Protein Phosphatase 2A Negatively Regulates Integrin α\textsubscript{IIb}β\textsubscript{3} Signaling*

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Integrin α\textsubscript{IIb}β\textsubscript{3} activation is critical for platelet physiology and is controlled by signal transduction through kinases and phosphatases. Compared with kinases, a role for phosphatases in platelet integrin α\textsubscript{IIb}β\textsubscript{3} signaling is less understood. We report that the catalytic subunit of protein phosphatase 2A (PP2Ac) associates constitutively with the integrin α\textsubscript{IIb}β\textsubscript{3} in resting platelets and in human embryonal kidney 293 cells expressing α\textsubscript{IIb}β\textsubscript{3}. The membrane proximal KVGFFKR sequence within the cytoplasmic domain of integrin α\textsubscript{IIb} is sufficient to support a direct interaction with PP2Ac. Fibrinogen binding to α\textsubscript{IIb}β\textsubscript{3} during platelet adhesion decreased integrin-associated PP2A activity and increased the phosphorylation of a PP2A substrate, vasodilator associated phosphoprotein. Over-expression of PP2Ac\textsubscript{c} in 293 cells decreased α\textsubscript{IIb}β\textsubscript{3}-mediated adhesion to immobilized fibrinogen. Conversely, small interferon RNA mediated knockdown of endogenous PP2Ac\textsubscript{c} expression in 293 cells, enhanced extracellular signal-regulated kinase (ERK1/2) and p38 activation, and accelerated α\textsubscript{IIb}β\textsubscript{3} adhesion to fibrinogen and von Willebrand factor. Inhibition of ERK1/2, but not p38 activation, abolished the increased adhesiveness of PP2Ac\textsubscript{c}-depleted 293 cells to fibrinogen. Furthermore, knockdown of PP2Ac\textsubscript{c} expression in bone marrow-derived murine megakaryocytes increased soluble fibrinogen binding induced by protease-activated receptor 4-activating peptide. These studies demonstrate that PP2Ac\textsubscript{c} can negatively regulate integrin α\textsubscript{IIb}β\textsubscript{3} signaling by suppressing the ERK1/2 signaling pathway.

Integrin cytoplasmic tails are devoid of any intrinsic catalytic activity. Nevertheless, integrins can transmit bidirectional signals across the plasma membrane of a cell and regulate several cellular processes, such as, adhesion, migration, and apoptosis. In the context of the major platelet integrin α\textsubscript{IIb}β\textsubscript{3}, emerging evidence indicates that cytoplasmic tails act as a molecular scaffold for intracellular enzymes and for both cytoskeletal and adaptor proteins and can either positively or negatively regulate signaling (1). For example, during an agonist-mediated inside-out signaling process, talin interacts with the integrin β\textsubscript{3} tail and induces integrin α\textsubscript{IIb}β\textsubscript{3} activation (2), whereas calcium and integrin-binding protein 1 binds to the α\textsubscript{IIb} tail and negatively regulates α\textsubscript{IIb}β\textsubscript{3} activation (3).

Subsequent binding of fibrinogen to the activated α\textsubscript{IIb}β\textsubscript{3} integrin initiates an outside-in signaling process that regulates platelet function. Outside-in signaling can be mediated by intricate interplay of a set of proteins that associate constitutively with the integrin and by others that either associate or dissociate with the integrin in response to fibrinogen binding. For instance, c-Src associates constitutively to the β\textsubscript{3} tail (4). Fibrinogen binding to α\textsubscript{IIb}β\textsubscript{3} induces association of protein tyrosine phosphatase 1B, spleen tyrosine kinase, and protein kinase Cβ to the β\textsubscript{3} tail (4–6) and calcium and integrin-binding protein 1 to the α\textsubscript{IIb} tail (7) and causes the dissociation of C terminus Src kinase from the β\textsubscript{3} tail (4) and the catalytic subunit of protein phosphatase 1 (PP1c)\textsuperscript{6} from the α\textsubscript{IIb} tail (8). Reversible phosphorylation of multiple effector proteins that are downstream of the integrin signaling pathway is one of the mechanisms by which these α\textsubscript{IIb}β\textsubscript{3}-associated proteins can initiate and/or transduce signals. The phosphorylation status of most signaling proteins is determined by a fine balance between the activities of kinases and phosphatases. Thus far, among the reported α\textsubscript{IIb}β\textsubscript{3}-associated signaling molecules, kinases have outnumbered phosphatases. Consequently, in contrast to integrin-associated kinases, a role for phosphatases in platelet signaling, with rare exception (5), is not well understood.

Protein phosphatase 2A (PP2A) is a ubiquitously expressed Ser/Thr phosphatase and is implicated in β\textsubscript{3} integrin function in cell types other than platelets (9). The PP2A holoenzyme consists of a ~36-kDa catalytic subunit C (PP2Ac) and a ~65-kDa structural subunit A (PP2Aa) that together form an AC core dimer (PP2Aac). The A subunit in the core dimer links multiple regulatory B subunits in a fashion that determines the substrate specificity, the subcellular location, and the catalytic activity of the phosphatase (10). Blockade by generic Ser/Thr phosphatase inhibitors like okadaic acid and calyculin A impair

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\textsuperscript{6} The abbreviations used are: PP1c, catalytic subunit of protein phosphatase 1; PARAP, protease-activated receptor 4-activating peptide; VASP, vasodilator-associated phosphoprotein; ERK, extracellular signal-regulated kinase; PP2Ac, catalytic subunit of protein phosphatase 2A; siRNA, short interference RNA; BSA, bovine serum albumin; VWF, von Willebrand factor; WT, wild type.
agonist-induced platelet aggregation, secretion (11–13), and $\alpha_{IIb}\beta_3$ outside-in signaling functions such as adhesion and spreading to immobilized fibrinogen. This act may be independent of $\beta_3$ Thr$^{\text{533}}$ phosphorylation status (14, 15); however, okadaic acid and calyculin A can inhibit multiple Ser/Thr phosphatases such as PP1, PP2A, and PP4 (16). Therefore, these agents are unable to specifically elucidate the role of PP2A in integrin $\alpha_{IIb}\beta_3$ signaling and function.

In this study, we show that a pool of the catalytic subunit of PP2A constitutively associates with integrin $\alpha_{IIb}\beta_3$. By using a genetic (gene knockdown and/or gene overexpression) approach in two distinct model systems, such as the 293 and the primary murine megakaryocytes, PP2Ac, was identified to negatively regulate $\alpha_{IIb}\beta_3$ adhesiveness to immobilized and soluble fibrinogen. PP2Ac can negatively regulate $\alpha_{IIb}\beta_3$ signaling by repressing the ERK1/2 activation pathway.

**MATERIALS AND METHODS**

**Immunoprecipitation and Western Blotting**—Blood was drawn in an acid/citrate/dextrose anticoagulant from normal, healthy, fasting donors. Each donor signed an informed consent approved by the Institutional Review Board of Baylor College of Medicine, Houston, TX. Washed platelets were prepared as previously described (17). Using either 750 μg/ml 1% Triton X-100 or Igepal CA-630, lysates were obtained from either resting platelets or 293 cells expressing $\alpha_{IIb}\beta_3$ or the various mutants. The lysates were immunoprecipitated using anti-$\alpha_{IIb}$ (Sew-8, gift from Dr. Newman, Blood Research Institute, Milwaukee, WI), anti-PP2Ac (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit IgG (Pierce) using Protein A-Sepharose (Amersham Biosciences). Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal antibody to $\alpha_{IIb}$, 7-amino-actinomycin D (BD Bioscience, San Jose, CA) and developed using the ECL system (Amersham Biosciences).

**Interaction of PP2Ac with the Integrin $\alpha_{IIb}$ Subunit**—Truncation of the cytoplasmic domain of integrin $\alpha_{IIb}$ at residue 985 was generated according to the manufacturer’s protocol for the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Integrin $\beta_3$ with truncation of the cytoplasmic domain at residue 716 was kindly provided by Dr. Michael H. Kroll (Baylor College of Medicine, Houston, TX). These constructs were sequenced to confirm the presence of the desired truncations. 293 cells were transiently transfected with wild-type $\alpha_{IIb}$, and $\beta_3$ or with truncated integrin tails using Lipofectamine (Invitrogen) for 48 h. Cells were lysed in 1% Triton X-100, and lysates were immunoprecipitated with anti-PP2Ac antibody and immunoblotted with anti-$\alpha_{IIb}$ antibody. In an alternate approach, biotinylated peptides corresponding to residues 985–995 of the integrin $\alpha_{IIb}$ (LAMWKVGFKKR) and a control with scrambled sequence (LWKRVAGPFKM) were synthesized at the Baylor College of Medicine Protein Sequencing Core Facility, Houston, TX. We mixed 25–50 μg/ml peptide with 1 μg/ml purified PP2A or 750 μg/ml platelet lysate, and the mixtures were precipitated using streptavidin-agarose beads. The beads were washed three times, and bound proteins were eluted using SDS sample buffer. Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-PP2Ac antibody, and signals were then detected using ECL.

**siRNA Construct, Transfection, and Adhesion**—The following SMARTpool siRNA reagents, purchased from Dharmacon (Thermo Fisher Scientific, Lafayette, CO), were used in this study: 1) catalog # M-003598 targeting human PP2A catalytic subunit, $\alpha$ isoforms (NM_002715); 2) catalog # M-04065700 targeting murine PP2A catalytic subunit, $\alpha$ isoforms (NM_019411); and 3) catalog # D-001206-13-05 a nonspecific control pool with no sequence homology to any human or mouse sequence. According to manufacturer’s instructions, we used silmiporter (Upstate Biotechnology) to transfect 293 $\alpha_{IIb}\beta_3$ cells with 100 nM siRNA. After 48 h, the cells were used for Western blotting or adhesion experiments. For the adhesion studies, 1 × 10$^5$ cells, suspended in Tyrode’s buffer containing 1.8 mM CaCl$_2$ and 0.49 mM MgCl$_2$, were incubated with either 5% BSA (control), 12.5 μg/ml fibrinogen (Enzyme Research Laboratories Inc., South Bend, IN), or 10 μg/ml VWF (gift from Dr. Jing-fei Dong, Baylor College of Medicine)-coated wells for varying time points. During certain experiments, the cells were pretreated with either control DMSO, 10 μM U0126 (ERK1/2 inhibitor), or 10 μM SB203580 (p38 inhibitor). Unbound cells were washed, and the adherent cells were quantified by assaying for acid phosphatase activity at 405 nm. The number of bound cells was obtained using a standard curve for absorbance versus cell number. Percent adhesion was calculated as the number of bound cells divided by the total number of cells added per well multiplied by 100. Specific fibrinogen binding was calculated after subtracting values obtained for BSA-coated wells. In some experiments, 293 $\alpha_{IIb}\beta_3$ cells were transiently transfected using Lipofectamine with cDNA for HA-tagged PP2Ac or the control vector (gift from Dr. A. Verin, University of Chicago, Chicago, IL). After 48 h, cells were analyzed for Western blotting and adhesion as described above. In some experiments, fibrinogen- or BSA-coated dishes were incubated with 2 × 10$^5$ platelets for 30 min at 37 °C. The fibrinogen-bound platelets and the non-adherent platelets from BSA-coated plates were lysed in a phosphate-free buffer, and $\alpha_{IIb}$ was immunoprecipitated. The integrin $\alpha_{IIb}$-associated PP2Ac activity assay was quantified using a PP2A phosphatase assay kit (Upstate Biotechnology). Using a malachite green assay, $\alpha_{IIb}$ immunoprecipitates were evaluated for PP2Ac activity by dephosphorylation of the phosphopeptide K-Rp-I-R-R.

**Megakaryocyte Culture, Transfection, and Flow Cytometry**—Megakaryocytes were obtained from the bone marrow cultures of BALB/c mice as described previously (18). At day 5, megakaryocytes were transfected with 100 nM control siRNA or PP2Ac, siRNA by using a transfecting agent from Mirus (Mirus Bio Corp., Madison, WI) for 48 h at 37 °C. On day 7, a portion of the differentiated megakaryocytes was used to assess the expression of PP2Ac. We mixed 50 μl of the megakaryocyte suspension with 2.5 mM agonist PAR4AP (AYPGKF, Protein Sequencing Core Facility, Baylor College of Medicine). Next, a non-blocking anti-$\alpha_{IIb}$ antibody, 7-amino-actinomycin D (BD Bioscience, San Jose, CA) and Alexa 488-conjugated fibrinogen (20 μg/ml final concentration, Invitrogen) were added in the presence or absence of 10 mM EDTA at room temperature for retraction.
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RESULTS

In resting platelets, a pool of the catalytic subunit of protein phosphatase 1 (PP1c) constitutively associates with αIIIbβ3 complex (8). Studies from other cell types have revealed that PP2Ac can associate with integrin β3 (19). Because the primary amino acid sequence within the catalytic subunits of PP1c and PP2A are closely related, we considered whether the catalytic subunit of PP2A (PP2Ac) could associate with αIIIbβ3 complex in platelets. During co-immunoprecipitation assays with lysates from resting human platelets, we detected the presence of PP2Ac in the αIII immunoprecipitate. Conversely, in a reciprocal co-immunoprecipitation assay, αIII was observed in the PP2Ac immunoprecipitate (Fig. 1A). Furthermore, the association of PP2Ac with integrin αIII was recapitulated in human embryonic kidney cell line 293 expressing αIII (FL2) and were viable (defined as negative for 7-amino-actinomycin D in the FL3 parameter).

In contrast to PP2Ac, the catalytic subunit of a structurally distinct Ser/Thr phosphatase, protein phosphatase 2C (PP2Cc) was not detected in the αIII immunoprecipitate, indicating the PP2Ac-αIIIβ3 interaction is specific (Fig. 1C). Although the phosphatase-integrin association may appear modest, its consequence on cellular function is not (see Fig. 4). PP2Acα and PP2Acβ are the two ubiquitously expressed isoforms of PP2Ac and share roughly 97% similarity in the primary amino acid sequence. The PP2Ac antibody used in these studies recognizes both isoforms. Thus, these studies indicate a specific and constitutive interaction of the catalytic subunit of PP2A with the resting integrin αIIIβ3.

15–20 min. Alexa-fibrinogen binding was measured using an EPICS-XL flow cytometer (Beckman Coulter, Miami, FL). The FL1 expression was evaluated from the gated population of only large megakaryocytes (size) that expressed αIIIβ3 (FL2) and were viable (defined as negative for 7-amino-actinomycin D in the FL3 parameter).

To identify whether αIII or the β3 cytoplasmic domains support PP2Ac interaction, 293 cells were transiently transfected with wild-type αIIIβ3 or with αIII and β3 cytoplasmic domain truncation mutants. The association of PP2Ac was then assessed by co-immunoprecipitation assays. Integrin αIII co-immunoprecipitated with PP2Ac in 293 cells transiently expressing the wild-type (WT) αIIIβ3 (Fig. 2A). Furthermore, cells expressing the WT αIII along with the β3 cytoplasmic truncation mutant (β3Δ716) also supported the interaction of αIII with PP2Ac. In contrast, αIII failed to associate with PP2Ac in 293 cells that expressed 1) no integrin αIIIβ3, 2) αIIIβ3 cytoplasmic truncation mutant (αIIIΔ989) along with the WT β3, and 3) αIII (αIIIΔ989) and β3 (β3Δ716) cytoplasmic truncation mutants. The apparent increased αIII association with PP2Ac in β3Δ716-expressing cells, seen in Fig. 2, was not consistently reproducible and may be due to an increased amount of immunoprecipitated PP2Ac. The inability of αIIIΔ989 mutant to support PP2A association was not due to a lack of αIII expression (Fig. 2A, 293 cell lysates). These studies suggest that the cytoplasmic domain of integrin αIII but not β3 supports the interaction of PP2Ac.

To ascertain whether PP2A could directly associate with the integrin, we examined the interaction of purified PP2A enzyme and purified αIIIβ3 cytoplasmic peptide. Because PP1c (a PP2A-related phosphatase) interacts with the αIIIβ3 cytoplasmic tail,
containing a PP1c binding motif, we considered whether the membrane proximal, as opposed to membrane distal, residues of the αIIb subunit could also support PP2Ac interaction. Purified PP2A enzyme and PP2Ac from the resting platelet lysates bound specifically to a biotinylated αIIb peptide containing the residues 985–995 of the integrin αIIb, but not to a control peptide with scrambled sequence (Fig. 2B). These studies suggest that the αIIb membrane proximal region containing the KVGFKKR sequence can support the direct interaction of PP2Ac.

Next, we examined if αIIbβ3 activation and ligand engagement regulates PP2Ac-αIIbβ3 association or αIIbβ3-associated PP2Ac activity. The association of PP2Ac with the integrin was evaluated following platelet adhesion to immobilized fibrinogen, an αIIbβ3-mediated event. The association of PP2Ac with αIIbβ3 was maintained regardless of whether the platelets were held in suspension over the BSA substrate or adhered to immobilized fibrinogen (Fig. 3A). Densitometric quantification revealed that a comparable (p = 0.642) amount of PP2Ac, associated with the integrin immunoprecipitates, was obtained from platelets that either adhered to fibrinogen or suspended over BSA (Fig. 3B). Similarly, a stable association of PP2Ac with the integrin was also observed during soluble fibrinogen binding induced by Mn²⁺ (data not shown). Next, the activity of PP2Ac associated with αIIbβ3 was quantified in the αIIb immunoprecipitates. Platelets that adhered to fibrinogen exhibited ~45% decreased (p = 0.02) αIIbβ3-associated PP2Ac activity compared with platelets that were maintained in suspension over the BSA substrate (Fig. 3C). Consistent with the decreased αIIbβ3-associated PP2Ac activity in fibrinogen-adhered platelets, we observed an increased Ser¹⁵⁷ phosphorylation of vasodilator-associated phosphoprotein (VASP), a PP2Ac substrate in fibrinogen adhered platelets (Fig. 3D). By densitometry, when compared with platelets suspended over BSA, fibrinogen-adhered platelets exhibited a ~2-fold increase of VASP phosphorylation (Fig. 3E). Thus, decreased integrin-associated PP2Ac activity in fibrinogen-adhered platelets correlated with the increased phosphorylation of PP2Ac substrate VASP in platelets. Collectively, these results indicate that the integrin-fibrinogen engagement may not significantly disrupt the association of a pool of phosphatase with the integrin, but rather decreases the phosphatase activity of PP2Ac associated with the αIIbβ3.

To explore a functional role for PP2Ac in integrin αIIbβ3 signaling, we overexpressed a HA-tagged PP2Acα in 293 αIIbβ3 cells and evaluated adhesion to immobilized fibrinogen. PP2Acα was chosen because of the reported 10-fold abundance over PP2Acβ in most tissues (21). Immunoblotting with anti-HA antibody confirmed the overexpression of PP2Acα (Fig. 4A). Compared with the vector control-treated cells, PP2Acα overexpression significantly decreased the adhesion of αIIbβ3 cells to fibrinogen (Fig. 4B). The αIIbβ3-specific blocking antibody, 10E5, inhibited the adhesion of 293 cells. Thus, indicating that the adhesion was primarily mediated through αIIbβ3. Comparable levels of integrin αIIb expression were observed by densitometry of αIIb, immunoblots in control vector and PP2Acα HA-overexpressed cells (54.59 ± 7.9 versus 55.10 ± 8.02, respectively). To further verify these findings, in complementary studies, we used short interference RNA (siRNA) to knockdown the expression of endogenous PP2Acα in 293 cells expressing αIIbβ3. Knockdown was maximal (~50–60%) and specific for PP2Ac, because PP1c and actin protein levels were comparable between the control and PP2ACα siRNA-treated cells (Fig. 4C). Compared with the control siRNA-treated cells, PP2ACα knockdown significantly increased the adhesion of αIIbαβ3 cells to fibrinogen (Fig. 4D). To further determine whether the increased adhesiveness exhibited by PP2ACα-depleted cells was specific to immobilized fibrinogen, we studied adhesion to immobilized VWF. PP2ACα knockdown significantly increased the adhesion of αIIbβ3 cells to VWF (Fig. 4E). This suggests the differential adhesion due to PP2ACα depletion is not ligand-specific. The mean fluorescence intensity for αIIbβ3 expression was 266.12 ± 55 and 321.3 ± 68 for control and PP2ACα siRNA-treated 293 cells, respectively (p = 0.89). Thus, integrin expression levels may not account for the
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FIGURE 4. PP2Acα negatively regulates αIIbβ3 adhesiveness in 293 cells. A, PP2Acα expression as revealed by anti-HA antibody in control and HA-tagged PP2Acα-overexpressing cells. This blot was reprobed for actin (loading control) and is a representative of four different experiments. B, effect of HA-PP2Acα overexpression on 293 cell adhesion. 293 cells transfected with control vector or vector with PP2Acα cDNA were allowed to adhere to fibrinogen in the presence and absence of 10E5 (blocking antibody to αIIbβ3). Error bars indicate that PP2Acα knockdown enhances αIIbβ3 adhesiveness of PP2Acα-depleted cells. Lysates obtained from 293 αIIbβ3 cells treated with either control or PP2Acα siRNA was separated by 10% SDS-PAGE. Mitogen-activated protein kinase activation was assessed by immunoblotting using antibodies specific for the active (dual tyrosine and threonine-phosphorylated) forms of activated p38 (p35) and p44/42 ERK (pERK1/2) respectively. Blots are representative of five different experiments.

To investigate a potential mechanism by which PP2Acα can negatively regulate cell adhesion, we explored the effect of depleting endogenous PP2Acα in 293 cells on p38 and ERK (PP2Acα effectors) signaling pathways. These pathways are implicated in the modulation of cellular cytoskeletal reorganization and cell adhesiveness (22). The siRNA-mediated depletion of PP2Acα resulted in an increased activation of p38 and ERK1/2 signaling (Fig. 5, A and C). Using densitometry, the comparison of control siRNA-treated cells and PP2Acα-depleted cells exhibited a ~2-fold (p = 0.046) increase for p38 activation and a ~2.5-fold (p = 0.0058) increase in ERK1/2 activation (Fig. 5, B and D). Next, we ascertained whether the increased adhesiveness of PP2Acα-depleted cells was due to increased ERK1/2 or p38 signaling. Compared with control siRNA-treated cells, PP2Acα depletion significantly (p = 0.0001) increased adhesion. This increase was abolished (p = 0.373) by the ERK1/2 inhibitor (U0126) (Fig. 5E). In contrast, the p38 inhibitor, SB203580, failed to repress the increased adhesiveness of PP2Acα-depleted cells (Fig. 5E). Similar results were obtained with another p38 inhibitor (SB202190) (not shown). These studies indicate that PP2Acα may suppress observed difference in adhesion. Taken together, these results indicate that PP2Acα negatively regulates αIIbβ3 outside-in signaling function in 293 cells.
Therefore, we chose to study the role of PP2Ac by agonists, and are amenable to genetic manipulation (3, 18). Here we show that PP2Ac influences agonist-induced fibrinogen binding, in part, by down-regulating ERK1/2 activation. Despite the similar binding motifs for PP1c and PP2Ac on integrin IIb3, it is likely that PP2Ac association may not be limited to the membrane proximal region containing KVGFFKR (24). The membrane proximal region of αIIb can host the binding of calcium and integrin-binding protein 1, PP1c, and ICIn (8, 25, 26); therefore, it is conceivable that only a subpopulation of αIIb3 may harbor PP2Ac. Because GFFKR sequence is conserved in other cytoplasmic peptides, have indicated that the αIIb membrane proximal region containing KVGFFKR is sufficient to mediate a direct interaction with PP2Ac (Fig. 2). This observation is consistent with a previous study that showed an association of the inhibitor for PP2A (11PP2A) with the membrane proximal GFFKR motif of integrin α3β3 (24). The membrane proximal region of αIIb can host the binding of calcium and integrin-binding protein 1, PP1c, and ICIn (8, 25, 26); therefore, it is conceivable that only a subpopulation of αIIb3 may harbor PP2Ac. Because GFFKR sequence is conserved in other α subunits, it is likely that PP2Ac association may not be limited to αIIb subunits.

Despite the similar binding motifs for PP1c and PP2Ac on integrin αIIb, integrin engagement resulted in a different effect for the two phosphatases. PP1c dissociated from the integrin complex (8), whereas a great extent of PP2A, remained associated with the integrin (Fig. 3A). This suggests that integrin engagement may differentially regulate the two phosphatases in platelets. Interestingly, fibrinogen binding during platelet adhesion to αIIbβ3 repressed the αIIbβ3-associated PP2Ac activity (Fig. 3B). It is conceivable that the decreased PP2Ac activity, following fibrinogen binding, may be due to an increased Tyr307 phosphorylation of PP2Ac that is mediated in part by αIIbβ3-

αIIbβ3 adhesiveness, in part, by down-regulating ERK1/2 activation pathway.

Next, we evaluated if genetic manipulation of PP2Ac negatively regulated αIIbβ3 signaling in an additional model system that has more direct relevance to platelet biology. It is not feasible to manipulate gene expression in platelets, because they are anucleate and mice lacking PP2Ac die around embryonic day 6.5, thus, precluding the study of platelets from PP2Ac−/− mice (23). In recent years megakaryocytes, from which platelets die around embryonic day 6.5, thus, precluding the study of platelets from PP2Ac−/− mice (23). In recent years megakaryocytes, from which platelets

To elucidate the role of PP2Ac in agonist-induced αIIbβ3 fibrinogen binding, we used murine siRNAs to knock down PP2Acα expression in megakaryocytes. Knockdown was maximal (∼40–50%) and specific for PP2Ac, because PP1c and actin protein levels were comparable between the control and PP2Acα-siRNA-treated megakaryocytes (Fig. 6B). The residual PP2Ac signal could represent incomplete PP2Acα knockdown or expression of PP2Acβ. Knockdown of PP2Acα significantly increased binding of soluble fibrinogen in response to PAR4AP (Fig. 6C) compared with the megakaryocytes treated with control siRNA (Fig. 6C). An increased trend that did not reach statistical significance was also noted for MnCl2-induced fibrinogen binding in PP2Acα-depleted megakaryocytes. This suggests that PP2Acα may also negatively regulate αIIbβ3 outside-in signaling in megakaryocytes (data not shown).

Surface expression of αIIbβ3 was not different between the control (mean fluorescence intensity, 94.67 ± 16) and PP2Acα (mean fluorescence intensity, 89.33 ± 13) siRNA-treated megakaryocytes and could not account for the observed difference in fibrinogen binding. These results suggest that PP2Acα negatively regulates integrin αIIbβ3 inside-out signaling in a murine megakaryocyte model system.

**DISCUSSION**

Signal transduction by kinases and phosphatases control integrin αIIbβ3 adhesiveness and activation events. However, a specific role for PP2A in integrin αIIbβ3 signaling is unclear. In this work, we show that a pool of PP2Ac associates constitutively with the integrin αIIbβ3 in resting platelets. Studies in 293 model systems revealed that PP2Acα can negatively regulate integrin αIIbβ3 adhesiveness, in part, via the suppression of ERK1/2, but not the p38 signaling pathway. Furthermore, PP2Acα negatively regulated PAR4AP-induced integrin αIIbβ3 inside-out signaling in a murine megakaryocyte model system.

Co-immunoprecipitation assays revealed a close proximal association of a pool of PP2Ac with integrin αIIbβ3. Additional studies, using integrin tail truncation mutants and integrin αIIb cytoplasmic peptides, have indicated that the αIIb membrane proximal region containing KVGFFKR is sufficient to mediate a direct interaction with PP2Acα (Fig. 2). This observation is consistent with a previous study that showed an association of the inhibitor for PP2A (11PP2A) with the membrane proximal GFFKR motif of integrin α3β3 (24). The membrane proximal region of αIIb can host the binding of calcium and integrin-binding protein 1, PP1c, and ICIn (8, 25, 26); therefore, it is conceivable that only a subpopulation of αIIb3 may harbor PP2Ac. Because GFFKR sequence is conserved in other α subunits, it is likely that PP2Ac association may not be limited to αIIb subunits.

**FIGURE 6.** PP2Acα negatively regulates αIIbβ3 activation in murine megakaryocytes. A, Alexa 488 fibrinogen binding in untransfected megakaryocytes. Large and alive megakaryocytes that expressed αIIb were analyzed for fibrinogen binding in response to 2.5 mM PAR4AP in the presence and absence of 10 mM EDTA by flow cytometry. B, expression of PAR4 in control and PP2Acα-transfected siRNA. The blot was stripped and analyzed for PAR1 and actin. These blots are representative of three experiments. C, increased fibrinogen binding in megakaryocytes transfected with murine PP2Acα siRNA. Fibrinogen binding was studied as described in panel A. Results are mean ± S.E. of six experiments, and the increased fibrinogen binding in megakaryocytes transfected with PP2Acα siRNA over the control siRNA was significant at +, p = 0.02 by analysis of variance.

**Inhibition of αIIbβ3 Signaling by PP2Acα**

PP2Acα expression in megakaryocytes. Knockdown was maximal (∼40–50%) and specific for PP2Ac, because PP1c and actin protein levels were comparable between the control and PP2Acα siRNA-treated megakaryocytes (Fig. 6B). The residual PP2Ac signal could represent incomplete PP2Acα knockdown or expression of PP2Acβ. Knockdown of PP2Acα significantly increased binding of soluble fibrinogen in response to PAR4AP (Fig. 6C) compared with the megakaryocytes treated with control siRNA (Fig. 6C). An increased trend that did not reach statistical significance was also noted for MnCl2-induced fibrinogen binding in PP2Acα-depleted megakaryocytes. This suggests that PP2Acα may also negatively regulate αIIbβ3 outside-in signaling in megakaryocytes (data not shown). Surface expression of αIIbβ3 was not different between the control (mean fluorescence intensity, 94.67 ± 16) and PP2Acα (mean fluorescence intensity, 89.33 ± 13) siRNA-treated megakaryocytes and could not account for the observed difference in fibrinogen binding. These results suggest that PP2Acα negatively regulates integrin αIIbβ3 inside-out signaling in a murine megakaryocyte model system.
Inhibition of $\alpha_{\text{III}} \beta_3$ Signaling by PP2Ac$_\alpha$

associated Src. In fact, fibrinogen binding to $\alpha_{\text{IIb}} \beta_3$ during platelet adhesion resulted in an increased $\alpha_{\text{IIb}} \beta_3$-associated Src activity (4). Also, phosphorylation of Tyr$^{753}$ residue, within the catalytic subunit of PP2A, by the tyrosine kinases PP60$^{c-src}$ or PP56$^{c-fak}$ resulted in a reduction of the PP2A activity (27). Decreased $\alpha_{\text{IIb}} \beta_3$-associated PP2A activity also correlates with the increased $\alpha_{\text{IIb}} \beta_3$-associated Ser/Thr kinase protein kinase C activity following fibrinogen binding (6), implying $\alpha_{\text{IIb}} \beta_3$-associated Ser/Thr kinase and phosphatase activity are tightly controlled.

It is difficult to ascertain a specific role for integrin-associated PP2A$\alpha$ in functional assays obtained from cells expressing either point mutations or deletions of the KVGFFKR region in $\alpha_{\text{IIb}}$ because multiple proteins dock in this region. Moreover, deletion of GFFKR sequence in $\alpha_{\text{IIb}}$ can lead to integrin activation via disruption of a salt bridge between the $\alpha_{\text{IIb}}$ and $\beta_3$ subunits (28). Therefore, in this study, we analyzed $\alpha_{\text{IIb}} \beta_3$ adhesive function in cells that are depleted of PP2A$\alpha$ by an siRNA approach. Although this approach can decipher a specific functional role for PP2A$\alpha$ independent of other KVGFFKR-binding proteins, we cannot stringently rule out the functional contribution of PP2A$\alpha$ that is also present in other subcellular locations. Nevertheless, our studies indicated that PP2A$\alpha$ knockdown in 293 $\alpha_{\text{IIb}} \beta_3$ model cells resulted in an increased $\alpha_{\text{IIb}} \beta_3$ adhesion to immobilized fibrinogen and VWF. These results are in contrast to an earlier study, wherein platelets treated with PP1/PP2A inhibitor calycin A produced a decreased adhesive phenotype to immobilized fibrinogen (14). This discrepancy could be due to inhibition of multiple phosphatases other than PP2A in calycin A-treated platelets or could merely reflect the differences between platelets and cell lines. The siRNA approach we have undertaken provides us an opportunity to evaluate, more specifically, a role for PP2A independent of PP1c, because PP1c expression was not decreased in PP2A$\alpha$ knockdown cells (Fig. 4C). Because platelets are anucleate and PP2A$\alpha$ null mice are embryonically lethal, we employed murine megakaryocytes as a comparable model to study platelet $\alpha_{\text{IIb}} \beta_3$ inside-out signaling process. We observed increased fibrinogen binding in PAR4AP-treated megakaryocytes that were treated with PP2A$\alpha$ siRNA (Fig. 6). Taken together with the results obtained from the 293 cell model system, these observations strengthen the conclusion that PP2A$\alpha$ negatively regulates integrin signaling.

How could integrin-associated PP2A$\alpha$ exert a negative regulation of integrin signaling? The association of PP2A$\alpha$ with the $\alpha_{\text{IIb}} \beta_3$ complex does not directly regulate the integrin affinity, because the basal fibrinogen binding in PP2A$\alpha$-depleted megakaryocytes was not statistically increased (Fig. 6C). Furthermore, the association of PP2A$\alpha$ with the $\alpha_{\text{IIb}} \beta_3$ complex does not regulate Thr$^{753}$ phosphorylation of integrin $\beta_3$, because we failed to observe $\beta_3$ Thr$^{753}$ phosphorylation in PP2A$\alpha$-depleted 293 cells (data not shown). Perhaps, multiple substrates for PP2A may exist in the focal adhesion protein complexes organized by $\alpha_{\text{IIb}}$ and $\beta_3$, cytoplasmic tails. Suppression of the phosphorylation or activation of these proteins within the complex by the $\alpha_{\text{IIb}} \beta_3$-associated PP2A$\alpha$ is likely to participate in limiting integrin activation and function. Indeed, we noticed that PP2A$\alpha$ repressed the activation of its effectors p38 and ERK1/2 (Fig. 5). Although activation of ERK1/2 is more intensely studied as a regulator of gene expression and cell proliferation, this pathway can also regulate cellular functions. For example, cell adhesion and spreading are inhibited by dominant-negative ERK (22) and promoted by ERK activation (29). ERK and p38 are required for platelet spreading on fibrinogen (30). Cell migration is inhibited by blocking p38 activation (31). We observed that PP2A$\alpha$-depleted cells exhibited increased ERK1/2 and p38 activation and increased adhesion to fibrinogen and VWF. Inhibition of ERK1/2, but not p38 signaling, abolished the increased adhesion of PP2A$\alpha$-depleted cells (Fig. 5E). Other investigators have reported that pharmacological inhibition of PP2A$\alpha$ leads to increased migration of endothelial and carcinoma cells (32, 33). Thus, the enhanced ERK1/2 and p38 signaling in PP2A$\alpha$-depleted cells would be predicted to exhibit greater adhesive and migratory properties.

Moreover, at the molecular level, evidence exists that ERK1/2 signaling can regulate focal adhesion complexes and integrin activation. Formation of peripheral actin microspikes are blocked by inhibiting ERK activation (34). After integrin engagement, active ERK is targeted to the newly forming focal adhesion via receptor for activated protein kinase C 1 (35). Interestingly, receptor for activated protein kinase C 1 also interacts with PP2A (36); therefore, it may provide the scaffold for cross-talk between PP2A and ERK signaling. Finally, the p38 and ERK signaling pathways are reported to play a critical role in the activation of integrin $\alpha_{\text{IIb}} \beta_3$ when induced by agonists like VWF and thrombin (20). Thus, suppression of ERK1/2 signaling by PP2A$\alpha$ could constitute a potential mechanism for limiting integrin function.

In summary, our understanding of the role for PP2A$\alpha$ in integrin signaling is fairly limited based on the use of pharmacological inhibitors. Using a genetic approach, our data indicate that PP2A$\alpha$ negatively regulates $\alpha_{\text{IIb}} \beta_3$ signaling. Given that platelets are derived from megakaryocytes, this finding could be directly relevant to platelets. Moreover, such functional interactions might also extend to other $\alpha$ integrin subunits, thereby providing additional regulatory mechanisms to control integrin activation.

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