The Tyrosine Phosphatase 1B Regulates Linker for Activation of T-cell Phosphorylation and Platelet Aggregation upon FcγRIIa Cross-linking*

Received for publication, April 7, 2003, and in revised form, July 1, 2003
Published, JBC Papers in Press, July 12, 2003, DOI 10.1074/jbc.M303602200

Ashraf Ragab, Stéphane Bodin, Cécile Viala, Hugues Chap, Bernard Payrastre, and Jeannie Ragab-Thomas‡

From INSERM U563, Centre de Physiopathologie de Toulouse-Purpan, Institut Fédératif de Recherche 30, Université Paul Sabatier, Hôpital Purpan, 31059 Toulouse Cedex, France

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, phospho-LAT 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.

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 hematic 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). Mouse 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 p59(Fyn) and p56(Lyn) 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 phosphatidylinositol (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 and protein-tyrosine phosphatases (PTPs). The mechanisms involved in protein dephosphorylation are still poorly known in platelets. Until now, no transmembrane PTP has been described at the platelet surface but 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 hematopoietic cells, where it is often defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 pp60c-src 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 such 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 for 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 dephosphorylation in platelets activated by FcγRIIα clustering. We found that the adaptor LAT, for 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 processes leading to irreversible platelet aggregation through dephosphorylation of proteins such as LAT.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies, and Fusion Proteins**—The anti-FcγRIIα 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 Fab’/2 fragment was from Jackson ImmunoResearch Laboratories. The monoclonal anti-HA antibody was from Eurogentec. The fluorescein-conjugated anti-mouse Ig secondary antibody (ALEXA 488) and rhodamine-conjugated phallolidin (ALEXA 594) were from Molecular Probes. Convulxin was purified from the venom of Crotalus durissus terrificus as previously described (33). Enhanced chemiluminescence Western blotting reagents were from Amersham Biosciences. Poly-(Glu-U-Tyr)_n calpains inh1, RGDS, and other chemical products were from Sigma. The phosphatase inhibitor PTP InhI was from Calbiochem. (γ-32P)ATP (3000 Ci/mmol) was from PerkinElmer Life Sciences. Ni-NTA ProBond resin was from Invitrogen.

GST-LAT cDNA was generated by inserting the 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 glutathione-Sepharose beads using Amersham Biosciences 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^115 to Ser mutation (GST-PTP1B C215S) was a gift from Dr. J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA). The full-length cDNA construct PTP1B (D181A) obtained from Dr. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was subcloned in pTAT-HA-His vector (24) as a Nod/EcoRI fragment. Isolation of LAT fusion protein was realized after sonication of bacteria in 8 x 10^8 units HEPESS (K17.2) 100 mM NaCl buffer, 20 mM imidazole; the clarified sonicate was applied to Ni-ProBond resin. LAT fusion protein elution was performed by the same buffer containing 100 mM imidazole. Detection of the LAT-HA-His PTP1B (D181A) fusion protein was performed by Western blotting using anti-HA or anti-PTP1B antibodies. Quantification of the purified proteins 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 FcγRIIα cross-linking were performed as previously described (35). Platelet aggregation was measured at 37 °C by a turbidimetric method using a dual-channel aggregometer (Payton Associates, Scarborough, Ontario, Canada). In some experiments, platelets were not shocked. To test the effect of inhibitors on LAT tyrosine phosphorylation, platelet suspensions (1 x 10^9 platelets/ml) were incubated for 3 min at 37 °C with 500 nM 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 the 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 of platelets, and cells were incubated at 37 °C for 20 min before stimulation.

Mouse platelets were prepared according to the following protocol: C57Bl/6 mice were anesthetized with a mix of Imalgene (ketamine) and Rompun, and blood was collected by cardiac puncture with a heparin-coated syringe (100 units/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 Na2HPO4, 20 mM HEPESS, pH 7.3, 1 mM MgCl2, 5 mM glucose) and finally suspended in the same buffer at a density of 8 x 10^9 platelets/ml. Stimulations of mouse platelets were carried out with 5 mM convulxin at 37 °C under stirring conditions.

**Isolation of Platelet Cytoskeleton**—500 μl of human platelets (1 x 10^9 platelets/ml) were activated by FcγRIIα cross-linking for indicated times; stimulation was stopped by addition of cytoskeleton buffer (CSB) to give a final concentration of 50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 1% Triton-X100, 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) and washed three times with CSK buffer. Cytoskeletal proteins were sonicated three times for 10 s in Lammeli sample buffer and then submitted to Western blotting using appropriate antibodies.

**Immunoprecipitation and Immunoblotting**—500 μl of 1 x 10^9/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 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. 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 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 (50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 1% SDS) and boiled for 2 min. The final volume was brought to 1.5 M PTP InhI added 1 min after FcγRIIα cross-linking. The reaction was stopped by 250 μl of 3 x RIPA buffer, and the clarified lysate was incubated with GST-PTP1B (C215S) for 2 h at 4 °C. Beads were washed, and the protein complexes from pulldown were analyzed by addition of 60 μl of RIPA buffer containing 6M urea, 12% SDS, 1% DTT, 1% SDS) and boiled for 2 min. The final volume was brought to 1.5 M by the addition of RIPA buffer containing 0.5% Triton X-100, and antiphosphotyrosine immunoprecipitation was performed with 4G10 antibody followed by Western blotting using LAT antibody.
Immune Complex Protein Phosphatase Assay and in Vitro LAT Dephosphorylation—Enzymatic activity of PTP1B in platelets was determined using paranitrophenyl phosphate as substrate. PTP1B immune complexes were washed twice with PTP assay buffer (62 mM HEPES, pH 7.5, 6.25 mM EDTA, 12.5 mM dithiothreitol) and incubated with 25 mM final concentration of paranitrophenyl phosphate for 30 min 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 of FcγRIIa stimulation as described above. PTP1B immune complex was realized 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 min of incubation at 30 °C under shaking, the reactions were stopped by the addition of sample buffer and detection of the phosphoproteins was performed by SDS-PAGE, followed by antiphosphotyrosine immunoblotting.

In-gel Phosphatase Assay—These experiments were performed according to the procedure of Burridge and Nelson (36) with some modifications. Briefly, 2 mg of poly(Glu4-Tyr1)n were phosphorylated by incubation overnight at 30 °C with ppp60⁰⁰⁰ kinase immunoprecipitated from thrombin-activated platelets and 20 μCi of [γ-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% trichloroacetic acid. After 30 min on ice, labeled poly(Glu4-Tyr1)n was dissolved in 200 μL of Tris buffer 0.75 M, pH 8.8, and incorporated in SDS-polyacrylamide running gel prior to polymerization at −10⁷ cpm/mL. Platelet lysates or LAT immunoprecipitates 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 fibronectin-coated coverslips at the concentration of 100 μg/mL during 1 h at 37 °C. Cells were washed with phosphate-buffered saline and fixed with formaldehyde 3%, 30 min at room temperature. Then they were permeabilized with 0.01% Triton X-100 in phosphate-buffered saline for 10 min at room temperature. Nonspecific sites were saturated with 3% bovine serum albumin in phosphate-buffered saline for 30 min. Platelets were incubated with monoclonal anti-HA antibody followed by fluorescein-conjugated anti-mouse Ig secondary antibody mixed with rhodamine-conjugated phalloidin. Slides were examined under a Zeiss confocal microscope (LSM 510, Axiovert 100) using immersion objective ×63.

**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 that 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 preincubation 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. Because calpains have been shown to regulate some tyrosine dephosphorylation events downstream of integrins in platelets, we investigated the effect of a calpains inhibitor (calpains inh1) on LAT phosphorylation status. As shown in Fig. 1D, LAT dephosphorylation was partly inhibited by this inhibitor, suggesting an implication of calpains-regulated PTPs downstream of integrins 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 under "Experimental Procedures."
Procedures.” Fig. 2A shows that resting platelets developed two basal PTP activities at 50 and 42 kDa. After 3 min of 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 mass between 40 and 55 kDa. Some other PTPs presenting higher molecular mass were also detected during platelet aggregation with modest activation level (data not shown). Moreover, experiments performed with RGDS preincubated platelets show that the increase in the 42-kDa species at 5 min of 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 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 Fig. 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, Fig. 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.

**Fig. 2.** PTP1B activation in FcγRIIa-stimulated human platelets. A, aliquots of whole platelet lysates were subjected to In-gel tyrosine phosphatase assay as described under “Experimental Procedures” (upper panel). Effects of RGDS (500 μM) or PTP InhI (12.5 μM) on tyrosine phosphatase activity were investigated at 5 min of 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 of stimulation was performed using PTP1B antibody. The supernatant was subjected to In-gel phosphatase analysis: cell lysate precleared with protein G-Sepharose beads (lane 1); cell lysate following one-way immunodepletion of PTP1B (lane 2). This experiment was performed twice with similar results. C, Western blotting of anti-PTP1B was 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 preincubated 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 pretreated with RGDS (500 μM) or not were stimulated by FcγRIIa cross-linking for 5 min. PTP1B activities were expressed in fold increase (mean ± S.D. of three independent experiments) compared with non-stimulated platelets (Control).
In gel phosphatase assay is not always appropriate to quantify platelets. Because 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 of platelet stimulation (Fig. 2). Moreover, pretreatment 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 regulate the cleavage of PTP1B and modulate 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 Fig. 3, the association with the cytoskeleton fraction upon FcγRIIa-mediated platelet activation reached a maximum at 2 min and persisted until 5 min. Interestingly, PTP1B was found in the same compartment after 2 min of 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 of stimulation (maximum of PTP1B activation). As shown in Fig. 4A, LAT was tyrosine-dephosphorylated by PTP1B in vitro. Interestingly, PTP1B immunoprecipitated from platelets pretreated with RGDS was less effective at dephosphorylating LAT. Moreover, pulldown experiments using the recombinant GST substrate-trapping PTP1B (C215S) (41), 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 Fig. 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 2–5 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 mouse platelets demonstrated that the PTPs associated with LAT were totally different in these species. Indeed, the in-gel phosphatase assay of immunoprecipitated LAT from mouse 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 characterize 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).

Because 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 pp60c-src, a known PTP1B substrate (27) (Fig. 6C). This treatment did not alter LAT tyrosine phosphorylation at 1 min of 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 permant molecule described as a powerful PTP1B inhibitor (43). Interestingly, addition of this inhibitor after 1, 2, or 3 min of 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 Fig. 7B, PTP Inh1 added 1 or 2 min after the agonist totally inhibited LAT dephosphorylation classically detected after 5 min of stimulation. This inhibition was less effective when the inhibitor was added after 3 or 4 min of 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 irreversible cell aggregation.

**DISCUSSION**

In T-lymphocyte, the docking protein LAT plays a critical role and is involved in PLCγ1 activation after T-cell receptor engagement (44). Platelets express LAT at a high level, allowing further investigations of this signaling protein in this peculiar hematopoietic cell where it has been shown to play a role in PLCγ2 activation (14). Here, we show that in human plate-
**FIG. 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 analyzed by anti-phosphotyrosine Western blotting (WB-Yp) using 4G10 antibody. In lane 3, IP-PTP1B was carried out from platelets pretreated with 500 μM RGDS. B, lysates of resting or FcγRIIa-stimulated platelets (5 min) in the absence or in the presence of PTP Inh I (12.5 μM) were incubated with GST-PTP1B (C215S) fusion protein. Proteins in the complex were subjected to re-immunoprecipitation with 4G10 antibody. These data were obtained three times with similar results.

**FIG. 5.** PTP1B is associated with LAT in FcγRIIa cross-linking or GPVI-activated human platelets but not in GPVI-activated mouse platelets. A, IP-LAT performed from resting or FcγRIIa-activated platelets were subjected to In-gel phosphatase assay as described under “Experimental Procedures.” The position of molecular mass is 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 mouse 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 under “Experimental Procedures.” Then, immunoprecipitated proteins were analyzed by Western blotting using anti-LAT antibody. Each profile is representative of three independent experiments showing similar results.
lets stimulated via FcγRIIa cross-linking LAT is strongly and rapidly tyrosine-phosphorylated independently of integrin engagement and platelet aggregation. The time course of LAT phosphorylation is in agreement with previous reports on 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. On 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 that could be the active form generated by calpains cleavage from the 50-kDa PTP1B. The identification of these PTPs (42 and 50 kDa) was obtained by immunodepletion experiments followed by an In-gel phosphatase assay. Immunoblotting experiments confirmed that FcγRIIa cross-linking leads to PTP1B proteolysis

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

A. Western blot anti-HA (WB-HA) was performed on a purified fraction of the fusion protein TAT-HA-PTP1B (D181A) eluted from Ni-ProBond resin. B. platelets were pretreated with purified TAT-HA-PTP1B (D181A) fusion protein. The localization of TAT-HA-PTP1B in platelets was performed using anti-HA antibody as described under "Experimental Procedures." The green fluorescence corresponds to the detection of TAT-HA-PTP1B (Anti-HA, left); red fluorescence to the actin cytoskeleton (phalloidin, right). Control immunofluorescence experiment with anti-HA antibody was performed on non-treated platelets (control). C. cell lysates containing equal amounts 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 analyzed by Western blotting with anti-Src antibody. D. platelets were preincubated (right part) or not (left part) with the cell permeant fusion protein TAT-HA-PTP1B (D181A) (10 µg) for 20 min followed by cell stimulation via FcγRIIa cross-linking at 1 or 5 min. LAT was immunoprecipitated and its tyrosine phosphorylation was analyzed by anti-phosphotyrosine Western blotting (WB-Yp). The amount of LAT immunoprecipitated was analyzed by Western blotting anti-LAT (WB-LAT). This experiment was performed twice with similar results.
from 50 to 42 kDa in platelets after 3 min of stimulation. The size of this generated fragment (42 kDa) and its absence in platelets pretreated with calpains inhibitor suggest that PTP1B proteolysis is because of calpains action as described upon thrombin stimulation (16). The comparison between the PTP activities observed in the In-gel PTPs assay and the PTP1B detection by Western blot seems to 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 PTP assay is not appropriate to compare enzymatic activities because some enzymes refold better than others and will appear more active (46). For this reason, we measured PTP activities from IP-PTP1B and we observed about a 2-fold increase in PTP1B activity, dependent on integrin \( \alpha IIb/\beta 3 \) engagement, after 5 min of Fc\( \gamma RIIa \) cross-linking. A similar result was previously reported in platelets activated with calcium ionophore A 23187 in which the activated calpains totally cleaved

**Fig. 7. Effect of PTP inhibition on platelet aggregation and LAT phosphorylation.** A, \( 5 \times 10^8 \) human platelets were activated by Fc\( \gamma RIIa \) cross-linking, and PTP Inh I (12.5 \( \mu M \)) was added at different times 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 of 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 in the left part of the panel. After stripping, the membrane was reprobed with anti-LAT antibody (lower panel). Data shown are representative of three independent experiments.

**Fig. 8. Proposed model highlighting the role of PTP1B in the regulation of LAT phosphorylation in Fc\( \gamma RIIa \)-mediated human platelets stimulation.** In resting platelets, PTP1B is maintained in a relative inactive form with the C-terminal tail anchored in the endoplasmic reticulum. Fc\( \gamma RIIa \) cross-linking leads to LAT 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 then participates in the dephosphorylation of LAT on some critical phosphotyrosine sites, resulting in protein multicomplex dissociation. Cytoskeletal rearrangement and focal adhesion organization will then allow platelet irreversible aggregation to take place (see “Discussion” for more details).
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 pretreated with a calpains inhibitor that 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 proteins containing the phosphotyrosine substrate trapping PTP1B (D181A) and the peptide TAT from human immunodeficiency virus 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 because of a limited quantity of fusion protein reaching phosphorylated LAT, resulting in a partial blocking effect of this molecule. However, because of the fact that LAT possesses ten potential phosphotyrosyl residues, it is also possible that other PTPs are involved in LAT dephosphorylation.

It is well demonstrated that PTPs exhibit strict substrate specificity, and the challenge concerning PTP 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 specific 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 Tyrp (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 as interacting 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” compartments that 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 because 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 maintenance of platelet aggregation, possibly through reinforcement of aggregates via reorganization 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 remodeling 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 in mediating cellular events and gives the notion that differential localization patterns of PTP1B serve a specific cellular function. At 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 PTP activities (data not shown). Finally, In-gel PTP assays performed on LAT immunoprecipitates 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 mouse 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 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 hemostatic disorders occurring in treated patients.

Acknowledgments—We are grateful to Dr. J. Chernoff for the gift of PTP1B (C215S), Dr. N. Tonks for the gift of PTP1B (D181A), and Dr. Dowdy for the TAT plasmid gift. We thank Dr. M. Jandrot-Perrus for providing purified convulxin and Dr. S. Muller-Valitutti for taking part in confocal fluorescence microscopy. We appreciate the helpful discussions with Dr. C. Racaud-Sultan. We thank C. Soulet for help in platelet preparation and advice in the preparation of the figures.

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