The heterotrimeric G protein $G\beta_1$ interacts with the catalytic subunit of protein phosphatase 1 and modulates G protein–coupled receptor signaling in platelets

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Thrombosis is caused by the activation of platelets at the site of ruptured atherosclerotic plaques. This activation involves engagement of G protein–coupled receptors (GPCR) on platelets that promote their aggregation. Although it is known that protein kinases and phosphatases modulate GPCR signaling, how serine/threonine phosphatases integrate with G protein signaling pathways is less understood. Because the subcellular localization and substrate specificity of the catalytic subunit of protein phosphatase 1 (PP1c) is dictated by PP1c-interacting proteins, here we sought to identify new PP1c interactors. GPCRs signal via the canonical heterotrimeric $G\alpha$ and $G\beta\gamma$ subunits. Using a yeast two-hybrid screen, we discovered an interaction between PP1cα and the heterotrimeric G protein $G\beta_1$ subunit. Co-immunoprecipitation studies with epitope-tagged PP1c and $G\beta_1$ revealed that $G\beta_1$ interacts with the PP1c α, β, and γ1 isoforms. Purified PP1c bound to recombinant $G\beta_1$-GST protein, and PP1c co-immunoprecipitated with $G\beta_1$ in unstimulated platelets. Thrombin stimulation of platelets induced the dissociation of the PP1c-$G\beta_1$ complex, which correlated with an association of PP1c with phospholipase C β3 (PLCβ3), along with a concomitant dephosphorylation of the inhibitory Ser^{1105} residue in PLCβ3. siRNA-mediated depletion of *GNB1* (encoding $G\beta_1$) in murine megakaryocytes reduced protease-activated receptor 4, activating peptide-induced soluble fibrinogen binding. Thrombin-induced aggregation was decreased in PP1c$\alpha^{-/-}$ murine platelets and in human platelets treated with a small-molecule inhibitor of $G\beta\gamma$. Finally, disruption of PP1c-$G\beta_1$ complexes with myristoylated $G\beta_1$ peptides containing the PP1c binding site moderately decreased thrombin-induced human platelet aggregation. These findings suggest that $G\beta_1$ protein enlists PP1c to modulate GPCR signaling in platelets.

The process of thrombosis entails activation of platelets at the site of ruptured atherosclerotic plaques. At these sites, platelets are likely exposed to several soluble agonists including thrombin, adenosine diphosphate (ADP), and thromboxane A2 (TXA2). These agonists engage distinct G protein–coupled receptors (GPCRs)[4] on platelets and initiate inside-out signaling (1, 2). This signaling process activates integrin $\alpha_{IIb}\beta_3$, enables the binding of plasma fibrinogen, and facilitates platelet aggregation. Binding of fibrinogen to activated $\alpha_{IIb}\beta_3$ in turn generates outside-in signals that modulate the rearrangement of platelet cytoskeleton and stabilizes the growing platelet clot (3).

An essential mediator of the GPCR signaling is the G protein $G\alpha\beta\gamma$ heterotrimers. Agonist binding to GPCR triggers the exchange of $G\alpha$ subunit–associated guanosine 5′-diphosphate (GDP) with guanosine 5′-triphosphate (GTP). This event induces a conformational change in the $G\alpha$ subunit, which may lead to the separation or dissociation of $G\alpha$ and $G\beta\gamma$ subunits, and thereby enable the $G\alpha$ and $G\beta\gamma$ subunits to engage with distinct downstream effectors (4). Propagation of signals involves the assembly and disassembly of multiprotein complexes that are regulated in part by protein phosphorylation in a spatial and temporal fashion. Although phosphorylation is regulated by both protein kinases and protein phosphatases, our understanding of how serine/threonine (Ser/Thr) protein phosphatases integrate with the GPCR signaling pathways has lagged behind that of the Ser/Thr protein kinases.

Protein phosphatase 1 (PP1) is a Ser/Thr phosphatase that regulates an entire range of cellular functions including cell division, cell metabolism, and cell death (5). The major isoforms of the catalytic subunit of PP1 (PP1cα, PP1cβ, and PP1cγ) share ~90% amino acid similarity and do not exist freely in cells. Instead, they associate with a wide range of proteins referred to as phosphatase interacting proteins (PIP) in a spatial

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[4] The abbreviations used are: GPCR, G protein–coupled receptor; PP1c, catalytic subunit of protein phosphatase 1; PAR4, protease-activated receptor 4; PAR4-AP, PAR4 activating peptide; Myr, myristoylated; PIP, phosphatase interacting proteins; RGS18, regulator of G protein signaling 18.

and temporal fashion. PP1 interacts with PIP via short peptide motifs, and many PIPs combine multiple docking motifs to form stable complex with PP1. The protein complexes of PP1c-PIP largely dictate where, when, and which substrates can be dephosphorylated by PP1c (6). We have noticed previously that PP1cγ$^{-/-}$ mice displayed moderately delayed time to occlusion in an *in vivo* thrombosis model and PP1cγ$^{-/-}$ platelets displayed decreased aggregation to low-dose thrombin but not collagen (7). The current study was undertaken to ascertain how PP1 couples with the GPCR-initiated signaling pathways by identifying unique PP1c interactors. To this end, we screened for the interaction partners of PP1cα by yeast two-hybrid analyses and identified the Gβ$_1$ subunit, a component of the heterotrimeric G protein as a novel PP1c binding protein. Depletion of Gβ$_1$ or PP1cα in murine megakaryocytes/platelets and disruption of PP1c-Gβ$_1$ complex by the myristoylated Gβ$_1$ peptide or blockade of Gβγ signaling in human platelets decreased GPCR signaling, suggesting a functional coupling of PP1c with Gβ$_1$ in platelets.

Results

Gβ$_1$ is a new PP1c interacting protein

To identify PP1c interacting partners in the GPCR signaling pathways, we utilized a yeast two-hybrid (Y2H) system with the full-length *PPP1Ca* (PP1cα) as a bait and screened the human bone marrow library. One of the interacting clones was identified as *GNB1* (Gβ$_1$ subunit). Gβ$_1$ is a component of the heterotrimeric G proteins that associate with GPCRs. To validate the findings from yeast two-hybrid studies, we performed co-immunoprecipitation assays in epitope-tagged cells. Gβ$_1$ co-immunoprecipitated with PP1cα, PP1cβ, and PP1cγ$_1$ from human kidney embryonal 293 cells transfected with HA-tagged PP1cα, PP1cβ, PP1cγ$_1$, or FLAG-tagged Gβ$_1$ plasmids (Fig. 1*A*). To ascertain the nature of PP1c-Gβ$_1$ interaction, we performed interaction studies with purified PP1c and Gβ$_1$-GST proteins (Fig. 1*B*). In a glutathione-*S*-transferase (GST) pull-down assay, purified PP1c bound to Gβ$_1$-GST fusion protein but not to GST protein (Fig. 1*C*), suggesting that Gβ$_1$ can interact directly with PP1c. These studies identify Gβ$_1$ as a novel PP1c interacting protein.

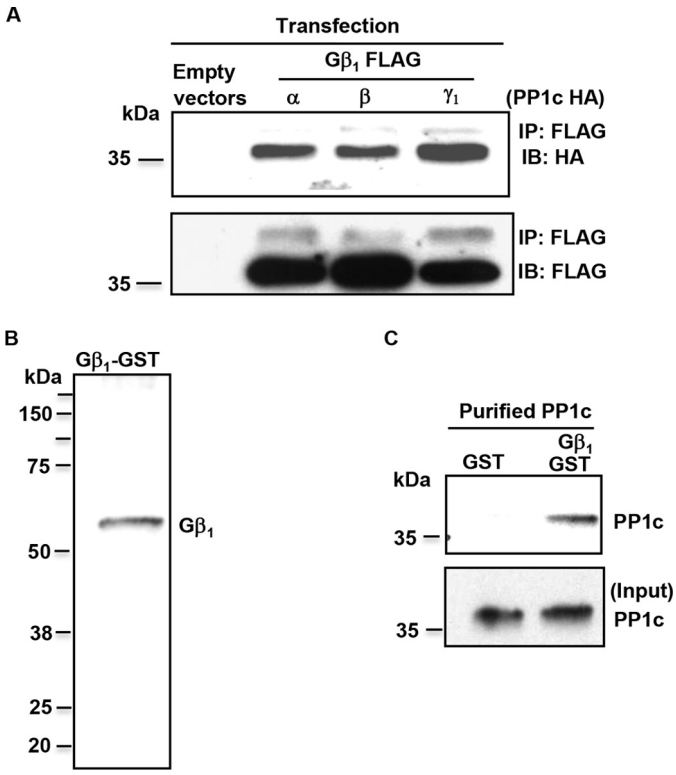

Figure 1. Gβ$_1$ is a novel PP1c interacting protein. *A*, lysate from 293 cells transfected with HA-tagged PP1c α, β, and γ and FLAG-tagged Gβ1 or empty vector were immunoprecipitated (*IP*) and immunoblotted (*IB*) with anti-HA and FLAG antibodies. Blots are representative of three experiments. *B*, characterization of Gβ$_1$-GST protein by immunoblotting with anti-Gβ$_1$ antibody. *C*, Gβ$_1$-GST or GST beads coupled to glutathione beads were used to pull down purified PP1c. Blots are representative of two experiments.

Presence of PP1c-Gβ$_1$ protein complex in platelets and its modulation during platelet activation

To examine if PP1c-Gβ$_1$ protein complex exists endogenously in platelets, we performed co-immunoprecipitation (Co-IP) assays with platelet lysate. Gβ$_1$ was immunoprecipitated using anti-Gβ$_1$ antibody from lysate obtained from unstimulated platelets and platelets treated with thrombin or ADP. Immunoprecipitated samples were immunoblotted with anti-PP1c antibody. Immunoblots of Gβ$_1$ immunoprecipitates from the resting (Res) platelets detected PP1c. However, the interaction of Gβ$_1$ with PP1c was decreased following platelet stimulation with GPCR agonist thrombin or ADP (Fig. 2, *A* and *B*). These studies suggest that PP1c-Gβ$_1$ protein complex may dissociate following the engagement of GPCRs.

One of the early consequences of GPCR signaling is the activation of the family of phospholipase C beta (PLCβ). Platelets deficient in both PLCβ$_2$ and PLCβ$_3$ showed decreased aggregation to low doses of thrombin and ADP (8). Among the PLCβ isoforms, we noticed that only PLCβ$_3$ contained one of the consensus PP1c binding motifs ((K/R)*X*(V/I)*X*(F/W)) ^{27}KFIKW^{31}. Because the subcellular localization of PP1c is regulated by its ability to partner with distinct proteins, some of which may bear the PP1c binding motifs, we evaluated whether PP1c could interact with PLCβ$_3$ in platelets using co-immunoprecipitation assays. Compared with the resting platelets, we observed an increased interaction of PLCβ$_3$ with PP1c from thrombin- or ADP-stimulated platelets (Fig. 2, *C* and *D*). These studies suggest that the engagement of GPCR could lead to an association of PP1c with PLCβ$_3$.

PLCβ$_3$ is phosphorylated, and in particular Ser^{1105} phosphorylation of PLCβ$_3$ by protein kinase A (PKA) inhibits PLCβ$_3$ activity (9). Therefore, we tested if agonist-induced association of PLCβ$_3$ with PP1c effected Ser^{1105} PLCβ$_3$ phosphorylation. Basal phosphorylation of Ser^{1105} PLCβ$_3$ was evident in unstimulated platelets, and agonist stimulation led to a decrease in the phosphorylation of Ser^{1105} PLCβ$_3$ (Fig. 2, *E* and *F*). Taken together, these studies suggest that thrombin or ADP stimulation of platelets induces the dissociation of PP1c-Gβ$_1$complex, which correlates with the association of PP1c with PLCβ$_3$ and the subsequent dephosphorylation of the inhibitory Ser^{1105} in PLCβ$_3$.

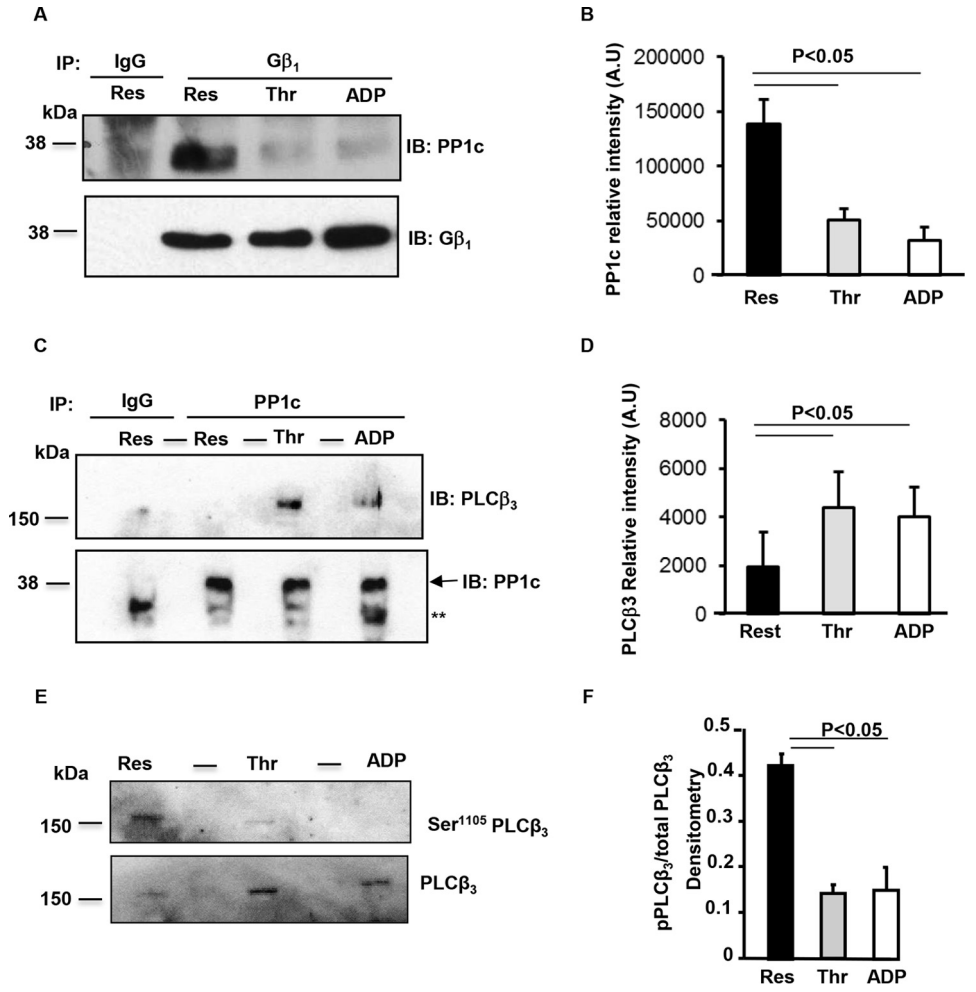

Figure 2. Platelet activation disrupts endogenous PP1c-Gβ$_1$ complex, facilitates PP1c-PLCβ$_3$ interaction, and dephosphorylates PLCβ$_3$ at Ser^{1105}. *A*, *C*, and *E*, platelets were maintained in resting (*Res*) state or treated with thrombin (*Thr*) (0.5 unit/ml) or ADP (10 μM) for 2 mins. *A*, lysate was immunoprecipitated with control IgG or Gβ$_1$ antibodies. Samples separated on SDS-PAGE were immunoblotted with anti-PP1c or Gβ$_1$ antibodies. *B*, densitometry of the relative intensity of PP1c in arbitrary units (AU) from four experiments. *C*, lysate was immunoprecipitated with control IgG or PP1c antibodies. Samples were loaded in alternate lanes and separated on SDS-PAGE and immunoblotted with anti-PLCβ$_3$ and PP1c antibodies. **, nonspecific bands. *D*, densitometry of the relative intensity of PLCβ$_3$ in arbitrary units (AU) from four experiments. *E*, lysate was loaded in alternate lanes and separated on SDS-PAGE and immunoblotted with anti-Ser ^{1105} PLCβ$_3$ or PLCβ$_3$ antibodies. *F*, densitometric quantification of PLCβ$_3$ Ser^{1105}/total PLCβ$_3$ from three to four experiments. *Error bars*, mean ± S. E.

Genetic and pharmacological inhibition of Gβ$_1$-reduced GPCR signaling

Compared with the Gα subunits, the contribution of Gβ and Gγ subunits in platelet GPCR signaling-dependent functions is unclear. Studies with Gβ$_1{}^{-/-}$ platelets were not pursued because Gβ$_1{}^{-/-}$ mice die within 2 days of birth (10). Megakaryocytes, from which platelets are derived, have emerged as an alternate system to study GPCR-induced signaling. Specifically, this model system is amenable to genetic manipulation and is most responsive to thrombin receptor protease-activated receptor 4 (PAR4)–dependent signaling (11, 12). Megakaryocytes cultured from the bone marrow of the wild-type mice were treated with murine *GNB1* siRNA. Depletion of Gβ$_1$ was evident in megakaryocytes (Fig. 3*A*). Compared with the control siRNA-treated megakaryocytes, Gβ$_1$-depleted megakaryocytes displayed decreased PAR4 activating peptide (PAR4-AP)–stimulated soluble fibrinogen binding (Fig. 3*B*). Fibrinogen binding was specific to integrin $\alpha_{IIb}\beta_3$ because addition of EDTA, a cation chelator, decreased PAR4-AP–dependent fibrinogen binding to base line (unstimulated). For the rest of the manuscript, all functional studies were performed with GPCR agonist thrombin. Next, we tested the effect of gallein (13), a small-molecule inhibitor of Gβγ, on human platelet aggregation. Thrombin-induced platelet aggregation was moderately reduced by gallein treatment (Fig. 3, *C* and *D*). Collectively, these studies suggest that genetic depletion of Gβ$_1$ in megakaryocytes and pharmacological blockade of Gβγ signaling in platelets can decrease PAR4/thrombin-induced inside-out integrin $\alpha_{IIb}\beta_3$ signaling.

Decreased thrombin-induced aggregation in PP1cα$^{-/-}$ platelets

To further study the contribution of PP1cα in thrombin signaling, we generated mice that lacked PP1cα in platelets. *PP1cα-loxp (fl)* mice (14) were crossed with megakaryocyte/platelet lineage–specific platelet factor 4 (*PF4) Cre* recombinase knock-in mice (15). The resulting PP1ca$^{-/-}$ mice displayed loss of PP1cα only in platelets but not in lymphocytes (Fig. 4*A*). Consistent with the previous study, wherein PP1cβ

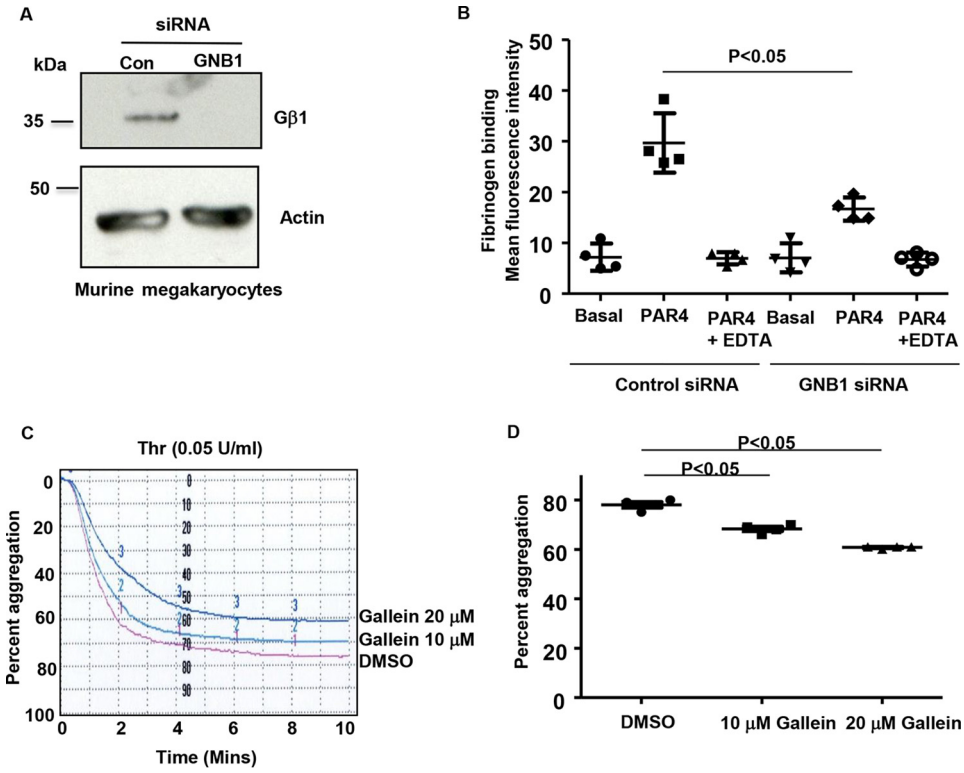

Figure 3. Genetic depletion of Gβ$_1$ and pharmacological inhibition of Gβγ signaling reduce thrombin receptor–induced fibrinogen binding and platelet aggregation. *A*, Gβ$_1$ expression in control and Gβ$_1$ siRNA-treated murine megakaryocytes. Blots were reprobed for actin (loading control). Blots are representative of two to three experiments. *B*, megakaryocytes were mixed with 1 mM PAR4-AP, nonblocking α_{IIb} antibody, 7-AAD dye, and Alexa 488 conjugated fibrinogen. Specific fibrinogen binding was evaluated as mean fluorescence intensity in a gated population of large megakaryocytes that are viable and expressed α_{IIb}. $n = 4$. *C*, aggregation profile of washed platelets preincubated with DMSO and 10 μM or 20 μM gallein and then stimulated with thrombin (0.05 unit/ml). The time to achieve 50% aggregation was increased by gallein treatment as seen by the rightward shift in the tracings. *D*, final platelet aggregation from four subjects is shown. *Error bars*, mean ± S. E.

isoform expression was enhanced in PP1cα$^{-/-}$ cardiac tissue (14), we also noticed ~2-fold up-regulation of PP1cβ isoform in PP1cα$^{-/-}$ platelets (Fig. 4*A*). Next, we assessed the role of PP1cα in thrombin-induced murine platelet aggregation. Compared with the *PP1cα-loxp fl/fl Cre* negative (wild type), *PP1cα-loxp fl/fl Cre* positive (PP1cα$^{-/-}$) platelets displayed decreased aggregation to low-dose (0.02 unit/ml) but not to high-dose (1 unit/ml) thrombin (Fig. 4, *B* and *C*). Moreover, 0.02 unit/ml thrombin-induced soluble fibrinogen binding was also reduced in PP1cα$^{-/-}$ platelets (Fig. 4*D*). Thus, loss of PP1cα decreased low-dose thrombin–induced soluble fibrinogen binding and platelet aggregation.

Disruption of PP1c-Gβ$_1$ complexes reduced thrombin-induced platelet aggregation

We noticed a variant of the RV*X*F PP1c binding motif in Gβ$_1$ at amino acids 78 through 82, ^{78}KLIIW^{82}. To investigate if this region in Gβ$_1$ can support the interaction of PP1c, we constructed full-length Gβ$_1$ plasmids with alanine substitutions at amino acids Lys, Ile, and Trp corresponding to positions 78, 80, and 82, respectively. In addition, a Gβ$_1$ plasmid with all three amino acids (KIW) mutated to alanine (triple mutant) K27A/I29A/W31A was also generated. FLAG-tagged alanine mutants of Gβ$_1$ were co-transfected with HA-tagged PP1c α, β, and γ$_1$ in HEK 293 cells and lysate subjected to co-immunoprecipitation assays. Cells expressing the wild-type Gβ$_1$ retained PP1c in the co-immunoprecipitation assay. Unexpectedly, PP1c co-immunoprecipitated with Gβ$_1$ containing the alanine substitutions at amino acids Lys, Ile, and Trp. However, Gβ$_1$ with the triple alanine mutations failed to support PP1c interaction (Fig. 5*A*). PP1c was adequately expressed under all transfection conditions (Fig. 5*A*, *lowest panel*). These studies reveal that simultaneous disruption of amino acids Lys, Ile, and Trp within the variant PP1c binding region on Gβ$_1$ can diminish PP1c interaction. Thus, this region in Gβ$_1$ may support an interaction with PP1c.

Based on the interaction studies in HEK 293 cells, we generated a synthetic cell permeable myristoylated (Myr) Gβ$_1$ peptide (^{75}QDGKLIIWDSY^{85}) and a scrambled control peptide. Thus, the Gβ$_1$ peptide contained the variant PP1c binding motif and encompassed amino acids 75–85 of the intact protein. We investigated whether the cell-permeable Gβ$_1$ peptide was capable of disrupting the endogenous PP1c-Gβ$_1$ complex by co-immunoprecipitation studies. PP1c was detected in Gβ$_1$ immunoprecipitates obtained from DMSO and the scrambled peptide-treated platelets. Treatment of platelets with the Gβ$_1$ peptide reduced the interaction of PP1c with Gβ$_1$ (Fig. 5*B*). These studies indicate that the myristoylated cell-permeable Gβ$_1$ peptide can decrease the interaction of PP1c with Gβ$_1$ complex in platelets.

To evaluate the consequence of perturbing PP1c-Gβ$_1$ complex on platelet GPCR signaling, we analyzed platelet aggregation in response to low-dose (0.02 unit/ml) thrombin. Pretreat-

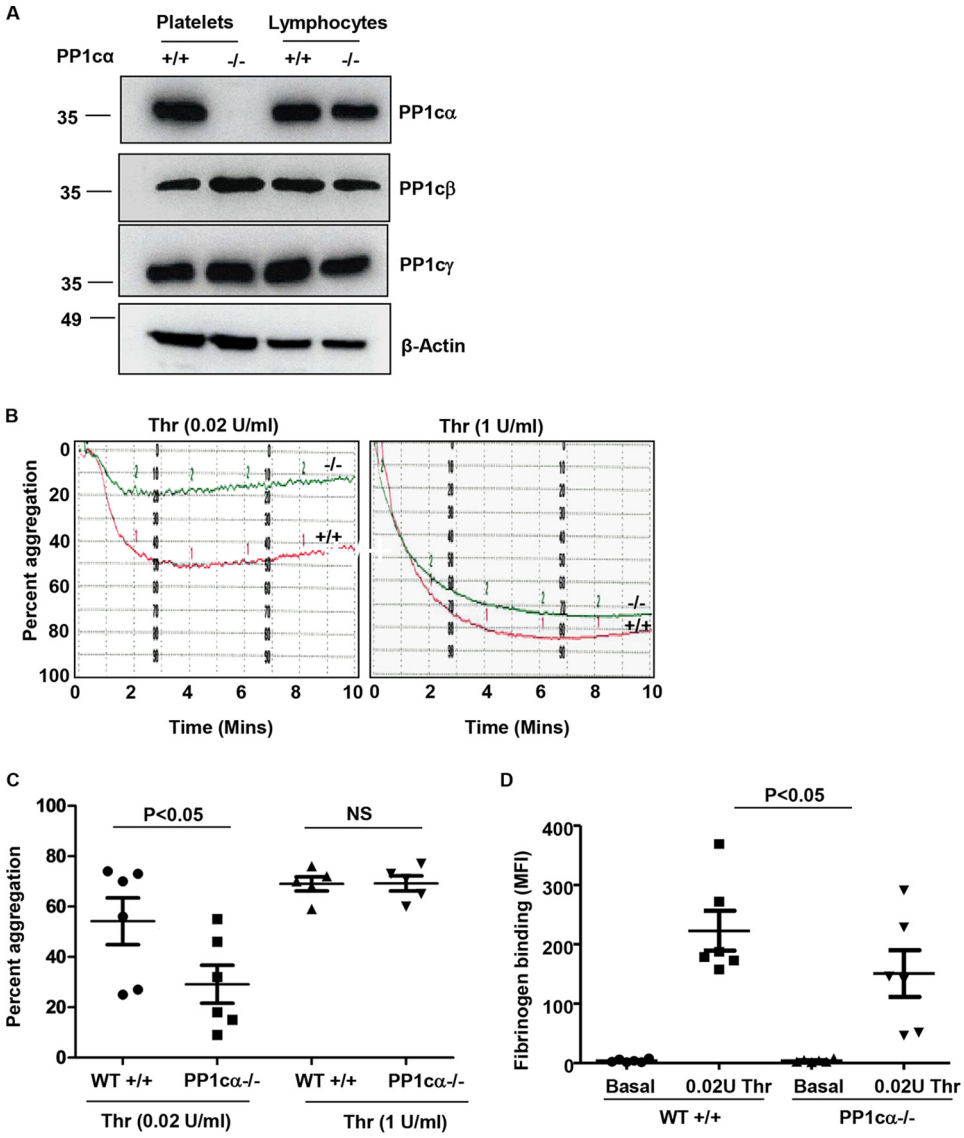

Figure 4. Knock-out of PP1cα in platelets decreases thrombin-induced platelet aggregation and soluble fibrinogen binding. *A*, characterization of platelet-specific PP1cα knock-out mice. Lysate from platelets and non-megakaryocytic tissue (lymphocytes) from *Pf4Cre*$^-$ and PP1c$\alpha^{fl/fl}$ (WT$^{+/+}$) and *Pf4Cre*$^+$ and PP1c$\alpha^{fl/fl}$ (PP1c$\alpha^{-/-}$) were immunoblotted with anti-PP1cα, PP1cβ, PP1cγ, and actin antibodies. *B*, aggregation profile of washed WT and PP1c$\alpha^{-/-}$ platelets stimulated with 0.02 unit/ml and 1 unit/ml thrombin. *C*, final platelet aggregation from six experiments is shown. *D*, platelets (basal, resting) stimulated with thrombin (0.02 unit/ml) in the presence of Alexa 488 conjugated fibrinogen were analyzed by flow cytometry, and soluble fibrinogen binding is shown as mean fluorescence intensity from six experiments. *Error bars*, mean ± S. E.

ment of human platelets with cell-permeable Gβ_1 peptide but not the scrambled peptide moderately decreased platelet aggregation in response to thrombin (Fig. 6, *A* and *B*). Gβ_1 peptide treatment delayed the time to achieve 50% thrombin-induced aggregation. These studies indicate that the complex of PP1c and Gβ_1 participates in platelet function.

Discussion

Platelet activation, in part, via GPCR signaling is critical for hemostasis and thrombosis. Heterotrimeric G proteins Gα and G$\beta\gamma$ subunits that associate with GPCRs transduce signals by engaging multiple effectors, including the protein kinases. Here, we show that Gβ_1 subunit interacts with the catalytic subunit of protein phosphatase 1 and modulates GPCR signaling in platelets. Specifically, GPCR signaling–dependent functions such as soluble fibrinogen binding and/or platelet aggregation was reduced if (*a*) Gβ_1 subunit was depleted by siRNA in murine megakaryocytes, (*b*) PP1cα was deleted by *Cre-lox* approach in murine platelets, (*c*) G$\beta\gamma$ signaling was blocked by a small-molecule inhibitor in human platelets, and (*d*) PP1c-Gβ_1 protein complex was disrupted by a cell-permeable Gβ_1 peptide bearing the PP1c binding motif in human platelets. These studies suggest the importance of coupling G protein–induced inside-out $\alpha_{IIb}\beta_3$ signaling with phosphatase-driven signal transduction during platelet activation.

Gα and G$\beta\gamma$ subunits engage in multiprotein complexes in a spatial and temporal fashion to facilitate transmission of signals from the GPCR. For example, after GPCR activation, the G$\beta\gamma$ subunits engage proteins including arrestin, G protein receptor kinase (GRK), PLCβ, PI3Kγ, etc. (16). In a yeast two-hybrid screen, we noticed a novel interaction of PP1cα with the Gβ_1 subunit. Co-immunoprecipitation assays in epitope-tagged

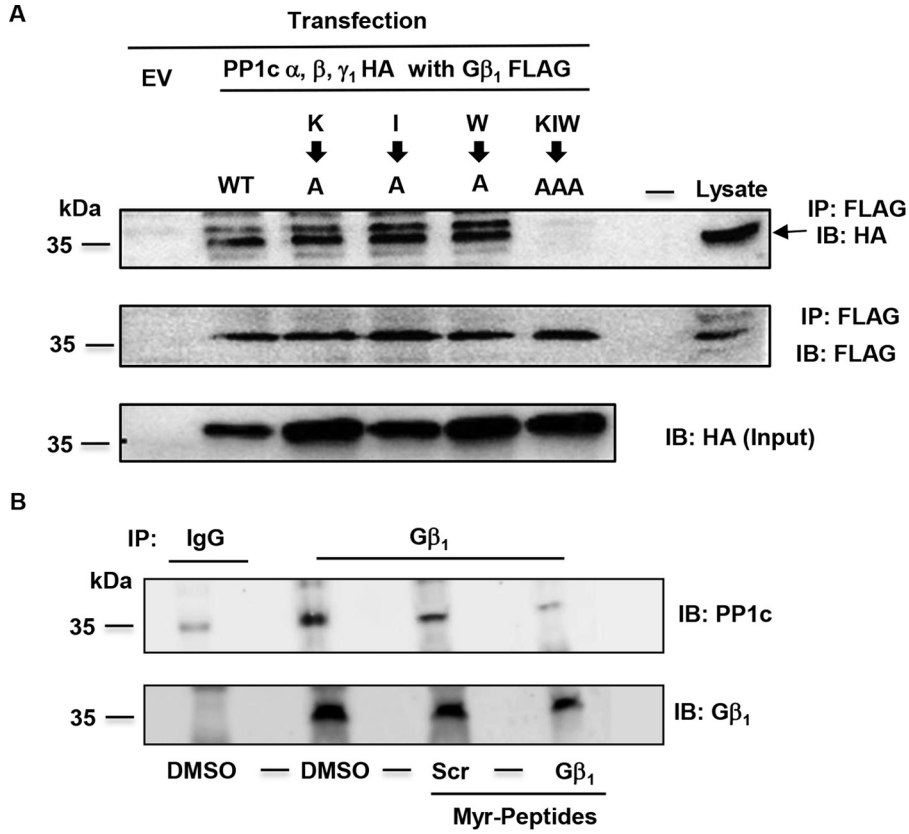

Figure 5. Identification of PP1c binding site on Gβ$_1$ and use of myristoylated Gβ$_1$ peptide to disrupt PP1c-Gβ$_1$ complex in platelets. *A*, 293 cells were transfected with either empty vector (*EV*), FLAG-tagged wild-type Gβ$_1$ (*WT*), or FLAG-tagged Gβ$_1$ with single alanine point mutations at residue Lys^{27}, Ile^{29}, Trp^{31}, or triple alanine mutations at these locations along with HA-tagged PP1c α, β, and γ. Lysate was immunoprecipitated (*IP*) with anti-FLAG antibody and immunoblotted (*IB*) with anti-FLAG and HA antibodies. *Input panel* shows PP1c HA expression in cells transfected with Gβ$_1$ full-length and mutant constructs. Similar results were obtained from two independent experiments. *B*, platelets were treated with vehicle DMSO, 200 μM Myr Gβ$_1$ peptide, or Myr control scrambled (*Scr*) peptide, and lysate was immunoprecipitated with control (*IgG*) and anti-PP1c antibodies and blotted with anti-Gβ$_1$ and PP1c antibodies. Blots are representative of three independent experiments.

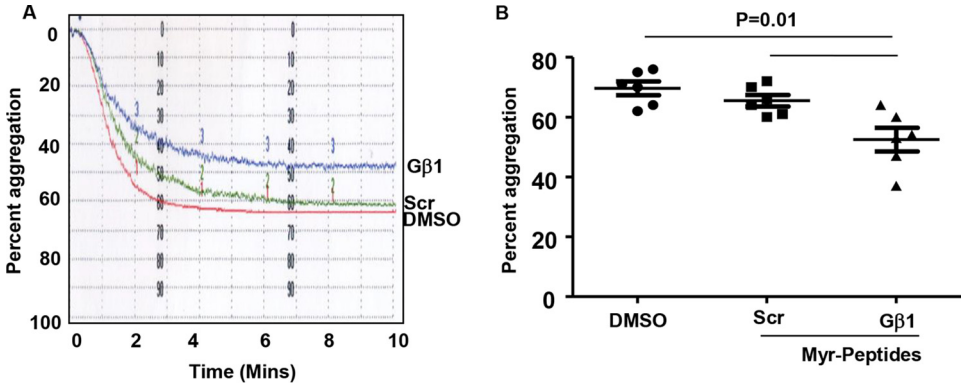

Figure 6. Myristoylated Gβ$_1$ peptide reduces thrombin-induced platelet aggregation. *A*, aggregation profile of washed platelets preincubated with DMSO, Myr control scrambled (*Scr*) peptide, and Myr Gβ$_1$ peptide and then stimulated with thrombin (0.02 unit/ml). *B*, final platelet aggregation from six subjects is shown. *Error bars*, mean ± SEM.

cells revealed that all isoforms of PP1c could interact with Gβ$_1$, whereas *in vitro* studies using purified proteins suggested that the interaction of Gβ$_1$ with PP1c was direct and independent of the Gα and Gγ subunits (Fig. 1). Similar to the ability of Gβ$_1$ to associate with PP1, Gβ-like (GβL) protein can interact with other subtypes of Ser/Thr phosphatases such as PP4, PP2A, and PP6 in transfected 293 cells (17). Such interactions likely facilitate the recruitment of these phosphatases to the IκB kinase (IKK) complex and regulate NF-κB signaling. Besides the Gβ subunit, Gα subunits (Gα$_{12}$ and Gα$_{13}$) can also interact with PP2A (18) and PP5 (19) and stimulate phosphatase activity in COS cells.

What is the significance of Ser/Thr phosphatase containing multiprotein complexes? Unlike Ser/Thr kinases that are diversified by gene duplication, the diversity of PP1 holoenzyme is achieved by the ability of PP1c to engage with a variety of proteins in a dynamic fashion to form separate PP1c-protein complexes. Indeed, over 200 distinct preassembled or signal-in-

duced phosphatase complexes have been identified, wherein, phosphatase-interacting proteins generally enable targeting of the catalytic subunit of the phosphatase to distinct subcellular locations and thereby provide specificity to the holoenzyme (6, 20). Our studies identify the existence of two phosphatase complexes (PP1c-Gβ_1 and PP1c-PLCβ_3) in platelets, not previously described in any cell types (Fig. 2). PP1-Gβ_1 complex, predominately noticed as a preformed complex in nonstimulated platelets, may facilitate the targeting of PP1 to the vicinity of GPCR and contribute to GPCR signaling. Indeed, we noticed that genetic depletion of Gβ1 in murine megakaryocytes reduced GPCR signaling (Fig. 3). However, depletion of Gβ1 is unlikely to ensure an unregulated PP1c that can indiscriminately dephosphorylate substrates. Rather, it is widely believed that PP1c does not exist freely in cell and is regulated by a multiprotein complex with PIP. Therefore, under conditions of Gβ1 depletion, PP1c might be interacting with other isoforms of Gβ or other PIP and respond to thrombin treatment. Pharmacological inhibition of G$\beta\gamma$ or disruption of PP1c-Gβ_1 complex also reduced GPCR signaling in human platelets (Figs. 3 and 6). Loss of PP1cα or PP1cγ (an isoform that also interacts with Gβ_1) reduced low-dose thrombin-induced platelet aggregation (Fig. 4) (7). Although we have not investigated the molecular target(s) of PP1c-Gβ_1 complex in platelets, previous studies in other cell types have noticed PP1cα- (21), PP1cβ- (22), and PP1cγ-mediated (23) dephosphorylation of GPCRs as one of the features for the GPCR resensitization process. It is important to note that besides PP1, PP2A (24), PP2B (25), and PP2C (26) are also reported to regulate GPCR phosphorylation.

PP1c-PLCβ_3 complex observed in agonist-stimulated platelets might target PP1 to regulate PLCβ_3 phosphorylation. In line with this argument, we noticed that Ser^{1105} PLCβ_3 phosphorylation was reduced in agonist-stimulated platelets (Fig. 2). Because previous studies suggest that Ser^{1105} phosphorylation of PLCβ_3 dampens its activity (9, 27), the dephosphorylation of Ser^{1105} PLCβ_3 upon agonist stimulation in platelets may represent one mechanism of PLCβ_3 activation to transmit GPCR signals. It is important to note that the expression of PLCβ_2 is higher than PLCβ_3 in human platelets (28). Moreover, deficiency of PLCβ_2 in human patients was associated with reduced GPCR-induced calcium mobilization, impaired aggregation, and bleeding diathesis (28–30). Although PLCβ_2 isoform is well recognized for relaying GPCR signals, it is possible that PLCβ_3 may have a redundant role in signal propagation downstream of GPCR. Indeed, platelets deficient in both PLCβ_2 and PLCβ_3 showed decreased aggregation to low doses of thrombin and ADP and reduced calcium mobilization and were unable to form stable clots *in vivo* (8).

Our studies bear some resemblance to previous observations in platelets and heterologous cells wherein PP1cα and PP1cγ formed a complex with the PP1 regulatory protein spinophilin (31), and loss of *PPP1R9B* (spinophilin) reduced platelet aggregation to thrombin and thromboxane A2 (32). In another study, PP1c associated with a protein complex containing at least 14–3-3γ and the regulator of G protein signaling 18 (RGS18) in platelets when the cyclic nucleotide levels were elevated (33). PP1c likely facilitated the dephosphorylation of RGS18 Ser^9 and Ser^{218} and the subsequent dissociation of RGS18 from 14–3-3γ complex that enabled RGS18 to dampen platelet activation. The detailed understanding of PP1c multiprotein complexes is not limited to academic interest because this knowledge may enable the development of peptides or small molecules that can bind to PP1 and thus disrupt specific protein complexes for functional studies and/or therapeutic modalities. Indeed, a cell-permeable PP1 disrupting peptide (PDP3) containing the PP1c binding motifs of nuclear inhibitor of PP1 (NIPP1) dephosphorylated mitotic substrates of PP1 and sensitized tumors to chemotherapy, which was induced by protein kinase inhibitors (34). In a similar approach, our studies revealed that Gβ_1 cell-permeable peptide disrupted PP1c-Gβ_1 interaction in platelets and reduced thrombin-induced platelet aggregation (Figs. 5 and 6).

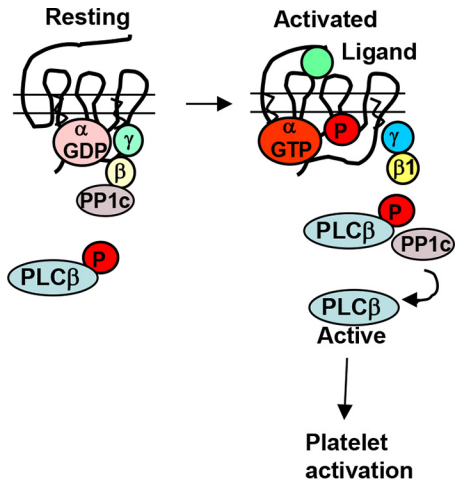

Figure 7. Proposed model depicting the role of PP1c-Gβ_1 and PP1c-PLCβ_3 complexes in GPCR-induced platelet activation. A multiprotein complex of Gβ_1 and PP1c exists presumably in the vicinity of GPCR. Engagement of GPCR with thrombin or ADP leads to the disruption of this complex. A new signal-induced complex of PP1c-PLCβ_3 with a concomitant dephosphorylation of PLCβ_3 Ser^{1105} occurs. Based on previous findings, dephosphorylation of PLCβ_3 may aid in signal transmission. Forced disruption of PP1c-Gβ_1 complex prior to thrombin stimulation reduced platelet aggregation. Collectively, these studies suggest that PP1 facilitates thrombin signaling by coupling with Gβ_1 and PLCβ_3 in the GPCR pathway.

There are some intrinsic limitations with this study. Gβ has five isoforms (16, 35), and one or more of the Gβ isoforms may have facilitated PAR4-dependent signaling in Gβ_1-depleted megakaryocytes. In the case of Gβ_1 peptide study, potential coupling of additional phosphatase subtypes PP2A or PP2B with Gβ_1 and/or other Gβ isoforms or Gα subunits may have facilitated signaling in human platelets. Multiple redundant signaling pathways triggered by the high dose of thrombin could have activated PP1cβ and PP1cγ isoforms in the absence of PP1cα and sustained platelet activation. These caveats may have contributed to the moderate functional phenotypes we noticed with the different approaches. In addition, the use of peptides that contain PP1c binding motifs can disrupt additional PP1c-PIP interactions. Nevertheless, our observations from several lines of investigation suggest that PP1c-Gβ_1 complex along with PP1c-PLCβ_3 complex may facilitate GPCR signaling as shown in a proposed model (Fig. 7).

In conclusion, using platelets as a model system, our studies provide a molecular basis for the functional coupling of PP1c with GPCR function/signaling and thus identify a novel phosphatase complex (PP1c-Gβ_1) as potential target for therapeutic

intervention in thrombotic and inflammatory conditions. Because GPCR, PP1c, and Gβ$_1$ are also ubiquitously expressed proteins that are involved in signal transduction in a variety of cell types, our findings will likely have relevance beyond platelets.

Experimental procedures

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using the Mate & Plate human bone marrow library from Clontech (Palo Alto, CA) (catalog no. 630477). Full-length PP1cα (amino acids 1–473) was generated by PCR using primers 5′-GCC-GAATTCATGGCGGATTTAGATAAAC-3′ (forward) and 3′-CGGGGATCCCTATTTCTTTGCTTGCTTTG-5′ (reverse) and ligated between *EcoR*1 and BamH1 sites of the bait vector pGBKT7. The pGBKT7-PP1cα vector was transformed into the yeast strain AH109 using lithium acetate. To study the interaction, AH109 yeasts containing the bait vector were mated with Y187 yeasts containing the cDNA libraries cloned in a prey vector pGADT7. The mated yeasts were plated on selection media lacking leucine (L) and tryptophan (W) and incubated at 30 °C until the colonies appear. Potential false positive colonies that grew on dropout medium (−LW) were ruled out by also qualitatively analyzing the expression of β-galactosidase (β-gal) marker gene activity by nitrocellulose filter lift assays. Briefly, colonies were transferred to Whatman No. 3 MM filter paper, permeated by brief immersion in liquid nitrogen, and incubated on filter paper saturated with Z-buffer containing 1 mg/ml X-gal at 30 °C for 0.5–8 h. β-gal–positive transformants were re-plated on synthetic medium lacking adenine, leucine, and tryptophan supplemented with histidine (-ALWH) to screen for *Ade*2 reporter gene activity assay. Plasmids from the positive colonies were recovered and amplified in *Escherichia coli* DH5α cells and selected for ampicillin and kanamycin resistance. Colonies which showed resistance for both the markers were analyzed by DNA sequencing using the T7 primers. Approximately 5×10^6 transformants were screened. Plasmids recovered from yeast were re-transformed into native AH109 yeast with the bait construct or nonspecific control selected on high stringency plates with SD/−Ade/−His/−Leu/−Trp/*X*-β-galactosidase to confirm the interaction.

Human platelet isolation

Blood from healthy donors was drawn in an acid citrate dextrose anticoagulant or 3.8% citrate after all the donors signed the informed consent, which was approved by the Institutional Review Board of Baylor College of Medicine. Blood was centrifuged at 189 *g* for 15 min at room temperature to obtain platelet-rich plasma (PRP). In some experiments, platelet-rich plasma supplemented with 75 nM prostaglandin E_1 was recentrifuged at 524 g for 10 min. The resultant platelet pellet was resuspended in Tyrode's buffer and centrifuged. Washed platelets were then suspended in Tyrode's buffer and counts adjusted to 2.5×10^8 platelets/ml.

GST pulldown assays

GST or Gβ$_1$GST (Abnova) (4 μg) pre-coupled with glutathione beads was mixed with 1 μg of recombinant polyhistidine-tagged PP1c (Santa Cruz Biotechnology) for 3 h at 4 °C. Beads were washed three times and the interacting proteins separated by SDS-PAGE and immunoblotted with anti-PP1c antibody.

Generation of DNA constructs and transfection assays

FLAG-tagged cDNA encoding full-length *GNB1* and cloned in pCDNA3.1 was purchased from the cDNA Resource Center, Bloomsburg University. Generation of Gβ$_1$ constructs with single alanine mutations (K27A, I29A, W31A) and triple alanine mutations (K27A/I29A/W31A) was performed using site-directed mutagenesis kit (QuikChange II XL) (Agilent Technologies). All constructs were sequenced and verified to contain the desired mutations. Full-length cDNAs for PP1c α, β, and γ cDNA were subcloned into pCMV vector between *EcoR1* and Not1 sites to generate HA-tagged PP1c. HEK 293 cells were co-transfected with HA-tagged PP1c α, β, γ and FLAG-tagged full-length Gβ$_1$ using Lipofectamine supplemented with PLUS reagent (Life Technologies). In some experiments, FLAG-tagged Gβ$_1$ alanine mutants were used. Cells transfected with empty FLAG and HA vector (EV) served as control. After 48 h, cells were washed with phosphate-buffered saline (PBS) and lysed with radioimmune precipitation assay buffer supplemented with 1 mM Na_3VO_4 and 1 μg/ml leupeptin.

Generation of platelet-specific PP1cα$^{-/-}$ mice

All experiments with mice were performed following approval by the Baylor College of Medicine Institutional Animal Care and Use Committee. *PP1cα-loxP* flox (*fl*) mice were generated in collaboration with Lexicon Genetics as described previously (14). Briefly, exon 3 of *PP1cα* was flanked with loxP sequence that can be recognized by Cre recombinase. *PP1cα flox/flox* homozygote mice were crossed with a megakaryocyte/platelet-specific *Pf4 Cre* knock-in mice (The Jackson Laboratory) (15) to obtain platelet-specific *PP1cα* knock-out mice (PP1cα$^{-/-}$). All studies were performed using male and female littermates (*PP1cα fl/fl* and *Cre* positive, referred to as PP1cα$^{-/-}$) and *PP1cα fl/fl* and *Cre* negative, referred to as wild type (WT$^{+/+}$).

Co-immunoprecipitation assays and immunoblotting

Lysate from transfected 293 cells were immunoprecipitated with anti-FLAG antibody. Samples were separated on a SDS-PAGE and immunoblotted with anti-HA and anti-FLAG antibodies. Platelets (resting) or following stimulation with thrombin (0.5 unit/ml) or ADP (10 μM) for 2 min under non-stirring conditions were lysed with radioimmune precipitation assay buffer and subjected to immunoprecipitation assays. In some experiments, platelets were treated with 0.2% vehicle DMSO, 200 μM myristoylated Gβ$_1$ peptide (^{75}QDGKLIIWDSY^{85}-NH_2) (Selleck Chemicals) or Myr-control scrambled peptide (DQI-LYDSKGWI) for 30 min at 37 °C before lysis. Platelet lysates were immunoprecipitated with control IgG, anti-Gβ$_1$ (Santa Cruz) or anti-PP1c (Santa Cruz) antibodies and immunoblotted with anti-PP1c or anti-PLCβ$_3$ antibodies, respectively. In some experiments, lysate of platelets simulated with agonist was separated on a SDS-PAGE gel and immunoblotted with anti-phospho PLCβ$_3$ (Ser^{1105}) (Cell Signaling) and anti-PLCβ3 (Cell Signaling) antibodies, followed by HRP-conjugated sec-

ondary antibodies, and blots were developed using SuperSignal chemiluminescent substrate (Thermo Scientific). Densitometry was performed using ImageJ software (National Institutes of Health).

Murine megakaryocytes, knockdown, and flow cytometry

Megakaryocytes were obtained from the bone marrow culture of the C57BL/6J black mice as we have described previously (12). Megakaryocytes on day 5 were transfected with 100 nM control siRNA or *GNB1* siRNA using Mirus transfecting agent (Mirus Bio) for 48 h at 37 °C. On day 7, megakaryocyte suspension was lysed and immunoblotted with anti-Gβ_1 and actin antibodies. An aliquot (50 μl) of megakaryocyte suspension was mixed with 1 mM PAR4-AP (AYPGKF) (protein sequencing Advanced Technology Core Laboratories, Baylor College of Medicine) along with nonblocking anti-α_{IIb} antibody, 7-amino-actinomycin D (7-AAD) (BD Biosciences), and Alexa 488 conjugated fibrinogen (20 μg/ml final concentration) (Thermo Scientific) in the presence and absence of 10 mM EDTA for 20 min. Using the Coulter EPICS XL Flow Cytometry System (Beckman Coulter), Alexa fibrinogen binding was measured as mean fluorescence intensity (MFI) in FL1 channel only from a gated population of large megakaryocytes (size) that expressed $\alpha_{IIb}\beta_3$ (FL2) and were viable (negative for 7-AAD in the FL3).

Aggregation assays and soluble fibrinogen binding

Platelet aggregation was analyzed under stirring conditions using eight-channel PAP-8C aggregometer (Bio/Data Corp.) following one of the treatments described below. Washed human platelets were preincubated with DMSO (0.2%), 10 μM and 20 μM gallein (Tocris Bioscience) for 30 min, and then challenged with thrombin (0.05 unit/ml). Washed murine platelets (WT and PP1c$\alpha^{-/-}$) were stimulated with low (0.02 unit/ml) and high (1 unit/ml) thrombin. In other studies, washed human platelets were treated with DMSO, 200 μM Gβ_1, or scrambled peptide for 30 min and then challenged with 0.02 unit/ml thrombin. To study the binding of soluble fibrinogen, washed resting (basal)- and thrombin (0.02 unit/ml)-stimulated murine platelets diluted to 2.5×10^7/ml were incubated with Alexa 488 conjugated fibrinogen (Thermo Scientific) and analyzed by flow cytometry as we have described previously (7).

Statistics

Data are expressed as mean ± S.E. and the statistical significance of the data analyzed using GraphPad Prism. For analysis of two groups, paired Student's *t* test was used, and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used for comparison of differences among multiple groups. $p < 0.05$ was considered statistically significant.

Author contributions—S. P. performed all studies related to human platelets and murine megakaryocytes and analyzed all data. T. K. performed the yeast two-hybrid studies, generated the platelet-specific PP1c knock-out mice, conducted murine platelet studies, and analyzed data. A. C. N. provided the PP1cα flox mice and edited the paper. K. V. V. conceived, designed, and interpreted the study and wrote the paper.

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