

PTP-1B is an essential positive regulator of platelet integrin signaling

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Outside-in integrin α IIb β 3 signaling is required for normal platelet thrombus formation and is triggered by c-Src activation through an unknown mechanism. In this study, we demonstrate an essential role for protein-tyrosine phosphatase (PTP)-1B in this process. In resting platelets, c-Src forms a complex with α IIb β 3 and Csk, which phosphorylates c-Src tyrosine 529 to maintain c-Src autoinhibition. Fibrinogen binding to α IIb β 3 triggers PTP-1B recruitment to the α IIb β 3-c-Src-Csk complex in a manner that is dependent on c-Src and specific tyrosine (tyrosine 152 and 153) and proline

(proline 309 and 310) residues in PTP-1B. Studies of PTP-1B-deficient mouse platelets indicate that PTP-1B is required for fibrinogen-dependent Csk dissociation from α IIb β 3, dephosphorylation of c-Src tyrosine 529, and c-Src activation. Furthermore, PTP-1B-deficient platelets are defective in outside-in α IIb β 3 signaling in vitro as manifested by poor spreading on fibrinogen and decreased clot retraction, and they exhibit ineffective Ca^{2+} signaling and thrombus formation in vivo. Thus, PTP-1B is an essential positive regulator of the initiation of outside-in α IIb β 3 signaling in platelets.

Introduction

Integrins mediate cell adhesion to extracellular matrix ligands. In addition to localizing cells for proper biological function, ligand binding to integrins initiates a process referred to as outside-in signaling (Hynes, 2002). Integrin signals collaborate with signals from growth factor, cytokine, and G protein-coupled receptors to regulate actin rearrangements and cell motility, growth, differentiation, and survival (Juliano et al., 2004). Because the cytoplasmic domains of integrin α and β subunits are devoid of catalytic activity, integrins must associate with intracellular enzymes to transduce signals. Associations between integrins and specific receptor and nonreceptor protein kinases have been demonstrated by biochemical, microscopic, and biophysical techniques (Brunton et al., 2004; de Virgilio et al., 2004). However, many of these associations take place relatively late after adhesive ligand binding, suggesting that they propagate rather than initiate outside-in signaling. One exception is in platelets, in which a constitutive association between integrin α IIb β 3 and c-Src is mediated by direct interaction of the β 3 cytoplasmic domain with the c-Src SH3 domain (Oberfell et al., 2002; Arias-Salgado et al., 2003). A similar relationship

may pertain to c-Src and the related integrin, α V β 3, in osteoclasts (Feng et al., 2001). Furthermore, in many cell types, a close functional, if not physical, relationship exists between Src family kinases and β 1 or β 2 integrins (Klinghoffer et al., 1999; Suen et al., 1999; Brunton et al., 2004).

α IIb β 3 mediates fibrinogen-dependent platelet aggregation and spreading on damaged vascular surfaces, whereas α V β 3 promotes osteoclast adhesion to vitronectin or osteopontin (Byzova et al., 1998; Shattil and Newman, 2004). Genetic deficiency of α IIb β 3 and α V β 3 leads to defects in hemostasis and bone remodeling, respectively (Hodivala-Dilke et al., 1999; Feng et al., 2001). Adhesive ligand binding to β 3 integrins leads to c-Src activation and tyrosine phosphorylation of c-Src substrates in platelets and osteoclasts (Feng et al., 2001; Oberfell et al., 2002; Arias-Salgado et al., 2003). The close relationship between β 3 integrins and c-Src is underscored by defective spreading of platelets that are deficient in multiple Src family kinases (Oberfell et al., 2002) and by overlapping bone remodeling phenotypes in mice that are deficient in c-Src or β 3 (Soriano et al., 1991; Hodivala-Dilke et al., 1999; McHugh et al., 2000). Consequently, attention is now focused on how β 3 integrins regulate c-Src to initiate outside-in signaling.

c-Src is maintained in an autoinhibited state by concerted intramolecular interactions of the SH2 domain with a COOH-terminal motif centered at phosphotyrosine 529 and of the SH3

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Abbreviation used in this paper: PTP, protein-tyrosine phosphatase.

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domain with a polyproline sequence in the linker region between the SH2 and kinase domains (Sicheri and Kuriyan, 1997; Young et al., 2001; Harrison, 2003). As c-Src appears to associate constitutively with $\beta 3$ integrins via the c-Src SH3 domain (Arias-Salgado et al., 2003), considerable reliance may be placed on the SH2-phosphotyrosine 529 interaction to help maintain low c-Src activity in nonadherent platelets. Thus, disruption of the SH2-phosphotyrosine 529 interaction by dephosphorylation of c-Src tyrosine 529 should facilitate c-Src activation during cell adhesion. Phosphorylation of c-Src tyrosine 529 is catalyzed by Csk, which is associated with the α IIb β 3-c-Src complex in resting platelets (Okada et al., 1991; Oberfell et al., 2002; Arias-Salgado et al., 2003). However, the identity of the protein-tyrosine phosphatase (PTP) that dephosphorylates c-Src tyrosine 529 to promote initiation of $\beta 3$ integrin signaling has remained unknown. In this study, we used biochemical and genetic approaches to unambiguously identify PTP-1B, which is a ubiquitous nonreceptor tyrosine phosphatase, as a phosphatase that is required for dephosphorylation of c-Src tyrosine 529 and for c-Src activation downstream of α IIb β 3. Moreover, we demonstrate that PTP-1B is required for outside-in signaling in platelets and for normal platelet thrombus formation in living mice.

Results

PTP-1B associates with α IIb β 3 and is required for integrin activation of c-Src

To explore how α IIb β 3 regulates c-Src, we sought to identify a PTP that localizes to the α IIb β 3-c-Src complex in response to fibrinogen binding to platelets. We reasoned that this might reverse phosphorylation of c-Src tyrosine 529 by Csk and, thereby, help to promote c-Src activation (Oberfell et al., 2002; Arias-Salgado et al., 2003). A previous study has demonstrated that PTP-1B is localized to internal membranes of resting platelets and is cleaved by calpain in a platelet aggregation-dependent manner (Frangioni et al., 1993). We found that PTP-1B coimmunoprecipitated with α IIb β 3 and c-Src from detergent lysates of human and mouse platelets. However, unlike the associations of c-Src and Csk with α IIb β 3, which are observed in resting platelets (Oberfell et al., 2002), the association of PTP-1B with α IIb β 3 and c-Src required fibrinogen binding to platelets. This was induced either by MnCl₂, which activates α IIb β 3 directly (Fig. 1 a; Litvinov et al., 2004), or by plating the cells on fibrinogen (not depicted). PTP-1B recruitment to α IIb β 3 in response to MnCl₂ and fibrinogen did not require PTP-1B cleavage by calpain because platelet aggregation was avoided under these unstirred conditions, and no such cleavage was observed. The interaction of PTP-1B with α IIb β 3 was specific and was observed whether immunoprecipitation was performed with antibodies to PTP-1B or α IIb β 3 (Fig. 1 b). The interactions of PTP-1B with α IIb β 3 and c-Src were prevented by pretreatment of platelets with 2 μ M SU6656 or 5 μ M PP2 to block Src kinase activity (Fig. 1 a) or with 2 mM RGDS (Arg-Gly-Asp-Ser) to inhibit fibrinogen binding.

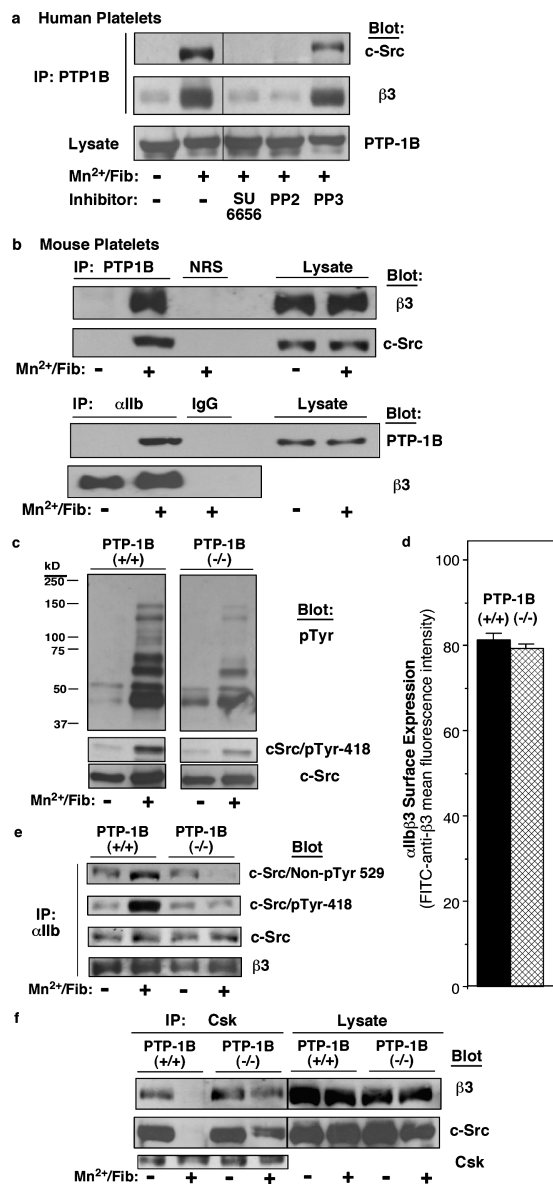


Figure 1. Interactions between PTP-1B, α IIb β 3, and c-Src in platelets. (a) Washed human platelets were incubated for 15 min at RT with 250 μ g/ml fibrinogen in the presence or absence of 0.5 mM MnCl₂. Some samples were preincubated for 15 min with 2 μ M SU6656, 5 μ M PP2, or 5 μ M PP3; the latter is an inactive congener of PP2. Clarified lysates were immunoprecipitated (IP) and probed on immunoblots as indicated. Vertical lines in the blots indicate grouping of images from different parts of the same gel. (b) Washed mouse platelets were incubated with MnCl₂ and fibrinogen, and immunoblots of immunoprecipitates were probed as in a. Control immunoprecipitations used normal rabbit serum (NRS) or rat IgG (IgG). (c) Role of PTP-1B in platelet tyrosine phosphorylation. Fibrinogen binding to PTP-1B^{+/+} and PTP-1B^{-/-} platelets was induced as in b. Lysates were immunoblotted with antibodies to phosphotyrosine (pTyr) or c-Src phosphotyrosine 418 and reprobbed with antibodies to c-Src. (d) α IIb β 3 surface expression in PTP-1B^{+/+} (black bar) and PTP-1B^{-/-} (hatched bar) platelets was quantified by flow cytometry. Mean fluorescence intensities are depicted in arbitrary units, and error bars represent means \pm SEM of three experiments. (e) PTP-1B is required for activation of integrin-associated c-Src. Fibrinogen binding to PTP-1B^{+/+} and PTP-1B^{-/-} platelets was induced as in b, and α IIb β 3 immunoprecipitates were probed on immunoblots as indicated. (f) PTP-1B is required for dissociation of Csk from the α IIb β 3-c-Src complex. Fibrinogen binding to PTP-1B^{+/+} and PTP-1B^{-/-} platelets was induced as in b, and Csk immunoprecipitates were probed on immunoblots as indicated. Each immunoblot panel is representative of three to five independent experiments.

Fibrinogen-dependent PTP-1B recruitment to α IIB β 3 and c-Src was also observed in response to platelet stimulation with traditional agonists, such as ADP and thrombin (unpublished data). However, in the studies that follow, MnCl₂ or platelet adhesion were used to induce fibrinogen binding to α IIB β 3 to prevent or minimize generalized signaling via G protein-coupled receptors and, thus, to facilitate direct assessment of outside-in α IIB β 3 signaling (Oberfell et al., 2002; Arias-Salgado et al., 2003). Overall, these results indicate that fibrinogen binding to α IIB β 3 triggers recruitment of PTP-1B to a plasma membrane complex of α IIB β 3 and c-Src in a manner that is dependent on Src kinase activity.

To establish whether PTP-1B is required for integrin activation of c-Src, platelets from knockout mice that were deficient in PTP-1B (PTP-1B^{-/-}) and wild-type (PTP-1B^{+/+}) littermates were studied (Klaman et al., 2000). Incubation of wild-type platelets with MnCl₂ and fibrinogen caused an increase in the tyrosine phosphorylation of numerous proteins. In contrast, PTP-1B^{-/-} platelets showed markedly reduced fibrinogen-dependent tyrosine phosphorylation (Fig. 1 c). Because several of the phosphorylated proteins, including Syk (72 kD) and adhesion and degranulation-promoting adaptor protein (130 kD), are substrates of c-Src during outside-in α IIB β 3 signaling, the catalytic activity of c-Src in α IIB β 3 immunoprecipitates was assessed indirectly by monitoring the phosphorylation of activation loop tyrosine 418. Whereas fibrinogen binding to PTP-1B^{+/+} platelets stimulated phosphorylation of c-Src tyrosine 418, this response was minimal or absent in PTP-1B^{-/-} platelets (Fig. 1, c and e). Platelets from heterozygous (PTP-1B^{+/-}) littermates responded normally (not depicted). The defective responses of PTP-1B^{-/-} platelets could not be explained by reduced surface expression of α IIB β 3 receptors (Fig. 1 d). These results suggest that PTP-1B^{-/-} platelets have a fundamental defect in α IIB β 3 activation of c-Src.

To determine whether PTP-1B is required for fibrinogen-dependent dephosphorylation of c-Src tyrosine 529, the phosphorylation state of tyrosine 529 was monitored with an antibody specific for nonphosphorylated tyrosine 529. Whereas fibrinogen binding to wild-type platelets stimulated dephosphorylation of c-Src tyrosine 529 (as indicated by increased immunoreactivity of the dephosphotyrosine 529 antibody), no such dephosphorylation was observed in PTP-1B^{-/-} platelets. In fact, the level of tyrosine 529 phosphorylation paradoxically increased upon fibrinogen binding (Fig. 1 e). Thus, PTP-1B is required for α IIB β 3-dependent dephosphorylation of c-Src tyrosine 529, likely explaining the defective activation of c-Src in PTP-1B^{-/-} platelets.

The finding of relatively increased phosphorylation of c-Src tyrosine 529 in fibrinogen-bound PTP-1B^{-/-} platelets suggested that PTP-1B may play some unexpected role in the phosphorylation of tyrosine 529 by Csk. Csk is normally associated with the α IIB β 3-c-Src complex in resting platelets and dissociates from it upon fibrinogen binding (Oberfell et al., 2002). However, Csk failed to fully dissociate from α IIB β 3 and c-Src after fibrinogen binding to PTP-1B^{-/-} platelets (Fig. 1 f). Thus, PTP-1B may not only dephosphorylate c-Src tyrosine 529 upon fibrinogen binding to α IIB β 3 (Arregui et al., 1998;

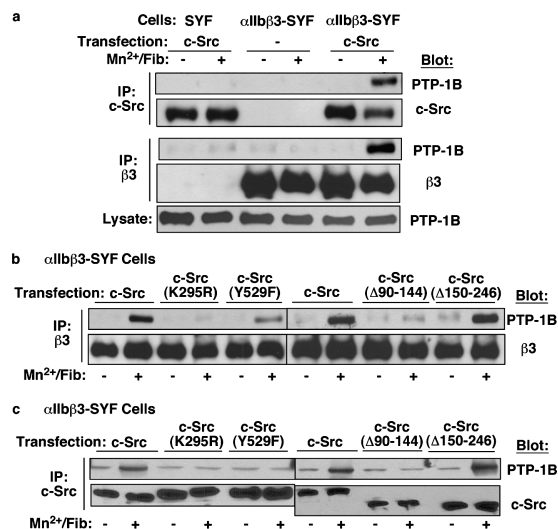


Figure 2. Structural features of c-Src that are required for interactions with PTP-1B and α IIB β 3. (a) SYF cells or SYF cells stably expressing human α IIB β 3 (α IIB β 3-SYF) were transiently transfected with wild-type c-Src or empty vector. After 48 h, transfected cells were incubated at 37°C for 15 min in the presence or absence of 1 mM MnCl₂ and 250 μ g/ml fibrinogen. Clarified lysates were immunoprecipitated with antibodies to c-Src or β 3, and immunoprecipitates were probed on immunoblots as indicated. Lysates were probed for PTP-1B as a loading control. (b and c) α IIB β 3-SYF cells were transfected with wild-type c-Src or an indicated c-Src mutant. After 48 h, transfected cells were incubated in the presence or absence of MnCl₂ and fibrinogen as in a. Clarified lysates were immunoprecipitated with antibodies to β 3 (b) or c-Src (c) and with the precipitates probed on immunoblots. Data are from a single experiment that was representative of three that were performed.

Dadke and Chernoff, 2002) but may also regulate the interaction between Csk and the α IIB β 3-c-Src complex.

Mechanism of PTP-1B- α IIB β 3-c-Src interactions

To better understand the basis for interactions between PTP-1B, α IIB β 3, and c-Src during outside-in α IIB β 3 signaling, mouse fibroblasts that were deficient in the ubiquitous Src family kinases c-Src, c-Yes, and Fyn (SYF cells; Klinghoffer et al., 1999) were stably transfected with α IIB β 3. These α IIB β 3-SYF cells express PTP-1B endogenously, enabling examination of PTP-1B interactions after transient transfection of c-Src. As observed with platelets, α IIB β 3-SYF cells expressing c-Src showed a fibrinogen-inducible association of PTP-1B with c-Src and α IIB β 3 (Fig. 2 a). However, PTP-1B failed to associate with c-Src in cells lacking α IIB β 3 or with α IIB β 3 in cells lacking c-Src. Thus, the fibrinogen-dependent interaction of PTP-1B with α IIB β 3 or c-Src requires both integrin and tyrosine kinase.

To determine what portions of the c-Src molecule are required for these PTP-1B interactions, α IIB β 3-SYF cells were transfected with selected c-Src mutants. Coimmunoprecipitation of PTP-1B with α IIB β 3 did not occur with catalytically inactive c-Src (K295R) or with c-Src lacking the SH3 domain (Δ 90-144). Identical results were obtained with c-Src SH3 domain mutants (W120F or Δ 90-92) that were incapable of interacting with polyproline type II motifs or the β 3 cytoplasmic domain (unpublished data). In contrast, c-Src tyrosine 529 (Y529F) and the c-Src SH2 domain (Δ 150-246) were dispensable for the interac-

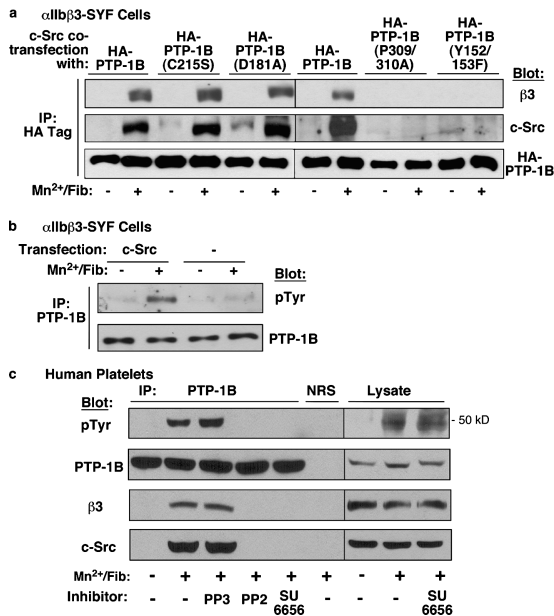


Figure 3. Structural features of PTP-1B that are required for interactions with α IIb β 3 and c-Src. (a) α IIb β 3-SYF cells were transiently cotransfected with c-Src and wild-type or mutant forms of HA-tagged human PTP-1B. After 48 h, transfected cells were incubated with or without MnCl₂ and fibrinogen as described in Fig. 2. Clarified lysates were immunoprecipitated with antibodies to the HA tag, and precipitates were probed on immunoblots as indicated. (b and c) PTP-1B is tyrosine phosphorylated in response to fibrinogen binding. α IIb β 3-SYF cells transfected with c-Src and empty vector (b) or human platelets (c) were incubated with or without 0.5 mM MnCl₂ and 250 μ g/ml fibrinogen for 10 min. Some platelet samples were preincubated for 15 min with c-Src inhibitors (5 μ M PP2 or 2 μ M SU6656) or 5 μ M PP3 as a control. Clarified lysates were immunoprecipitated with an antibody to PTP-1B, and immunoprecipitates and lysates were probed on immunoblots. Data are from a single experiment that was representative of three that were performed. NRS, normal rabbit serum.

tion of PTP-1B with α IIb β 3 (Fig. 2 b). Similar results were obtained for the interaction of PTP-1B with c-Src except that c-Src tyrosine 529 was also required (Fig. 2 c). The requirement for the c-Src SH3 domain might be explained by the direct binding of SH3 to the β 3 cytoplasmic domain (Arias-Salgado et al., 2003) rather than binding of c-Src SH3 to PTP-1B. Together with the platelet results (Fig. 1 a), these outcomes indicate that association of PTP-1B with the α IIb β 3–c-Src complex is regulated by c-Src catalytic activity and by a process that requires tyrosine 529.

To establish what regions of PTP-1B are required for these interactions, HA-tagged PTP-1B was cotransfected with c-Src into α IIb β 3-SYF cells. Wild-type PTP-1B and two different phosphatase-inactive “substrate-trapping” mutants (C215S and D181A) each interacted with α IIb β 3 and c-Src (Fig. 3 a). Interestingly, the interaction with c-Src was somewhat greater with the D181A PTP-1B mutant, which is known to exhibit a higher affinity for binding to PTP-1B substrates than the C215S mutant (Flint et al., 1997). These data are consistent with a direct dephosphorylation of c-Src tyrosine 529 by PTP-1B. In contrast to substrate-trapping mutants, the double mutation of proline 309 and 310 to alanine prevented PTP-1B interaction with α IIb β 3 and c-Src, as did the double mutation of tyrosine 152 and 153 to phenylalanine. These amino acid

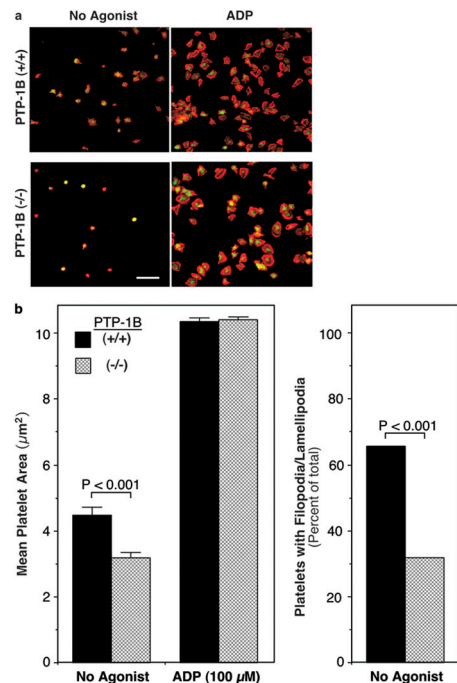


Figure 4. PTP-1B^{-/-} platelets are defective in α IIb β 3-dependent spreading on fibrinogen. (a) Platelets from PTP-1B^{+/+} and PTP-1B^{-/-} mice were plated on fibrinogen-coated coverslips for 40 min at RT in the presence or absence of 100 μ M ADP. Adherent cells were fixed, permeabilized, and stained with rhodamine-phalloidin (F-actin, red) and antiphosphotyrosine antibodies (green). Images were acquired with a confocal fluorescence microscope. Bar, 10 μ m. (b) Platelet surface areas from at least 25 images were analyzed and depicted in the left panel as means \pm SEM. The right panel depicts the percentage of platelets containing one or more filopodia and/or lamellipodia. At least 80 cells each were analyzed.

residues may help to mediate interactions of PTP-1B with one or more members of the integrin signaling complex during the early phase of outside-in signaling (Dadke and Chernoff, 2002). In addition, they may enable the phosphorylation of PTP-1B by c-Src because PTP-1B can phosphorylate c-Src in vitro (Jung et al., 1998), and fibrinogen binding to α IIb β 3-SYF cells (Fig. 3 b) or platelets (Fig. 3 c) stimulated tyrosine phosphorylation of PTP-1B in a Src-dependent manner.

PTP-1B regulates platelet functions that are dependent on outside-in α IIb β 3 signaling

Outside-in signaling via α IIb β 3 facilitates platelet spreading on fibrinogen and platelet thrombus formation under conditions of flow (Phillips et al., 2001; Nesbitt et al., 2002; Shattil and Newman, 2004). Therefore, these responses were compared in PTP-1B^{-/-} and PTP-1B^{+/+} platelets. PTP-1B^{-/-} platelets attached but failed to spread on fibrinogen over 45 min, whereas PTP-1B^{+/+} platelets exhibited cytoskeletal reorganization, filopodial and lamellipodial extensions, and varying degrees of spreading (Fig. 4 a, no agonist). When spreading was assessed by computer analysis of mean platelet areas and the percentage of platelets with filopodia or lamellipodia was quantified, the differences between PTP-1B^{-/-} and PTP-1B^{+/+} platelets were statistically significant ($P < 0.001$; Fig. 4 b).

The stimulation of platelets with a G protein-coupled receptor agonist such as ADP results in more rapid and uniform platelet spreading on fibrinogen when compared with cells incubated without agonist (Haimovich et al., 1993). Thus, in addition to α Ib β 3 signaling, costimulatory pathways are involved in full platelet spreading. In contrast to the spreading defect of untreated PTP-1B^{-/-} platelets, costimulation with ADP resulted in uniform, full spreading (Fig. 4, a and b). PTP-1B^{-/-} platelets adhered normally to fibrinogen (Fig. 5 a), and they bound soluble fibrinogen normally in response to either ADP, PAR4 receptor-activating peptide, or convulxin, which is a glycoprotein VI agonist (Fig. 5 b). In addition, stirred PTP-1B^{-/-} platelets that were incubated with 1–10 μ M ADP or 250 μ M PAR4 receptor-activating peptide exhibited an initial rate and extent of aggregation that was equivalent to those of PTP-1B^{+/+} platelets (unpublished data). On the other hand, PTP-1B^{-/-} platelets mediated less fibrin clot retraction than PTP-1B^{+/+} platelets ($P < 0.05$); this response is dependent, in part, on α Ib β 3-triggered changes in the actin cytoskeleton (Fig. 5 c; Phillips et al., 2001; Shattil and Newman, 2004). Collectively, these results indicate that PTP-1B is required for normal outside-in α Ib β 3 signaling in platelets. However, PTP-1B appears to be dispensable for agonist induction of soluble fibrinogen binding to α Ib β 3 and for ADP costimulation of platelet spreading.

Thrombus formation can be studied in living mice by real-time fluorescence and brightfield microscopy of cremaster muscle arterioles that were subjected to laser injury (Falati et al., 2002). Platelets from PTP-1B^{-/-} and PTP-1B^{+/+} mice were labeled with the Ca²⁺-sensitive fluorescent dye Fura 2 and were reinfused into PTP-1B^{-/-} and PTP-1B^{+/+} mice, respectively. Labeled donor platelets accounted for ~20% of total platelets in the recipients. This enabled quantification of fluorescent platelet accumulation and mobilization of intracellular Ca²⁺ in developing thrombi at sites of laser injury (Fig. 6 and Videos 1 and 2, available at <http://www.jcb.org/cgi/content/full/jcb.200503125/DC1>). As described previously for other normal mouse platelets (Falati et al., 2002), PTP-1B^{+/+} platelets accumulated into a growing thrombus for 60–120 s, and some platelets detached over the course of several minutes. Platelet calcium mobilization increased over roughly the same time course. In contrast, the quantity of PTP-1B^{-/-} platelets that incorporated into a growing thrombus was markedly reduced, and those platelets that did become incorporated tended to detach rapidly and exhibited little calcium mobilization (Fig. 6 and Videos 1 and 2). Similar results were obtained when labeled PTP-1B^{-/-} platelets were reinfused into PTP-1B^{+/+} mice, indicating that the defect was intrinsic to PTP-1B^{-/-} platelets (17 thrombi were analyzed in three PTP-1B^{+/+} mice; not depicted). Thus, in this model of vascular injury, PTP-1B is required for calcium mobilization and stable platelet accumulation into growing thrombi.

PTP-1B^{-/-} mice did not exhibit spontaneous bleeding, but their mean tail bleeding times were nominally longer than those of controls (although this difference was not statistically significant: PTP-1B^{-/-}, 254 \pm 53 s; PTP-1B^{+/+}, 198 \pm 19 s; $P < 0.06$, $n = 14$ mice each). However, rebleeding from tail

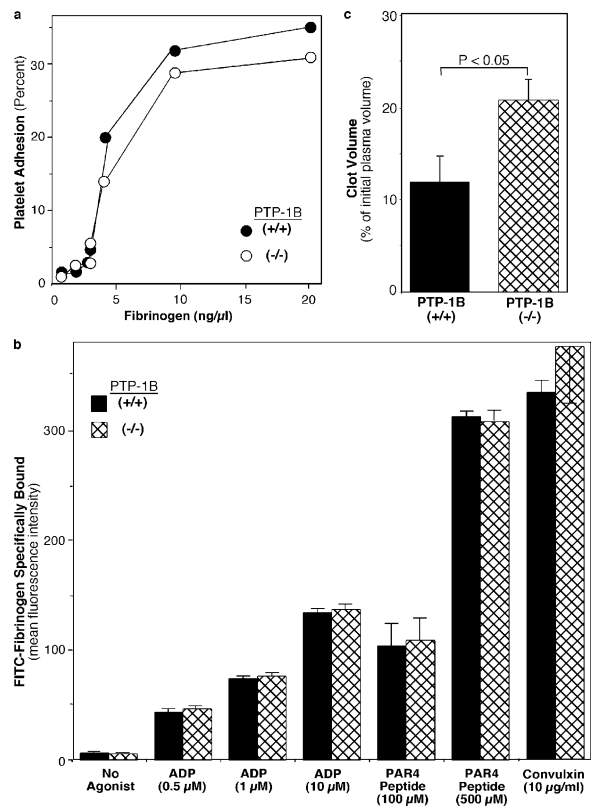


Figure 5. Role of PTP-1B in the interaction of platelets with fibrinogen and fibrin. (a) Platelet adhesion. Washed platelets (1.5×10^6 in 50 μ l incubation buffer) were incubated in fibrinogen-coated microtiter wells for 1 h at RT, and platelet adhesion was quantified. Platelets from at least four mice were used to generate duplicate points at each fibrinogen concentration. (b) Soluble fibrinogen binding. Platelets were incubated at RT for 20 min with 150 μ g/ml FITC-fibrinogen in the presence or absence of ADP, convulxin, or PAR4 receptor-activating peptide (AYPGKF; Faruqi et al., 2000). Fibrinogen binding was analyzed by flow cytometry. Data are the means \pm SEM of quadruplicate determinations from an experiment that was representative of three that were performed. (c) Fibrin clot retraction was assessed 2 h after the addition of thrombin and CaCl₂ to platelet-rich plasma. Clot volumes, expressed as a percentage of the initial volume of platelet-rich plasma, were significantly greater in PTP-1B^{-/-} than in PTP^{+/+} samples, indicating less clot retraction. Data represent means \pm SEM of seven experiments.

wounds after initial bleeding had stopped occurred in 28% of PTP-1B^{-/-} mice but in none of the controls. This pattern of rebleeding has also been observed in mice with a defect in outside-in signaling as a result of tyrosine-to-phenylalanine mutations in the β 3 cytoplasmic domain (Law et al., 1999a).

Discussion

Src family kinases are key components of outside-in integrin signaling to the actin cytoskeleton in hematopoietic and nonhematopoietic cells (Klinghoffer et al., 1999; Obergfell et al., 2002; Lowell, 2004). In particular, c-Src, the most abundant Src family member that is expressed in platelets, can bind directly to the integrin β 3 subunit, and fibrinogen binding to α Ib β 3 triggers c-Src activation (Obergfell et al., 2002; Arias-Salgado et al., 2003). We sought to determine the mechanism by which fibrinogen binding leads to c-Src activation and ex-

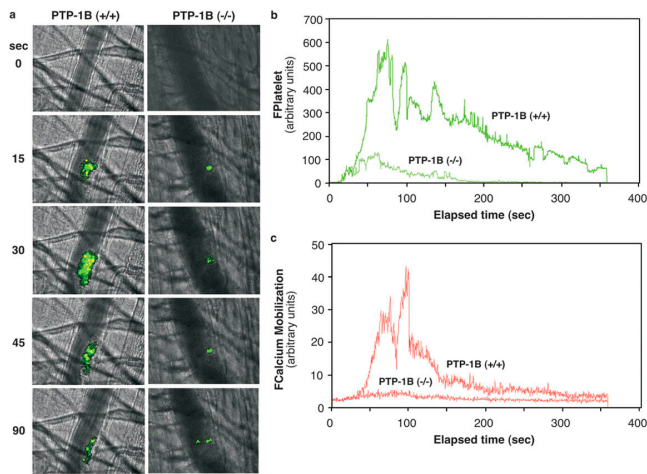


Figure 6. Defective thrombus formation in PTP-1B^{-/-} mice. PTP-1B^{+/+} and PTP-1B^{-/-} platelets were labeled *ex vivo* with Fura 2-AM and were reinfused into PTP-1B^{+/+} and PTP-1B^{-/-} recipient mice, respectively. Then, vessel walls of arterioles in recipient cremaster muscles were subjected to laser injury, and the accumulation of fluorescent platelets into developing thrombi was assessed. (a) Representative composite brightfield and fluorescence images of Fura 2-labeled platelets up to 90 s after laser injury of an arteriole. Green represents labeled platelets and yellow represents cytoplasmic free calcium. Blood flow is from bottom to top. See Videos 1 and 2 (available at <http://www.jcb.org/cgi/content/full/jcb.200503125/DC1>) for examples of thrombus formation in PTP-1B^{+/+} and PTP-1B^{-/-} mice, respectively. (b) Accumulation of fluorescent platelets into the developing thrombus. Fluorescent signal was detected at 510 nm after excitation at 380 nm. FPlatelet is defined as the integrated fluorescence intensity associated with platelets. (c) Calcium mobilization within fluorescent platelets of the developing thrombus. Fluorescent signal was detected at 510 nm after excitation at 340 nm. FCalcium mobilization is defined as the integrated fluorescence intensity associated with calcium mobilization. Each curve in b and c is a composite of 18 independent thrombi generated in three mice (six thrombi per mouse). To analyze these data, all 18 curves were plotted versus time, and median values were determined at each time point and depicted in the figure.

plore the physiological significance of this process. The results establish that (1) fibrinogen binding to platelets leads to PTP-1B recruitment to an α IIB β 3-based signaling complex that includes c-Src and Csk; (2) recruitment of PTP-1B is required for the dissociation of Csk from the complex, dephosphorylation of c-Src tyrosine 529, and c-Src activation; (3) PTP-1B is required for α IIB β 3-dependent platelet spreading on fibrinogen and for normal fibrin clot retraction but not for the agonist-induced activation of α IIB β 3; and (4) deficiency of PTP-1B results in defective platelet thrombus formation in an *in vivo* model of vascular injury.

Although PTPs frequently exert negative regulation of signaling pathways, positive regulation has also been described previously (Neel et al., 2003; Tonks, 2003). In fact, receptor tyrosine phosphatases such as RPTP- α or nonreceptor phosphatases such as Shp2 promote outside-in integrin signaling in fibroblasts, in some cases by dephosphorylating c-Src tyrosine 529 or the equivalent residue in another Src family kinase (Oh et al., 1999; Su et al., 1999). Although PTP-1B has been implicated in β 1 integrin-dependent c-Src activation, this has been observed only in immortalized fibroblasts and not in a primary cell type (Cheng et al., 2001), raising the question as to its physiological significance. Our data establish the *in vivo* rele-

vance of PTP-1B activation of c-Src downstream of a β 3 integrin. PTP-1B may also exert negative regulation of integrin signaling by dephosphorylating c-Src substrates such as p130 Cas (Arregui et al., 1998; Liu et al., 1998; Cheng et al., 2001). Multiple substrates for PTP-1B may exist in platelets, although our results indicate that the dominant action of PTP-1B is the positive regulation of α IIB β 3 signaling through activation of integrin-associated c-Src. In contrast to these results for PTP-1B, platelets from motheaten viable mice that were deficient in Shp1 catalytic function displayed normal α IIB β 3-dependent activation of c-Src (unpublished data) and a morphology upon attachment to fibrinogen that is similar to wild-type platelets (Lin et al., 2004; unpublished data).

The fibrinogen-dependent association of PTP-1B with α IIB β 3 was observed in human and mouse platelets and in a fibroblast model system, enabling examination of its structural basis. PTP-1B recruitment to α IIB β 3 required catalytic competence and the SH3 domain of c-Src (Figs. 1 and 2). Moreover, specific proline (proline 309 and 310) and tyrosine (tyrosine 152 and 153) residues in PTP-1B were necessary (Fig. 3), suggesting that a protein (or proteins) with SH3, SH2, and/or phosphotyrosine-binding domains is involved in mediating linkage of PTP-1B to the integrin complex. Although the linker protein in platelets could be c-Src itself, there is no evidence that the c-Src SH2 domain binds to PTP-1B, and the c-Src SH3 domain may not be available to PTP-1B when it engages the integrin β 3 cytoplasmic domain (Arias-Salgado et al., 2003). Thus, a model is proposed in which PTP-1B is localized in resting platelets to internal membranes (Frangioni et al., 1993). Then, fibrinogen binding induces α IIB β 3 oligomerization (Simmons et al., 1997; Buen-suceso et al., 2003), triggering transautophosphorylation of integrin-associated c-Src. This event might not be sufficient for full c-Src activation (Harrison, 2003), but low level activation might enable c-Src to phosphorylate a protein that is capable of recruiting PTP-1B to the α IIB β 3 complex. After recruitment, PTP-1B may become a substrate for c-Src (Fig. 3, b and c; Jung et al., 1998) and induce further c-Src activation by promoting Csk dissociation from the integrin complex and dephosphorylation of tyrosine 529 (Fig. 1 f).

Although additional studies will be required to determine the mode of PTP-1B linkage to the α IIB β 3 complex in platelets, work in other cells has implicated scaffold or adaptor molecules, such as SHPS-1, PAG/Cbp, and Dok-1, in mediating interactions between Src kinases, PTPs, and/or Csk in response to growth factors or cell adhesion (Timms et al., 1999; Dube et al., 2004; Zhang et al., 2004). However, SHPS-1 is poorly expressed in platelets, and PAG/Cbp does not interact with α IIB β 3 (Wonerow et al., 2002). Intriguingly, Dok-1 contains a phosphotyrosine-binding domain and potential SH2-binding sites and is a substrate for PTP-1B (Dube et al., 2004). Furthermore, Dok-1 binds directly to Csk (Shah and Shokat, 2002) and may associate with integrin β cytoplasmic domains (Calderwood et al., 2003). Dok-2, a homologue of Dok-1, is expressed in platelets (Garcia et al., 2004). In preliminary studies, we have found that Dok-2 coimmunoprecipitates with PTP-1B from resting platelets. In addition, fibrinogen binding to plate-

lets stimulates tyrosine phosphorylation of Dok-2, dissociation of Dok-2 from PTP-1B, and its association with Csk (unpublished data). However, a role for Dok-2 or any other Csk-binding protein (Thomas et al., 1999) in regulating PTP-1B recruitment to α IIB β 3 and outside-in signaling remains to be determined.

Altogether, these studies have established a new function for PTP-1B by uncovering requirements for PTP-1B in integrin-dependent c-Src activation, platelet spreading on fibrinogen, and clot retraction and platelet thrombus formation. Defects in both platelet spreading and clot retraction may be adequately explained by the c-Src activation defect in PTP-1B^{-/-} platelets because both responses require outside-in α IIB β 3 signaling (Phillips et al., 2001; Shattil and Newman, 2004). However, although thrombus formation under flow conditions depends on signaling inputs from multiple platelet receptors, including α IIB β 3 (Ruggeri, 2002; Jackson et al., 2003), it is legitimate to ask whether the impairment of c-Src activation in PTP-1B^{-/-} platelets is the cause of defects in platelet calcium mobilization and thrombus formation, which were observed in the microcirculation of PTP-1B^{-/-} mice (Fig. 6). Although we cannot exclude the possibility of additional unstudied signaling pathways that are affected by a deficiency of PTP-1B, we found no defect in agonist-induced α IIB β 3 activation, platelet aggregation, or agonist costimulation of platelet spreading. Furthermore, the ligation of α IIB β 3 is known to induce calcium transients that are required for the formation of stable platelet aggregates under conditions of flow and wall shear stress, which are typical within arterioles (Mazzucato et al., 2002; Nesbitt et al., 2002). In particular, IP₃ production and calcium mobilization are triggered by Src-dependent activation of phospholipase C γ during outside-in α IIB β 3 signaling (Wonerow et al., 2003). Thus, links between defective integrin activation of c-Src and reduced calcium mobilization provide a plausible explanation for the reduced thrombus formation observed in PTP-1B^{-/-} mice.

In contrast to the reduced platelet thrombus formation in cremasteric vessels of PTP-1B^{-/-} mice, there was no spontaneous bleeding, although rebleeding from tail bleeding time wounds was more frequent than in control mice. Thus, the degree of any abnormality in hemostasis imposed by PTP-1B deficiency may be dictated by the type, location, and extent of vascular injury. The same might be true in the case of pharmacological inhibition of PTP-1B. Interestingly, one PTP-1B antagonist of questionable specificity has been shown to reverse platelet aggregation that is stimulated by cross-linking the Fc γ RIIIa receptor (Ragab et al., 2003). Although the selectivity of PTP-1B antagonists is still an issue, they are being evaluated for the treatment of type 2 diabetes and obesity because PTP-1B negatively regulates insulin and leptin receptor signaling in nonhematopoietic tissues (Elchebly et al., 1999; Klamon et al., 2000; Cheng et al., 2002; Zabolotny et al., 2002; Tonks, 2003; Hooft van Huijsduijnen et al., 2004). Assuming that PTP-1B antagonists with appropriate selectivity and toxicity profiles can be developed, current studies indicate that these compounds should be analyzed for their effects on platelet outside-in α IIB β 3 signaling.

Materials and methods

Reagents and antibodies

Mouse mAb to human PTP-1B and rabbit pAb to murine PTP-1B were obtained from Calbiochem and Upstate Biotechnology, respectively. Antibodies against c-Src (327 and 1671) and the integrin β 3 subunit (SSA6 and 8053) were described previously (Arias-Salgado et al., 2003). Antibodies to Csk (C-20) and the COOH terminus of c-Src (B-12) were obtained from Santa Cruz Biotechnology, Inc. Phosphospecific antibody to Src tyrosine 418 was obtained from Biosource International, and antibody specific for the nonphosphorylated form of c-Src tyrosine 529 was obtained from Cell Signaling Technology, Inc. Rat monoclonal anti-mouse CD41 (integrin α Ib subunit), FITC-conjugated hamster anti-mouse CD61 (integrin β 3 subunit), and mouse mAb to Csk were from BD Biosciences. Mouse mAbs 4G10 and PY20 to phosphotyrosine were obtained from Upstate Biotechnology and BD Biosciences, respectively. Antibody HA.11 against the HA epitope tag was obtained from Covance. HRP-conjugated secondary antibodies and HRP-conjugated protein A-Sepharose beads were purchased from Bio-Rad Laboratories. HRP-conjugated anti-mouse IgG TrueBlot (eBioscience) was used when necessary to eliminate interference by the heavy chain of immunoprecipitating antibodies. FITC-conjugated anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories. Purified human fibrinogen was purchased from Enzyme Research Laboratories, Inc. Rhodamine-phalloidin was obtained from Molecular Probes. Src kinase inhibitors PP2 and SU6656 and the control compound PP3 were obtained from Calbiochem. Protein A- and protein G-Sepharose beads were purchased from GE Healthcare. All other reagents were obtained from Sigma-Aldrich.

Mouse strains

PTP-1B^{+/+} and PTP-1B^{-/-} mice (SV129/C57BL6/J) were described previously (Klamon et al., 2000). Age- and sex-matched littermates were used for each experiment. Mice were housed and handled in accordance with institutional guidelines.

Cell lines, plasmids, and transfections

SYF cells (mouse embryonic fibroblasts deficient in c-Src, Fyn, and c-Yes) were obtained from American Type Tissue Collection. Cells were maintained at 37°C with 6% CO₂ in DME supplemented with 10% FBS, L-glutamine, and antibiotics. The vector pCDM8/ α Ib has been described previously (Hughes et al., 1995). Integrin β 3 cDNA was subcloned into HindIII-XhoI sites of pcDNA3.1/Zeo (Invitrogen). Expression vectors containing human α Ib and β 3 subunits were cotransfected into SYF cells with LipofectAMINE (Invitrogen). Stable transfectants (α Ib β 3-SYF cells) were isolated by selective growth in medium containing 125 μ g/ml Zeocin (Invitrogen), and clones expressing α Ib β 3 were isolated by single cell sorting. A single clone (A29) was used for the studies reported in this article, but similar results were obtained with three other independent clones.

Expression vectors for wild-type and mutant c-Src (K295R, Y529F, Δ 90-144, and Δ 150-246) and HA-tagged PTP-1B have been described previously (Sells and Chernoff, 1995; Arias-Salgado et al., 2003). Mutant PTP-1B constructs (C215S, D181A, P309/310A, and Y152/153F) were generated using the Site-Directed Mutagenesis Kit (Stratagene), and mutations were confirmed by direct DNA sequencing. Transient transfections of SYF cells were performed with LipofectAMINE. After 24 h, cells were serum starved in 0.5% FBS and were cultured for an additional 24 h before further use.

Platelet isolation and functional assays

Human and mouse platelets were obtained from fresh anticoagulated whole blood, washed, and resuspended to 3×10^8 cells/ml in a platelet incubation buffer (Law et al., 1999b). A pool of platelets from at least four mice was used for each experiment. FITC-fibrinogen binding to platelets and platelet aggregation were measured as described previously (Law et al., 1999b). Surface expression of α Ib β 3 in mouse platelets was monitored with a FITC-conjugated anti-mouse β 3 antibody. Platelet spreading was assessed by confocal microscopy after plating cells on immobilized fibrinogen (100 μ g/ml of coating concentration) for 40–90 min. Fluorescence images were acquired with a laser scanning confocal microscope (model MRC 1024; Bio-Rad Laboratories) using a 60 \times oil immersion objective (Nikon). Platelet surface areas were measured using Image Pro Plus software (Media Cybernetics, Inc.). Platelet adhesion was quantified by an acid phosphatase assay after incubating 1.5×10^6 cells (50 μ l) for 1 h at RT in fibrinogen-coated microtiter wells (Law et al., 1999b). The percentage of adherent platelets was determined by calculating the ratio

of bound/maximal signal at 405 nm, with maximal signal obtained from wells with platelets not subjected to washing. Fibrin clot retraction was studied by incubating 150 μ l of mouse platelet-rich plasma (2.2×10^7 platelets) in the presence of 1 U/ml thrombin and 3 mM CaCl_2 for 2 h at RT in an aggregometer cuvette. A paper clip was added to facilitate clot removal at the termination of the experiment. The volume of residual clot-free plasma was determined, and clot volume was taken as 150 μ l minus this value. Clot volume was expressed as a percentage of the original 150- μ l plasma volume.

Immunoprecipitation and immunoblotting

Cells were lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM sodium vanadate, 0.5 mM sodium fluoride, 1 mM leupeptin, and complete protease inhibitor cocktail (Roche Applied Science). Lysates were clarified by centrifugation at 13,000 g for 10 min at 4°C, and 100–500 μ g of protein from the soluble fraction was immunoprecipitated using a relevant primary antibody and protein A- or protein G-Sepharose beads. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting, and immunoreactive bands were detected by enhanced chemiluminescence (SuperSignal West Pico Substrate; Pierce Chemical Co.).

Platelet thrombus formation in vivo

Fluorescence and brightfield microscopy was used to capture real-time digital images of Fura 2-AM-labeled platelets in developing thrombi of living mice after a laser-induced injury to the arteriole wall in the cremaster muscle (Falati et al., 2002). In brief, platelets from PTP-1B^{-/-} and PTP-1B^{+/+} mice were loaded with Fura 2-AM. Then, 250–300 $\times 10^6$ labeled platelets, corresponding to ~20% of the total endogenous platelet number, were infused into the circulation of an anesthetized mouse. PTP-1B^{-/-} and PTP-1B^{+/+} donor platelets were infused into PTP-1B^{-/-} or PTP-1B^{+/+} recipient mice as indicated in each particular experiment. Vascular injury was induced 10 min after platelet infusion. Real-time multichannel intravital microscopy was used to monitor two fluorescence channels and one brightfield channel almost simultaneously. The accumulation of labeled platelets within a developing thrombus was monitored at 510 nm after excitation at 380 nm, and calcium mobilization within those platelets was monitored at 510 nm after excitation at 340 nm. Mouse tail bleeding times and the occurrence of rebleeding from tail wounds were assessed as described previously (Law et al., 1999a).

Online supplemental material

Videos show thrombus formation in a cremasteric arteriole of a living PTP-1B^{+/+} (Video 1) or PTP-1B^{-/-} (Video 2) mouse at three frames/s for 3 min. PTP-1B^{+/+} and PTP-1B^{-/-} platelets were labeled with Fura 2, an arteriole in a recipient cremaster muscle was subjected to laser injury, and the accumulation of fluorescent platelets into the developing thrombus was assessed as described above and in Fig. 6 a. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200503125/DC1>.

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