Dominant Role of the Protein-Tyrosine Phosphatase CD148 in Regulating Platelet Activation Relative to Protein-Tyrosine Phosphatase-1B

Jun Mori, Ying-Jie Wang, Stuart Ellison, Silke Heising, Benjamin G. Neel, Michel L. Tremblay, Steve P. Watson, Yotis A. Senis

Objective—The receptor-like protein-tyrosine phosphatase (PTP) CD148 and the nontransmembrane PTP1-B have been shown to be net positive regulators of Src family kinases in platelets. In the present study, we compared the relative contributions of these PTPs in platelet activation by the major glycoprotein, glycoprotein VI, α\textsubscript{IIb}β\textsubscript{3}, and CLEC-2.

Methods and Results—PTP-1B–deficient mouse platelets responded normally to the glycoprotein VI–specific agonist collagen-related peptide and antibody-mediated CLEC-2 activation. However, they exhibited a marginal reduction in α\textsubscript{IIb}β\textsubscript{3}-mediated Src family kinase activation and tyrosine phosphorylation. In contrast, CD148-deficient platelets exhibited a dramatic reduction in activation by glycoprotein VI and α\textsubscript{IIb}β\textsubscript{3} and a marginal reduction in response to activation by CLEC-2, which was further enhanced in the absence of PTP-1B. These defects were associated with reduced activation of Src family kinase and Syk, suggesting a causal relationship. Under arteriolar flow conditions, there was defective aggregate formation in the absence of PTP-1B and, to a greater extent, CD148 and a severe abrogation of both adhesion and aggregation in the absence of both PTPs.

Conclusion—Findings from this study demonstrate that CD148 plays a dominant role in activating Src family kinases in platelets relative to PTP-1B. Both PTPs are required for optimal platelet activation and aggregate formation under high arterial shear rates. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: CD148 ■ platelets ■ protein-tyrosine phosphatase ■ protein-tyrosine phosphatase-1B ■ Src tyrosine kinase

Platelets are small anucleate blood cells that plug holes in damaged blood vessels. They do so by adhering to exposed extracellular matrix proteins at sites of injury and forming an aggregate. Secondary mediators released by activated platelets, including ADP and thromboxane A\textsubscript{2}, amplify activation signals and attract other platelets to the growing platelet plug. The surface of activated platelets supports the localized generation of thrombin, a powerful platelet agonist that stabilizes the platelet aggregate by catalyzing fibrin polymerization and deposition. Platelets can also have a detrimental effect on health by blocking blood vessels that supply the brain and heart, leading to stroke and myocardial infarction, respectively. Antiplatelet drugs are widely used to protect against these pathologies; however, they have the added risk of causing increased bleeding. Thus, there is a need for development of novel antiplatelet drugs that protect against thrombosis with little or no bleeding side effects.

Src family kinases (SFKs) are essential for initiating and propagating signal via the SFK-Syk-PLC\gamma2 pathway. This signaling pathway is shared by many major platelet surface receptors, including the collagen activation receptor glycoprotein VI (GPVI)-Fc receptor γ-chain, the integrin α\textsubscript{IIb}β\textsubscript{3}, and the podoplanin receptor CLEC-2.1–3 Classically, SFK activity is regulated by tyrosine phosphorylation. Phosphorylation of the C-terminal tyrosine by C-terminal Src kinase inhibits SFK activity by mediating an intramolecular interaction with the SH2 domain. Conversely, dephosphorylation of this site by a protein-tyrosine phosphatase (PTP) disrupts this interaction and allows the SFK to access and phosphorylate substrates.4 Maximal catalytic activity is achieved after trans-autophosphorylation of the activation loop tyrosine.4

We recently demonstrated that CD148 is a critical net positive regulator of SFKs in platelets,5 in association with
the C-terminal inhibitory tyrosine of SFKs. More recently, CD148 was shown to also dephosphorylate the activation loop tyrosine of SFKs, suggesting that it can also attenuate SFK activity. Targeted deletion of CD148 in mice results in reduced platelet reactivity to collagen and fibrinogen and reduced thrombus formation after vessel injury. The only other PTP shown to positively regulate SFKs in platelets is the structurally distinct nontransmembrane PTP, PTP-1B. In this study, we compared the functional responses of platelets from PTP-1B and CD148 single and double knockout (DKO) mice on the same genetic background (C57BL/6) to determine the relative contributions of these 2 PTPs in the regulation of SFKs and platelet activation by the surface glycoprotein receptors GPVI, αIIbβ3, and CLEC-2.

Methods

Mice
Mice lacking CD148 and PTP-1B on a C57BL/6 background were crossed; progeny were interbred to generate the 4 genotypes used in this study: wild-type, CD148-deficient (CD148 knockout [KO]), PTP-1B-deficient (PTP-1B KO), and deficient in both PTPs (DKO). All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986.

Antibodies and Reagents
Anti-mouse phosphotyrosine, anti-Rac1, anti-Arp, and agarose-conjugated anti-phosphotyrosine antibody (clone: 4G10) were purchased from Millipore (Billerica, MA). Alexa Fluor 488-conjugated phaloidin, Alexa Fluor 488-conjugated fibrinogen, and PTP-1B-deficient (PTP-1B KO) and in deficient in both PTPs (DKO). All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986.

Platelet Preparation and Aggregation
Mouse blood was collected from terminally CO2-narcotized mice by cardiac puncture into 1:10 (vol:vol) acid citrate dextrose (120 mmol/L sodium citrate, 110 mmol/L glucose, 80 mmol/L citric acid). Washed platelets were prepared, as previously described. Aggregation of washed platelets (2×107/mL) was measured using a lumi-aggregometer (Chrono-Log, Havertown, PA).

Flow Cytometry
Platelets (2×107/mL) stimulated with collagen-related peptide (CRP), thrombin, or CLEC-2 antibody for 20 minutes at room temperature were fixed with 1% ice cold formaldehyde and stained with fluorescein isothiocyanate–conjugated anti–P-selectin antibody or fluorescein isothiocyanate–conjugated fibrinogen. Filamentous actin was measured in resting and activated washed platelets using a modified method to that previously described. Samples were analyzed using FACS Calibur flow cytometer and CellQuest software (BD; Becton Dickinson, Franklin Lakes, NJ).

Platelet Biochemistry
Washed mouse platelet whole-cell lysates (3–5×107/mL) and immunoprecipitations were prepared and Western blotted, as previously described. In integrin signaling studies, tyrosine-phosphorylated proteins were immunoprecipitated from equal amounts of total protein using agarose-conjugated anti-phosphotyrosine antibody. Agarose-conjugated nonimmune mouse IgG was used as a control. Total protein concentrations of whole-cell lysates were measured using the Bio-Rad Protein Assay (Hemel Hempstead, United Kingdom). Band intensities of Western blots were quantified using Adobe Photoshop CS software (San Jose, CA). SFK activation loop and Syk tyrosine phosphorylation was normalized to actin and Syk loading controls, respectively, in each experiment.

Static Adhesion Assay
Washed platelets (2×107/mL) were placed on fibrinogen-coated coverslips for 45 minutes at 37°C, fixed, and imaged, as previously described. For immunofluorescence microscopy, adherent platelets were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were permeabilized with 0.1% Triton-X 100 in PBS and stained with appropriate antibodies or Alexa Fluor 488–conjugated phalloidin for 60 minutes. Cells were imaged using a confocal microscope (Leica DM IRE2, Milton Keynes, United Kingdom).

Platelet Flow Adhesion Assay
Blood was collected into sodium heparin (5 U/mL) and PPACK (40 μmol/L), stained with 2 μmol/L DiOC6, and flowed through collagen-coated glass microslides (1×10 mm) at 3000 s−1 for 3 minutes at 37°C, as previously described. Adherent platelets were washed, fixed, and images captured by differential interference contrast microscopy.

Statistical Analysis
The Student t test (1 sample or independent samples) and 2-way ANOVA were used to compare sample means and determine statistical significance. P<0.05 was considered significant.

Results

Mice Lacking PTP-1B and CD148 Are Viable and Have Normal Platelet Counts
PTP-1B and CD148 single and DKO mouse models were used to investigate the relative contributions of PTP-1B and CD148 to platelet activation. All mouse models were on a C57BL/6 background to allow for direct comparisons to be made. Platelets from PTP-1B KO mice had no detectable PTP-1B by Western blotting and expressed normal levels of CD148 and vice versa. DKO mice, generated by crossing PTP-1B−/− CD148−/− mice, were born at normal Mendelian frequency with no overt developmental defects. Neither PTP-1B nor CD148 was detectable in platelets from DKO mice by Western blotting, as expected (Figure IA in the online-only Data Supplement). Platelet counts and volumes were normal in all 3 KO mouse models (Table I in the online-only Data Supplement).

PTP-1B Does Not Regulate GPVI-Mediated Functional Responses or Signaling
Consistent with previous results, we found that PTP-1B–deficient platelets respond normally to GPVI agonists. PTP-1B–deficient platelets stimulated with low concentrations of the GPVI-specific agonist CRP (1 μg/mL) and the physiological ligand collagen (3 μg/mL), which binds to GPVI and the integrin α2β1, aggregated, expressed surface P-selectin, and
bound fibrinogen normally (Figure 1A–1C). PAR-4–mediated responses were also normal to low concentration (0.04 U/mL) of thrombin (Figure 1A–1C). In contrast, CD148-deficient platelets did not respond to the same concentration of CRP and underwent only a very weak, delayed aggregation to collagen (Figure 1A) in agreement with previous results.\(^5,^6\) Similar results were observed in mice deficient in PTP-1B and CD148, including the very weak and delayed onset of aggregation to collagen (Figure 1A–1C). These findings demonstrate that CD148 is the major PTP regulating GPVI-mediated functional responses.

To determine whether there were any additional effects of ablating PTP-1B, we analyzed tyrosine phosphorylation. As expected, whole-cell tyrosine phosphorylation was normal in PTP-1B–deficient platelets and reduced in CD148- and PTP-1B/CD148-deficient platelets (Figure 1D). CRP-mediated whole-cell tyrosine phosphorylation was markedly reduced, by comparable amounts, in CD148- and PTP-1B/CD148-deficient platelets (Figure 1D). SFK activity was indirectly measured by Western blotting using a phospho-specific antibody to the SFK activation loop tyrosine. Phosphorylation of this site is a trans-autophosphorylation...
event that is commonly used as an indicator of SFK activity.\(^4\) Phosphorylation of this site was normal in resting and CRP-stimulated PTP-1B–deficient platelets but markedly reduced in CD148- and PTP-1B/CD148–deficient platelets, consistent with reduced whole-cell tyrosine phosphorylation in these platelets (Figure 1D). The level of phosphorylation of the SFK activation loop tyrosine was the same in platelets lacking either CD148 alone or PTP-1B and CD148, demonstrating no functional redundancy between PTP-1B and CD148 in regulating SFKs in resting platelets or downstream of GPVI.

Dominant Role of CD148 in Regulating Platelet Spreading on Fibrinogen

We next compared the relative contributions of PTP-1B and CD148 to integrin \(\alpha_{\text{IIb}}\beta_3\)-mediated responses. Integrin \(\alpha_{\text{IIb}}\beta_3\) surface expression was normal in all mutant platelets (Table I in the online-only Data Supplement). Unexpectedly, filopodia formation and mean platelet surface area of PTP-1B–deficient platelets were normal on a fibrinogen-coated surface under basal conditions (Figure 2A and 2B). In contrast, CD148-deficient platelets showed minimal spreading under basal conditions, reduced spreading in the presence of thrombin (Figure 2A and 2B), and further reduced spreading in PTP-1B/CD148–deficient platelets (Figure 4A and 4B). Interestingly, a 23-kDa band was hyperphosphorylated in both single- and double-deficient platelets (Figure 4A), possibly representing a substrate of CD148.

Impaired CLEC-2 Signaling in CD148- and PTP-1B/CD148–Deficient Platelets

We next investigated whether PTP-1B regulates CLEC-2–mediated functional responses and signaling. PTP-1B–deficient platelets aggregated and secreted ATP normally in response to intermediate (10 \(\mu\)g/mL) and low (3 \(\mu\)g/mL) concentrations of anti–CLEC-2 antibody (Figure 5A and 5B). Consistent with our previous findings,\(^5\) CD148-deficient platelets responded normally to 10 \(\mu\)g/mL anti–CLEC-2 antibody (Figure 5A and 5B) but exhibited marginal reductions in aggregation, ATP secretion, P-selectin expression, and fibrinogen binding to 3 \(\mu\)g/mL anti–CLEC-2 antibody (Figure 5A–5D). Interestingly, platelets lacking both PTP-1B and CD148 responded less well than CD148-deficient platelets to intermediate and low concentrations of anti–CLEC-2 antibody (Figure 5A–5D), demonstrating that PTP-1B plays a minor role in regulating CLEC-2–mediated platelet activation that is masked in the presence of CD148.

We next investigated the molecular basis of CLEC-2–mediated functional defects. Consistent with our functional findings (Figure 5A–5D), whole-cell tyrosine phosphorylation was normal in PTP-1B–deficient platelets, reduced in CD148-deficient platelets, and further reduced in PTP-1B/CD148-deficient platelets, in response to 3 \(\mu\)g/mL anti–CLEC-2 antibody (Figure 5E). Furthermore, phosphorylation of the SFK activation loop tyrosine and Syk phosphorylation were similarly reduced in CD148-deficient platelets (Figure 5E and 5F), demonstrating additive effects of deleting PTP-1B and CD148 in CLEC-2 signaling. In addition, there was a reduction in phosphoproteins coimmunoprecipitating with Syk, including a doublet migrating at 32 kDa (Figure 5F).

PTP-1B and CD148 Are Essential for Optimal Platelet Aggregation Under Flow

Based on our findings demonstrating additive effects of PTP-1B and CD148 in SFK and Syk activation downstream of the integrin \(\alpha_{\text{IIb}}\beta_3\) and CLEC-2, we investigated the ability of single and DKO platelets to adhere and aggregate to...
collagen under arterial shear (3000 s⁻¹). Despite exhibiting marginal functional defects under static and low shear conditions, PTP-1B–deficient platelets failed to form large platelet aggregates on collagen at 3000 s⁻¹ (Figure 6), consistent with previous in vivo results demonstrating reduced thrombus formation in PTP-1B–deficient mice using the laser injury model. Indeed, platelet aggregates were flatter, resembling platelet monolayers, consistent with 

Figure 2. Reduced spreading of protein-tyrosine phosphatase (PTP)-1B/CD148-deficient platelets on fibrinogen. A, Washed platelets pretreated with PBS (basal), 0.1U/mL thrombin, or 10 μmol/L PP1 were plated on a fibrinogen-coated surface. Representative images captured by differential interference contrast microscopy from 3 experiments. Scale bar, 5 μm. B, The mean platelet surface area (μm²) of individual platelets was quantified using ImageJ software. C, The percentage of spiky platelets present per field of view on fibrinogen in the presence of thrombin was quantified. D, The mean platelet perimeter length (μm) of individual platelets was quantified using ImageJ software (n=250–500 platelets per condition; mean±SD; *P<0.05, ***P<0.001). WT indicates wild type; KO, knockout; DKO, double KO.
PTP-1B regulating αIIbβ3 signaling, but not GPVI signaling. This was in striking contrast to control platelets (Figure 6). As expected, a much more dramatic phenotype was seen with CD148-deficient platelets, which formed only small clusters of platelets on the collagen-coated surface (Figure 6).

PTP-1B/CD148–deficient platelets exhibited the most striking defect, failing to form any aggregates under these conditions (Figure 6). These findings demonstrate that both PTP-1B and CD148 are required for optimal platelet aggregate formation on collagen under arterial shear conditions.
Discussion

In the present study, we show several new findings on the functional roles of PTP-1B and CD148 in platelet activation. The main finding is that PTP-1B is a selective regulator of SFKs downstream of the integrin $\alpha_{IIb}\beta_3$, whereas CD148 is a global regulator of SFKs in resting and activated platelets. As a result, deletion of PTP-1B has much milder effects than deletion of CD148 and additive effects in some instances, such as downstream of $\alpha_{IIb}\beta_3$ and CLEC-2, but not GPVI. The other major finding of this study is that PTP-1B is essential for optimal platelet aggregation on collagen under high shear rates, an experience in stenotic arteries. Ablation of PTP-1B results in a dramatic reduction in large aggregate formation but still allows platelets to stick together and form monolayers and smaller aggregates. This is in striking contrast to CD148-deficient platelets, which form very few and small clusters of platelets under the same conditions. These findings have important implications for targeting PTP-1B and CD148 in thrombotic conditions.

Although previously reported that PTP-1B is essential for activating $\alpha_{IIb}\beta_3$-associated Src and initiating outside-in integrin signaling, we found only a marginal reduction in SFK activity and whole-cell tyrosine phosphorylation in fibrinogen-adhered PTP-1B–deficient platelets. We suspect that differences between our findings and those of Shattil and coworkers probably reflect differences in experimental design, rather than strain variation of the KO mouse models. In particular, it is notable that the Shattil study reported minor lamellipodia formation on adhesion of control platelets to fibrinogen, suggesting that the platelets were in a more reactive state. In addition, Src phosphorylation was measured in suspension after stimulation with fibrinogen and MnCl$_2$ in the Shattil study, whereas SFK phosphorylation was measured in fibrinogen-adhered platelets in our study. Despite these differences, both studies concur that PTP-1B is a positive and selective regulator of SFKs downstream of $\alpha_{IIb}\beta_3$. 

Figure 4. Impaired $\alpha_{IIb}\beta_3$ signaling in protein-tyrosine phosphatase (PTP)-1B– and CD148-deficient platelets. Washed platelets in the presence of 2U/mL apyrase and 10 $\mu$mol/L indomethacin were plated on either BSA- or fibrinogen (fib)-coated surfaces for 45 minutes at 37°C. Tyrosine-phosphorylated proteins were immunoprecipitated (IP) from equal amounts of whole-cell lysates (WCLs). Samples for IP (A) or WCLs (B) were analyzed for Western blotting. Blots are representative of 3 experiments. Data presented are means (±SEM) of 3 independent experiments (***P<0.01, **P<0.001). WT indicates wild type; KO, knockout; DKO, double KO.
Figure 5. Impaired CLEC-2–mediated functional responses and signaling in CD148- and protein-tyrosine phosphatase (PTP)-1B/CD148-deficient platelets. Washed platelet aggregation (A) or ATP secretion (B) by 10 or 3 μg/mL CLEC-2 antibody (Ab). Images are representative of 3 experiments. Data presented are means (±SEM) of 3 independent experiments (*P<0.05, **P<0.01, ***P<0.001). Platelets stimulated with 3 μg/mL CLEC-2 Ab were stained with fluorescein isothiocyanate (FITC)–P-selectin Ab (C) or FITC–fibrinogen (D). Mean fluorescence intensity (MFI) was measured by flow cytometry and quantified (geometric mean±SD; **P<0.01, ***P<0.001). Whole-cell lysates (WCLs; E) or immunoprecipitated (IP) Syk (F) from platelets stimulated with 3 μg/mL CLEC-2 Ab for 10 minutes were analyzed for Western blotting. Images are representative of 3 experiments. Data presented are means (±SEM) of 3 independent experiments (*P<0.05, **P<0.01, ***P<0.001). WT indicates wild type; KO, knockout; DKO, double KO.
Interestingly, despite exhibiting marginal or no functional defects under static or low shear conditions, PTP-1B–deficient platelets failed to form large aggregates on collagen under high arterial shear rates. This is probably caused by PTP-1B playing a minor role in regulating the SFK-Syk-PLCγ2 signaling pathway, which is shared by GPVI, αIIbβ3, and GPIb-IX-V, all of which are essential for thrombus formation. Thus, it is only when the platelets are put under more extreme conditions, such as shear rates in stenotic arteries, that this defect becomes apparent. CLEC-2, which also signals via this pathway, has been implicated in regulating thrombus stability; although this is controversial,18–20 our flow adhesion findings are consistent with the in vivo observations of the Shattil group,8 demonstrating reduced thrombus formation in arterioles of PTP-1B–deficient mice using the laser injury model. Interestingly, PTP-1B–deficient mice did not exhibit a bleeding diathesis,8 making PTP-1B an attractive antiplatelet drug target for prevention of thrombus formation. In contrast, CD148-deficient mice form almost no thrombi under high shear conditions and have a bleeding diathesis,5 suggesting targeting it may have bleeding complications. However, it may be a more appropriate target as a thrombus buster.

Our study revealed additive effects of PTP-1B and CD148 downstream of αIIbβ3 and CLEC-2. This was mainly at the level of SFK and Syk activation. Although we have previously reported that CD148 is not essential for CLEC-2 signaling to rhodocytin and high concentrations of the same CLEC-2 monoclonal antibody that was used in this study,12 here we show that CD148 plays a minor role in activating SFKs and Syk downstream of CLEC-2 in response to weak stimulation. We suspect that PTP-1B also plays a role in initiating or propagating CLEC-2 signaling that is masked by the presence of CD148. This is supported by a further reduction in SFK and Syk activation downstream of CLEC-2 in DKO platelets compared with CD148-deficient platelets. Reduced Syk-associated phosphoproteins suggest altered compartmentalization in DKO and CD148-deficient platelets.

Differences in PTP-1B- and CD148-mediated SFK activation probably reflect differential compartmentalization of these 2 PTPs. PTP-1B is associated with the cytoplasmic surface of the endoplasmic reticulum (ER),21 whereas CD148 is localized to the plasma membrane.22 For ER-localized PTP-1B to active SFKs at the plasma membrane, it must either be liberated from the ER and translocate to the plasma membrane or the ER must come into close proximity with the plasma membrane, both of which are reported to occur.21,23 Release of PTP-1B from the ER is a calpain-mediated event.24 In contrast, CD148 is constitutively plasma membrane–associated, where it can readily access SFKs.

In conclusion, findings from this study fit a model in which CD148 lies upstream of SFKs and plays a dominant role in maintaining a pool of active SFKs in platelets, whereas PTP-1B plays a more specialized role regulating SFKs downstream of αIIbβ3 and possibly CLEC-2. This study highlights the unique roles played by these structurally distinct PTPs in regulating platelet activation and platelet function under arterial flow conditions. Indeed, PTP-1B and CD148 may prove to be attractive novel antiplatelet targets in the prevention and treatment of thrombosis, either alone or in combination.

Acknowledgments

We thank all members of the Birmingham Biomedical Sciences Unit, especially Jenny Ullah, for exceptional technical assistance. J.M. is a British Heart Foundation (BHF) Postdoctoral Researcher, S.P.W. is a BHF Chair, and Y.A.S. is a BHF Intermediate Research Fellow.

Sources of Funding

This work was supported by British Heart Foundation (BHF) Project grants PG/07/034/22775 and PG/11/108/29237, and BHF Intermediate Fellowship FS/08/034/25085.

Disclosures

None.
References


Dominant Role of the Protein-Tyrosine Phosphatase CD148 in Regulating Platelet Activation Relative to Protein-Tyrosine Phosphatase-1B

Jun Mori, Ying-Jie Wang, Stuart Ellison, Silke Heising, Benjamin G. Neel, Michel L. Tremblay, Steve P. Watson and Yotis A. Senis

Arterioscler Thromb Vasc Biol. published online October 11, 2012; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/10/11/ATVBAHA.112.300447

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/10/11/ATVBAHA.112.300447.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Supplement Material

Methods

Mice

Mice lacking CD148\textsuperscript{1,2} and PTP-1B\textsuperscript{3} on a C57BL/6 background were crossed; progeny were interbred to generate the four genotypes used in this study—wild-type (WT), CD148-deficient (CD148 KO), PTP-1B-deficient (PTP-1B KO), and deficient in both PTPs (DKO). All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986.

Antibodies and reagents

Anti-mouse PTP-1B antibody was purchased from Millipore (Billerica, MA). Rat anti-mouse CLEC-2 antibody was purchased from AbD Serotec (Oxford, UK). Alexa Fluor® 488 goat anti-rat IgG (H+L) was purchased from Invitrogen (Camarillo, CA). All other antibodies and reagents were obtained from previously described sources.\textsuperscript{2,4,5}

Platelet preparation

Mouse blood was collected from terminally CO\textsubscript{2}-narcosed mice by cardiac puncture into 1:10 (v:v) acid citrate dextrose (ACD: 120 mM sodium citrate, 110 mM glucose, 80 mM citric acid). Washed platelets were prepared, as previously described.\textsuperscript{4}

Flow cytometry

Surface glycoprotein expression was measured in resting washed platelets (2x10\textsuperscript{7}/mL) by flow cytometry using FITC-conjugated Abs, or in the case of CLEC-2, a primary antibody and a fluorescently-conjugated secondary antibody. Samples were analyzed using FACSCalibur flow cytometer and CellQuest software (BD; Becton Dickinson, Franklin Lakes, NJ).

Platelet biochemistry
$5 \times 10^8$/mL of washed mouse platelet whole-cell lysates (WCLs) were prepared and western blotted, as previously described.$^4,^5$

**Statistical analysis**

Student’s $t$ test (one sample or independent samples) and 2-way analysis of variance were used to compare sample means and determine statistical significance. $P$ values < 0.05 were considered significant.

**References**

Supplemental Table I

<table>
<thead>
<tr>
<th>GP</th>
<th>WT</th>
<th>PTP-1B KO</th>
<th>CD148 KO</th>
<th>DKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPVI</td>
<td>100 ± 5.23</td>
<td>102.71 ± 3.87</td>
<td>48.21 ± 8.56 ***</td>
<td>65.9 ± 7.41 ***</td>
</tr>
<tr>
<td>αIIbβ3</td>
<td>100 ± 11.47</td>
<td>102.81 ± 14.7</td>
<td>86.98 ± 16.98</td>
<td>85.09 ± 28.54</td>
</tr>
<tr>
<td>CLEC-2</td>
<td>100 ± 20.18</td>
<td>98.62 ± 21.36</td>
<td>87.68 ± 7.63</td>
<td>106.8 ± 19.71</td>
</tr>
</tbody>
</table>

Supplemental Table I. Platelet surface glycoprotein expression in knockout mouse models

Platelet surface glycoprotein (GP) expression in C57BL/6 strain was determined by flow cytometry. Washed platelets from wild-type (WT), PTP-1B and CD148 single and double knockout (KO and DKO) mice were stained with indicated FITC-conjugated antibodies or CLEC-2 antibody and Alexa Fluor 488-conjugated secondary antibody. Geometric mean fluorescence intensities were measured for each sample and presented as percent of litter-matched WT (n = 6-10 mice/genotype; mean ± standard deviation; ***P < 0.001 versus WT).
Supplemental Figure I

A

Whole cell lysates prepared of washed platelets (5 × 10^8/mL) from wild-type (WT), PTP-1B and CD148 single and double knockout (KO and DKO) mice were western blotted with anti-CD148, -PTP-1B and -actin antibodies. Images are representative of three experiments. Platelet count (B) and mean platelet volume (MPV) (C) in whole blood. (n = 9-11 mice/genotype; mean ± standard deviation).

B

C