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Distinct Roles for the α , β and $\gamma 1$ isoforms of Protein Phosphatase 1 in the Outside-in $\alpha_{IIb}\beta_3$ Integrin Signaling Dependent Functions

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Abstract

Although protein kinases and phosphatases participate in integrin $\alpha_{IIb}\beta_3$ signaling, whether integrin functions are regulated by the catalytic subunit of protein phosphatase 1 (PP1c) isoforms are unclear. We show that siRNA mediated knockdown of all PP1c isoforms (α , β and $\gamma 1$) in 293 $\alpha_{IIb}\beta_3$ cells decreased adhesion to immobilized fibrinogen and fibrin clot retraction. Selective knockdown of only PP1c $\gamma 1$ did not alter adhesion or clot retraction, while depletion of PP1c β decreased both functions. Unexpectedly, knockdown of PP1c α enhanced $\alpha_{IIb}\beta_3$ adhesion to fibrinogen and clot retraction. Protein interaction studies revealed that all PP1c isoforms can interact with the integrin α_{IIb} subunit. Phosphoproteomic studies revealed an enhanced activation of mitogen-activated protein kinase (MAPK) p38 in the PP1c α depleted cells. Enhanced adhesive phenotype displayed by the PP1c α depleted 293 $\alpha_{IIb}\beta_3$ cells was blocked by pharmacological inhibition of p38. Conversely, the decreased adhesion of PP1c α overexpressing cells was rescued by the expression of constitutively active p38 α or p38 γ . Thus, PP1c isoforms have distinct contribution to the outside-in $\alpha_{IIb}\beta_3$ signaling-dependent functions in 293 $\alpha_{IIb}\beta_3$ cells. Moreover, PP1c α negatively regulates integrin function by suppressing the p38 pathway.

Keywords

Protein phosphatase 1; Adhesion; Clot retraction; Fibrinogen

INTRODUCTION

Hemostatic response at the site of vascular injury entails stable platelet-platelet and platelet-extracellular matrix adhesion that is mediated in part by platelet integrin $\alpha_{IIb}\beta_3$. Engagement of integrin $\alpha_{IIb}\beta_3$ generates outside-in signals, which facilitates the rearrangement of cytoskeletal compartment and contributes to processes like stable adhesion, spreading and fibrin clot retraction in platelets [1-2]. Outside-in $\alpha_{IIb}\beta_3$ signaling encompasses reversible phosphorylation of multiple effectors (cytoskeletal, signaling and adaptor proteins) in the pathways that are downstream of the integrin receptor. Interplay between the activities of protein kinases and phosphatases that associate with the cytoplasmic tails of the $\alpha_{IIb}\beta_3$ complex can initiate outside-in $\alpha_{IIb}\beta_3$ signaling [3]. We and others have reported that the catalytic subunit of protein phosphatase 1 (PP1c) associates with the integrin $\alpha_{IIb}\beta_3$ complex in platelets and in heterologous model cells overexpressing $\alpha_{IIb}\beta_3$ [4-5].

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Protein phosphatase 1 is a major serine/threonine (Ser/Thr) phosphatase that regulates a variety of cellular functions ranging from protein synthesis, cell division, metabolism and apoptosis [6-7]. The catalytic subunit of PP1 (PP1c) associates with a wide variety of proteins called PP1c interacting proteins (PIPs). These proteins can either target PP1c to a subcellular location or act as substrates for PP1c or regulate the PP1c activity [6-7]. Three isoforms of PP1c that share ~90% amino acid sequence similarity have been identified and includes PP1 α , PP1 β / δ and PP1 γ [6]. PP1 γ has two splice variants; PP1 γ 1 (ubiquitous expression) and PP1 γ 2 (testis specific expression).

Generic Ser/Thr phosphatase inhibitors like calyculin A and okadaic acid block PP1, PP2A and impair agonist-induced platelet aggregation, adhesion and spreading on immobilized fibrinogen [8-11]. In contrast to the studies using generic Ser/Thr phosphatase inhibitors, we noticed a moderate decrease in only low-dose thrombin-induced $\alpha_{IIb}\beta_3$ activation, fibrinogen binding and aggregation in PP1 $\gamma^{-/-}$ platelets. Importantly, outside-in $\alpha_{IIb}\beta_3$ signaling-dependent functions like adhesion, spreading and clot retraction were largely unaltered in PP1 $\gamma^{-/-}$ platelets [12]. A milder phenotype noted in the PP1 $\gamma^{-/-}$ platelets may reflect functional redundancies by other PP1c isoforms or represent a true PP1 γ isoform specific phenotype. Our ability to test whether platelet functions are regulated in a PP1c isoform specific manner is hampered by the lack of PP1 $\alpha^{-/-}$ and PP1 $\beta^{-/-}$ mice models and the non-availability of isoform specific pharmacological inhibitors. Here, we assessed the role of PP1c in regulating the outside-in $\alpha_{IIb}\beta_3$ functions by utilizing genetic approaches in $\alpha_{IIb}\beta_3$ overexpressing human kidney embryonal 293 cell model system. We report that the depletion of all PP1c isoforms in the 293 $\alpha_{IIb}\beta_3$ cells moderately decreased cell adhesion and clot retraction. Unexpectedly, analysis of the individual PP1c isoforms revealed opposing roles for the PP1 α and PP1 β isoforms in outside-in $\alpha_{IIb}\beta_3$ signaling-dependent functions. Moreover, PP1 α negatively regulated $\alpha_{IIb}\beta_3$ adhesion via the regulation of p38 mitogen-activated protein kinase (MAPK) pathway.

MATERIALS AND METHODS

Reagents

SiImporter, PP2Ac and PP1 β antibodies were from Millipore (Millipore, Billerica, MA). Antibodies to PP1 α , PP1 γ 1 and HA were from Santacruz Biotechnology (Santacruz, CA). Antibodies to integrin α_{IIb} (SEW-8 and 132.1) were kindly provided by Dr. P. Newman, Blood Research Institute (Milwaukee, WI). Antibody 10E5 and integrilin were gifts from Drs. B. Coller, Rockefeller University (New York, NY) and D. Phillips (Portola Pharmaceuticals Inc., San Francisco, CA). Human Phospho-Kinase Array kit was from BD BioScience (San Jose, CA). Fibrinogen was from Enzyme Research Laboratories Inc. (South Bend, IN). Fibronectin and lipofectamine were from Invitrogen (Carlsbad, CA). ECL system was from GE Healthcare Life Science (Piscataway, NJ). Phospho-p38 (Thr¹⁸⁰, Tyr¹⁸²) and p38 antibodies were from Cell Signaling Technologies (Beverly, MA). SB203580 hydrochloride was from Tocris Bioscience (Ellisville, MO).

Adhesion and clot retraction in 293 $\alpha_{IIb}\beta_3$ cells

Human embryonal kidney 293 cells overexpressing $\alpha_{IIb}\beta_3$ (293 $\alpha_{IIb}\beta_3$) were generated as we have previously described [13]. 293 $\alpha_{IIb}\beta_3$ cells were transfected with 100 nM siRNA using siImporter. A (smart pool) of four independent siRNAs targeting PP1 α , PP1 β , PP1 γ 1 along with a non-specific control siRNA (Dharmacon, Thermo Fisher Scientific, Lafayette, CO) were used in this study. For overexpression studies, 293 $\alpha_{IIb}\beta_3$ cells were transiently transfected with cDNAs for haemagglutinin (HA)-tagged PP1 α or the control vector (gift from Dr. M. Eto, Thomas Jefferson University, Philadelphia, PA) using lipofectamine [14]. In certain experiments, cells were also transfected with empty vector or

HA tagged constitutively active p38 α (MKK6-p38 α) or p38 γ (MKK6-p38 γ) fusion constructs (gift from Dr. G. Chen, Medical College of Wisconsin, Milwaukee, WI) [15]. After 48-72 hours, the cells were used for either Western blotting or cell adhesion experiments. For adhesion experiments, 1×10^5 cells in Tyrode's buffer were incubated for 15 or 30 minutes on a ninety six well plate coated with 12.5 $\mu\text{g/ml}$ fibrinogen or 12.5 $\mu\text{g/ml}$ fibronectin and blocked with 5% BSA. Control wells were coated with 5% BSA. Wells were washed three times and adhered cells were calorimetrically assayed for acid phosphatase at 405 nm as we have described [16]. The absorbance value obtained for BSA coated wells was subtracted from that of fibrinogen and fibronectin coated wells. A standard curve for absorbance versus cell numbers was used to obtain the number of adhered cells. Percent adhesion was calculated as the number of fibrinogen/fibronectin bound cells divided by the total cells added per well and multiplied by 100. In certain experiments, cells were pretreated with 10 μM water soluble p38 inhibitor (SB203580) for 30 minutes prior to adhesion studies. Integrin expression levels in PP1c depleted/overexpressing cells were monitored by flow cytometric studies using anti- $\alpha_{\text{IIb}}\beta_3$ -PE and anti- $\alpha_{\text{IIb}}\beta_3$ (P2) antibodies as described before [13].

Fibrin clot retraction assays were performed as in our previous study [13]. 293 $\alpha_{\text{IIb}}\beta_3$ cells (4×10^6) treated with either control, PP1c α , PP1c β , PP1c γ 1 or PP1c (α , β , γ 1) siRNAs were suspended in 300 μl DMEM containing 28 mM CaCl $_2$ and 25 mM HEPES. Clot formation was initiated by the addition of thrombin (2.5 U). After incubation at 37°C for varying time periods, clot retraction was quantified by measuring the volume of liquid not incorporated into the clot. The percentage of clot retraction was calculated as the (amount of liquid collected)/ (the amount of liquid before the clot) \times 100. Control siRNA treated cells pretreated with 5 μM Integrilin ($\alpha_{\text{IIb}}\beta_3$ blocker) were also used. In certain experiments, cells were treated with 10 μM SB203580 prior to clot retraction assays.

GST pulldown, co-immunoprecipitation and immunoblotting

The cDNAs for PP1c α , PP1c β and PP1c γ 1 in pCMV vector was amplified by PCR and subcloned into a glutathione S-transferase (GST) vector pGEX 4T-1. GST or GST tagged PP1c α , PP1c β , PP1c γ 1 proteins were expressed in *Escherichia coli* following induction with isopropyl β -D-thiogalactopyranoside and purified using glutathione beads. Purified GST proteins were characterized by Commassie staining. Purified GST or GST-PP1c α , β or γ 1 were pre-coupled with glutathione beads and mixed with lysate from 293 $\alpha_{\text{IIb}}\beta_3$ cells (300 μg) and platelets (150 μg) for 3 hours at 4°C. Beads were washed and the PP1c α , PP1c β and PP1c γ 1 interacting proteins separated by SDS-PAGE and immunoblotted with anti- α_{IIb} antibody. For co-immunoprecipitation assays, lysate (\sim 300 μg) from 293 $\alpha_{\text{IIb}}\beta_3$ cells was immunoprecipitated with rabbit IgG or α_{IIb} (Sew-8) antibodies using protein A sepharose beads, and immunoblotted with antibodies to all PP1c isoforms and α_{IIb} . In some experiments, forty μg of lysate from 293 cells were separated by SDS-PAGE and immunoblotted with either anti-PP1c α , PP1c β , PP1c γ 1, PP2Ac, phospho-p38, p38, HA or actin antibodies using ECL. The signals on the films were scanned and the densitometric quantification performed using Image J software from NIH.

Phosphoproteomics studies

Lysate (\sim 500 μg in 150 μl) from control and PP1c α depleted cells were diluted with the buffer in the Phospho-kinase antibody array kit and incubated with the nitrocellulose membrane spotted with various phospho kinase antibodies. The membrane was washed and incubated with the biotinylated detection antibody cocktail, followed by streptavidin-HRP and detected by ECL as per the manufacturer's instructions.

Statistics

Statistical significance of the data was analyzed by using a paired Student's t test. Data is expressed as mean \pm SEM. $P < 0.05$ was considered as significant.

3. RESULTS

PP1c depletion moderately decreased adhesion of 293 $\alpha_{IIb}\beta_3$ cells to fibrinogen

Since the pharmacological inhibitors that block PP1 also exert some inhibition on PP2A, one approach to study the role of PP1c in $\alpha_{IIb}\beta_3$ signaling is to utilize PP1c knockdown or overexpression strategies. However, such genetic approaches are challenging in anucleate platelets. Cell model system overexpressing integrin $\alpha_{IIb}\beta_3$ are amenable to genetic manipulations and have immensely contributed to the understanding of integrin $\alpha_{IIb}\beta_3$ outside-in signaling and function [17-19]. Therefore, in this study, we utilized the previously characterized 293 $\alpha_{IIb}\beta_3$ cell model [13].

PP1c isoforms in 293 $\alpha_{IIb}\beta_3$ cells were depleted by siRNA and evaluated for the adhesion to immobilized fibrinogen. PP1c knockdown assessed by an antibody that recognizes all PP1c isoforms was specific, since the levels of PP2Ac and actin were comparable between the control and PP1c depleted cells (Figure 1A). Compared to the control siRNA treated cells, PP1c depleted cells exhibited a moderate but significant decrease in adhesion to fibrinogen (Figure 1B). Antibody 10E5 inhibited the adhesion of 293 $\alpha_{IIb}\beta_3$ cells to fibrinogen, suggesting that the adhesion assays were integrin $\alpha_{IIb}\beta_3$ specific (Figure 1B). In contrast, adhesion of control and PP1c depleted cells to fibronectin, a ligand for the endogenous $\alpha_5\beta_1$ on 293 cells was not significantly different (Figure 1C). This suggests that the differential adhesion due to PP1c depletion is integrin ($\alpha_{IIb}\beta_3$) and ligand (fibrinogen) specific. Mean fluorescence intensity (MFI) for the $\alpha_{IIb}\beta_3$ expression was 26.9 ± 6.7 (control) and 29.35 ± 4.8 (PP1c depleted) and could not account for the differences in adhesion. These studies suggest that PP1c in part stabilized $\alpha_{IIb}\beta_3$ mediated adhesive interactions to fibrinogen.

3.2 Isoform specific roles for PP1c in $\alpha_{IIb}\beta_3$ outside-in signaling-dependent functions

To clarify the contribution of PP1c isoforms in integrin function, we selectively depleted each of the PP1c isoforms and evaluated $\alpha_{IIb}\beta_3$ outside-in signaling dependent functions in 293 $\alpha_{IIb}\beta_3$ cells. PP1c γ_1 siRNA depleted PP1c γ_1 but not PP1c α and PP1c β isoforms in 293 cells (Figure 2A). Functional studies revealed a comparable adhesion between the control and PP1c γ_1 depleted cells (Figure 3A). PP1c β depleted cells (Figure 2B) showed a modest but significant reduction in the adhesion to fibrinogen (Figure 3B). MFI for the $\alpha_{IIb}\beta_3$ expression was comparable between the control (28.4 ± 8.1) and PP1c β depleted cells (28.9 ± 10.8). Unexpectedly, loss of PP1c α (Figure 2C) moderately enhanced the adhesion of 293 $\alpha_{IIb}\beta_3$ cells to fibrinogen (Figure 3C). Integrin $\alpha_{IIb}\beta_3$ expression was not significantly ($p > 0.05$) altered in the PP1c α depleted cells (MFI of 23.3 ± 0.8 and 21.1 ± 1.4 for control and PP1c β depleted cells). Compared to the PP1c α depleted cells, an opposing adhesive phenotype displayed by the PP1c α depleted cells prompted us to further validate the PP1c α knockdown data. In a complementary approach, we overexpressed a HA-tagged PP1c α in 293 $\alpha_{IIb}\beta_3$ cells and evaluated adhesion to fibrinogen. Immunoblotting with anti-HA and anti-PP1c α antibodies confirmed the overexpression of PP1c α (Figure 2D). Consistent with the PP1c α knockdown studies, overexpression of PP1c α in 293 $\alpha_{IIb}\beta_3$ cells moderately decreased the adhesion to fibrinogen (Figure 3D). Integrin expression levels were unaltered in these cells (MFI of 28.1 ± 4.3 and 29.3 ± 5.4 for control and PP1c α HA cells).

To further validate the PP1c isoform specific phenotypes, we examined fibrin clot retraction, an independent $\alpha_{IIb}\beta_3$ outside-in signaling function that involves the rearrangement of actin

cytoskeleton. Compared to the control siRNA treated cells, depletion of all PP1c isoforms led to a decreased fibrin clot retraction at all time points (Figure 4A). Loss of PP1c α moderately increased clot retraction compared to the control siRNA at 30 and 60 minutes. In contrast, depletion of PP1c β decreased clot retraction. Loss of PP1c γ 1 did not significantly alter clot retraction process (Figure 4A). Pretreatment of control siRNA cells with integrilin blocked fibrin clot retraction, indicating that clot retraction by 293 α _{IIb} β ₃ cells was α _{IIb} β ₃ dependent. Taken together, these studies indicate that the PP1c isoforms distinctly contribute to the α _{IIb} β ₃ outside-in signaling functions in 293 α _{IIb} β ₃ cells.

Interaction of all PP1c isoforms with the α _{IIb} integrin subunit

We previously showed that α _{IIb} integrin subunit co-immunoprecipitates with PP1c using an antibody that recognizes all isoforms [4]. Because of the distinct role for each PP1c isoforms in the α _{IIb} β ₃ outside-in signaling, we examined if specific isoforms of PP1c associates with α _{IIb}. PP1c α , PP1c β and PP1c γ were expressed as a GST fusion protein in *E.coli* (Figure 4B). Integrin α _{IIb} from the lysate of 293 α _{IIb} β ₃ cells **and platelets** interacted with the GST-PP1c α , GST-PP1c β and GST-PP1c γ 1 but not with GST alone in a GST pull down assays (Figure 4C). Moreover, co-immunoprecipitation assays revealed that all PP1c isoforms can associate with α _{IIb} in 293 α _{IIb} β ₃ cells (Figure 4D). These studies indicate that all the PP1c isoforms can interact with the α _{IIb} β ₃ complex.

PP1c α negatively regulates α _{IIb} β ₃ functions by suppressing the p38 signaling

It is possible that PP1c isoforms distinctly modulate integrin function by engaging discrete effectors downstream of the integrin pathway. Therefore, we sought to identify potential PP1c α specific effectors that participate in negatively regulating integrin function. PP1c α depleted cell lysate was subjected to phosphoproteomics studies using a phospho-kinase antibody array kit. Compared to the control siRNA cells, PP1c α depleted cells showed altered phosphorylation response to several signaling proteins, including a robust enhancement in the phosphorylation of residues Thr¹⁸⁰ and Tyr¹⁸² of the MAPK p38, which is a surrogate marker for p38 activation (Supplementary data, Table 1). A ~1.5 fold increased p38 activation in the PP1c α depleted cells by immunoblotting studies validated the phosphoproteomics studies (Figure 5A). Interestingly, depletion of PP1c β or PP1c γ did not overtly impact p38 activation (Figure 5A), suggesting that p38 may be a PP1c specific effector.

To ascertain whether the increased p38 activation in PP1c α depleted cells contributed to their enhanced functional phenotype, adhesion and clot retraction assays were performed with a p38 pharmacological inhibitor. The enhanced adhesiveness and the clot retraction phenotype displayed by the PP1c α depleted cells were abrogated in the presence of p38 inhibitor SB203580 (Figure 5B and 5C). In a complementary approach, we transfected the constitutively active p38 α (HA-tagged MKK6-p38 α) or p38 γ (HA-tagged MKK6-p38 γ) fusion constructs [15] into 293 α _{IIb} β ₃ cells overexpressing HA-PP1c α and examined cell adhesion to immobilized fibrinogen. MAPK kinase 6 (MKK6) is an upstream activator of p38 and fusing the cDNA of p38 in frame with its activator MKK6 via a decapeptide linker (Gly-Glu)₅ results in a constitutively active p38 form [15]. Such strategies have been used previously to generate constitutively active ERK [20] and constitutively active Jun [21]. Compared to the cells transfected with either control vectors, HA-MKK6-p38 α or HA-PP1c α , dual transfection of HA-MKK6-p38 α and HA-PP1c α showed expression of both MKK6-p38 α fusion protein (~80 kDa) and the PP1c α (~38 kDa) (Figure 5D). Similar results were noticed for the combination of HA-PP1c α and HA-MKK6-p38 γ (Figure 5D). MKK6-p38 α is predominantly localized in the cytoplasm, while MKK6-p38 γ tends to be localized to both the cytosol and the nucleus [15]. Such distribution patterns may account for the lower intensity of MKK6-p38 γ that we observed in our studies, compared to the MKK6-

p38 α . Compared to the cells expressing empty vectors (for HA-PP1 α and HA-MKK6-p38), overexpression of PP1 α decreased $\alpha_{IIb}\beta_3$ adhesion to fibrinogen (Figure 5E). More importantly, dual expression of PP1 α and MKK6-p38 α or PP1 α and MKK6-p38 γ rescued the decreased adhesiveness of the PP1 α overexpressing cells to fibrinogen (Figure 5E). Thus, PP1 α negatively regulates $\alpha_{IIb}\beta_3$ -fibrinogen adhesion by suppressing the p38 signaling pathway.

DISCUSSION

Previous studies have identified that calyculin A can inhibit platelet adhesion and spreading to fibrinogen [8]. However, since this compound can block Ser/Thr phosphatases like PP1, PP2A and PP4 [22], a specific role for PP1 and its isoforms in the functions of $\alpha_{IIb}\beta_3$ remain unclear. Although tautomycin or its close analogue tautomycetin was reported to be a PP1 preferential inhibitor [23-24], we noticed that tautomycin moderately inhibited platelet PP2A activity (not shown), thereby precluding the use of tautomycin in platelet studies. To overcome the challenges posed by the pharmacological agents and the inability to use platelets in studies to evaluate the role of PP1, we utilized genetic approach in heterologous cells expressing $\alpha_{IIb}\beta_3$. Depletion of all PP1c isoforms in 293 $\alpha_{IIb}\beta_3$ cells revealed a moderate decrease in adhesion to fibrinogen and clot retraction (Figures 1B and 4A). Furthermore, loss of PP1 γ 1 did not alter adhesion or clot retraction (Figures 3A and 4A), while loss of PP1 β decreased both functions (Figures 3B and 4A). Surprisingly, loss of PP1 α enhanced adhesion and clot retraction (Figures 3C and 4A). Thus, isoform specific roles for PP1c in outside-in $\alpha_{IIb}\beta_3$ signaling dependent functions were identified. Indeed, PP1c isoform specific roles have been described to modulate morphological and cell cycle changes in Hela cells [25] and sarcoplasmic reticulum mediated Ca^{+2} cycling in rat cardiomyocytes [26]. Interestingly, like PP1 γ depleted cells, PP1 $\gamma^{-/-}$ platelets also exhibited normal $\alpha_{IIb}\beta_3$ mediated adhesion and clot retraction [12]. Despite these observations, findings from the 293 $\alpha_{IIb}\beta_3$ cells should be cautiously interpreted in the context of platelets. Future studies with the platelets from PP1 α and PP1 β null mice will be needed to validate the observed phenotypes.

It is not clear how PP1c isoforms selectively regulate $\alpha_{IIb}\beta_3$ functions. Studies in Figure 4C and 4D suggest that the distinct PP1c phenotypes are not due to isoform specific localization of PP1c with the cytoplasmic tail of the integrin α_{IIb} subunit. However, specific or preferential interactions of PP1c isoforms with effectors exist. For example, PP1 α associates with a focal adhesion protein tensin [27]. PP1 β interacts with focal adhesion protein (FAK) [28] and myosin phosphatase targeting subunit (MYPT) [29], while PP1 γ 1 can interact with spinophilin, an actin binding scaffolding protein [30]. Isoform specific interactions of PP1c with distinct effectors in 293 $\alpha_{IIb}\beta_3$ cells could temporally place a particular PP1c isoform in a certain milieu within the focal adhesions. This in turn could facilitate a temporal dephosphorylation of Ser/Thr residues of specific substrates and thereby regulate the assembly and disassembly of cellular focal adhesions. Such mechanisms could support the biological relevance for an isoform specific PP1c role. It is not apparent how combined loss of all PP1c isoforms showed a moderate reduction in $\alpha_{IIb}\beta_3$ functions, despite the positive and negative contribution of PP1 β and PP1 α isoforms, respectively. PP1c interacts with ~200 phosphatase interacting proteins, some of which are endogenous inhibitors that are regulated by phosphorylation. The complex nature of phosphatase interactome and their ability to integrate with other signaling networks, may have precluded the functional outcome of multiple PP1c knock down to be a mere additive of the outcomes displayed by single PP1c knockdown. Such observations were also noted with other signaling molecules. Broad spectrum non-isoform selective PKC inhibitors block platelet functions yet, several PKC isoforms have distinct and opposing roles in platelet activation events [31].

How could loss of PP1 α negatively regulate outside-in α IIb β 3 functions? Phosphoproteomics and immunoblot analysis revealed increased p38 activation in PP1 α depleted cells (Figure 5A). p38 is required for α IIb β 3 functions such as clot retraction [32] and spreading of platelets pretreated with PAR4-AP to fibrinogen and collagen [33]. Thus, enhanced p38 activation could be predicted to potentiate outside-in α IIb β 3 signaling function. Indeed, p38 activation in PP1 α depleted cells was responsible for potentiating adhesive and clot retraction functions in the PP1 α depleted cells. p38 inhibitor blocked the increased adhesion and clot retraction displayed by the PP1 α depleted cells (Figures 5B and 5C). Conversely, the constitutively active p38 α or p38 γ rescued the decreased adhesive phenotype showed by PP1 α overexpressing cells (Figure 5E). Interestingly, p38 phosphorylates and regulates the activity of PP1 α interacting protein tensin [34].

In summary, a genetic approach revealed for the first time that outside-in α IIb β 3 signaling-dependent functions are regulated in part by PP1c signaling, with an opposing role for PP1 α and PP1 β . Mechanistically, regulation of the p38 pathway underpins the PP1 α mediated negative regulation of α IIb β 3 adhesion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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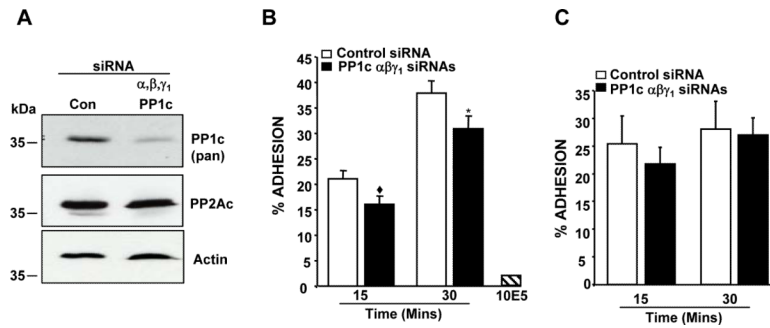


Figure 1. Effect of depleting the PP1c isoforms on 293 $\alpha_{IIb}\beta_3$ cell adhesion

(A) Lysate from the control siRNA and PP1c depleted cells were immunoblotted with anti-PP1c pan antibody (recognizes all PP1c isoforms), anti-PP2Ac and anti-actin (loading). (B) Adhesion of 293 $\alpha_{IIb}\beta_3$ cells treated with either control or PP1c α , β or γ_1 siRNAs to fibrinogen. Compared to control siRNA, the decreased adhesion of all PP1c isoform depleted cells were significant at $\blacklozenge p < 0.001$, $*p = 0.002$. $n = 4-7$. (C) Adhesion of 293 $\alpha_{IIb}\beta_3$ cells treated with control or PP1c α , β or γ_1 siRNAs to fibronectin. The adhesion difference between control and PP1c depleted cells from 3 experiments were not significant ($P > 0.05$).

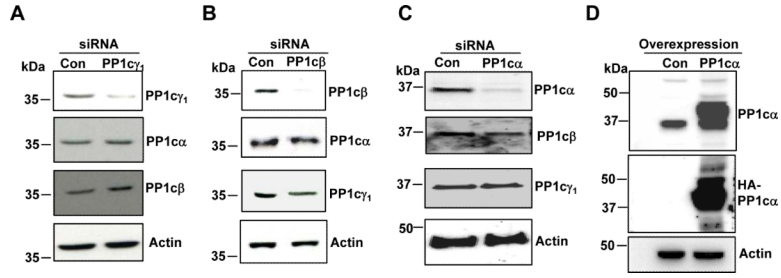


Figure 2. Knockdown of PP1c isoforms and overexpression of PP1 α in 293 $\alpha_{IIb}\beta_3$ cells
 (A) Lysate from the control siRNA and PP1 γ_1 siRNA-treated cells were immunoblotted with PP1 γ_1 , PP1 α , PP1 β and actin antibodies. (B) Lysate from control and PP1 β siRNA-treated cells were immunoblotted with anti-PP1 β , anti-PP1 α , anti-PP1 γ_1 and actin antibodies. (C) PP1 α , PP1 β , PP1 γ_1 and actin expression in 293 $\alpha_{IIb}\beta_3$ cells treated with control and PP1 α siRNAs. (D) Immunoblotting with anti-PP1 α and HA antibodies in the control vector and HA-tagged PP1 α overexpressing cells. Knockdown and overexpression blots are representative of 2-3 experiments.

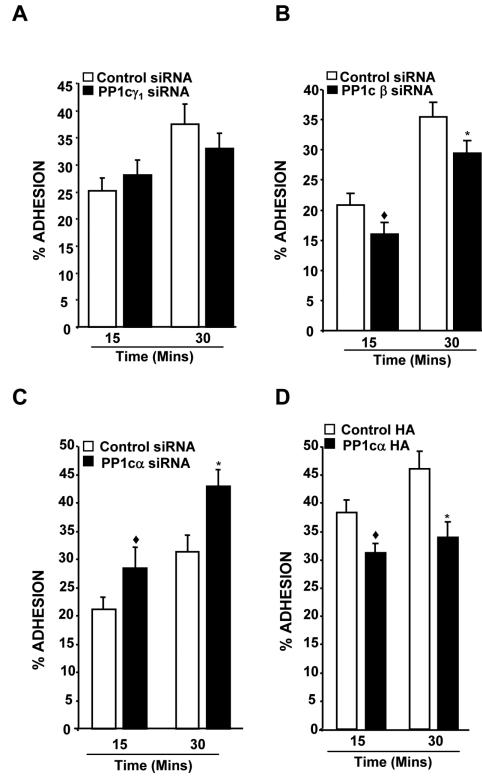


Figure 3. Effect of depleting the PP1c isoforms and overexpression of PP1c α on cell adhesion
 (A) Depletion of PP1c γ_1 did not affect fibrinogen adhesion. n=4. (B) Compared to control siRNA, the decreased adhesion of PP1c β depleted cells were significant at ♦p=0.005, *p=0.001. n=5-6. (C). Compared to control siRNA, the increased adhesion of PP1c α depleted cells were significant at ♦p=0.01, *p<0.0001. n=8-12. (D) Compared to control HA, the decreased adhesion of PP1c α HA overexpressing cells were significant at ♦p<0.001, *p=0.006. n=5-9.

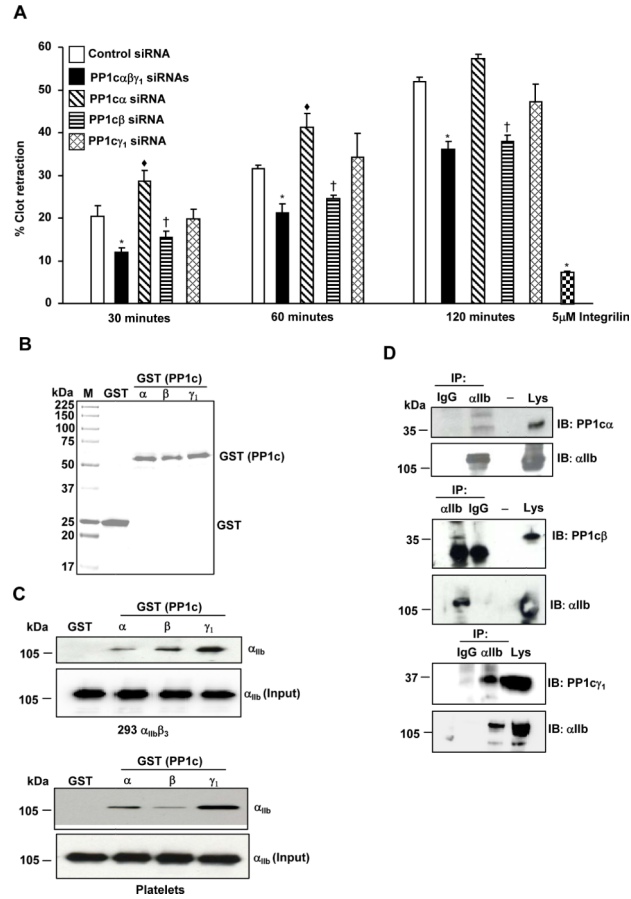


Figure 4. Effect of depleting the PP1c isoforms on fibrin clot retraction

(A) The decreased fibrin clot retraction of PP1c (α , β , γ_1) depleted cells compared to the control siRNA treated cells was significant at * $p=0.03$, * $p=0.01$, * $p=0.001$ for 30, 60 and 120 minutes, respectively. The increased fibrin clot retraction of PP1c α depleted cells compared to the control siRNA treated cells was significant at $\blacklozenge p=0.05$, $\blacklozenge p=0.04$, $p=0.07$ for 30, 60 and 120 minutes, respectively. The decreased fibrin clot retraction of PP1c β depleted cells compared to the control siRNA treated cells was significant at $\dagger p=0.04$, $\dagger p=0.03$, $\dagger p=0.005$ for 30, 60 and 120 minutes, respectively. Integrilin (5 μ M) blocked the clot retraction of control siRNA cells at 120 minutes * $p=0.003$, $n=3$. (B) PP1c α , β and γ_1 GST proteins separated on a SDS-PAGE gel were detected by Coomassie Blue staining. M is Molecular weight marker. (C). α_{IIb} in the lysate from the 293 $\alpha_{IIb}\beta_3$ cells and platelets interact with PP1c α -GST, PP1c β -GST, PP1c γ_1 -GST but not GST. Comparable levels of α_{IIb} in lysate (input) was used for pull down studies. $n=2-3$. (D) 293 $\alpha_{IIb}\beta_3$ cell lysate was immunoprecipitated (IP) with IgG (control) or α_{IIb} and immunoblotted (IB) with anti-PP1c α , PP1c β , PP1c γ_1 and α_{IIb} . Lys is cell lysate. $n=2$.

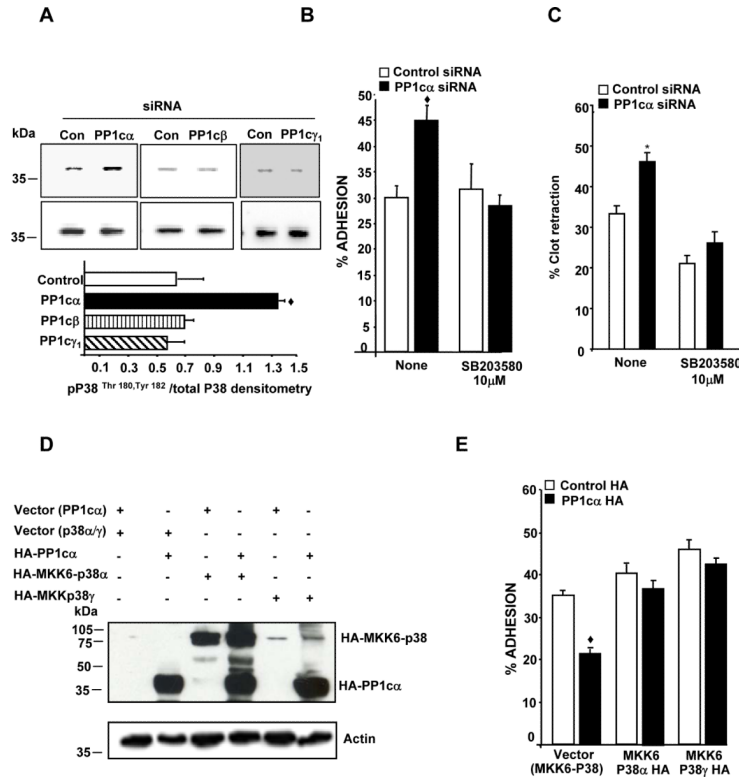


Figure 5. PP1 α negatively regulates α _{IIb} β ₃ adhesion by suppressing the p38 signaling pathway (A) Lysate from the 293 cells treated with either control, PP1c (α , β , γ) siRNAs were immunoblotted with anti-phospho-Thr¹⁸⁰, Tyr¹⁸² p38 (pp38) and p38 antibodies. Densitometric quantification of the activation of p38 was expressed as pp38/total p38 from 2-3 experiments in arbitrary units. The increased P38 activation in PP1 α depleted cells compared to the control siRNA treated cells was significant at \blacklozenge p=0.04. Effect of the water soluble p38 inhibitor on the increased fibrinogen adhesion (B) and clot retraction (C) displayed by the PP1 α depleted cells. Compared to control siRNA, the increased adhesion of PP1 α depleted cells at 30 minutes and the increased clot retraction at 60 minutes were significant at \blacklozenge p=0.001 and p=0.03. Treatment with SB203580 abolished the increased adhesion and clot retraction differences p=0.618 and p=.138 n=4-5. (D) Expression of PP1 α with either MKK6-P38 α or MKK6-p38 γ fusion proteins detected by immunoblotting with anti-HA antibody in 293 α _{IIb} β ₃ cells overexpressing the appropriate constructs. The blot is a representative of 2-3 experiments. (E) Effect of MKK6-p38 α and MKK6-p38 γ on the decreased adhesion of PP1 α overexpressing cells to fibrinogen at 15 minutes. Compared to the control HA, the decreased adhesion of PP1 α HA overexpressing cells were significant at \blacklozenge p<0.001. These differences were lost in cells expressing MKK6-p38 α (p>0.05) or MKK6-p38 γ (p>0.0.1) *p=0.006. n=4.