

The Catalytic Subunit of Protein Phosphatase 1 Gamma Regulates Thrombin-Induced Murine Platelet $\alpha_{IIb}\beta_3$ Function

Francisca C. Gushiken¹, Han Hyojeong¹, Subhashree Pradhan¹, Kimberly W. Langlois², Nawaf Alrehani¹, Miguel A. Cruz¹, Rolando E. Rumbaut^{1,2,3}, K. Vinod Vijayan^{1*}

1 Department of Medicine, Baylor College of Medicine, Houston, Texas, United States of America, **2** Department of Pediatrics, Baylor College of Medicine, Houston, Texas, United States of America, **3** Medical Care Line, Michael E DeBakey VA Medical Center, Houston, Texas, United States of America

Abstract

Background: Hemostasis and thrombosis are regulated by agonist-induced activation of platelet integrin $\alpha_{IIb}\beta_3$. Integrin activation, in turn is mediated by cellular signaling via protein kinases and protein phosphatases. Although the catalytic subunit of protein phosphatase 1 (PP1c) interacts with $\alpha_{IIb}\beta_3$, the role of PP1c in platelet reactivity is unclear.

Methodology/Principal Findings: Using γ isoform of PP1c deficient mice (PP1c $\gamma^{-/-}$), we show that the platelets have moderately decreased soluble fibrinogen binding and aggregation to low concentrations of thrombin or protease-activated receptor 4 (PAR4)-activating peptide but not to adenosine diphosphate (ADP), collagen or collagen-related peptide (CRP). Thrombin-stimulated PP1c $\gamma^{-/-}$ platelets showed decreased $\alpha_{IIb}\beta_3$ activation despite comparable levels of $\alpha_{IIb}\beta_3$, PAR3, PAR4 expression and normal granule secretion. Functions regulated by outside-in integrin $\alpha_{IIb}\beta_3$ signaling like adhesion to immobilized fibrinogen and clot retraction were not altered in PP1c $\gamma^{-/-}$ platelets. Thrombus formation induced by a light/dye injury in the cremaster muscle venules was significantly delayed in PP1c $\gamma^{-/-}$ mice. Phosphorylation of glycogen synthase kinase (GSK3) β -serine 9 that promotes platelet function, was reduced in thrombin-stimulated PP1c $\gamma^{-/-}$ platelets by an AKT independent mechanism. Inhibition of GSK3 β partially abolished the difference in fibrinogen binding between thrombin-stimulated wild type and PP1c $\gamma^{-/-}$ platelets.

Conclusions/Significance: These studies illustrate a role for PP1c γ in maintaining GSK3 β -serine9 phosphorylation downstream of thrombin signaling and promoting thrombus formation via fibrinogen binding and platelet aggregation.

Citation: Gushiken FC, Hyojeong H, Pradhan S, Langlois KW, Alrehani N, et al. (2009) The Catalytic Subunit of Protein Phosphatase 1 Gamma Regulates Thrombin-Induced Murine Platelet $\alpha_{IIb}\beta_3$ Function. PLoS ONE 4(12): e8304. doi:10.1371/journal.pone.0008304

Editor: Karl-Wilhelm Koch, University of Oldenburg, Germany

Received: September 10, 2009; **Accepted:** November 19, 2009; **Published:** December 15, 2009

Copyright: © 2009 Gushiken et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from National Institutes of Health (NIH) HL081613 (K.V.V.), HL079368 (R.E.R) and a Grant-In Aid 09GRNT2260224 from the American Heart Association (AHA) to K.V.V. F.C.G. is supported by the Scientist Development Grant 060180N from the AHA. S.P. was supported in part by NIH grant T-32HL072754. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: vvijayan@bcm.tmc.edu

Introduction

Agonist-induced platelet activation is critical for maintaining hemostasis at the site of vascular injury. Activation of platelets also contributes to the process of thrombosis following the rupture of atherosclerotic plaques. Thrombin generated at the site of injury binds to protease activated receptors (PAR)1 or PAR4 on human or mouse platelets, triggers protein phosphorylation on serine/threonine (Ser/Thr) residues and facilitates integrin $\alpha_{IIb}\beta_3$ activation [1]. In particular, Ser/Thr kinases such as mitogen-activated protein kinase (MAPK) [2], protein kinase G (PKG) [3,4], protein kinase B/AKT [5,6], protein kinase C α (PKC α) [7], PKC θ [8] and glycogen synthase kinase 3 β (GSK3 β) [9] play a key role in $\alpha_{IIb}\beta_3$ activation by thrombin or PAR4 activating peptide (PAR4-AP). Importantly, thrombin-induced and Ser/Thr kinase-mediated activation of signaling circuitry in platelets is reversible. Since protein phosphatases can fine-tune kinase-mediated signaling processes, we hypothesized that Ser/Thr phosphatases participate in hemostasis/thrombosis by regulating agonist-induced platelet activation.

Platelets express several Ser/Thr phosphatases, and a pool of the catalytic subunits of protein phosphatase 1 (PP1c) and protein phosphatase 2A (PP2Ac) associates with integrin $\alpha_{IIb}\beta_3$ [10,11]. Generic Ser/Thr phosphatase inhibitors like okadaic acid and calyculin A inhibited agonist-induced platelet aggregation, secretion [12–14], adhesion and spreading to immobilized fibrinogen [15]. Since these pharmacological agents inhibit several closely related phosphatases (namely, PP1, PP2A and PP4) [16], it is difficult to interpret the specific contribution of protein phosphatase 1 (PP1) and/or its isoforms in agonist-induced integrin $\alpha_{IIb}\beta_3$ signaling. Our goal was to address a role for PP1 in platelets using a genetic approach.

PP1 is a major eukaryotic Ser/Thr protein phosphatase that regulates a variety of functions like glycogen metabolism, muscle contraction, transcription, translation and cell division [17,18]. PP1 is a multimeric enzyme formed by the assembly of catalytic and regulatory subunits. PP1c is a 35–38 kDa protein that exists as three isoforms: α , β/δ and γ sharing greater than 90% amino acid sequence similarity. Two splice variants of PP1c γ (PP1c $\gamma 1$ and

PP1 γ 2) have also been identified. All PP1c isoforms are ubiquitously expressed, except for PP1 γ 2 that is testis specific. Cellular PP1 activity is regulated by multiple factors: 1) reversible phosphorylation of the regulatory subunits 2) dissociation of the regulatory and the catalytic subunits 3) allosteric regulation of the regulatory subunits and 4) inducible expression of the regulatory subunits [17].

Among the various isoforms of PP1c, mice bearing a targeted deletion of the gene for PP1 γ [*Ppp1cc*^{-/-}] are viable and are considered in this study [19]. Using PP1 γ ^{-/-} platelets, we report a moderate decrease in low dose thrombin-induced $\alpha_{IIb}\beta_3$ activation, soluble fibrinogen binding, platelet aggregation and thrombus formation. GSK3 β -Ser9 phosphorylation that promotes platelet function was also decreased in thrombin-stimulated PP1 γ ^{-/-} platelets. GSK3 β inhibitor partially abrogated the difference in fibrinogen binding between thrombin-stimulated wild type and PP1 γ ^{-/-} platelets. These studies suggest a positive role for PP1 γ in thrombin-induced platelet functional responses.

Materials and Methods

Materials

Unless stated, all reagents were from Sigma-Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated antibodies (anti-mouse CD41 (α_{IIb}) and CD62 (P-selectin) were from BD Bioscience (San Jose, CA). Phycoerythrin-conjugated JON/A (recognizes activated murine $\alpha_{IIb}\beta_3$) and thrombin were gifts from Dr. B. Nieswandt (University of Wurzburg, Germany) and Dr. J. Fenton (New York State Department of Health, Albany), respectively. ADP and collagen were from Helena Laboratories (Beaumont, TX). GSK3 β inhibitor VIII was from Calbiochem EMD4Biosciences (Darmstadt, Germany). Protease-activated receptor 4-activating peptide (PAR4AP) AYPGKF was synthesized by the Protein Core Facility at Baylor College of Medicine. Collagen-related peptide (CRP) was synthesized at Baylor College of Medicine and cross-linked by glutaraldehyde. Alexa 488-conjugated fibrinogen was from Invitrogen, (Carlsbad, CA) while human fibrinogen was from Enzyme Research Laboratories Inc. (South Bend, IN). Antibodies to phospho-AKT Ser473, phospho-GSK3 β Ser9 and AKT were obtained from Cell signaling (Boston, MA). Antibodies to the α , and γ isoforms of PP1c, PAR3 and PAR4 were purchased from Santacruz Biotechnology (Santacruz, CA). β isoform of PP1c was from Upstate Biotechnology/Millipore (Billerica, MA).

Mice Platelet Preparation

All animal studies were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine. CD-1 mice carrying the PP1 γ targeted deletion [19] and subsequently backcrossed for 10 generations onto Balb/C background were obtained from Dr. S. Varmuza (University of Toronto). For most studies, 8–14 weeks old wild type (WT) and PP1 γ ^{-/-} littermate mice matched for age and gender were used. Blood was collected from the inferior vena cava of isoflurane-anesthetized mice into acid-citric acid-dextrose (ACD) at a ratio of 1:10 (vol/vol). Whole blood was further diluted with an equal volume of Dulbecco's phosphate buffered saline (D-PBS) (Invitrogen), [2.6 mM KCl, 1.4 mM KH₂PO₄, 137 mM NaCl, 8 mM Na₂HPO₄] containing ACD (1:9 parts ACD/D-PBS). Platelet rich plasma (PRP) was obtained by centrifugation of the diluted blood at 68 g for 10 minutes at room temperature. PRP was further centrifuged at 754 g for 10 minutes and the resulting platelet pellet was washed once in D-PBS and resuspended in D-PBS containing 0.005 U/ml

apyrase to avoid desensitization of ADP receptors. Platelets were counted using a Coulter counter (Beckman-Coulter (Z1), Miami, FL) and adjusted to a final concentration of 2.5×10^8 /ml and then allowed to rest for at least 1 hour.

Immunoblotting Studies

Resting platelets were lysed with 1% Triton X-100 containing lysis buffer supplemented with a cocktail of protease and phosphatase inhibitors. Forty μ g of protein lysate were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with antibodies to different isoforms of PP1c, PAR3, PAR4 and actin. In some experiments, washed platelet treated with varying concentrations of thrombin for 2 minutes were lysed and immunoblotted with antibodies to phospho-AKT Ser 473, phospho-GSK3 β Ser9, and AKT. Binding of primary antibodies were detected by HRP-conjugated secondary antibodies developed using ECL-system (Amersham Biosciences).

Fibrinogen Binding, Flow Cytometry Analysis and Platelet Aggregation

To study fibrinogen binding, washed platelets were diluted to a concentration of 2.5×10^7 /ml with Tyrode's buffer containing 1.8 mM CaCl₂ and 0.49 mM MgCl₂. Platelets were stimulated with various concentrations of the following agonists: thrombin (0.005, 0.01, 0.02, 0.1 U/ml), adenosine diphosphate (ADP) (5, 10, 20 μ M), collagen (1, 2.5 and 5 μ g/ml), collagen-related peptide (CRP) (0.1, 0.2 μ g/ml), PAR4-AP (100, 500 μ M) followed by addition of Alexa 488-conjugated fibrinogen (50 μ g/ml final concentration) at room temperature for 15–20 minutes. In some studies, platelets were pretreated with control (DMSO) or 10 μ M GSK3 β inhibitor VIII for 30 minutes before stimulation with thrombin (0.02 U/ml). In other studies, platelets (resting or thrombin-stimulated) were incubated with FITC-conjugated anti-mouse CD41, PE-conjugated anti-mouse GPIIb α , PE-labeled JON/A, and FITC-conjugated anti-mouse P-selectin antibodies to detect $\alpha_{IIb}\beta_3$, GPIIb α expression, activation of integrin $\alpha_{IIb}\beta_3$, and α granule secretion, respectively. Fluorescence-activated cell sorting (FACS) analysis of platelets was performed using an EPICS-XL flow cytometry (Beckman Coulter). Platelet aggregation in response to various agonists was initiated by addition of various agonists to a 225 μ l aliquot of washed platelets in a four channel Bio/Data PAP-4C aggregometer (Biodata Corporation, Horsham, PA)

Platelet Adhesion and Clot Retraction

Ninety six well plates were coated with 100 μ g/ml of fibrinogen and blocked with 5 mg/ml of Bovine Serum Albumin (BSA). Control wells were coated with only BSA. Fibrinogen or BSA coated wells were incubated with 1×10^7 washed platelets in D-PBS supplemented with 1.8 mM CaCl₂ and 0.49 mM MgCl₂ for varying time periods at 37°C. Unbound platelets were washed and the attached platelets were quantified by assaying for acid phosphatase activity at 405 nM. The number of platelets attached was obtained by using a standard curve for absorbance versus cell number. Fibrin-clot retraction, was initiated by the addition of 1 U/ml thrombin to 150 μ l of mouse platelet-rich plasma containing (2.5×10^8 platelets/ml) supplemented with 3 mM CaCl₂. After incubating at RT for varying time periods, the amount of liquid not incorporated into the clot is measured. The volume of the clot was determined by subtracting the measured volume from 150 μ l (initial volume). Clot volume was expressed as a percentage of the initial volume.

In Vivo Platelet Thrombus Formation

Microvascular thrombosis was examined in the cremaster venules using a previously well described model of light/dye – induced endothelial injury by intravital microscopy [20,21]. Briefly, male mice anesthetized with 50 mg/kg phenobarbital sodium were placed on a custom plexiglas tray maintained at 37°C with a homeothermic blanket. To assist in breathing, a tracheotomy was performed. Later, an internal jugular vein and common carotid artery were cannulated to facilitate administration of various preparations and monitoring of blood pressure/heart rate, respectively. The cremaster microvascular bed was prepared, perfused with bicarbonate-buffered saline solution (pH range, 7.35–7.45) at 35°C and transferred to the stage of an upright intravital video microscope (BX-50; Olympus, Tokyo, Japan). After equilibration for 30 minutes, 10 mL/kg 5% FITC-labeled dextran (150 kD) was injected intravenously through the jugular vein. A suitable venule was selected after survey of the vascular bed with a 4 \times objective (NA 0.13) lens. Following the measurement of the diameter and blood flow velocity of the vessel (Doppler velocimeter; Microcirculation Research Institute, College Station, TX), a photochemical injury was initiated by exposing approximately 100 μ m of the vessel to filtered excitation light at 0.6 W/cm² (from a 175W xenon lamp; Sutter Instrument, Novato, CA; and an HQ-FITC filter cube; Chroma Technology, Brattleboro, VT). Epi-illumination was applied continuously, and the time of onset of platelet aggregates (thrombus onset) and the time of flow cessation (for at least 60 seconds) were monitored and recorded using a 40 \times water-immersion objective (NA 0.8) by an observer who was blinded to the genotype of the animals. Thrombi were induced in 1–2 venules per animal, and the results for each animal were averaged.

Tail-Bleeding Times

Tails of anesthetized mice (WT and PP1c γ ^{-/-}) were severed with a scalpel 1 mm from the tip and immediately immersed in a tube containing PBS solution at 37°C. Thirty seconds later, the tail was immersed into a new tube with PBS and this process of transferring the tail to a new tube was repeated until the bleeding stopped. The time from the incision to cessation of blood (as revealed by visual examination of blood in the tubes) were recorded as tail bleeding times.

Statistical Analysis

Results are expressed as means \pm SEM. Experimental conditions were compared by using paired student's *t* test.

Results

PP1c γ ^{-/-} Platelets Exhibited Comparable Expression of Other PP1c Isoforms

Immunoblotting studies with an antibody specific for the γ isoform of PP1c confirmed the absence of PP1c γ protein in PP1c γ ^{-/-} platelets (Figure 1C). Furthermore, there was no evidence for a compensatory up-regulation of α and β isoforms of PP1c in the platelets from PP1c γ ^{-/-} mice (Figure 1A and 1B). These blots were stripped and reprobed with actin to ensure equal protein loading (Figure 1A, Band C lower panel).

PP1c γ ^{-/-} Platelets Exhibited a Mild Agonist-Specific Impairment in Fibrinogen Binding and Aggregation

To determine if the loss of *Ppp1cc* gene affected platelet reactivity washed platelets from the WT and PP1c γ ^{-/-} mice were stimulated with platelet agonists and analyzed for soluble

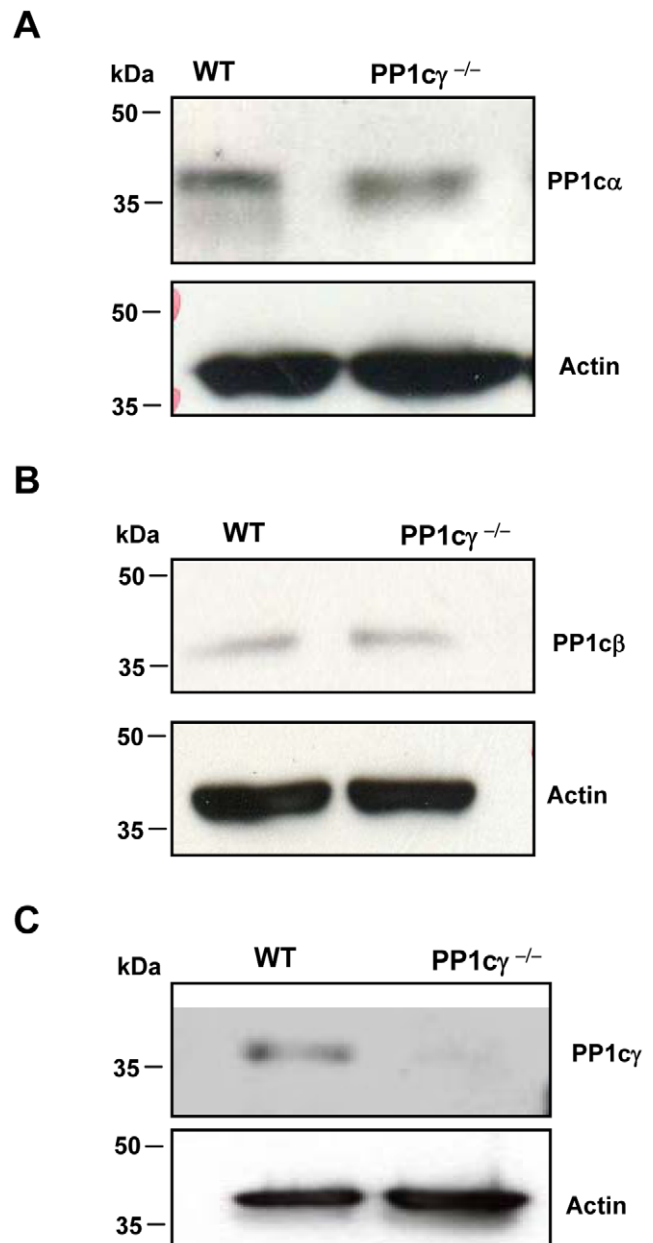


Figure 1. PP1c isoforms in platelets. (A–C) Platelet lysate from wild type (WT) or PP1c γ ^{-/-} mice were evaluated by SDS-PAGE and Western blotting using isoform specific antibodies to α , β and γ (recognizes γ 1 and γ 2 splice variants). These membranes were stripped and reprobed with actin to demonstrate equal protein loading (lower panels). Blots are representative of three independent experiments. doi:10.1371/journal.pone.0008304.g001

fibrinogen binding. Low concentrations of strong agonists like thrombin, collagen and dilute suspension of platelets were used to ensure that platelets did not aggregate in this assay. ADP, collagen and CRP induced-soluble fibrinogen binding were comparable between the WT and PP1c γ ^{-/-} platelets (Figure 2A). In contrast, addition of low doses of thrombin to PP1c γ ^{-/-} platelets resulted in a modest but significant decrease in the soluble fibrinogen binding. A ~42% ($p=0.02$) and ~45% ($p=0.01$) decreased fibrinogen binding in response to 0.01 U/ml and 0.02 U/ml of thrombin was observed in PP1c γ ^{-/-} platelets (Figure 2B). However, the difference in fibrinogen binding between WT and

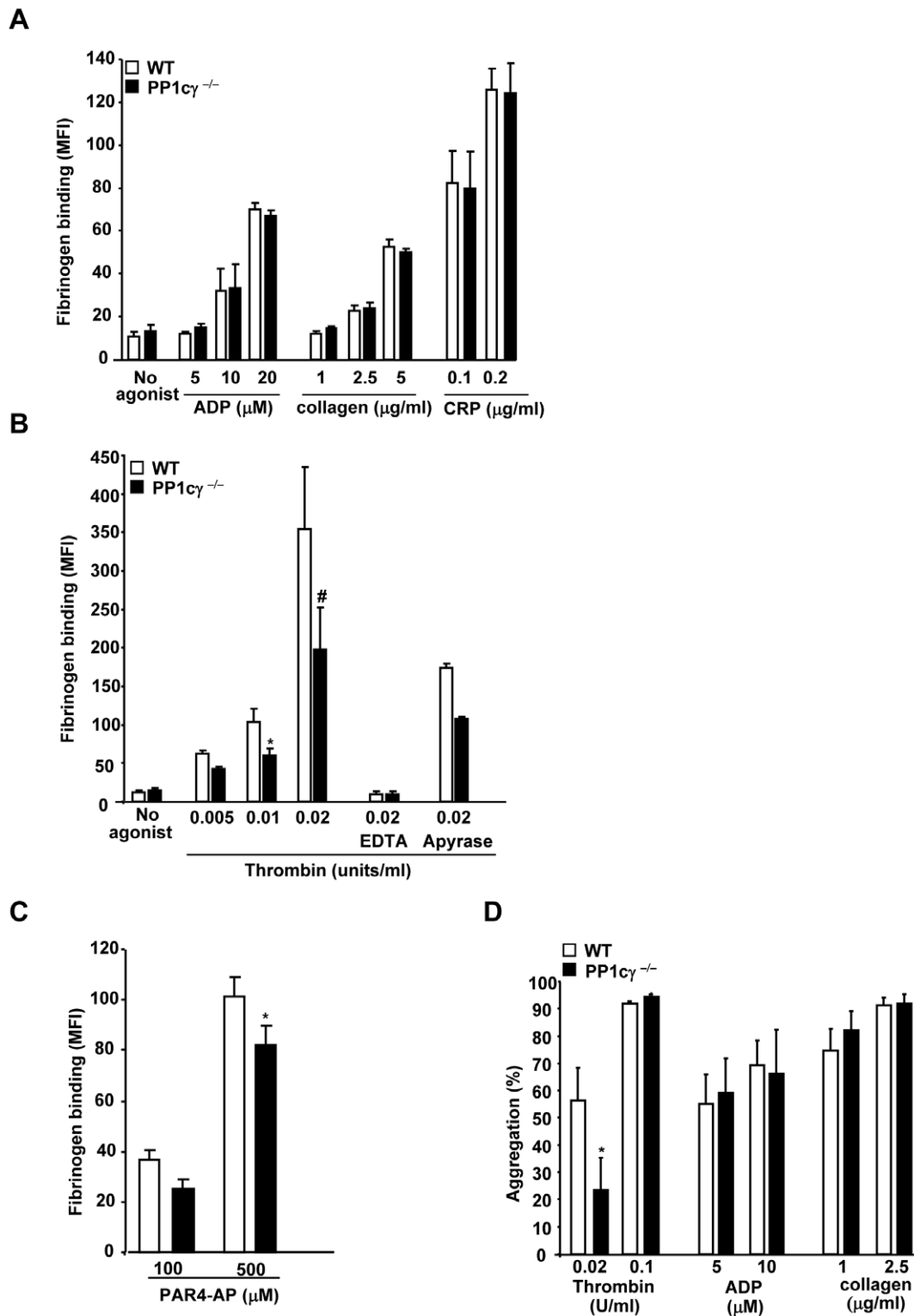


Figure 2. Thrombin specific impairment in soluble fibrinogen binding and aggregation in PP1c γ ^{-/-} platelets. Washed platelets from WT or PP1c γ ^{-/-} mice were stimulated with ADP, collagen or CRP (A), thrombin (B) or PAR4-AP (C) in the presence of Alexa 488 fibrinogen and bound fibrinogen analyzed by flow cytometry as mean fluorescence intensity (MFI). Data are \pm SEM of 9–11 experiments for ADP, thrombin and collagen and 13–14 experiments for PAR4-AP. As compared to the WT platelets, the decreased fibrinogen binding in PP1c γ ^{-/-} platelets were significant at * $p=0.02$ (for 0.01 U/ml thrombin); # $p=0.01$ (for 0.02 U/ml thrombin); * $p=0.05$ (for 200 μ M PAR4-AP). In other experiments ($n=3$), platelets were pretreated with 0.5 mM EDTA or 2 mM apyrase (ADP scavenger) prior to stimulation with 0.02 U/ml thrombin. (D) Percentage aggregation for WT and PP1c γ ^{-/-} platelets in response to the indicated doses of thrombin, ADP and collagen. Studies are indicated as \pm SEM of 5–6 experiments. The decreased aggregation in 0.02 U/ml thrombin stimulated PP1c γ ^{-/-} platelets compared to WT platelets was significant at * $p=0.04$. doi:10.1371/journal.pone.0008304.g002

PP1 γ ^{-/-} platelets was lost with increasing concentrations of thrombin (not shown). Addition of cation chelator EDTA known to block $\alpha_{IIb}\beta_3$ function inhibited fibrinogen binding in WT and PP1 γ ^{-/-} platelets to values obtained in unstimulated platelets. Many, but not all responses of thrombin in human platelets could be reproduced by thrombin receptor activating peptide [22–24]. To explore whether PP1 γ participated in thrombin signaling downstream of mouse thrombin receptor PAR4, we stimulated platelets with protease-activated receptor 4 activating peptide (PAR-4AP) and assessed fibrinogen binding. Compared with wild type platelets, PP1 γ ^{-/-} platelets showed only marginally decreased soluble fibrinogen binding in response to PAR-4AP (P = 0.05) (Figure 2C).

Soluble fibrinogen binding under stirring conditions can lead to platelet aggregation. Next, we determined whether platelet aggregation in response to agonist was altered in PP1 γ ^{-/-} platelets. Compared with WT platelets, PP1 γ ^{-/-} platelets demonstrated ~60% decreased aggregation to 0.02 U/ml thrombin. Aggregation in response to ADP and collagen was comparable between the WT and PP1 γ ^{-/-} platelets (Figure 2D). Since moderate differences in soluble fibrinogen binding and aggregation between the WT and PP1 γ ^{-/-} platelets were noticed only at low thrombin concentrations and not with other agonists, we conducted the rest of the studies in this report with only low dose thrombin.

PP1 γ ^{-/-} Platelets Exhibited Decreased Thrombin-Induced Activation of $\alpha_{IIb}\beta_3$ despite Normal Expression of $\alpha_{IIb}\beta_3$, Thrombin Receptors and Normal Granule Secretion

To identify potential mechanisms for the decreased reactivity in thrombin stimulated PP1 γ ^{-/-} platelets, we analyzed the expression of thrombin receptors PAR3 and PAR4. Because reliable antibodies that detect PAR4 by immunofluorescence were not readily available, we subjected platelet lysate to immunoblotting experiments with anti-PAR3 and PAR4 antibodies. Comparable expression of PAR3 and PAR4 receptors was observed between the WT and PP1 γ ^{-/-} platelets (Figure 3A). Besides PARs, GPIIb α serves as high affinity thrombin binding site [25]. However, GPIIb α expression was not different in WT and PP1 γ ^{-/-} platelets (data not shown). Next, we studied the activation status of integrin $\alpha_{IIb}\beta_3$ in thrombin-stimulated WT and PP1 γ ^{-/-} platelets by flow cytometry using a murine $\alpha_{IIb}\beta_3$ activation-specific JON/A antibody. Under resting conditions, there was no difference in JON/A binding between the WT and PP1 γ ^{-/-} platelets. In contrast, addition of thrombin (0.02 U/ml) to PP1 γ ^{-/-} platelets resulted in a moderate but significant decrease in JON/A binding (Figure 3B). Expression levels of $\alpha_{IIb}\beta_3$ in the resting and thrombin stimulated platelets were comparable in the WT and PP1 γ ^{-/-} mice (Figure 3C). This indicates that the decreased JON/A binding in thrombin stimulated PP1 γ ^{-/-} platelets were not due to the altered integrin expression. Potential alterations in platelet granule secretion events in PP1 γ ^{-/-} mice could account for the decreased $\alpha_{IIb}\beta_3$ activation at low doses of thrombin. However, secretion of P-selectin (α granule content) was not altered between WT and PP1 γ ^{-/-} platelets (Figure 3D). Furthermore, addition of exogenous fibrinogen did not rescue the decreased aggregation of thrombin stimulated PP1 γ ^{-/-} platelets (not shown). ADP scavenger apyrase did not abolish thrombin-induced fibrinogen binding difference between the WT and PP1 γ ^{-/-} platelets (Figure 2B). This observation suggests that the fibrinogen binding difference between the WT and PP1 γ ^{-/-} platelets is not dependent on dense granule ADP. Thus, these

studies suggest that decreased $\alpha_{IIb}\beta_3$ activation and not altered receptor expression or granule secretion may have contributed to the decreased fibrinogen binding in thrombin-treated PP1 γ ^{-/-} platelets.

PP1 γ ^{-/-} Platelet Exhibited Normal Outside in Integrin $\alpha_{IIb}\beta_3$ Signaling

We assessed if functions mediated by outside-in integrin $\alpha_{IIb}\beta_3$ signaling were altered in the PP1 γ ^{-/-} mice. In contrast to the data from soluble fibrinogen, adhesion of PP1 γ ^{-/-} platelets to immobilized fibrinogen was comparable to the WT platelets (Figure 4A). Thrombin-induced clot retraction of platelet rich plasma samples was also not altered between the WT and PP1 γ ^{-/-} mice (Figure 4B). Collectively, these studies imply that outside-in integrin $\alpha_{IIb}\beta_3$ signaling processes were not altered by the lack of PP1 γ in platelets.

PP1 γ ^{-/-} Mice Exhibited Delayed Thrombus Formation in a Light-Dye Injury Thrombosis Model

To establish if the defect in PP1 γ ^{-/-} platelets observed *in vitro* might affect thrombus formation *in vivo*, we studied the responses of WT and PP1 γ ^{-/-} mice to a light/dye-induced injury model that has been previously shown not to denude the endothelium [20]. The initiation time for thrombus formation was not different between the WT and PP1 γ ^{-/-} mice (Fig. 5A). In contrast, we noticed a moderate but significant (P = 0.03) delay in the time for complete cessation of blood flow (occlusion time) in PP1 γ ^{-/-} mice (Fig. 5A). Microvascular diameter and wall shear rate was comparable between the WT and PP1 γ ^{-/-} mice (23.3 ± 0.3 μ m vs. 24.6 ± 0.5 μ m and 515 ± 28 sec⁻¹ vs. 516 ± 29 sec⁻¹, respectively). Similarly, blood pressure and heart rate was not significantly different between the WT and PP1 γ ^{-/-} mice (82.8 ± 1.9 mmHg vs. 81.5 ± 2.1 mmHg and 436 ± 14 min⁻¹ and 465 ± 12 min⁻¹, respectively) suggesting that the delayed thrombus formation in PP1 γ ^{-/-} mice was independent of these above mentioned factors. Thus, in this thrombosis model, PP1 γ participates modestly in stabilizing thrombus formation. In contrast, tail bleeding times were not different between the WT and PP1 γ ^{-/-} mice (Figure 5B).

Thrombin-Stimulated PP1 γ ^{-/-} Platelets Exhibited Decreased Glycogen Synthase Kinase3 β -Serine 9 Phosphorylation in an AKT Independent Manner

Platelet functional responses to thrombin are mediated in part by activation of signaling molecules in the PI3K-AKT-GSK-3 β circuitry. Interestingly, there exist some similarity in the phenotype of PP1 γ ^{-/-} mice when compared to AKT2^{-/-}, AKT1^{-/-} and GSK3 β ^{+/-} mice. All four null mice predominantly showed thrombin or PAR4 specific impairment in platelet function except AKT1^{-/-} mice, which also exhibited defects in platelet responses to collagen [6]. Therefore, we investigated if PP1 γ regulates thrombin-induced signaling to AKT. Activation of AKT as measured by phosphorylation of AKT-Ser473 was comparable between the thrombin stimulated WT and PP1 γ ^{-/-} platelets (Figure 6A and 6B). Next, we assessed the activation of GSK3 β (AKT substrate) because PP1 can regulate GSK-3 β Ser9 phosphorylation, independent of AKT, under *in vitro* conditions [26]. GSK3 β is a suppressor of platelet function and agonist-induced Ser9 phosphorylation decreases GSK3 β kinase activity and promotes platelet function [9]. Consistent with the earlier report [9], thrombin stimulation in WT platelets resulted in sustained phosphorylation of GSK-3 β -Ser9. Unexpectedly, thrombin-stimulated PP1 γ ^{-/-} platelets showed a maximum of ~1.9

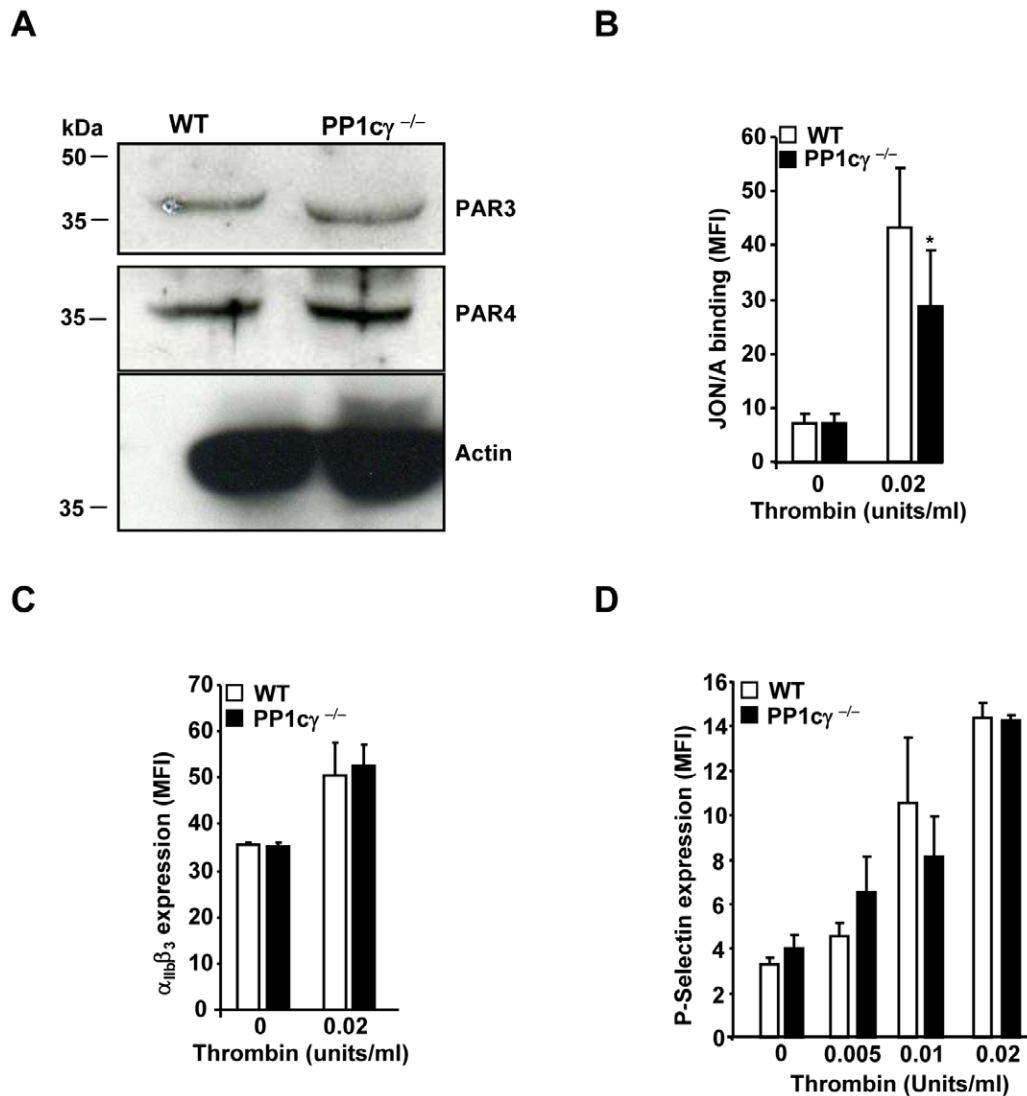


Figure 3. Expression of thrombin receptors, $\alpha_{IIb}\beta_3$ and P-selectin in PP1 γ ^{-/-} platelets. (A) Lysate from resting washed platelets was evaluated by SDS-PAGE and immunoblotted with anti-PAR3 antibody (~43 kDa). The blot was stripped and reprobed with anti-PAR4 (~38 kDa) and then with anti-actin (loading) antibodies. Blots are representative of two experiments. Washed platelets were incubated with anti-JON/A PE antibody (B) or anti- α_{IIb} FITC antibody (C) or anti-P-selectin antibody (D) and subjected to flow cytometry. Results are expressed as \pm SEM of 5–6 experiments. Compared to thrombin stimulated WT platelets, the decreased binding of JON/A antibody to PP1 γ ^{-/-} platelets was significant at * $p=0.003$. doi:10.1371/journal.pone.0008304.g003

fold decreased GSK-3 β -Ser9 phosphorylation (6A and 6C). These observations imply a role for PP1 γ in GSK-3 β -Ser9 phosphorylation downstream of thrombin signaling by an AKT independent pathway. It should be emphasized that although a majority of PAR4-stimulated GSK3 β -Ser9 phosphorylation was previously shown to be dependent on PI3K/AKT signaling, the same study also reported a fraction of GSK3 β -Ser9 phosphorylation that was independent of PI3K/AKT pathway [9]. Thus, a pathway involving PP1 γ might represent one of the non-AKT dependent mechanisms that could potentially regulate GSK3 β -Ser9 phosphorylation.

Since GSK3 β -Ser9 phosphorylation inhibits the constitutive GSK3 β activity, we examined whether the decreased GSK3 β -Ser9 phosphorylation (active GSK3 β) upon thrombin stimulation could contribute to the decreased fibrinogen binding in PP1 γ ^{-/-} platelets. Compared with WT platelets, PP1 γ ^{-/-} platelets exhibited ~40% decreased fibrinogen binding upon

stimulation with 0.02 U/ml thrombin in the presence of DMSO (control). However, in the presence of GSK3 β inhibitor VIII, PP1 γ ^{-/-} platelets exhibited ~23% decreased fibrinogen binding compared to the WT platelets, suggesting that the difference in fibrinogen binding between the thrombin-stimulated WT and PP1 γ ^{-/-} platelets were partially abolished by GSK3 β inhibitor. Thus, these studies imply that GSK3 activity, in part, contributes to the decreased fibrinogen binding in PP1 γ ^{-/-} platelets.

Discussion

Compared with Ser/Thr kinases, a specific role for PP1 γ in platelet activation is unclear. Although a compensatory up-regulation of PP1 α and PP1 β isoforms was not particularly evident in PP1 γ ^{-/-} platelets, a potential redundancy between the individual PP1c isoforms in PP1 γ ^{-/-} platelet function is

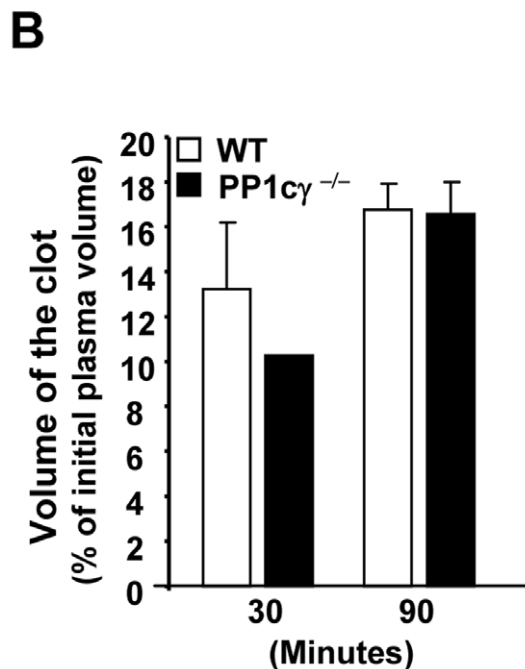
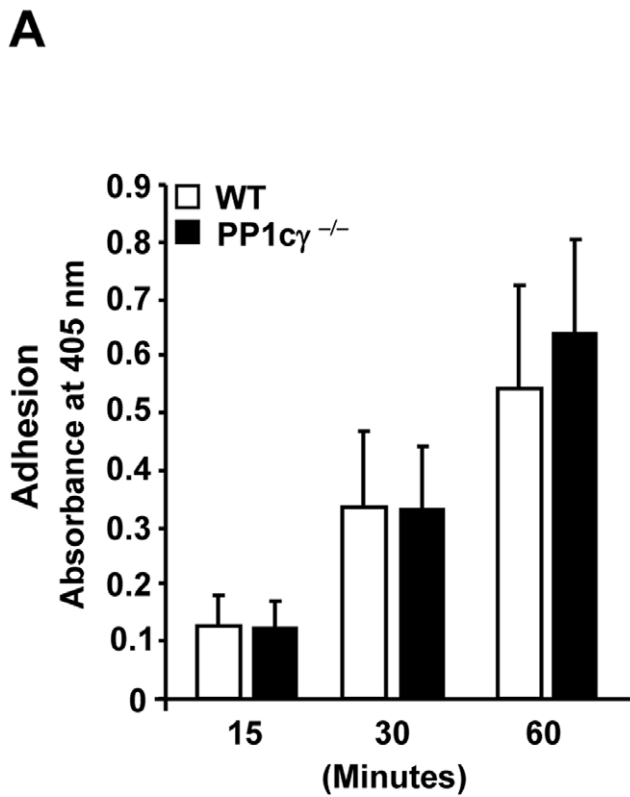


Figure 4. Outside-in integrin $\alpha_{IIb}\beta_3$ signaling functions in PP1c γ ^{-/-} platelets. (A) Washed platelets were incubated with immobilized fibrinogen-coated micro titer wells for the indicated periods of time and adhesion quantified. The data are expressed as \pm SEM of 3 experiments. (B) Fibrin clot retraction was examined following the addition of thrombin to platelet-rich plasma. Volume of the clot is expressed as \pm SEM of 3 experiments. doi:10.1371/journal.pone.0008304.g004

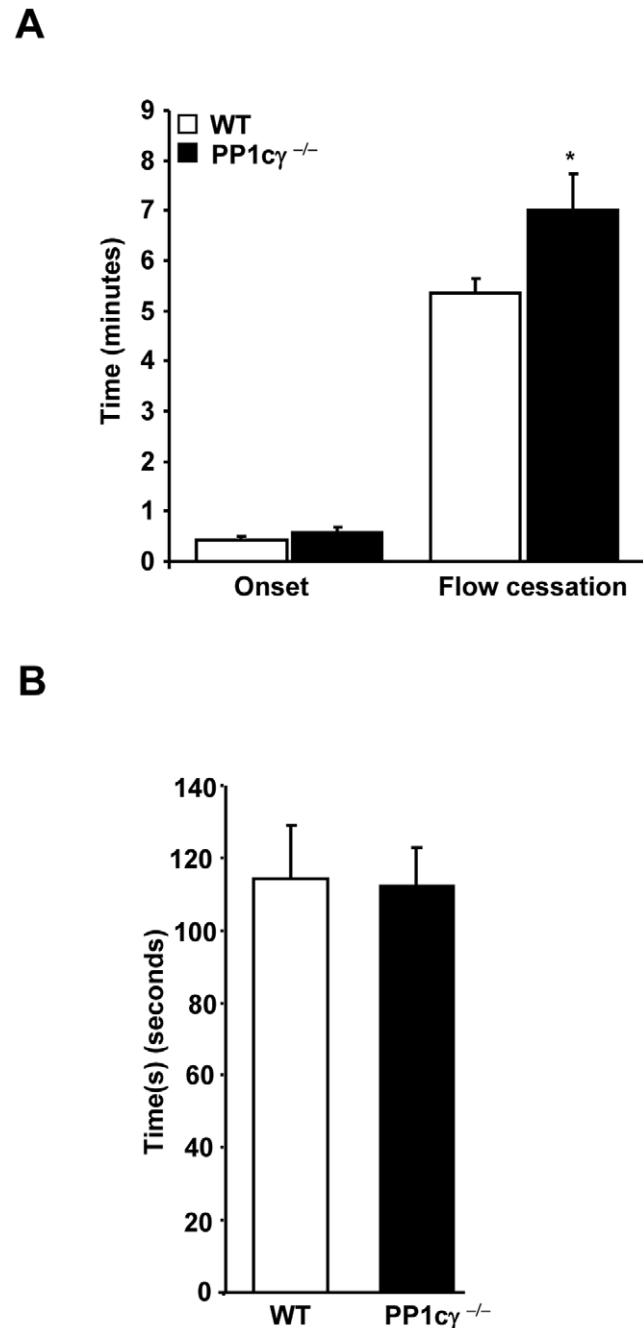


Figure 5. Role of PP1c γ in thrombosis and hemostasis injury models. (A) Light/dye-induced microvascular thrombosis was studied in the venules of the cremaster muscles of WT and PP1c γ ^{-/-} mice by intra vital microscopy. The onset of thrombosis (onset) and the cessation of blood flow following the injury were monitored. Results are expressed as \pm SEM of 13 experiments. Compared to the WT, the delayed time for flow cessation in PP1c γ ^{-/-} mice was significant at *p=0.03. (B). Tail vein bleeding times from 13 WT and PP1c γ ^{-/-} mice are shown. doi:10.1371/journal.pone.0008304.g005

to be expected and may underlie the mild platelet phenotype seen in PP1c γ ^{-/-} mice. Despite this constrain, our studies uncovers two novel aspects of PP1c γ with regards to the platelet biology. 1) PP1c γ regulates low dose thrombin-induced integrin $\alpha_{IIb}\beta_3$ activation, soluble fibrinogen binding, platelet

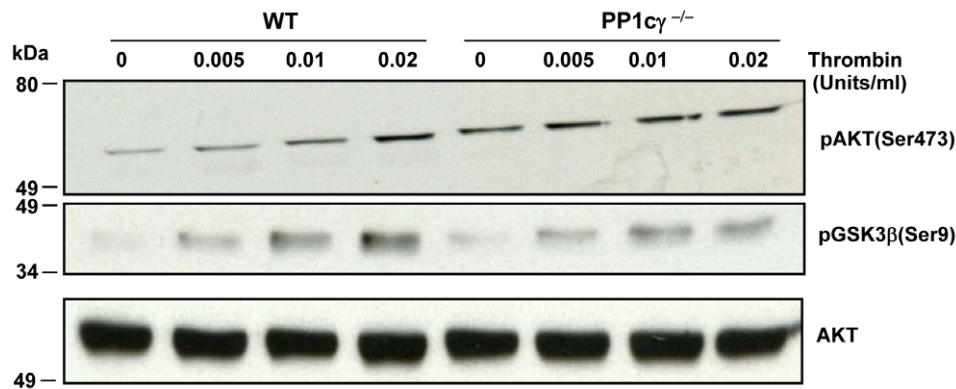
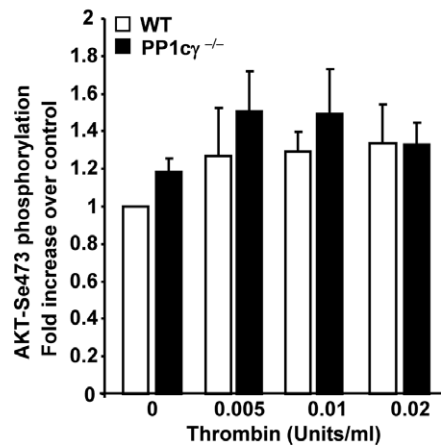
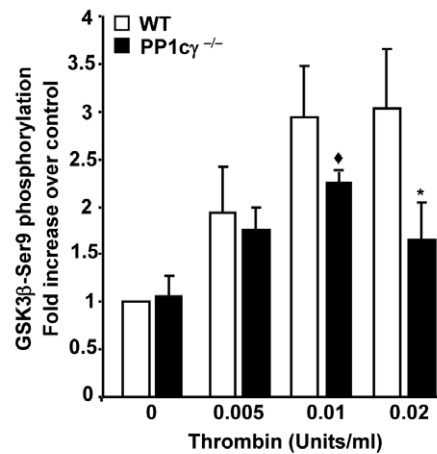
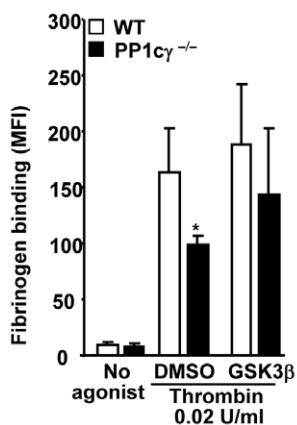
A**B****C****D**

Figure 6. Decreased phosphorylation of GSK3 β -Ser9 in thrombin stimulated PP1 $\gamma^{-/-}$ platelets. (A). Platelet lysate from resting (0) and thrombin activated (0.005, 0.01 and 0.02 U/ml) was separated on an SDS-PAGE and immunoblotted with antibodies to AKT-Ser473, GSK3 β -Ser9 and AKT (total). (B and C) Densitometric quantification of phosphorylation levels for AKT-Ser473 and GSK3 β -Ser9 in WT and PP1 $\gamma^{-/-}$ stimulated platelets. The decreased phosphorylation of GSK3 β -Ser9 in thrombin-stimulated PP1 $\gamma^{-/-}$ platelets was significant at $\diamond p=0.05$ (for 0.01 U/ml thrombin); $*p=0.02$ (for 0.02 U/ml thrombin). Data are expressed as \pm SEM of 5 experiments. (D) Effect of GSK3 β inhibitor VIII on thrombin-induced fibrinogen binding on WT and PP1 $\gamma^{-/-}$ platelets. Compared with WT platelets, PP1 $\gamma^{-/-}$ platelets displayed \sim 40% decreased fibrinogen binding in response to 0.02 U/ml thrombin in the presence of solvent control (DMSO) ($*P=0.048$) and GSK3 inhibitor VIII (GSK3 β) partially reduced the difference to 23%. Data are expressed as \pm SEM of 3–4 experiments. doi:10.1371/journal.pone.0008304.g006

aggregation and *in vivo* thrombus formation. 2) PP1 γ is needed for a sustained thrombin-stimulated GSK3 β -Ser9 phosphorylation in platelets.

Targeted disruption of the *Ppp1c* gene in mice led to impaired spermiogenesis and male infertility [19]. Although PP1 γ is more intensely studied in the area of reproductive biology, our studies indicate that PP1 γ can also regulate platelet activation in an agonist-specific manner. In particular, a subtle role for PP1 γ was noted in $\alpha_{IIb}\beta_3$ activation, fibrinogen binding and aggregation response to low dose thrombin but not to ADP, collagen and CRP (Figures 2). Perhaps, activation of multiple redundant signaling pathways by higher concentrations of thrombin and/or other agonists might favor activation of PP1 α or PP1 β in the absence of PP1 γ to sustain activation of platelets. More importantly, thrombus formation induced by light-dye injury was significantly delayed in the cremasteric venules of the PP1 $\gamma^{-/-}$ mice (Figure 5A). Since PP1 $\gamma^{-/-}$ is not a platelet specific knockout, contribution of PP1 γ from other cell types within the vasculature towards *in vivo* thrombosis cannot be ruled out. Nevertheless, this *in vivo* study is consistent and correlates well with the *in vitro* platelet data. In contrast to the thrombosis model, normal hemostasis was not altered in PP1 $\gamma^{-/-}$ mice as revealed by normal tail bleeding times (Figure 5B). It is not known whether the differential role played by PP1 γ in hemostasis versus thrombosis models was due to the intrinsic differences in the type and extent of injury in the two models, or due to the compensatory mechanisms, which was sufficient to drive hemostasis but not thrombosis.

Previous studies have identified that outside-in integrin $\alpha_{IIb}\beta_3$ signaling mechanisms facilitated the dissociation of $\alpha_{IIb}\beta_3$ bound PP1c, with the ability to dephosphorylate platelet myosin light chain (MLC) [10]. Following the engagement of integrin $\alpha_{IIb}\beta_3$, PP1 γ was shown to be involved in the dephosphorylation of ADP-induced nPKC η activation [27]. These observations imply that PP1 γ might regulate platelet functions mediated by outside-in integrin $\alpha_{IIb}\beta_3$ signaling processes. However, outside-in integrin $\alpha_{IIb}\beta_3$ signaling dependent functions like adhesion and spreading (not shown) to immobilized fibrinogen and clot retraction were not altered in PP1 $\gamma^{-/-}$ mice. Since all PP1c isoforms can interact with $\alpha_{IIb}\beta_3$ (unpublished observation), one possibility is that the PP1 γ isoform does not participate in the functional responses initiated by outside-in integrin $\alpha_{IIb}\beta_3$ signaling. Indeed, platelet MLC is dephosphorylated by PP1 β/δ (myosin phosphatase) but not PP1 γ [28]. Another possibility relates to an easy compensation by other PP1c isoforms following engagement of integrin $\alpha_{IIb}\beta_3$ to alter platelet outside-in signaling functions. Finally, the platelet functional data from PP1 $\gamma^{-/-}$ mice was not completely consistent with the studies in human platelets treated with Ser/Thr protein phosphatase inhibitors. The latter studies employed calyculin A and okadaic acid and observed inhibition of platelet aggregation and secretion to various agonists and adhesion to immobilized fibrinogen [12–15]. These differences may stem from the fact that the platelet phenotype obtained in response to the pharmacological agents might be due to inhibition of multiple phosphatases as opposed to inhibition of only PP1 γ in this study.

Decreased platelet responses to thrombin observed in the PP1 $\gamma^{-/-}$ mice was not due to altered expression of PAR3, PAR4, $\alpha_{IIb}\beta_3$ or decreased platelet counts. In fact, we noticed a moderate but significant increase in platelet numbers in PP1 $\gamma^{-/-}$ mice [Mean \pm SEM $596.78 \times 10^3/\text{mm}^3 \pm 17.94$ in WT compared with $693.71 \times 10^3/\text{mm}^3 \pm 19.87$ in PP1 $\gamma^{-/-}$ mice; N = 12, P = 0.0003]. However, we cannot rule out the possibility that the lack of PP1 γ isoform could alter PAR4 activation associated proximal signaling events such as binding of GTP to the G proteins that couple to PAR4. Perhaps, multiple substrates of

PP1 γ may exist in the signaling pathways that control $\alpha_{IIb}\beta_3$ activation downstream of thrombin signaling. Absence of PP1 γ may alter the phosphorylation of one or more of these cellular proteins, thereby suppressing the platelet function. In fact, we noticed that the GSK3 β -Ser9 phosphorylation was significantly reduced in thrombin stimulated PP1 $\gamma^{-/-}$ platelets (Figures 6A and 6C). GSK3 β is Ser/Thr kinase and phosphorylation of Ser9 residue inhibits its constitutively active kinase activity [29]. Although an initial study using a more generic and non selective GSK3 inhibitor implied a positive role for GSK3 β in platelet function [30], a more recent comprehensive genetic analysis using GSK3 β heterozygote mice revealed that GSK3 β is a negative regulator of platelet function [9]. Results from the latter study indicated that thrombin stimulated GSK3 β Ser 9 phosphorylation and removed the suppressive role of GSK3 β on platelet function. Thus, a decreased GSK3 β -Ser9 phosphorylation upon thrombin stimulation is predicted to maintain a constitutive GSK3 β activity, thereby inhibiting the platelet function. Indeed, our studies revealed that the PP1 $\gamma^{-/-}$ platelets stimulated with thrombin had decreased GSK3 β -Ser9 phosphorylation along with decreased $\alpha_{IIb}\beta_3$ activation, fibrinogen binding, aggregation and delayed thrombus formation. Moreover, pharmacological inhibition of GSK3 β partially abolished the difference in fibrinogen binding between WT and PP1 $\gamma^{-/-}$ platelets (Figure 6D). These observations suggest that additional mechanisms that underpin the decreased fibrinogen binding in thrombin-stimulated PP1 $\gamma^{-/-}$ platelets exist and remain to be identified.

The molecular basis for the relationship between the loss of PP1 γ and decreased GSK3 β -Ser9 phosphorylation is currently unclear. PP1 γ does not appear to directly dephosphorylate Ser9 on GSK3 β , because GSK3 β -Ser9 phosphorylation under basal conditions was not significantly increased in PP1 $\gamma^{-/-}$ platelets. Although AKT has a major role in GSK3 β Ser9 phosphorylation, PP1 γ does not appear to regulate GSK3 β phosphorylation via AKT, since we observed comparable level of AKT-Ser473 phosphorylation in thrombin stimulated WT and PP1 $\gamma^{-/-}$ platelets. A potential for AKT independent regulation of GSK3 β -Ser9 phosphorylation was also noted in the previous study [9]. Since PKC, PKA or p90Rsk can also phosphorylate GSK3 β at Ser(9) [31], it is likely that PP1 γ could affect GSK3 β Ser9 phosphorylation by regulating the activation of one or more of these Ser/Thr kinases. Alternatively, PP1 γ may inhibit other Ser/Thr phosphatases such as PP2A [32], which may participate in dephosphorylating Ser9 on GSK3 β under conditions of thrombin stimulation thereby ensuring increased GSK3 β -Ser9 phosphorylation.

In summary, our studies indicate that under conditions of low thrombin stimulation, platelet PP1 γ is required for GSK3 β -Ser9 phosphorylation, $\alpha_{IIb}\beta_3$ activation, soluble fibrinogen binding, platelet aggregation and stabilization of thrombus formation. The agonist specific role for PP1 γ is intriguing and future studies will be needed to address how PP1 γ couples specifically to signaling by PAR receptors and not to other G-protein coupled receptors.

Acknowledgments

We thank Dr. Susannah Varmuza (University of Toronto) for providing us with PP1 $\gamma^{-/-}$ mice.

Author Contributions

Conceived and designed the experiments: FCG MAC RER KVV. Performed the experiments: FCG HH SP KWL NA KVV. Analyzed the data: FCG HH SP KWL NA MAC RER KVV. Wrote the paper: FCG MAC RER KVV. Maintained the animal colony and generated the experimental animals: NA.

References

- Shattil SJ, Newman PJ (2004) Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* 104: 1606–1615.
- Li Z, Xi X, Du X (2001) Mitogen-activated Protein Kinase-dependent Signaling Pathway in the Activation of Platelet Integrin $\alpha_{IIb}\beta_3$. *J Biol Chem* 276: 42226–42232.
- Li Z, Zhang G, Feil R, Han J, Du X (2006) Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin $\alpha_{IIb}\beta_3$. *Blood* 107: 965–972.
- Li Z, Zhang G, Marjanovic JA, Ruan C, Du X (2004) A platelet secretion pathway mediated by cGMP-dependent protein kinase. *J Biol Chem* 279: 42469–42475.
- Woulfe D, Jiang H, Morgans A, Monks R, Birnbaum M, et al. (2004) Defects in secretion, aggregation, and thrombus formation in platelets from mice lacking Akt2. *J Clin Invest* 113: 441–450.
- Chen J, De S, Damron DS, Chen WS, Hay N, et al. (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. *Blood* 104: 1703–1710.
- Konopatskaya O, Gilio K, Harper MT, Zhao Y, Cossemans JM, et al. (2009) PKC α regulates platelet granule secretion and thrombus formation in mice. *J Clin Invest* 119: 399–407.
- Nagy B Jr, Bhavaraju K, Getz T, Bynagari YS, Kim S, et al. (2009) Impaired activation of platelets lacking protein kinase C- θ isoform. *Blood* 113: 2557–2567.
- Li D, August S, Woulfe DS (2008) GSK3 β is a negative regulator of platelet function and thrombosis. *Blood* 111: 3522–3530.
- Vijayan KV, Liu Y, Li TT, Bray PF (2004) Protein phosphatase 1 associates with the integrin α_{IIb} subunit and regulates signaling. *J Biol Chem* 279: 33039–33042.
- Gushiken FC, Patel V, Liu Y, Pradhan S, Bergeron AL, et al. (2008) Protein phosphatase 2A negatively regulates integrin $\alpha_{IIb}\beta_3$ signaling. *J Biol Chem* 283: 12862–12869.
- Nishikawa M, Toyoda H, Saito M, Morita K, Tawara I, et al. (1994) Calyculin A and okadaic acid inhibit human platelet aggregation by blocking protein phosphatases types 1 and 2A. *Cell Signal* 6: 59–71.
- Hoyt CH, Lerea KM (1995) Aggregation-dependent signaling in human platelets is sensitive to protein serine/threonine phosphatase inhibitors. *Biochemistry* 34: 9565–9570.
- Higashihara M, Takahata K, Kurokawa K, Ikebe M (1992) The inhibitory effects of okadaic acid on platelet function. *FEBS Lett* 307: 206–210.
- Lerea KM, Cordero KP, Sakariassen KS, Kirk RI, Fried VA (1999) Phosphorylation sites in the integrin β_3 cytoplasmic domain in intact platelets. *J Biol Chem* 274: 1914–1919.
- McCluskey A, Sim AT, Sakoff JA (2002) Serine-threonine protein phosphatase inhibitors: development of potential therapeutic strategies. *J Med Chem* 45: 1151–1175.
- Cohen PT (2002) Protein phosphatase 1—targeted in many directions. *J Cell Sci* 115: 241–256.
- Ceulemans H, Bollen M (2004) Functional diversity of protein phosphatase-1, a cellular economizer and reset button. *Physiol Rev* 84: 1–39.
- Varmuza S, Jurisicova A, Okano K, Hudson J, Boekelheide K, et al. (1999) Spermiogenesis is impaired in mice bearing a targeted mutation in the protein phosphatase 1c γ gene. *Dev Biol* 205: 98–110.
- Rumbaut RE, Randhawa JK, Smith CW, Burns AR (2004) Mouse cremaster venules are predisposed to light/dye-induced thrombosis independent of wall shear rate, CD18, ICAM-1, or P-selectin. *Microcirculation* 11: 239–247.
- Patel KN, Soubra SH, Bellera RV, Dong JF, McMullen CA, et al. (2008) Differential role of von Willebrand factor and P-selectin on microvascular thrombosis in endotoxemia. *Arterioscler Thromb Vasc Biol* 28: 2225–2230.
- Lau LF, Pumiglia K, Cote YP, Feinstein MB (1994) Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. *Biochem J* 303 (Pt 2): 391–400.
- Lasne D, Donato J, Falet H, Rendu F (1995) Different abilities of thrombin receptor activating peptide and thrombin to induce platelet calcium rise and full release reaction. *Thromb Haemost* 74: 1323–1328.
- Satoh K, Ozaki Y, Asazuma N, Yatomi Y, Ruomei Q, et al. (1996) Differential mobilization of tyrosine kinases in human platelets stimulated with thrombin or thrombin receptor agonist peptide. *Biochem Biophys Res Commun* 225: 1084–1089.
- De ML, Mazzucato M, Masotti A, Fenton JW, Ruggeri ZM (1991) Function of glycoprotein Ib alpha in platelet activation induced by alpha-thrombin. *J Biol Chem* 266: 23776–23783.
- Zhang F, Phiel CJ, Spece L, Gurvich N, Klein PS (2003) Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *J Biol Chem* 278: 33067–33077.
- Bynagari YS, Nagy B Jr, Tuluc F, Bhavaraju K, Kim S, et al. (2009) Mechanism of activation and functional role of protein kinase C ϵ in human platelets. *J Biol Chem* 284: 13413–13421.
- Watanabe Y, Ito M, Kataoka Y, Wada H, Koyama M, et al. (2001) Protein kinase C-catalyzed phosphorylation of an inhibitory phosphoprotein of myosin phosphatase is involved in human platelet secretion. *Blood* 97: 3798–3805.
- Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378: 785–789.
- Barry FA, Graham GJ, Fry MJ, Gibbins JM (2003) Regulation of glycogen synthase kinase 3 in human platelets: a possible role in platelet function? *FEBS Lett* 553: 173–178.
- Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 359: 1–16.
- Sutherland C, Leighton IA, Cohen P (1993) Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem J* 296 (Pt 1): 15–19.