 50 % (w/v) protein A/G sepharose beads for 1 h at 4°C. After washing, the immunoprecipitated proteins were resolved by 10% SDS-PAGE, electrotransferred to nitrocellulose membranes and detected by immunoblotting with the appropriate antibodies using the enhanced chemiluminescence lighting (ECL) system. For re-immunoprecipitation technique, the first immunoprecipitate (IP-PTP1B) obtained from control or 5 min stimulated platelets was incubated with 60 µl of buffer (50mM Tris-HCl, pH 7.5, 10mM DTT, 1% SDS) and boiled for 2 min. The final volume was brought to 1.5 ml by the addition of RIPA buffer containing 0.5% triton X-100 and a second IP was performed with LAT antibody.

In some experiments TAT-HA-PTP1B (D181A) treated or non treated human platelets were subjected to HA immunoprecipitation as described above and analysed by western blot using polyclonal anti Src kinases antibody (SRC-2).
Binding assays using GST-fusion proteins.

For LAT re-immunoprecipitation from PTP1B (C215S) pull-down complex, 500 µl of platelet suspension (5x10^8) were stimulated for 5 min in the absence or in the presence of 12.5 µM PTP InhI added 1 min after FcγRIIa cross-linking. The reaction was stopped by 250 µl 3X RIPA buffer and the clarified lysate was incubated with GST-PTP1B (C215S) for 2 h at 4°C. Beads were washed and the proteins complexes from pull down assay were denatured by addition of 60 µl of buffer (50mM Tris-HCl, pH 7.5 10mM DTT, 1% SDS) and boiled for 2 min. The final volume was brought to 1.5 ml by the addition of RIPA buffer containing 0.5% triton X-100 and antiphosphotyrosine immunoprecipitation were performed with 4 G10 antibody followed by western blotting using LAT antibody.

Immune complex protein phosphatase assay and in vitro LAT dephosphorylation.

Enzymatic activity of PTP1B in platelets were determined using paranitrophenyl phosphate as substrate. PTP1B immune complexes were washed twice with PTP assay buffer (62 mM HEPES pH.5, 6.25 mM EDTA, 12.5 mM dithiothreitol) and incubated with 25mM final concentration of paranitrophenyl phosphate for 30 minutes at 30°C under shaking. Reactions were terminated by adding 800 µl of 1N NaOH. After centrifugation at 13,000 rpm for 3 min, optical density of supernatants was measured at 410 nm. For LAT dephosphorylation in vitro, LAT immunoprecipitation was performed from platelets after 1 min FcγRIIa stimulation as described above. PTP1B immune complex was realised at 5 min stimulation. The dephosphorylation reaction was performed by mixing the two washed immune-complexes in 50 µl of the same buffer. After 30 minutes incubation at 30°C under shaking, the reactions were stopped by the addition of sample buffer, detection of the phosphoproteins was performed by SDS-PAGE followed by anti-phosphotyrosine immunoblotting.
In-gel phosphatase assay.

These experiments were performed according to Burridge and Nelson procedure (36) with some modifications. Briefly, 2 mg of poly (glu-tyr)\textsubscript{n} were phosphorylated by incubation overnight at 30°C with pp60\textsuperscript{src} kinase immunoprecipitated from thrombin activated platelets and 20 \(\mu\)Ci of [\(\gamma\)\textsuperscript{32P}] ATP. The kinase reaction was terminated by centrifugation at 4°C (13,000 rpm; 5 min). The supernatant of agarose beads was mixed with an equal volume of 20% trichloracetic acid. After 30 min on ice, labelled poly (glu-tyr) was sedimented. The precipitate was dissolved in 200 \(\mu\)l tris buffer 0.75 M, pH 8.8 and incorporated in SDS polyacrylamide running gel prior to polymerization at about 10\textsuperscript{6} cpm/ml. Platelet lysates or LAT immunoprecipitated were submitted to SDS-PAGE according to the standard protocol.

Confocal immunofluorescence microscopy.

Resting human platelets were incubated or not with TAT-HA-PTP1B (D181A) fusion protein as described above. Platelets were allowed to adhere on fibrinogen–coated coverslips at the concentration of 100 \(\mu\)g/ml during 1 H at 37°C. Cells were washed with phosphate buffered saline (PBS) and fixed with formaldehyde 3 %, 30 min at room temperature. Then, they were permeabilized with 0.01 % triton X100 in PBS for 10 min at room temperature. Non specific sites were saturated with 3 % bovine serum albumin in PBS for 30 min. Platelets were incubated with monoclonal anti-HA antibody followed by fluorescein-conjugated anti-mouse Ig secondary antibody mixed with rhodamin-conjugated phalloidin. Slides were examinated under a Zeiss confocal microscope (LSM 510, Axiovert 100) using immersion objective 63X.
LAT regulation by PTP1B in human FcγRIIa activated platelets.

Results

The rapid tyrosine phosphorylation of LAT is followed by an integrin-mediated dephosphorylation in FcγRIIa stimulated platelets.

FcγRIIa cross-linking led to a rapid LAT phosphorylation reaching a maximum at 1 min followed by a dephosphorylation which was complete after 5 min of stimulation (Fig. 1A). When platelet aggregation was prevented by the absence of shaking during stimulation (Fig. 1B), or by cell pre-incubation with RGDS peptide (Fig. 1C), we observed a sustained tyrosine phosphorylation of LAT. In some but not all experiments, a small delay in LAT phosphorylation was observed in non shaking conditions of stimulation (Fig. 1B). Overall, these data indicate that the phosphorylation of LAT did not require platelet aggregation and integrin engagement whereas its dephosphorylation was strongly dependent on these processes. As calpains have been shown to regulate some tyrosine dephosphorylation events downstream of integrins in platelets, we investigated the effect a calpains inhibitor (calpains inh1.) on LAT phosphorylation status. As reported in figure 1D, LAT dephosphorylation was partly inhibited by this inhibitor suggesting an implication of calpains regulated PTPs downstream of integrin in this mechanism.

PTP1B is activated in human platelets upon FcγRIIa cross-linking.

To investigate the PTPs involved in LAT dephosphorylation in human platelets via FcγRIIa cross-linking, lysates obtained from resting or stimulated platelets were submitted to an In-gel phosphatase assay as described in Experiment procedure. Figure 2A shows that resting platelets developed two basal PTP activities at 50 and 42 kDa. Since 3min FcγRIIa cross-linking, the level of phosphatase activity of the 42 kDa species strongly increased with the appearance of several new bands detected at a molecular weight between 40 and 55 kDa. Some other PTPs presenting higher molecular weight were also detected during platelet
aggregation with modest activation level (data not shown). Moreover, experiments performed with RGDS pre-incubated platelets show that the increase in the 42 kDa species at 5 min stimulation was blocked indicating a crucial role of integrins in this PTP activation. As expected, the tyrosine phosphatase inhibitor (PTP InhI) inhibited this enzymatic activation.

The proteins detected in the In-gel phosphatase assay at about 40 kDa and 50 kDa could be the active fragment of PTP1B and its full-length respectively. To determine the identity of these proteins, we performed immunodepletion of the whole lysates obtained from 5 min stimulated platelets. As shown in figure 2B, the supernatant of PTP1B immunoprecipitate (lane 2) exhibited only a weak phosphatase activity at 42 kDa and a significant decrease at 50 kDa (about 50 %) indicating that these two bands corresponded to the two forms of PTP1B present in stimulated platelets. Moreover, the figure 2C shows that, under basal conditions, PTP1B was essentially present as a full length 50 kDa protein, whereas 3 min after FcγRIIa cross-linking, the truncated form of PTP1B (42 kDa) was generated. This cleavage decreased when platelets were pre-incubated with a calpains inhibitor (calpains inh1) suggesting that calpains catalyzed PTP1B proteolysis in FcγRIIa stimulated platelets. As some enzymes refold better than others, In-gel phosphatase assay is not always appropriate to quantify a specific PTP activity. Therefore, we immunoprecipitated PTP1B from platelet lysates and measured its activity by an in vitro phosphatase assay using paranitrophenyl phosphate as a substrate. PTP1B developed a 2.3 fold increase in enzymatic activity after 5 min platelet stimulation (Fig. 2D). Moreover, pre-treatment of cells with RGDS totally prevented integrin-induced PTP1B cleavage (not shown) and decreased the activity detected in PTP1B immunoprecipitate (Fig. 2D). These data demonstrate that αIIb/β3 integrin and calpains pathway regulated the cleavage of PTP1B and modulates its global activity measured in vitro, in human platelets stimulated by FcγRIIa cross-linking as previously observed in thrombin activated platelets (16).
The actin cytoskeleton plays an important role in cell physiology and numerous signaling proteins have been found to associate with this cellular compartment after platelet aggregation (17,37-40). As shown in figure 3, LAT associated with the cytoskeleton fraction upon FcγRIIa-mediated platelet activation. This association reached a maximum at 2 min and persisted until 5 min. Interestingly, PTP1B was found in the same compartment after 2 min stimulation. The co-localization of LAT and PTP1B in the platelet cytoskeleton correlated with the time-course of LAT dephosphorylation suggesting that PTP1B could dephosphorylate LAT in this cell compartment.

**Phosphorylated LAT is a PTP1B substrate in FcγRIIa activated human platelets.**

To investigate the potential participation of PTP1B in LAT dephosphorylation, we performed in vitro experiments using a mix of LAT immunoprecipitated from 1 min stimulated platelets (maximum of LAT phosphorylation) and PTP1B immunoprecipitated from 5 min stimulation (maximum of PTP1B activation). As shown in figure 4A, LAT was tyrosine dephosphorylated by PTP1B in vitro. Interestingly, PTP1B immunoprecipitated from platelets pre-treated with RGDS was less effective to dephosphorylate LAT. Moreover, pull-down experiments using the recombinant GST-substrate trapping PTP1B (C215S) followed by immunoprecipitation of phosphotyrosyl-proteins with 4G10 antibody, indicated that phospho LAT was present in the pool of proteins associated with GST-PTP1B (C215S) only when cells were incubated with PTP inhibitor to prevent LAT dephosphorylation (Fig. 4B). The purity of the fusion protein GST-PTP1B (C215S) is indicated in the right of figure 4B. This result strongly suggests a specific enzyme–substrate association. Furthermore, the In-gel phosphatase assay performed on immunoprecipitated LAT shows that LAT co-immunoprecipitated with a 42 kDa PTP at 2 to 5 min of platelet stimulation (Fig. 5A). This result indicates an association of the truncated form of PTP1B (42 kDa) with LAT in human
platelets upon FcγRIIa cross-linking. The same association was found in human platelets activated by the GPVI agonist, convulxin (Fig. 5B, left panel). Thus, the regulation of LAT phosphorylation by PTP1B in human platelets is likely not restrained to Fc receptor activation but may also be an important regulatory pathway via GPVI in collagen signaling. It is noteworthy that the same protocol performed from mice platelets demonstrated that the PTPs associated with LAT were totally different in these species. Indeed, the In-gel phosphatase assay of immunoprecipitated LAT from mice platelets activated by convulxin shows an activation of 120; 66 and 60 kDa PTPs and the lack of 42 kDa active PTP (Fig. 5B, right panel).

To further characterise the association of PTP1B with LAT in human platelets, we performed double-immunoprecipitations (proteins immunoprecipitated with the anti-PTP1B antibody were submitted to immunoprecipitation with anti-LAT antibody). These experiments indicated that PTP1B was associated with LAT after FcγRIIa-mediated platelet stimulation (Fig. 5C). As direct genetic approaches are not possible in platelets, we used the cell permeant fusion protein TAT-substrate trapping PTP1B (D181A) to trap PTP1B substrate leading to a dominant negative effect (42). We controlled the integrity of this fusion protein TAT-HA-PTP1B (D181A) purified on Ni beads (Fig. 6A). We verified, by immunofluorescence technique, that it was indeed able to enter the platelets (Fig. 6B) and was actually able to trap pp60<sup>src</sup> a known PTP1B substrate (27) (Fig. 6C). This treatment did not alter LAT tyrosine phosphorylation at 1 min platelet stimulation but partly inhibited LAT dephosphorylation (55% inhibition) at 5 min of platelet stimulation (Fig. 6D). Altogether, these experiments provide strong evidence that PTP1B is implicated in LAT dephosphorylation occurring in the late phase of FcγRIIa-mediated human platelet activation.
PTP activities are critical for irreversible platelet aggregation.

As a first attempt to assess the functional role of PTP activation downstream of integrins in stimulated platelets, we used the PTP Inh1, a permeant molecule described as a powerful PTP1B inhibitor (43). Interestingly, addition of this inhibitor after 1; 2 or 3 min stimulation led to a reversion of platelet aggregation (Fig. 7A). In these experimental conditions, we performed LAT immunoprecipitations followed by a western blot analysis using a specific antibody anti-Yp. As shown in figure 7B, PTP Inh1 added 1 or 2 min after the agonist, totally inhibited LAT dephosphorylation classically detected after 5 min stimulation. This inhibition was less effective when the inhibitor was added after 3 or 4 min platelet stimulation because, at these times, PTP1B was already activated. Altogether, our results suggest that PTP1B plays an important role in the negative regulation of LAT tyrosine phosphorylation in human platelets, a mechanism correlated with the irreversible cell aggregation.

Discussion

In T lymphocyte, the docking protein LAT plays a critical role and is involved in PLCγ1 activation after TCR engagement (44). Platelets express LAT at a high level allowing further investigations of this signaling protein in this peculiar haematopoietic cell where it has been shown to play a role in PLCγ2 activation (14). Here, we show that, in human platelets stimulated via FcγRIIa cross-linking, LAT is strongly and rapidly tyrosine phosphorylated independently of αIIb/β3 integrin engagement and platelet aggregation. The time-course of LAT phosphorylation is in agreement with previously reports in collagen or convulxin stimulated platelets (12,14).

Our data indicate that LAT phosphorylation is transient, followed by its complete dephosphorylation. Interestingly, LAT dephosphorylation is strictly dependent on platelet
aggregation and does not occur when the integrin αIIb/β3 is blocked by RGDS peptide. Our results clearly demonstrate that the PTPs implicated in LAT dephosphorylation are activated through an integrin outside-in signaling. Until now, only a few reports have been published on the characterization of PTPs involved in platelet functions. We have previously shown that thrombin stimulation led to SHP-1 activation in human platelets (19). However, this PTP is implicated before integrin engagement (45) and is therefore not a good candidate for dephosphorylating LAT. In the other hand, it has been shown that the 50 kDa PTP1B is activated by calpains-mediated proteolysis via a calcium dependent-process downstream of integrin αIIb/β3 engagement in thrombin stimulated platelets, (15,16). As we found that LAT dephosphorylation is absolutely dependent on platelet aggregation, we focused our investigations on this PTP. The In-gel phosphatase assay performed with lysates obtained from FcγRIIa stimulated human platelets indicates that the major PTP developing a phosphatase activity coincides with a 42 kDa protein which could be the active form generated by calpains cleavage from the 50 kDa PTP1B. The identification of these PTP (42 and 50 kDa) as the two forms of PTP1B was obtained by immunodepletion experiments followed by an In-gel phosphatase assay. Immunoblotting experiments confirmed that FcγRIIa cross-linking leads to PTP1B proteolysis from 50 kDa to 42 kDa in platelets after 3 min stimulation. The size of this generated fragment (42 kDa) and its absence in platelets pre-treated with calpains inhibitor suggest that PTP1B proteolysis is due to calpains action as described upon thrombin stimulation (16). The comparison between the PTPs activities observed in the In-gel PTPs assay and the PTP1B detection by WB seem indicate that the 42 kDa form of PTP1B developed a higher specific activity than the 50 showing an enzymatic activation of the PTP1B when this PTP was cleaved by calpains. However, In-gel PTPs assay is not appropriate to compare enzymatic activities because some enzymes refold better than other and will appear more active (46). For this reason, we measured PTP activities from IP-
PTP1B and we observed about two-fold increase in PTP1B activity, dependent on integrin αIIb/β3 engagement, after 5 min FcγRIIa cross-linking. A similar result was previously reported in platelets activated with calcium ionophore A 23187 in which the activated calpains totally cleaved PTP1B (16). Altogether, our data suggest that, in FcγRIIa stimulated human platelets, the calpains activated via integrins lead to PTP1B activation by proteolysis (see Fig. 8).

The positive correlation between the time-course of PTP1B activation and LAT dephosphorylation suggested that LAT could be a major substrate of PTP1B in human platelets activated via FcγRIIa cross-linking. The partial inhibition of LAT dephosphorylation in platelets pre-treated with a calpains inhibitor which prevents PTP1B proteolysis and activation is in agreement with this hypothesis. This inhibition is only partial (no more than 50%) but it is known that the activity of calpains is difficult to totally abolish in activated platelets (16). In addition, using fusion protein containing the phosphatase “substrate trapping” PTP1B (D181A) and the peptide TAT from HIV allowing cell penetration (34), we could demonstrate the implication of PTP1B in LAT dephosphorylation. The fact that only 55% of LAT dephosphorylation was inhibited by the “substrate trapping” TAT-PTP1B incubation may be due to a limited quantity of fusion protein reaching phosphorylated LAT resulting in a partial blocking effect of this molecule. However, due to the fact that LAT posses ten potential phosphotyrosyl residues, it is also possible that others PTPs are involved in LAT dephosphorylation.

It is well demonstrated that PTPs exhibit strict substrate specificity and the challenge concerning PTPs studies is now to identify specific physiological substrates for each enzyme. The development of the In-gel phosphatase technique to detect PTP activities associated with a given phospho-protein from immunoprecipitates could be a powerful tool for the identification of specifics substrates. Using this technique, we obtained evidence that LAT is
associated with PTP1B in FcγRIIa stimulated human platelets strongly suggesting that phosphorylated LAT is a substrate of activated PTP1B in these cells. A consensus substrate recognition motif for PTP1B containing a tandem Tyr (E/D-pY-pY-R/K) was described in previously identified PTP1B substrates (47,48). LAT does not possess this consensus sequence, but other PTP1B substrates lacking this motif were also described and its presence might not be essential for protein dephosphorylation by PTP1B (42). Moreover, PTP1B, which displays a proline-rich region, has been described to interact directly with the adaptor protein Grb2 via its SH3 domains (49). Grb2 and Gad have been shown to associate with phosphorylated LAT in human platelets (13). It is possible that the association PTP1B-LAT may be connected by Grb2 or Gad or other SH3 containing signaling proteins in stimulated platelets. This is in agreement with the fact that we found PTP1B and LAT both located in the platelet cytoskeleton. Moreover, preliminary results obtained in our laboratory indicate that these two proteins are also present in lipid “rafts” compartment which represent a signaling platform in platelets (50).

The physiological implications of the PTPs activated downstream of integrins in platelets is still poorly documented. Dephosphorylation mechanisms could play a critical role in cellular functions since incubation of platelets with PTP inhibitor, 1 or 2 min after agonist addition, leads to reversible platelet aggregation. These data suggest that the dephosphorylation steps may be critical to the maintain of platelet aggregation possibly through reinforcement of aggregates via reorganisation of the actin cytoskeleton. In agreement, some data suggest that integrin-dependent tyrosine dephosphorylation is involved in fibrin clot retraction in which the actin cytoskeleton plays a crucial role (51). It is conceivable that the cytoskeleton remodelling leading to irreversible phase of aggregation may require dephosphorylation of several signaling proteins involved in the very early phase of platelet activation, including LAT. The identification of targets of PTP1B clarifies its role
LAT regulation by PTP1B in human FcγRIIa activated platelets.

in mediating cellular events and gives the notion that differential localization patterns of PTP1B serve a specific cellular function. At the first glance, PTP1B −/− mice, generated in two laboratories (52,53), would be useful to estimate the impact of this PTP in platelet aggregation via FcγRIIa cross-linking, however, mouse platelets do not express this Fc receptor. Moreover, the In-gel phosphatase assays performed on homogenates obtained from human or mouse platelets activated by the GPVI agonist convulxin (using signaling pathways similar to FcγRIIa) clearly show a totally different pattern of PTPs activities (data not shown). Finally, In-gel PTP assays performed on LAT immunoprecipitated indicate a dramatically different pattern of PTPs associated with LAT upon convulxin stimulation, in these two species. Particularly, PTP1B does not interact with LAT in mouse platelets. These data strongly suggest that the PTP involved in the regulation of LAT phosphorylation might be different between human and mice platelets. Thus, PTP1B −/− mice platelets are likely not appropriate to study the role of PTP1B in the regulatory pathway of LAT phosphorylation occurring in human platelets. Accordingly, PTP1B −/− mice do not develop any serious bleeding phenotype suggesting a minor role for this PTP in mouse platelet aggregation. In contrast, our results indicate that the role of PTP1B in human platelet functions may be considered with attention. Indeed, the use of PTP1B inhibitors as a therapeutic drugs for type 2 diabetes treatment could induce side effects on platelet aggregation. It will be interesting to study the impact of these potential pharmacological molecules on platelet reactivity in order to prevent some eventual haemostatic disorders occurring in treated patients.

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References

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Footnotes

*Abbreviations:* CSK, cytoskeleton; Cvx, convulxin; FcγRIIa, Fc γ receptor for immunoglobulin G; GPVI, glycoprotein VI; GST, glutathione S transferase; HA, hemagglutinin; HIV, human immunodeficiency virus; ITAM, Immunoreceptor Tyrosine-based Activation Motif; LAT, Linker for Activation of T cell; PI 3-kinase, phosphatidylinositol 3 kinase; PLC, phospholipase C; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SH2, Src homology 2; TCR, T cell receptor.
Figure Legends

**Figure 1:** *Time course of LAT tyrosine phosphorylation in FcγRIIa stimulated human platelets.*

Platelets were stimulated by FcγRIIa cross-linking as described in Experimental procedures under shaking (A) or no shaking conditions (B). In some experiments, platelets were pre-incubated with 500 µM RGDS peptide (C), or with a calpains inhibitor (calpains inh1, 10 µM) (D). Equivalent amount of whole platelet lysate (5x10⁸ platelets) were subjected to LAT immunoprecipitation. The immunoprecipitates were separated by SDS-PAGE and analysed by immunoblotting with 4G10 (upper panel) or LAT (lower panel) antibodies. Each profile is a representative experiment independently performed four times.

**Figure 2:** *PTP1B activation in FcγRIIa stimulated human platelets.*

(A) Aliquots of whole platelet lysates were subjected to In-gel tyrosine phosphatase assay as described in Experimental procedures (upper panel). Effects of RGDS (500 µM) or PTP InhI (12.5 µM) on tyrosine phosphatase activity were investigated at 5 min stimulation. Lower panel shows coomassie blue staining of total protein loaded on the gel; MW: molecular weight markers. This pattern is representative of three experiments (B) PTP1B immunodepletion from platelet lysates after 5 min stimulation was performed using PTP1B antibody and the supernatant was subjected to In-gel phosphatase analysis: cell lysate precleared with protein G sepharose beads (lane1); cell lysate following one way immunodepletion of PTP1B (lane 2). This experiment were performed twice with similar results.(C) Western blotting anti PTP1B were performed from lysates of FcγRIIa stimulated platelets during indicated times. The full length of PTP1B corresponds to the 50 kDa protein and the band at 42 kDa is its truncated form. In the last lane, platelets were pre-incubated with calpains inh1 (10 µM). (D) PTP1B activity was measured by *in vitro* phosphatase assay.
performed on PTP1B immunoprecipitates using paranitrophenyl phosphate as exogenous substrate. Platelets pre-treated with RGDS (500 µM) or not were stimulated by FcγRIIa cross-linking for 5 min. PTP1B activities were expressed in fold increase (mean +/- SD of three independent experiments) compared with non stimulated platelets (control).

**Figure 3:** Translocation of LAT and PTP1B to the cytoskeleton of FcγRIIa stimulated platelets.

Washed human platelets were stimulated by FcγRIIa cross-linking as described in Experimental procedures at the indicated time. The cytoskeleton was extracted and resuspended in SDS-PAGE sample buffer for immunoblotting analysis. Revelation of LAT by the polyclonal anti LAT antibody (upper panel); identification of PTP1B using PTP1B antibody (middle panel); membrane was stripped off and reprobed with anti-actin antibody (lower panel). This figure is representative of two different experiments with similar results.

**Figure 4:** Phospho LAT is a substrate of PTP1B in vitro.

(A) LAT was immunoprecipitated from platelets 1 min after FcγRIIa cross-linking (IP-LAT) and these immunoprecipitates were mixed with PTP1B immunoprecipitates (IP-PTP1B) performed from 5 min stimulated platelets. The phosphorylated state of LAT was analysed by anti-phosphotyrosine western blotting (WB-Yp) using 4G10 antibody. In the third lane, IP-PTP1B was carried out from platelets pre-treated with 500 µM RGDS. (B) Lysates of resting or FcγRIIa stimulated platelets (5 min) in the absence or in the presence of PTP InhI (12.5 µM) were incubated with GST-PTP1B (C215S) fusion protein. Proteins in the complex were subjected to re-immunoprecipitation with 4G10 antibody. The immunoprecipitates were analysed by western blotting using anti LAT antibody. Coomassie blue of the GST and GST-
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PTP1B(C215S) are shown in the right of the picture. These data were obtained three times with similar result.

**Figure 5:** *PTP1B is associated with LAT in FcγRIIa cross-linking or GPVI activated human platelets but not in GPVI activated mice platelets.*

(A) IP-LAT performed from resting or FcγRIIa activated platelets were subjected to In-gel phosphatase assay as described in Experimental procedures. The position of molecular weight was indicated to the right. Western blots anti LAT (WB-LAT) were performed on a part of each IP-LAT to evaluate the quantity of LAT loaded on the gel. (B) The same protocol was performed from human or mice platelets stimulated with 5 nM convulxin (Cvx). (C) Platelets were activated or not by FcγRIIa cross-linking for 5 min. PTP1B was immunoprecipitated with specific anti PTP1B antibody and the immune complex was subjected to re-immunoprecipitation with anti LAT antibody as described in Experimental procedures. Then, immunoprecipitate proteins were analysed by western blotting using anti LAT antibody. Each profiles are representative of three independent experiments showing similar results.

**Figure 6:** *PTP1B is involved in LAT dephosphorylation in human platelets upon FcγRIIa cross-linking.*

(A) Western blot anti HA (WB-HA) was performed on purified fraction of the fusion protein TAT-HA-PTP1B (D181A) eluted from Ni-ProBond resin. (B) platelets were pre-treated with purified TAT-HA-PTP1B (D181A) fusion protein. The localization of TAT-HA-PTP1B in platelets were performed using anti HA antibody as described in Experimental procedures. The green fluorescence corresponds to the detection of TAT-HA-PTP1B (left; Anti-HA); red fluorescence to the actin cytoskeleton (right; phalloidin); Control IF experiment with anti HA antibody was performed on non treated platelets (control). (C) Cell lysates containing equal
amount of protein from control resting platelets (lane 1) or TAT-HA-PTP1B incubated platelets (lane 2) were immunoprecipitated with anti-HA antibody. Protein complexes were analysed by western blotting with anti Src antibody. (D) Platelets were pre-incubated (right part) or not (left part) with the cell permeant fusion protein TAT-HA-PTP1B (D181A) (10μg) for 20 min followed by cell stimulation via FcγRIIa cross-linking at 1 or 5 min. LAT was immunoprecipitated and its tyrosine phosphorylation was analysed by anti-phosphotyrosine western blotting (WB-Yp). The amount of LAT immunoprecipitated was analysed by western blotting anti-LAT (WB-LAT). This experiment was performed twice with similar results.

**Figure 7:** *Effect of PTP inhibition on platelet aggregation and LAT phosphorylation.*

(A) 5.10^8 human platelets were activated by FcγRIIa cross-linking and PTP Inh I (12.5 μM) was added at different time of stimulation. Aggregation of control and different treated platelets was measured by an aggregometer. Arrowhead indicates the time of agonist addition and kinetic addition of PTP Inh I. (B) After 5 min stimulation, each reaction was stopped by addition of RIPA buffer, LAT was immunoprecipitated with polyclonal anti LAT antibody and the immunoprecipitates were submitted to immunoblotting with 4G10 antibody (upper panel). Time course of LAT phosphorylation performed from platelet control (without PTP Inh I) is indicated on the left of the panel. After stripping, the membrane was re-probed with anti LAT antibody (lower panel). Data shown are representative of three independent experiments.

**Figure 8:** *Proposed model highlighting the role of PTP1B in the regulation of LAT phosphorylation in FcγRIIa-mediated human platelets stimulation.*

In resting platelets, PTP1B is maintained in a relative inactive form with carboxyl terminal tail anchored in the endoplasmic reticulum. FcγRIIa cross-linking leads to LAT
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...phosphorylation *via* probably the tyrosine kinase Syk during the early phase of platelet aggregation independently of integrin engagement. In the second phase of platelet aggregation, after integrin $\alpha$IIb $\beta$3 activation and fibrinogen binding to this integrin, PTP1B is truncated by activated calpains and localizes near its substrate LAT. PTP1B participates then to the dephosphorylation of LAT on some critical phosphotyrosine sites resulting in proteins multi-complex dissociation. Cytoskeletal rearrangement and focal adhesion organisation will then allow platelet irreversible aggregation to take place (see discussion for more details).
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Fig. 1
LAT regulation by PTP1B in human FcγRIIa activated platelets.

Fig. 2
LAT regulation by PTP1B in human FcγRIIa activated platelets.

Fig. 3
LAT regulation by PTP1B in human FcγRIIa activated platelets.

**Figure 4**

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WB: Yp

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GST-PTP1B(C215S)

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Fig. 5
LAT regulation by PTP1B in human FcγRIIa activated platelets.

Fig. 7
LAT regulation by PTP1B in human FcγRIIa activated platelets.
The tyrosine phosphatase 1B regulates LAT phosphorylation and platelet aggregation upon Fc γRIIa cross-linking
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