The heterotrimeric G protein $G_B\gamma_1$ 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 Go and G$\beta\gamma$ subunits. Using a yeast two-hybrid screen, we discovered an interaction between PP1c and the heterotrimeric G protein $G_B\gamma_1$ subunit. Co-immunoprecipitation studies with epitope-tagged PP1c and $G_B\gamma_1$ revealed that $G_B\gamma_1$ interacts with the PP1c α, β, and γ1 isoforms. Purified PP1c bound to recombinant $G_B\gamma_1$-GST protein, and PP1c co-immunoprecipitated with $G_B\gamma_1$ in unstimulated platelets. Thrombin stimulation of platelets induced the dissociation of the PP1c-$G_B\gamma_1$ complex, which correlated with an association of PP1c with phospholipase C β3 (PLCβ3), along with an upstream dephosphorylation of the inhibitory Ser$^{1103}$ residue in PLCβ3. siRNA-mediated depletion of GNB1 (encoding $G_B\gamma_1$) in murine megakaryocytes reduced protease-activated receptor 4, activating peptide-induced fibrinogen binding. Thrombin-induced aggregation was decreased in PP1cα$^-/-$ murine platelets and in human platelets treated with a small-molecule inhibitor of G$\beta\gamma$. Finally, disruption of PP1c-$G_B\gamma_1$ complexes with myristoylated $G_B\gamma_1$ peptides containing the PP1c binding site moderately decreased thrombin-induced human platelet aggregation. These findings suggest that $G_B\gamma_1$ 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 $\alpha_{IIb}\beta_3$, enabling 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 Ga$\beta\gamma$ heterotrimers. Agonist binding to GPCR triggers the exchange of Ga subunit–associated guanosine 5’-diphosphate (GDP) with guanosine 5’-triphosphate (GTP). This event induces a conformational change in the Ga subunit, which may lead to the separation or dissociation of Ga and G$\beta\gamma$ subunits, and thereby enable the Go 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 (PP1αc, PP1cβ, and PP1cy) 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

$^4$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 PI/Ps 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 PP1c yγ- mice displayed moderately delayed time to occlusion in an in vivo thrombosis model and PP1c yγ- 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 Gβγ subunit, a component of the heterotrimeric G protein as a novel PP1c binding protein. Depletion of Gβγ, or PP1cα in murine megakaryocytes/platelets and disruption of PP1c-Gβγ complex by the myristoylated Gβγ peptide or blockade of Gβγ signaling in human platelets decreased GPCR signaling, suggesting a functional coupling of PP1c with Gβγ in platelets.

Results

Gβγ 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 PPP1Cc (PP1co) as a bait and screened the human bone marrow library. One of the interacting clones was identified as GNB1 (Gβγ subunit). Gβγ 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. Gβγ co-immunoprecipitated with PP1co, PP1cβ, and PP1cγ1 from human kidney embryonal 293 cells transfected with HA-tagged PP1co, PP1cβ, PP1cγ1, or FLAG-tagged Gβγ plasmids (Fig. 1A). To ascertain the nature of PP1c-Gβγ interaction, we performed interaction studies with purified PP1c and Gβγ-GST proteins (Fig. 1B). In a glutathione-S-transferase (GST) pull-down assay, purified PP1c bound to Gβγ-GST fusion protein but not to GST protein (Fig. 1C), suggesting that Gβγ can interact directly with PP1c. These studies identify Gβγ as a novel PP1c interacting protein.

Presence of PP1c-Gβγ protein complex in platelets and its modulation during platelet activation

To examine if PP1c-Gβγ protein complex exists endogenously in platelets, we performed co-immunoprecipitation (Co-IP) assays with platelet lysate. Gβγ was immunoprecipitated using anti-Gβγ antibody from lysate obtained from unstimulated platelets and platelets treated with thrombin or ADP. Immunoprecipitated samples were immunoblotted with anti-PP1c antibody. Immunoblots of Gβγ immunoprecipitates from the resting (Res) platelets detected PP1c. However, the interaction of Gβγ with PP1c was decreased following platelet stimulation with GPCR agonist thrombin or ADP (Fig. 2, A and B). These studies suggest that PP1c-Gβγ 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 PLCβ2 and PLCβ3 showed decreased aggregation to low doses of thrombin and ADP (8). Among the PLCβ isoforms, we noticed that only PLCβ3 contained one of the consensus PP1c binding motifs ((K/R)(V/I)(F/W)) 22KFIKW 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 PLCβ3 in platelets using co-immunoprecipitation assays. Compared with the resting platelets, we observed an increased interaction of PLCβ3 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 PLCβ3.

PLCβ3 is phosphorylated, and in particular Ser 1105 phosphorylation of PLCβ3 by protein kinase A (PKA) inhibits PLCβ3 activity (9). Therefore, we tested if agonist-induced association of PLCβ3 with PP1c effected Ser 1105 PLCβ3 phosphorylation. Basal phosphorylation of Ser 1105 PLCβ3 was evident in unstimulated platelets, and agonist stimulation led to a decrease in the phosphorylation of Ser 1105 PLCβ3 (Fig. 2, E and F). Taken together, these studies suggest that thrombin or ADP stimulation of platelets induces the dissociation of PP1c-Gβγ complex, which correlates with the association of PP1c with PLCβ3 and the subsequent dephosphorylation of the inhibitory Ser 1105 in PLCβ3.
Genetic and pharmacological inhibition of Gβγ-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 Gβ1−/− platelets were not pursued because Gγ1−/− 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 Gβ2 was evident in megakaryocytes (Fig. 3A). Compared with the control siRNA-treated megakaryocytes, Gβ2–depleted megakaryocytes displayed decreased PAR4 activating peptide (PAR4-AP)–stimulated soluble fibrinogen binding (Fig. 3B). Fibrinogen binding was specific to integrin αIIbβ3 because addition of EDTA, a cation chelator, decreased PAR4-AP–dependent fibrinogen binding to baseline (unstimulated). For the rest of the manuscript, all functional studies were performed with GPCR agonist thrombin. Next, we tested the effect of galiein (13), a small-molecule inhibitor of Gβγ on human platelet aggregation. Thrombin-induced platelet aggregation was moderately reduced by galiein treatment (Fig. 3, C and D). Collectively, these studies suggest that genetic depletion of Gβ2 in megakaryocytes and pharmacological blockade of Gβγ signaling in platelets can decrease PAR4/thrombin-induced inside-out integrin αIIbβ3 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 PP1cα−/− mice displayed loss of PP1cα only in platelets but not in lymphocytes (Fig. 4A). Consistent with the previous study, wherein PP1cβ...
isoform expression was enhanced in PP1cα−/− cardiac tissue (14), we also noticed ~2-fold up-regulation of PP1cβ isoform in PP1cα−/− platelets (Fig. 4A). 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. 4D). Thus, loss of PP1cα decreased low-dose thrombin–induced soluble fibrinogen binding and platelet aggregation.

Disruption of PP1c-Gβ1 complexes reduced thrombin–induced platelet aggregation

We noticed a variant of the RVXF PP1c binding motif in Gβ1 at amino acids 78 through 82, KLIIW. To investigate if this region in Gβ1 can support the interaction of PP1c, we constructed full-length Gβ1 plasmids with alanine substitutions at amino acids Lys, Ile, and Trp corresponding to positions 78, 80, and 82, respectively. In addition, a Gβ1 plasmid with all three amino acids (KIW) mutated to alanine (triple mutant) was also generated. FLAG-tagged alanine mutants of Gβ1 were co-transfected with HA-tagged PP1c α, β, and γ, in HEK 293 cells and lysate subjected to co-immunoprecipitation assays. Cells expressing the wild-type Gβ1 retained PP1c in the co-immunoprecipitation assay. Unexpectedly, PP1c co-immunoprecipitated with Gβ1 containing the alanine substitutions at amino acids Lys, Ile, and Trp. However, Gβ1 with the triple alanine mutations failed to support PP1c interaction (Fig. 5A).

Based on the interaction studies in HEK 293 cells, we generated a synthetic cell permeable myristoylated (Myr) Gβ1 peptide (QDGKLIWDSY) and a scrambled control peptide. Thus, the Gβ1 peptide contained the variant PP1c binding motif and encompassed amino acids 75–85 of the intact protein. We investigated whether the cell-permeable Gβ1 peptide was capable of disrupting the endogenous PP1c-Gβ1 complex by co-immunoprecipitation studies. PP1c was detected in Gβ1 immunoprecipitates obtained from DMSO and the scrambled peptide-treated platelets. Treatment of platelets with the Gβ1 peptide reduced the interaction of PP1c with Gβ1 (Fig. 5B). These studies indicate that the myristoylated cell-permeable Gβ1 peptide can decrease the interaction of PP1c with Gβ1 complex in platelets.

To evaluate the consequence of perturbing PP1c-Gβ1 complex on platelet GPCR signaling, we analyzed platelet aggregation in response to low-dose (0.02 unit/ml) thrombin. Pretreati-
ment of human platelets with cell-permeable G\(\beta_1\) peptide but not the scrambled peptide moderately decreased platelet aggregation in response to thrombin (Fig. 6, A and B). G\(\beta_1\) peptide treatment delayed the time to achieve 50% thrombin-induced aggregation. These studies indicate that the complex of PP1c and G\(\beta_1\) participates in platelet function.

**Discussion**

Platelet activation, in part, via GPCR signaling is critical for hemostasis and thrombosis. Heterotrimeric G proteins \(\alpha\) and G\(\beta\gamma\) subunits that associate with GPCRs transduce signals by engaging multiple effectors, including the protein kinases. Here, we show that G\(\beta_1\) 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) G\(\beta_1\) subunit was depleted by siRNA in murine megakaryocytes, (b) PP1c\(\alpha\) 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-G\(\beta_1\) protein complex was disrupted by a cell-permeable G\(\beta_1\) peptide bearing the PP1c binding motif in human platelets. These studies suggest the importance of coupling G protein–induced inside-out IIb/IIIa signaling with phosphatase-driven signal transduction during platelet activation.

\(\alpha\) 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), PLC\(\beta\), PI3K\(\gamma\), etc. (16). In a yeast two-hybrid screen, we noticed a novel interaction of PP1c\(\alpha\) with the G\(\beta_1\) subunit. Co-immunoprecipitation assays in epitope-tagged
cells revealed that all isoforms of PP1c could interact with Gβ₁, whereas in vitro studies using purified proteins suggested that the interaction of Gβ₁ with PP1c was direct and independent of the Gα and Gγ subunits (Fig. 1). Similar to the ability of Gβ₁ 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 (Gα₁₂ and Gα₁₃) 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-

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**Figure 5. Identification of PP1c binding site on Gβ₁, and use of myristoylated Gβ₁ peptide to disrupt PP1c-Gβ₁ complex in platelets.**

A. 293 cells were transfected with either empty vector (EV), FLAG-tagged wild-type Gβ₁ (WT), or FLAG-tagged Gβ₁, with single alanine point mutations at residue Lys²⁷, Ile²⁹, Trp³¹, 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 Gβ₁ full-length and mutant constructs. Similar results were obtained from two independent experiments. B. Platelets were treated with vehicle DMSO, 200 μM Myr Gβ₁ peptide, or Myr control scrambled (Scr) peptide, and lysate was immunoprecipitated with control (IgG) and anti-PP1c antibodies and blotted with anti-Gβ₁ and PP1c antibodies. Blots are representative of three independent experiments.

**Figure 6. Myristoylated Gβ₁ peptide reduces thrombin-induced platelet aggregation.**

A. Aggregation profile of washed platelets preincubated with DMSO, Myr control scrambled (Scr) peptide, and Myr Gβ₁ peptide and then stimulated with thrombin (0.02 unit/ml). B. Final platelet aggregation from six subjects is shown. Error bars, mean ± SEM.

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**Table 1. Transfection with PP1c α, β, γ, HA with Gβ₁, FLAG**

| Transfection | EV | PP1c α, β, γ, HA with Gβ₁, FLAG |
|--------------|----|---------------------------------|
|              |    |                                 |
| kDa          |    |                                 |
| WT           | 35 |                                 |
| A            | 35 |                                 |
| AAA          | 35 |                                 |

**Figure 5. Identification of PP1c binding site on Gβ₁, and use of myristoylated Gβ₁ peptide to disrupt PP1c-Gβ₁ complex in platelets.**

**Figure 6. Myristoylated Gβ₁ peptide reduces thrombin-induced platelet aggregation.**

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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-Gβ1 and PP1c-PLCβ3) in platelets, not previously described in any cell types (Fig. 2). PP1-GB3 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 GB1 in murine megakaryocytes reduced GPCR signaling (Fig. 3). However, depletion of GB1 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 GB1 depletion, PP1c might be interacting with other isoforms of GB or other PIP and respond to thrombin treatment. Pharmacological inhibition of GBγ or disruption of PP1c-Gβ1 complex also reduced GPCR signaling in human platelets (Figs. 3 and 6). Loss of PP1cα or PP1cγ (an isoform that also interacts with Gβγ) reduced low-dose thrombin-induced platelet aggregation (Fig. 4) (7). Although we have not investigated the molecular target(s) of PP1c-Gβ3 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-PLCβ3 complex observed in agonist-stimulated platelets might target PP1 to regulate PLCβ3 phosphorylation. In line with this argument, we noticed that Ser1105 PLCβ3 phosphorylation was reduced in agonist-stimulated platelets (Fig. 2). Because previous studies suggest that Ser1105 phosphorylation of PLCβ3 dampens its activity (9, 27), the dephosphorylation of Ser1105 PLCβ3 upon agonist stimulation in platelets may represent one mechanism of PLCβ3 activation to transmit GPCR signals. It is important to note that the expression of PLCβ3 is higher than PLCβ1 in human platelets (28). Moreover, deficiency of PLCβ1 in human patients was associated with reduced GPCR-induced calcium mobilization, impaired aggregation, and bleeding diathesis (28–30). Although PLCβ2 isoform is well recognized for relaying GPCR signals, it is possible that PLCβ3 may have a redundant role in signal propagation downstream of GPCR. Indeed, platelets deficient in both PLCβ2 and PLCβ3 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 Ser3 and Ser218 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 GB1 cell-permeable peptide disrupted PP1c-Gβ1 interaction in platelets and reduced thrombin-induced platelet aggregation (Figs. 5 and 6).

There are some intrinsic limitations with this study. GB has five isoforms (16, 35), and one or more of the GB isoforms may have facilitated PAR4-dependent signaling in GB-depleted megakaryocytes. In the case of GB1 peptide study, potential coupling of additional phosphatase subtypes PP2A or PP2B with GB1 and/or other GB isoforms or GBα 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-Gβ1 complex along with PP1c-PLCβ3 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-Gβ1) as potential target for therapeutic...
PP1c-GBP₁ complex regulates platelet physiology

intervention in thrombotic and inflammatory conditions. Because GPCR, PP1c, and GBP₁ 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-GAATTCATGGCGATTACATAAAC-3’ (forward) and 3’-CGGGGATCCCTATTCTTTGCTTGCTTGG-5’ (reverse) and ligated between EcoRI and BamH1 sites of the bait vector pGBK7. The pGBK7-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 Ade2 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 × 10⁶ 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 E₁, was recen- trifuged 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 counted adjusted to 2.5 × 10⁶ platelets/ml.

GST pulldown assays

GST or Gβ₁,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 GBP₁ and cloned in pCDNA3.1 was purchased from the cDNA Resource Center, Bloomsburg University. Generation of GBP₁ 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 EcoRI and Not1 sites to generate HA-tagged PP1c. HEK 293 cells were co-transfected with HA-tagged PP1c α, β, and γ and FLAG-tagged full-length GBP₁, using Lipofectamine supplemented with PLUS reagent (Life Technologies). In some experiments, FLAG-tagged Gβ₁, 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 Na₃VO₄ 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-loxpP 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 GBP₁ peptide (79-QDGKLIWDSY85-NH₂) (Selleck Chemicals) or Myr-control scrambled peptide (DQILYDSDKGW1) for 30 min at 37 °C before lysis. Platelet lysates were immunoprecipitated with control IgG, anti-Gβ₁ (Santa Cruz) or anti-PP1c (Santa Cruz) antibodies and immunoblotted with anti-PP1c or anti-PLCB₁ antibodies, respectively. In some experiments, lysate of platelets stimulated with agonist was separated on a SDS-PAGE gel and immunoblotted with anti-phospho PLCβ₁ (Ser185) (Cell Signaling) and anti-PLCβ3 (Cell Signaling) antibodies, followed by HRP-conjugated sec-
ordinary 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-αGp, 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-αthb, 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 αthbβ3 (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 galien (Tocris Bioscience) for 30 min, and then challenged with thrombin (0.05 unit/ml). Washed murine platelets (WT and PP1cα−/−) 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 Gβγ, 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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