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Protein Phosphatase 2A Negatively Regulates Integrin $\alpha_{IIb}\beta_3$ Signaling*

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Integrin $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ signaling is less understood. We report that the catalytic subunit of protein phosphatase 2A (PP2Ac) associates constitutively with the integrin $\alpha_{IIb}\beta_3$ in resting platelets and in human embryonal kidney 293 cells expressing $\alpha_{IIb}\beta_3$. The membrane proximal KVGFFKR sequence within the cytoplasmic domain of integrin α_{IIb} is sufficient to support a direct interaction with PP2Ac. Fibrinogen binding to $\alpha_{IIb}\beta_3$ during platelet adhesion decreased integrin-associated PP2A activity and increased the phosphorylation of a PP2A substrate, vasodilator associated phosphoprotein. Overexpression of PP2Ac$_\alpha$ in 293 cells decreased $\alpha_{IIb}\beta_3$-mediated adhesion to immobilized fibrinogen. Conversely, small interference RNA mediated knockdown of endogenous PP2Ac$_\alpha$ expression in 293 cells, enhanced extracellular signal-regulated kinase (ERK1/2) and p38 activation, and accelerated $\alpha_{IIb}\beta_3$ adhesion to fibrinogen and von Willebrand factor. Inhibition of ERK1/2, but not p38 activation, abolished the increased adhesiveness of PP2Ac $_\alpha$-depleted 293 cells to fibrinogen. Furthermore, knockdown of PP2A$_{c\alpha}$ expression in bone marrow-derived murine megakaryocytes increased soluble fibrinogen binding induced by protease-activated receptor 4-activating peptide. These studies demonstrate that PP2Ac $_\alpha$ can negatively regulate integrin $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$, 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_3 tail and induces integrin $\alpha_{IIb}\beta_3$ activation (2), whereas calcium and integrin-binding protein 1 binds to the α_{IIb} tail and negatively regulates $\alpha_{IIb}\beta_3$ activation (3).

Subsequent binding of fibrinogen to the activated $\alpha_{IIb}\beta_3$ 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_3 tail (4). Fibrinogen binding to $\alpha_{IIb}\beta_3$ induces association of protein tyrosine phosphatase 1B, spleen tyrosine kinase, and protein kinase Cβ to the β_3 tail (4–6) and calcium and integrin-binding protein 1 to the α_{IIb} tail (7) and causes the dissociation of C terminus Src kinase from the β_3 tail (4) and the catalytic subunit of protein phosphatase 1 (PP1c)[6] from the α_{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 $\alpha_{IIb}\beta_3$-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 $\alpha_{IIb}\beta_3$-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_1 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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[6] The abbreviations used are: PP1c, catalytic subunit of protein phosphatase 1; PAR4AP, 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 β_3 Thr^{753} 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$_\alpha$ was identified to negatively regulate $\alpha_{IIb}\beta_3$ adhesiveness to immobilized and soluble fibrinogen. PP2Ac$_\alpha$ 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-α_{IIb} (Sew-8, gift from Dr. Newman, Blood Research Institute, Milwaukee, WI), anti-PP2A$_c$ (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit IgG (Pierce) using Protein A-Sepharose beads (Amersham Biosciences). Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose, probed with monoclonal antibodies to α_{IIb} (132.1), PP2A$_c$ (Upstate Biotechnology/Millipore, Billerica, MA), or PP2C$_c$ (Alexis Biochemicals, San Diego, CA), and developed using the ECL system (Amersham Biosciences).

Interaction of PP2Ac with the Integrin α_{IIb} Subunit—Truncation of the cytoplasmic domain of integrin α_{IIb} at residue 989 was generated according to the manufacturer's protocol for the QuikChange^{TM} site-directed mutagenesis kit (Stratagene, La Jolla, CA). Integrin β_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 α_{IIb} and β_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-PP2A$_c$ antibody and immunoblotted with anti-α_{IIb} antibody. In an alternate approach, biotinylated peptides corresponding to residues 985–995 of the integrin α_{IIb} (LAMWKVGFFKR) 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, α isoforms (NM_002715); 2) catalog # M-04065700 targeting murine PP2A catalytic subunit, α 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 siImporter (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$_\alpha$ 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 2×10^8 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 α_{IIb} was immunoprecipitated. The integrin α_{IIb}-associated PP2Ac activity assay was quantified using a PP2A phosphatase assay kit (Upstate Biotechnology). Using a malachite green assay, α_{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$_\alpha$ 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$_\alpha$. 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-α_{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

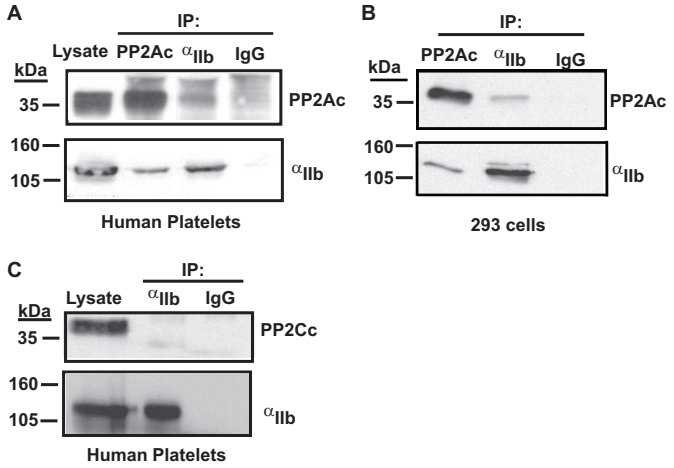

FIGURE 1. **Association of PP2Ac, but not PP2Cc, with integrin $\alpha_{IIb}\beta_3$.** Integrin α_{IIb} was immunoprecipitated from lysates of washed platelets (*A*) or 293 cells expressing $\alpha_{IIb}\beta_3$ (*B*) with control antibodies: rabbit IgG, anti-α_{IIb}, or anti-PP2Ac. Immunoprecipitates were immunoblotted with antibodies to α_{IIb} or PP2Ac. *C*, α_{IIb} was immunoprecipitated, as described above, and the membrane was probed with anti-PP2Cc antibody. Results are representative of three to four experiments.

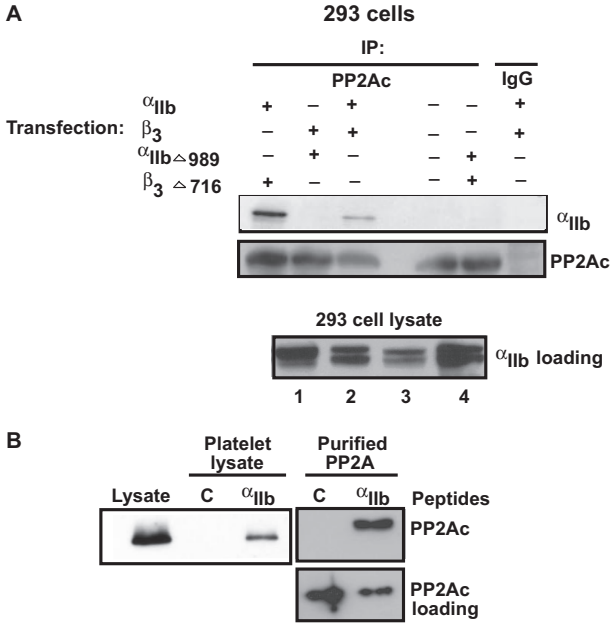

FIGURE 2. **Direct interaction of PP2Ac with α_{IIb}.** *A*, 293 cells were transiently transfected with either wild-type $\alpha_{IIb}\beta_3$ or truncated α_{IIb} or β_3 mutants as indicated. After 48 h, transfected cells were lysed and immunoprecipitated with control IgG or anti-PP2Ac antibody and immunoblotted with anti-α_{IIb} and anti-PP2Ac antibodies. To demonstrate the presence of α_{IIb} in each of the different transfections performed above, lysates obtained from 293 cells transfected with α_{IIb}- and β_3-truncated mutants (*1*), WT α_{IIb}- and β_3-truncated mutants (*2*), α_{IIb}-truncated mutant and WT β_3 (*3*), and WT $\alpha_{IIb}\beta_3$ (*4*) were immunoblotted with anti-α_{IIb} antibody. *B*, biotinylated α_{IIb} cytoplasmic peptide (designated "α_{IIb}") or control (designated *C*) peptide was incubated with either purified PP2A enzyme or platelet lysates. Proteins were precipitated using streptavidin-agarose beads, separated by SDS-PAGE, and immunoblotted with an anti-PP2Ac antibody. An equal aliquot of peptide/PP2Ac mixture was run simultaneously to demonstrate the presence of PP2Ac among reactions (*PP2Ac loading*). Blots are representative of three different experiments.

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 $\alpha_{IIb}\beta_3$ (FL2) and were viable (defined as negative for 7-amino-actinomycin D in the FL3 parameter).

RESULTS

In resting platelets, a pool of the catalytic subunit of protein phosphatase 1 (PP1c) constitutively associates with $\alpha_{IIb}\beta_3$ complex (8). Studies from other cell types have revealed that PP2Ac can associate with integrin β_1 (19). Because the primary amino acid sequence within the catalytic subunits of PP1 and PP2A are closely related, we considered whether the catalytic subunit of PP2A (PP2Ac) could associate with $\alpha_{IIb}\beta_3$ complex in platelets. During co-immunoprecipitation assays with lysates from resting human platelets, we detected the presence of PP2Ac in the α_{IIb} immunoprecipitate. Conversely, in a reciprocal co-immunoprecipitation assay, α_{IIb} was observed in the PP2Ac immunoprecipitate (Fig. 1*A*). Furthermore, the association of PP2Ac with integrin $\alpha_{IIb}\beta_3$ could be recapitulated in human embryonal kidney cell line 293 expressing $\alpha_{IIb}\beta_3$. Thus, suggesting the phosphatase-integrin association is intrinsic to integrin $\alpha_{IIb}\beta_3$ (Fig. 1*B*).

In contrast to PP2Ac, the catalytic subunit of a structurally distinct Ser/Thr phosphatase, protein phosphatase 2C (PP2Cc) was not detected in the α_{IIb} immunoprecipitate, indicating the PP2Ac-$\alpha_{IIb}\beta_3$ interaction is specific (Fig. 1*C*). Although the phosphatase-integrin association may appear modest, its consequence on cellular function is not (see Fig. 4). PP2Ac$_\alpha$ and PP2Ac$_\beta$ 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 $\alpha_{IIb}\beta_3$.

To identify whether α_{IIb} or the β_3 cytoplasmic domains support PP2Ac interaction, 293 cells were transiently transfected with wild-type $\alpha_{IIb}\beta_3$ or with α_{IIb} and β_3 cytoplasmic domain truncation mutants. The association of PP2Ac was then assessed by co-immunoprecipitation assays. Integrin α_{IIb} co-immunoprecipitated with PP2Ac in 293 cells transiently expressing the wild-type (WT) $\alpha_{IIb}\beta_3$ (Fig. 2*A*). Furthermore, cells expressing the WT α_{IIb} along with the β_3 cytoplasmic truncation mutant ($\beta_3\Delta716$) also supported the interaction of α_{IIb} with PP2Ac. In contrast, α_{IIb} failed to associate with PP2Ac in 293 cells that expressed 1) no integrin $\alpha_{IIb}\beta_3$, 2) α_{IIb} cytoplasmic truncation mutant ($\alpha_{IIb}\Delta989$) along with the WT β_3, and 3) α_{IIb} ($\alpha_{IIb}\Delta989$) and β_3 ($\beta_3\Delta716$) cytoplasmic truncation mutants. The apparent increased α_{IIb} association with PP2Ac in $\beta_3\Delta716$-expressing cells, seen in Fig. 2, was not consistently reproducible and may be due to an increased amount of immunoprecipitated PP2Ac. The inability of $\alpha_{IIb}\Delta989$ mutant to support PP2A$_c$ association was not due to a lack of α_{IIb} expression (Fig. 2*A*, 293 cell lysates). These studies suggest that the cytoplasmic domain of integrin α_{IIb}, but not β_3, 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 α_{IIb} cytoplasmic peptide. Because PP1c (a PP2Ac-related phosphatase) interacts with the α_{IIb} cytoplasmic tail,

containing a PP1c binding motif, ^{989}KVXF^{992}, we considered whether the membrane proximal, as opposed to membrane distal, residues of the α_{IIb} subunit could also support PP2Ac interaction. Purified PP2A enzyme and PP2Ac from the resting platelet lysates bound specifically to a biotinylated α_{IIb} peptide containing the residues 985–995 of the integrin α_{IIb}, but not to a control peptide with scrambled sequence (Fig. 2*B*). These studies suggest that the α_{IIb} membrane proximal region containing the KVGFFKR sequence can support the direct interaction of PP2Ac.

Next, we examined if $\alpha_{IIb}\beta_3$ activation and ligand engagement regulates $PP2A_c$-$\alpha_{IIb}\beta_3$ association or $\alpha_{IIb}\beta_3$-associated PP2A activity. The association of PP2Ac with the integrin was evaluated following platelet adhesion to immobilized fibrinogen, an $\alpha_{IIb}\beta_3$-mediated event. The association of PP2Ac with $\alpha_{IIb}\beta_3$ was maintained regardless of whether the platelets were held in suspension over the BSA substrate or adhered to immobilized fibrinogen (Fig. 3*A*). 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. 3*B*). Similarly, a stable association of PP2Ac with the integrin was also observed during soluble fibrinogen binding induced by Mn^{+2} (data not shown). Next, the activity of PP2Ac associated with $\alpha_{IIb}\beta_3$ was quantified in the α_{IIb} immunoprecipitates. Platelets that adhered to fibrinogen exhibited ~45% decreased ($p = 0.02$) $\alpha_{IIb}\beta_3$-associated PP2Ac activity compared with platelets that were maintained in suspension over the BSA substrate (Fig. 3*C*). Consistent with the decreased $\alpha_{IIb}\beta_3$-associated PP2Ac activity in fibrinogen-adhered platelets, we observed an increased Ser^{157} phosphorylation of vasodilator-associated phosphoprotein (VASP), a PP2Ac substrate in fibrinogen adhered platelets (Fig. 3*D*). By densitometry, when compared with platelets suspended over BSA, fibrinogen-adhered platelets exhibited a ~2-fold increase of VASP phosphorylation (Fig. 3*E*). 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 $\alpha_{IIb}\beta_3$.

To explore a functional role for PP2Ac in integrin $\alpha_{IIb}\beta_3$ signaling, we overexpressed a HA-tagged $PP2Ac_\alpha$ in 293 $\alpha_{IIb}\beta_3$ cells and evaluated adhesion to immobilized fibrinogen. $PP2Ac_\alpha$ was chosen because of the reported 10-fold abundance over $PP2Ac_\beta$ in most tissues (21). Immunoblotting with anti-HA antibody confirmed the overexpression of $PP2Ac_\alpha$ (Fig. 4*A*). Compared with the vector control-treated cells, $PP2Ac_\alpha$ overexpression significantly decreased the adhesion of $\alpha_{IIb}\beta_3$ cells to fibrinogen (Fig. 4*B*). The $\alpha_{IIb}\beta_3$-specific blocking antibody, 10E5, inhibited the adhesion of 293 cells. Thus, indicating that the adhesion was primarily mediated through $\alpha_{IIb}\beta_3$. Comparable levels of integrin α_{IIb} expression were observed by densitometry of α_{IIb} immunoblots in control vector and $PP2Ac_\alpha$ HA-overexpressed cells (54.59 ± 7.9 *versus* 55.10 ± 8.02, respectively). To further verify these findings, in

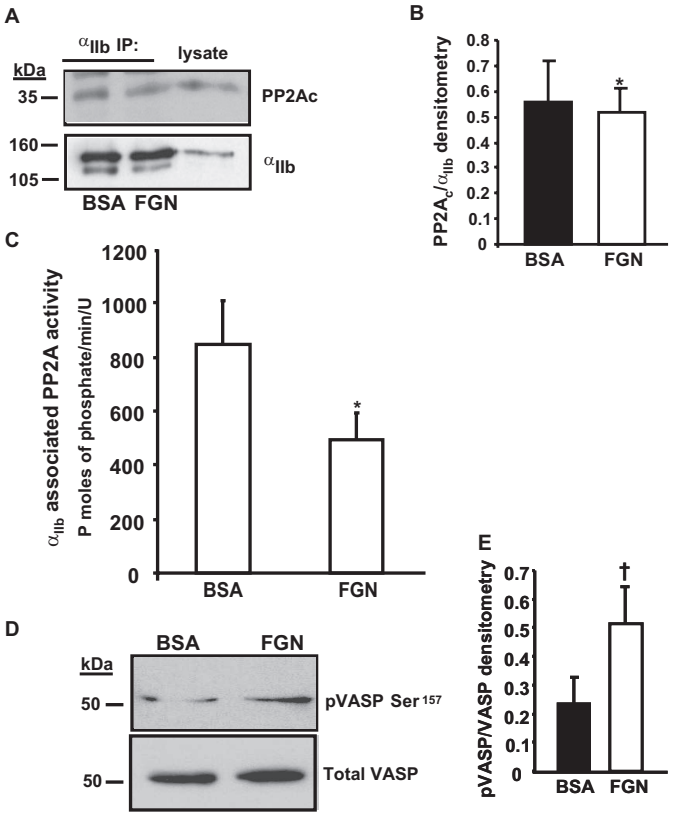

FIGURE 3. **Fibrinogen binding decreases integrin-associated PP2A activity.** *A*, platelets were either allowed to adhere to fibrinogen (*FGN*) or maintained in suspension over BSA substrate. Lysates were prepared and immunoprecipitated with an α_{IIb} antibody and immunoblotted with anti-α_{IIb} or anti-$PP2A_c$ antibodies. *B*, densitometric quantification of PP2Ac associated with the integrin α_{IIb} (ratio of co-immunoprecipitated PP2Ac to α_{IIb} in arbitrary units) in lysates obtained from platelets that were suspended on BSA or adhered to fibrinogen. Results are mean ± S.E., $n = 3$, *, $p = 0.642$. *C*, α_{IIb} immunoprecipitates were washed in phosphatase assay buffer and evaluated for PP2Ac activity by quantifying the dephosphorylation of the phosphopeptide RKpTIRR using a malachite green PP2A phosphatase assay kit. Results are expressed as mean ± S.E. of five experiments; *, $p = 0.02$ by paired *t* test. *D*, lysates from platelets suspended on BSA or adhered to fibrinogen were separated by 10% SDS-PAGE and immunoblotted with anti-phospho-VASP (Ser^{157}). The same blot was stripped and reprobed for total VASP (to assess for loading). *E*, densitometric quantification of VASP phosphorylation (ratio of pVASP to total VASP in arbitrary units) from suspended platelets or fibrinogen adherent platelets. Results are mean ± S.E., $n = 3$, †, $p = 0.05$.

complementary studies, we used short interference RNA (siRNA) to knock down the expression of endogenous $PP2Ac_\alpha$ in 293 cells expressing $\alpha_{IIb}\beta_3$. Knockdown was maximal (~50–60%) and specific for PP2Ac, because PP1c and actin protein levels were comparable between the control and PP2Ac siRNA-treated cells (Fig. 4*C*). Compared with the control siRNA-treated cells, $PP2Ac_\alpha$ knockdown significantly increased the adhesion of $\alpha_{IIb}\beta_3$ cells to fibrinogen (Fig. 4*D*). To further determine whether the increased adhesiveness exhibited by $PP2Ac_\alpha$-depleted cells was specific to immobilized fibrinogen, we studied adhesion to immobilized VWF. $PP2Ac_\alpha$ knockdown significantly increased the adhesion of $\alpha_{IIb}\beta_3$ cells to VWF (Fig. 4*E*). This suggests the differential adhesion due to $PP2Ac_\alpha$ depletion is not ligand-specific. The mean fluorescence intensity for $\alpha_{IIb}\beta_3$ expression was 266.12 ± 55 and 321.3 ± 68 for control and $PP2Ac_\alpha$ siRNA-treated 293 cells, respectively ($p = 0.89$). Thus, integrin expression levels may not account for the

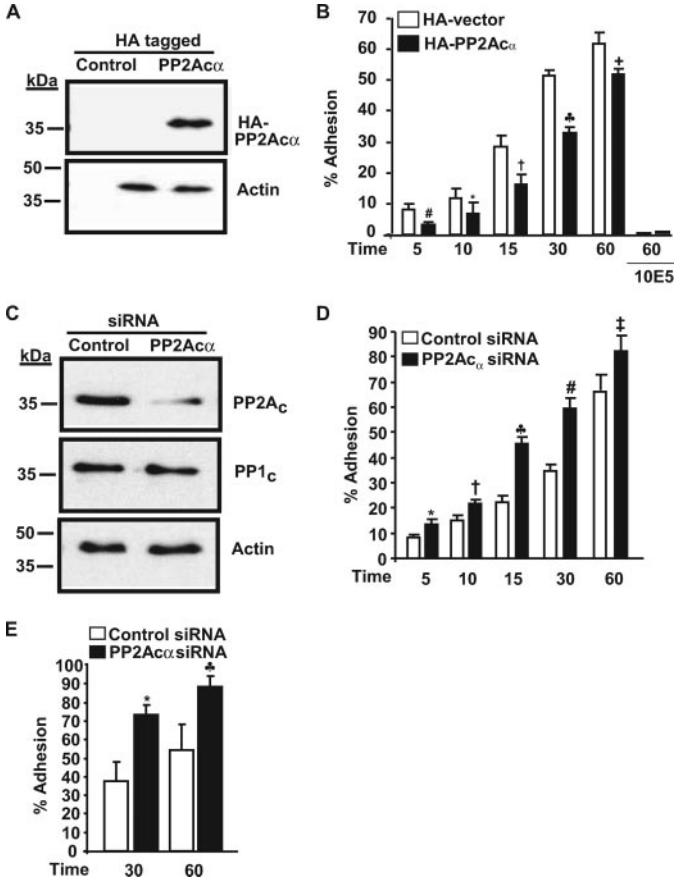

FIGURE 4. **PP2Ac$_\alpha$ negatively regulates $\alpha_{IIb}\beta_3$ adhesiveness in 293 cells.** *A*, PP2Ac$_\alpha$ expression as revealed by anti-HA antibody in control and HA-tagged PP2Ac$_\alpha$-overexpressing cells. This blot was reprobed for actin (loading control) and is a representative of four different experiments. *B*, effect of HA-PP2Ac$_\alpha$ overexpression on 293 cell adhesion. 293 cells transfected with control vector or vector with PP2Ac$_\alpha$ cDNA were allowed to adhere to fibrinogen in the presence and absence of 10E5 (blocking antibody to $\alpha_{IIb}\beta_3$), and adhesion was measured by absorbance at 405 nm. Results are mean ± S.E. of 4–7 experiments in triplicate for various time points and 2 experiments for 10E5 blocking studies. Results were significant at #, $p = 0.008$; *, $p = 0.0031$; †, $p < 0.0001$; ♣, $p < 0.0001$; +, $p = 0.002$ for 5-, 10-, 15-, 30-, and 60-min adhesion. *Error bars* were too narrow to be seen with the 10E5 inhibition. *C*, PP2Ac expression in control and PP2Ac$_\alpha$-transfected siRNA. The membrane was reprobed for PP1c and actin to demonstrate siRNA specificity and equal loading respectively. Blots are representative of five different experiments. *D*, effect of PP2Ac$_\alpha$ knockdown on 293 cell adhesion to fibrinogen. Results are mean ± S.E. of 5–6 experiments each performed in triplicate and was significant at *, $p = 0.001$; †, $p = 0.006$; ♣, $p < 0.001$; #, $p < 0.0001$; ‡, $p = 0.01$ for 5-, 10-, 15-, 30-, and 60-min adhesion by *t* test. *E*, effect of PP2Ac$_\alpha$ knockdown on 293 cell adhesion to von Willebrand factor. Results are mean ± S.E. of three experiments each performed in triplicate and was significant at *, $p = 0.0084$; ♣, $p = 0.0086$, for 30 and 60 min.

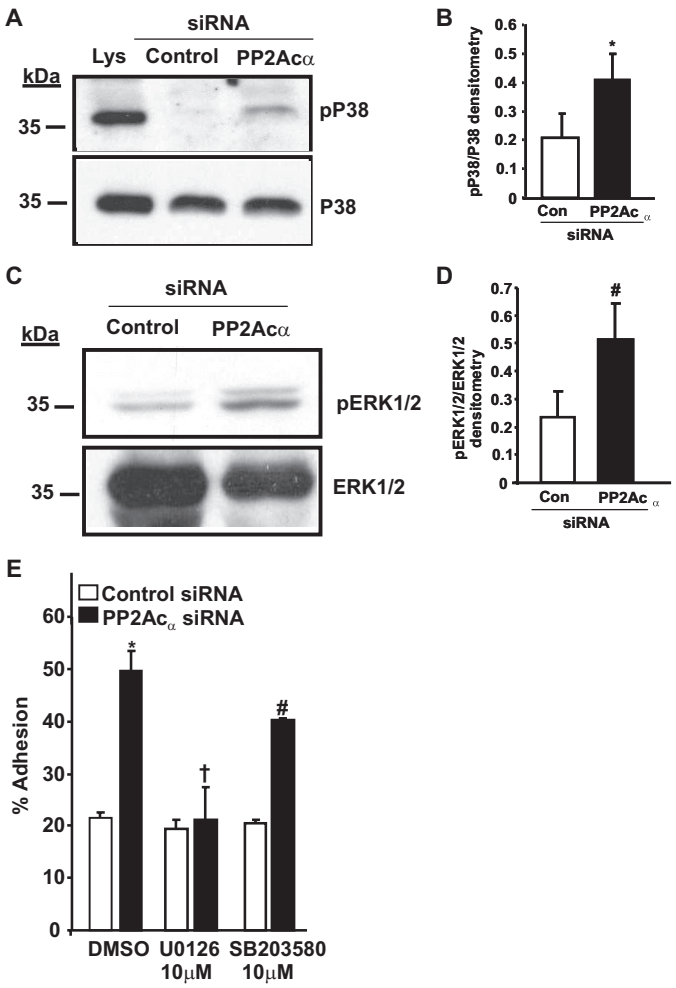

FIGURE 5. **PP2Ac$_\alpha$ knockdown enhances p38 and ERK1/2 activation in 293 cells and blockade of ERK1/2 signaling abolishes the increased adhesiveness of PP2Ac$_\alpha$-depleted cells.** Lysates obtained from 293 $\alpha_{IIb}\beta_3$ cells treated with either control or PP2Ac$_\alpha$, and siRNA 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 (pP38) (*A*) and p44/42 ERK (pERK1/2) (*C*). Cells treated with 0.5 M sorbitol (*Lys*) in *A* serves as positive control for p38 activation. The blots were stripped and reprobed for total p38 (*p38*) or ERK1/2 to assess the equivalency of loading. Densitometric quantification of the enhanced activation of p38 (*B*) and ERK1/2 (*D*) in PP2Ac$_\alpha$ siRNA-treated cells compared with the control treated cells. Data are mean ± S.E. of three experiments for p38 and five experiments for ERK1/2 and was significant at *, $p = 0.04$ for p38 and #, $p = 0.005$ for ERK1/2. *E*, effect of ERK1/2 inhibitor (U0126) or p38 inhibitor (SB203580) on the increased adhesion of PP2Ac$_\alpha$-depleted cells to fibrinogen. Results are mean ± S.E. of three experiments performed in triplicate with the following *p* values: *, $p = 0.0001$; †, $p = 0.373$; #, $p = 0.012$.

observed difference in adhesion. Taken together, these results indicate that PP2Ac$_\alpha$ negatively regulates $\alpha_{IIb}\beta_3$ outside-in signaling function in 293 cells.

To investigate a potential mechanism by which PP2Ac$_\alpha$ can negatively regulate cell adhesion, we explored the effect of depleting endogenous PP2Ac$_\alpha$ in 293 cells on p38 and ERK (PP2Ac$_\alpha$ effectors) signaling pathways. These pathways are implicated in the modulation of cellular cytoskeletal reorganization and cell adhesiveness (22). The siRNA-mediated depletion of PP2Ac$_\alpha$ 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 PP2Ac$_\alpha$-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 PP2Ac$_\alpha$-depleted cells was due to increased ERK1/2 or p38 signaling. Compared with control siRNA-treated cells, PP2Ac$_\alpha$ depletion significantly ($p = 0.0001$) increased adhesion. This increase was abolished ($p = 0.373$) by the ERK1/2 inhibitor (U0126) (Fig. 5*E*). In contrast, the p38 inhibitor, SB203580, failed to repress the increased adhesiveness of PP2Ac$_\alpha$-depleted cells (Fig. 5*E*). Similar results were obtained with another p38 inhibitor (SB202190) (not shown). These studies indicate that PP2Ac$_\alpha$ may suppress

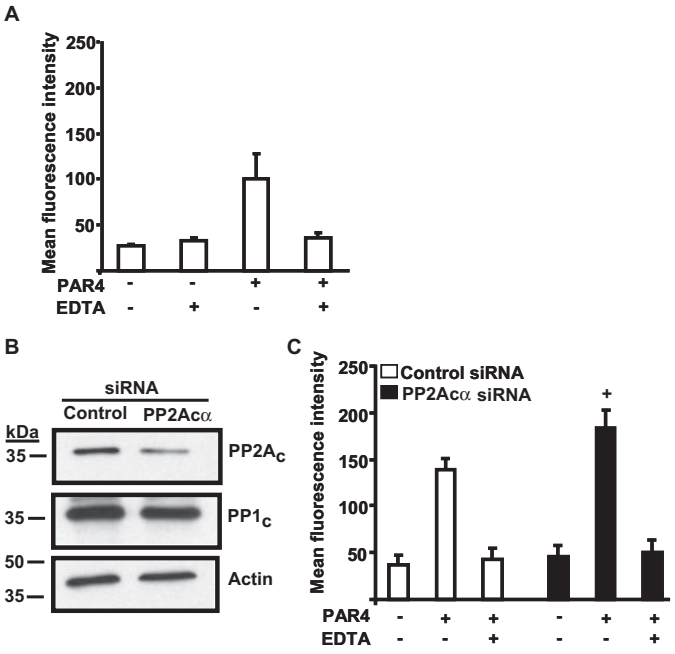

FIGURE 6. **PP2Ac$_\alpha$ negatively regulates $\alpha_{IIb}\beta_3$ activation in murine megakaryocytes.** *A*, Alexa 488 fibrinogen binding in untransfected megakaryocytes. Large and alive megakaryocytes that expressed α_{IIb} were analyzed for fibrinogen binding in response to 2.5 mM PAR4AP in the presence and absence of 10 mM EDTA by flow cytometry. *B*, expression of PP2A$_c$ in control and PP2Ac$_\alpha$-transfected siRNA. The blot was stripped and analyzed for PP1c and actin. These blots are representative of three experiments. *C*, increased fibrinogen binding in megakaryocytes transfected with murine PP2Ac$_\alpha$ 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 PP2Ac$_\alpha$ siRNA over the control siRNA was significant at +, $p = 0.02$ by analysis of variance.

$\alpha_{IIb}\beta_3$ adhesiveness, in part, by down-regulating ERK1/2 activation pathway.

Next, we evaluated if genetic manipulation of PP2Ac$_\alpha$ negatively regulated $\alpha_{IIb}\beta_3$ 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$_\alpha$ die around embryonic day 6.5, thus, precluding the study of platelets from PP2Ac$_\alpha$ null mice (23). In recent years megakaryocytes, from which platelets are derived, have emerged as an attractive system for studying integrin inside-out signaling. They express $\alpha_{IIb}\beta_3$, are activated by agonists, and are amenable to genetic manipulation (3, 18). Therefore, we chose to study the role of PP2Ac$_\alpha$ in murine megakaryocytes, which is a physiologically relevant model comparable to platelets. In concurrence with the previously published reports using this model system, we noticed increased fibrinogen binding in protease-activated receptor 4 activating peptide (PAR4AP)-stimulated murine megakaryocytes compared with the unstimulated megakaryocytes. Addition of EDTA, a divalent cation chelator, decreased fibrinogen binding in PAR4AP-treated megakaryocytes to the level of the unstimulated megakaryocytes. This indicates that the increased fibrinogen binding in response to PAR4AP is specific to integrin activation (Fig. 6*A*).

To elucidate the role of PP2Ac$_\alpha$ in agonist-induced $\alpha_{IIb}\beta_3$ fibrinogen binding, we used murine siRNAs to knock down PP2Ac$_\alpha$ expression in megakaryocytes. Knockdown was maximal (~40–50%) and specific for PP2Ac, because PP1c and actin protein levels were comparable between the control and PP2Ac$_\alpha$ siRNA-treated megakaryocytes (Fig. 6*B*). The residual PP2Ac signal could represent incomplete PP2Ac$_\alpha$ knockdown or expression of PP2Ac$_\beta$. Knockdown of PP2Ac$_\alpha$ significantly increased binding of soluble fibrinogen in response to PAR4AP (Fig. 6*C*) compared with the megakaryocytes treated with control siRNA (Fig. 6*C*). An increased trend that did not reach statistical significance was also noted for $MnCl_2$-induced fibrinogen binding in PP2Ac$_\alpha$-depleted megakaryocytes. This suggests that PP2Ac$_\alpha$ may also negatively regulate $\alpha_{IIb}\beta_3$ outside-in signaling in megakaryocytes (data not shown). Surface expression of $\alpha_{IIb}\beta_3$ was not different between the control (mean fluorescence intensity, 94.67 ± 16) and PP2A$_{c\alpha}$ (mean fluorescence intensity, 89.33 ± 13) siRNA-treated megakaryocytes and could not account for the observed difference in fibrinogen binding. These results suggest that PP2Ac$_\alpha$ negatively regulates integrin $\alpha_{IIb}\beta_3$ inside-out signaling in a murine megakaryocyte model system.

DISCUSSION

Signal transduction by kinases and phosphatases control integrin $\alpha_{IIb}\beta_3$ adhesiveness and activation events. However, a specific role for PP2A in integrin $\alpha_{IIb}\beta_3$ signaling is unclear. In this work, we show that a pool of PP2Ac associates constitutively with the integrin $\alpha_{IIb}\beta_3$ in resting platelets. Studies in 293 model systems revealed that PP2Ac$_\alpha$ can negatively regulate integrin $\alpha_{IIb}\beta_3$ adhesiveness, in part, via the suppression of ERK1/2, but not the p38 signaling pathway. Furthermore, PP2Ac$_\alpha$ negatively regulated PAR4AP-induced integrin $\alpha_{IIb}\beta_3$ inside-out signaling in a murine megakaryocyte model system.

Co-immunoprecipitation assays revealed a close proximal association of a pool of PP2Ac with integrin $\alpha_{IIb}\beta_3$. Additional studies, using integrin tail truncation mutants and integrin α_{IIb} cytoplasmic peptides, have indicated that the α_{IIb} membrane proximal region containing KVGFFKR is sufficient to mediate a direct interaction with PP2A$_c$ (Fig. 2). This observation is consistent with a previous study that showed an association of the inhibitor for PP2A (I1PP2A) with the membrane proximal GFFKR motif of integrin $\alpha_{3A}\beta_1$ (24). The membrane proximal region of α_{IIb} 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 $\alpha_{IIb}\beta_3$ may harbor PP2Ac. Because GFFKR sequence is conserved in other α subunits, it is likely that PP2Ac association may not be limited to α_{IIb} subunits.

Despite the similar binding motifs for PP1c and PP2Ac on integrin α_{IIb}, integrin engagement resulted in a different effect for the two phosphatases. PP1c dissociated from the integrin complex (8), whereas a great extent of PP2A$_c$ remained associated with the integrin (Fig. 3*A*). This suggests that integrin engagement may differentially regulate the two phosphatases in platelets. Interestingly, fibrinogen binding during platelet adhesion to $\alpha_{IIb}\beta_3$ repressed the $\alpha_{IIb}\beta_3$-associated PP2Ac activity (Fig. 3*B*). It is conceivable that the decreased PP2Ac activity, following fibrinogen binding, may be due to an increased Tyr^{307} phosphorylation of PP2Ac that is mediated in part by $\alpha_{IIb}\beta_3$-

associated Src. In fact, fibrinogen binding to $\alpha_{IIb}\beta_3$ during platelet adhesion resulted in an increased $\alpha_{IIb}\beta_3$-associated Src activity (4). Also, phosphorylation of Tyr^{307} residue, within the catalytic subunit of PP2A, by the tyrosine kinases $PP60^{Src}$ or $PP56^{Lck}$ resulted in a reduction of the PP2A activity (27). Decreased $\alpha_{IIb}\beta_3$-associated PP2A activity also correlates with the increased $\alpha_{IIb}\beta_3$-associated Ser/Thr kinase protein kinase C activity following fibrinogen binding (6), implying $\alpha_{IIb}\beta_3$-associated Ser/Thr kinase and phosphatase activity are tightly controlled.

It is difficult to ascertain a specific role for integrin-associated PP2Ac in functional assays obtained from cells expressing either point mutations or deletions of the KVGFFKR region in α_{IIb}, because multiple proteins dock in this region. Moreover, deletion of GFFKR sequence in α_{IIb} can lead to integrin activation via disruption of a salt bridge between the α_{IIb} and β_3 subunits (28). Therefore, in this study, we analyzed $\alpha_{IIb}\beta_3$ adhesive function in cells that are depleted of PP2Ac$_\alpha$ by an siRNA approach. Although this approach can decipher a specific functional role for PP2Ac$_\alpha$ independent of other KVGFFKR-binding proteins, we cannot stringently rule out the functional contribution of PP2Ac$_\alpha$ that is also present in other subcellular locations. Nevertheless, our studies indicated that PP2Ac$_\alpha$ knockdown in 293 $\alpha_{IIb}\beta_3$ model cells resulted in an increased $\alpha_{IIb}\beta_3$ adhesion to immobilized fibrinogen and VWF. These results are in contrast to an earlier study, wherein platelets treated with PP1/PP2A inhibitor calyculin A produced a decreased adhesive phenotype to immobilized fibrinogen (14). This discrepancy could be due to inhibition of multiple phosphatases other than PP2A in calyculin 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 PP2Ac independent of PP1c, because PP1c expression was not decreased in PP2Ac knockdown cells (Fig. 4*C*). Because platelets are anucleate and PP2Ac$_\alpha$ null mice are embryonically lethal, we employed murine megakaryocytes as a comparable model to study platelet $\alpha_{IIb}\beta_3$ inside-out signaling process. We observed increased fibrinogen binding in PAR4AP-treated megakaryocytes that were treated with PP2Ac$_\alpha$ siRNA (Fig. 6). Taken together with the results obtained from the 293 cell model system, these observations strengthen the conclusion that PP2Ac$_\alpha$ negatively regulates integrin signaling.

How could integrin-associated PP2Ac exert a negative regulation of integrin signaling? The association of PP2Ac with the $\alpha_{IIb}\beta_3$ complex does not directly regulate the integrin affinity, because the basal fibrinogen binding in PP2Ac$_\alpha$-depleted megakaryocytes was not statistically increased (Fig. 6*C*). Furthermore, the association of PP2Ac with the $\alpha_{IIb}\beta_3$ complex does not regulate Thr^{753} phosphorylation of integrin β_3, because we failed to observe β_3 Thr^{753} phosphorylation in PP2Ac$_\alpha$-depleted 293 cells (data not shown). Perhaps, multiple substrates for PP2Ac may exist in the focal adhesion protein complexes organized by α_{IIb} and β_3 cytoplasmic tails. Suppression of the phosphorylation or activation of these proteins within the complex by the $\alpha_{IIb}\beta_3$-associated PP2Ac$_\alpha$ is likely to participate in limiting integrin activation and function. Indeed, we noticed that PP2Ac$_\alpha$ 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$_{c\alpha}$-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$_{c\alpha}$-depleted cells (Fig. 5*E*). Other investigators have reported that pharmacological inhibition of PP2Ac leads to increased migration of endothelial and carcinoma cells (32, 33). Thus, the enhanced ERK1/2 and p38 signaling in PP2Ac$_\alpha$-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 $\alpha_{IIb}\beta_3$ when induced by agonists like VWF and thrombin (20). Thus, suppression of ERK1/2 signaling by PP2A could constitute a potential mechanism for limiting integrin function.

In summary, our understanding of the role for PP2A in integrin signaling is fairly limited based on the use of pharmacological inhibitors. Using a genetic approach, our data indicate that PP2Ac$_\alpha$ negatively regulates $\alpha_{IIb}\beta_3$ 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 α integrin subunits, thereby providing additional regulatory mechanisms to control integrin activation.

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