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

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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 αIIbβ3 and c-Src is mediated by direct interaction of the β3 cytoplasmic domain with the c-Src SH3 domain (Obergfell et al., 2002; Arias-Salgado et al., 2003). A similar relationship may pertain to c-Src and the related integrin, αVβ3, in osteoclasts (Feng et al., 2001). Furthermore, in many cell types, a close functional, if not physical, relationship exists between Src family kinases and β3 integrins and c-Src is underscored by defective spreading on damaged vascular surfaces, whereas αVβ3 promotes osteoclast adhesion to vitronectin or osteopontin (Byzova et al., 1998; Shattil and Newman, 2004). Genetic deficiency of αIIbβ3 and αVβ3 leads to defects in hemostasis and bone remodeling, respectively (Hodivala-Dilke et al., 1999; Feng et al., 2001). Adhesive ligand binding to β3 integrins leads to c-Src activation and tyrosine phosphorylation of c-Src substrates in platelets and osteoclasts (Feng et al., 2001; Obergfell et al., 2002; Arias-Salgado et al., 2003). The close relationship between β3 integrins and c-Src is underscored by defective spreading of platelets that are deficient in multiple Src family kinases (Obergfell et al., 2002) and by overlapping bone remodeling phenotypes in mice that are deficient in c-Src or β3 (Soriano et al., 1991; Hodivala-Dilke et al., 1999; McHugh et al., 2000). Consequently, attention is now focused on how β3 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 (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 αIIbβ3, dephosphorylation of c-Src tyrosine 529, and c-Src activation. Furthermore, PTP-1B–deficient platelets are defective in outside-in αIIbβ3 signaling in vitro as manifested by poor spreading on fibrinogen and decreased clot retraction, and they exhibit ineffective Ca²⁺ signaling and thrombus formation in vivo. Thus, PTP-1B is an essential positive regulator of the initiation of outside-in αIIbβ3 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 αIIbβ3 and c-Src is mediated by direct interaction of the β3 cytoplasmic domain with the c-Src SH3 domain (Obergfell et al., 2002; Arias-Salgado et al., 2003). A similar relationship may pertain to c-Src and the related integrin, αVβ3, in osteoclasts (Feng et al., 2001). Furthermore, in many cell types, a close functional, if not physical, relationship exists between Src family kinases and β3 integrins and c-Src is underscored by defective spreading on damaged vascular surfaces, whereas αVβ3 promotes osteoclast adhesion to vitronectin or osteopontin (Byzova et al., 1998; Shattil and Newman, 2004). Genetic deficiency of αIIbβ3 and αVβ3 leads to defects in hemostasis and bone remodeling, respectively (Hodivala-Dilke et al., 1999; Feng et al., 2001). Adhesive ligand binding to β3 integrins leads to c-Src activation and tyrosine phosphorylation of c-Src substrates in platelets and osteoclasts (Feng et al., 2001; Obergfell et al., 2002; Arias-Salgado et al., 2003). The close relationship between β3 integrins and c-Src is underscored by defective spreading of platelets that are deficient in multiple Src family kinases (Obergfell et al., 2002) and by overlapping bone remodeling phenotypes in mice that are deficient in c-Src or β3 (Soriano et al., 1991; Hodivala-Dilke et al., 1999; McHugh et al., 2000). Consequently, attention is now focused on how β3 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 (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 αIIbβ3, dephosphorylation of c-Src tyrosine 529, and c-Src activation. Furthermore, PTP-1B–deficient platelets are defective in outside-in αIIbβ3 signaling in vitro as manifested by poor spreading on fibrinogen and decreased clot retraction, and they exhibit ineffective Ca²⁺ signaling and thrombus formation in vivo. Thus, PTP-1B is an essential positive regulator of the initiation of outside-in αIIbβ3 signaling in platelets.

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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 β3 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 αIIbβ3–c-Src complex in resting platelets (Okada et al., 1991; Obergfell 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 β3 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 αIIbβ3. 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 αIIbβ3 and is required for integrin activation of c-Src

To explore how αIIbβ3 regulates c-Src, we sought to identify a PTP that localizes to the αIIbβ3–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 (Obergfell 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 αIIbβ3 and c-Src from detergent lysates of human and mouse platelets. However, unlike the associations of c-Src and Csk with αIIbβ3, which are observed in resting platelets (Obergfell et al., 2002), the association of PTP-1B with αIIbβ3 and c-Src required fibrinogen binding to platelets. This was induced either by MnCl2, which activates αIIbβ3 directly (Fig. 1 a; Litvinov et al., 2004), or by plating the cells on fibrinogen (not depicted). PTP-1B recruitment to αIIbβ3 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 αIIbβ3 was specific and was observed whether immunoprecipitation was performed with antibodies to PTP-1B or αIIbβ3 (Fig. 1 b). The interactions of PTP-1B with αIIbβ3 and c-Src were prevented by pretreatment of platelets with 2 μM SU6656 or 5 μM 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, αIIbβ3, and c-Src in platelets. (a) Washed human platelets were incubated for 15 min at RT with 250 μg/ml fibrinogen in the presence or absence of 0.5 mM MnCl2. Some samples were preincubated for 15 min with 2 μM SU6656, 5 μM PP2, or 5 μM 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 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 αIIbβ3 was specific and was observed whether immunoprecipitation was performed with antibodies to PTP-1B or αIIbβ3 (Fig. 1 b). The interactions of PTP-1B with αIIbβ3 and c-Src were prevented by pretreatment of platelets with 2 μM SU6656 or 5 μM PP2 to block Src kinase activity (Fig. 1 a) or with 2 mM RGDS (Arg-Gly-Asp-Ser) to inhibit fibrinogen binding.
Fibrinogen-dependent PTP-1B recruitment to αIbβ3 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 αIbβ3 to prevent or minimize generalized signaling via G protein–coupled receptors and, thus, to facilitate direct assessment of outside-in αIbβ3 signaling (Obergfell et al., 2002; Arias-Salgado et al., 2003). Overall, these results indicate that fibrinogen binding to αIbβ3 triggers recruitment of PTP-1B to a plasma membrane complex of αIbβ3 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 αIbβ3 signaling, the catalytic activity of c-Src in αIbβ3 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 αIbβ3 receptors (Fig. 1 d). These results suggest that PTP-1B−/− platelets have a fundamental defect in αIbβ3 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 dephosphorytrosine 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 αIbβ3-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 αIbβ3–c-Src complex in resting platelets and dissociates from it upon fibrinogen binding (Obergfell et al., 2002). However, Csk failed to fully dissociate from αIbβ3 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 αIbβ3 (Arregui et al., 1998;
tion of PTP-1B with αIIbβ3 (Fig. 2 b). Similar results were obtained for the interaction of PTP-1B with c-Src except that c-Src tyrosine 529 was alsorequired (Fig. 2 c). The requirement for the c-Src SH3 domain might be explained by the direct binding of SH3 to the β3 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 αIIbβ3–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 αIIbβ3-SYF cells. Wild-type PTP-1B and two different phosphatase-inactive “substrate-trapping” mutants (C215S and D181A) each interacted with αIIbβ3 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 αIIbβ3 and c-Src, as did the double mutation of tyrosine 152 and 153 to phenylalanine. These amino acid 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 αIIbβ3-SYF cells (Fig. 3 b) or platelets (Fig. 3 c) facilitated tyrosine phosphorylation of PTP-1B in a Src-dependent manner.

PTP-1B regulates platelet functions that are dependent on outside-in αIIbβ3 signaling

Outside-in signaling via αIIbβ3 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 αIIbβ3 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 µM ADP or 250 µM 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 αIIbβ3-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 αIIbβ3 signaling in platelets. However, PTP-1B appears to be dispensable for agonist induction of soluble fibrinogen binding to αIIbβ3 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 ± 53 s; PTP-1B+/+, 198 ± 19 s; P < 0.06, n = 14 mice each). However, rebleeding from tail 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 β3 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; Oberfeld 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 β3 subunit, and fibrinogen binding to αIIbβ3 triggers c-Src activation (Oberfeld 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-
explore the physiological significance of this process. The results establish that (1) fibrinogen binding to platelets leads to PTP-1B recruitment to an αIIbβ3-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 αIIbβ3-dependent platelet spreading on fibrinogen and for normal fibrin clot retraction but not for the agonist-induced activation of αIIbβ3; 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 β1 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 relevance of PTP-1B activation of c-Src downstream of a β3 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 αIIbβ3 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 αIIbβ3-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 αIIbβ3 was observed in human and mouse platelets and in a fibroblast model system, enabling examination of its structural basis. PTP-1B recruitment to αIIbβ3 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 β3 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 αIIbβ3 oligomerization (Simmons et al., 1997; Buen-suco 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 αIIbβ3 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 αIIbβ3 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 αIIbβ3 (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 β3 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 αIIbβ3 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/− mice because both responses require outside-in αIIbβ3 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 αIIbβ3 (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 αIIbβ3 activation, platelet aggregation, or agonist costimulation of platelet spreading. Furthermore, the ligation of αIIbβ3 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 shear stress, which are typical within arterioles (Mazzucato et al., 2002), are involved in the regulation of c-Src activation in platelets (Bos et al., 1989; Tschopp et al., 2000; Cheng et al., 2002; Zabolotny et al., 2002; Tonks, 2003), it is legitimate to ask whether the impairment 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 FcγRIIIa 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 antagonists inhibit c-Src activation and reduced calcium mobilization in nonhematopoietic tissues (Elchebly et al., 1999; Klaman 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 αIIbβ3 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 β3 subunit (SA6 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. Phospho-specific antibodies to c-Src tyr 418 was obtained from Biosource International, and antibody specific for the nonphosphorylated form of c-Src tyrine 529 was obtained from Cell Signaling Technology, Inc. Rat monoclonal anti–mouse CD41 (integrin αIIbβ3 subunit), FITC-conjugated hamster anti–mouse CD61 (integrin β3 subunit), and mouse mAb to Csk were from BD Biosciences. Mouse mAbs 4G510 and PY20 were phospho-specific antibodies to the 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, 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 (Klaman 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/αIIb has been described previously (Hughes et al., 1995). Integrin β3 CDNA was subcloned into HindIII/XhoI sites of pcDNA3.1/Zeo (Invitrogen). Expression vectors containing human αIIb and β3 subunits were cotransfected into SYF cells with LipofectAMINE (Invitrogen). Stable transfectants (αIIbβ3-SYF cells) were isolated by selective growth in medium containing 125 μg/ml Zeocin (Invitrogen), and clones expressing αIIbβ3 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, D90–144, and D150–246) 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 × 108 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 αIIbβ3 in mouse platelets was monitored with a FITC-conjugated anti–mouse β3 antibody. Platelet spreading was assessed by confocal microscopy after plating cells on immobilized fibrinogen (100 μg/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 × 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 × 108 cells (50 μl) for 1 h 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 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 was described as described previously [Low et al., 1999a].

Online supplemental material

Videos show thrombus formation in a cremaster artery 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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