Cross-talk between Serine/Threonine Protein Phosphatase 2A and Protein Tyrosine Phosphatase 1B Regulates Src Activation and Adhesion of Integrin αIIbβ3 to Fibrinogen*

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Integrin αIIbβ3 signaling mediated by kinases and phosphatases participate in hemostasis and thrombosis, in part, by supporting stable platelet adhesion. Our previous studies indicate that the genetic manipulation of PP2Aα (α isoform of the catalytic subunit of protein phosphatase 2A) negatively regulate the adhesion of human embryonal kidney 293 cells expressing αIIbβ3 to fibrinogen. Here, we demonstrated that small interference RNA (siRNA) mediated knockdown of PP2Aα in 293 αIIbβ3 cells led to the dephosphorylation of Src Tyr-529, phosphorylation of Src Tyr-418 and an increased Src kinase activity. Conversely, overexpression of PP2Aα decreased the basal Src activity. Pharmacological inhibition of PP2A in human platelets or PP2Aα knockdown in primary murine megakaryocytes resulted in Src activation. PP2Aα-depleted 293 αIIbβ3 cells did not alter the serine (Ser) phosphorylation of Src but enhanced the Ser-50 phosphorylation of protein tyrosine phosphatase 1B (PTP-1B) with a concomitant increase in the PTP-1B activity. Src activation in the PP2Aα-depleted 293 αIIbβ3 cells was abolished by siRNA mediated knockdown of PTP-1B. Pharmacological inhibition of Src or knockdown of Src, PTP-1B blocked the enhanced activation of extracellular signal-regulated kinase (ERK1/2) and the increased adhesiveness of PP2Aα-depleted 293 αIIbβ3 cells to fibrinogen, respectively. Thus, inactivation of PP2Aα promotes hyperphosphorylation of PTP-1B Ser-50, elevates PTP-1B activity, which dephosphorylates Src Tyr-529 to activate Src and its downstream ERK1/2 signaling pathways that regulate αIIbβ3 adhesion. Moreover, these studies extend the notion that a cross-talk between Ser/Thr and Tyr phosphatases can fine-tune αIIbβ3 outside-in signaling.

Stable platelet-platelet and platelet-extracellular matrix interactions play a critical role in hemostasis and thrombosis. These interactions can be mediated by integrin αIIbβ3 signaling at the sites of vascular injury. The binding of αIIbβ3 to an extracellular ligand-like fibrinogen triggers outside-in signals in platelets via modulation of the activities of several kinases and phosphatases. These signals in turn regulate the cytoskeletal reorganization, which contributes to stable platelet adhesion

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The abbreviations used are: PTP-1B, protein tyrosine phosphatase 1B; PP2Aα, α isoform of the catalytic subunit of protein phosphatase 2A; OA, okadaic acid; PP2Aα, scaffolding A subunit of PP2A; PP1c, catalytic subunit of α, β, and γ isoforms of protein phosphatase 1.
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MATERIALS AND METHODS

Interaction of Src with PP2Ac.

**FIGURE 1.** Interaction of Src with PP2Ac. A, characterization of GST proteins by Coomassie Blue staining. Molecular weight markers (lane 1), GST (lane 2), and PP2Ac-GST (lane 3). PP2Aa (B) and Src (C) interact with PP2Ac-GST. PP2Aa-GST or GST coupled to glutathione beads were used to pull-down PP2Aa subunit or Src from the 293 αIIbβ3 cell lysate. The lower panel (input) shows comparable levels of PP2Aa or Src in the lysate used for pull-down assays. Blots are representative of 2–3 experiments. D, Src interacts with PP2Ac in co-immunoprecipitation assays. Lysate from 293 αIIbβ3 cells or platelets were immunoprecipitated (IP) with control (IgG) or PP2Ac antibody and immunoblotted with anti-Src and PP2Ac antibodies. Blots are representative of 2–3 different experiments.

siRNA Construct, Transfection, and Adhesion—Stable human embryonal kidney 293 cells overexpressing αIIbβ3 were generated by flow cytometric sorting using monoclonal antibodies specific to αIIbβ3 and cultured in DMEM with 10% FBS as previously described (7). A preformed mix of four independent (SMART pool) siRNAs targeting human PP2Acα, PTP-1B, c-Src, PP1cα, PP1cβ, and mouse PP2Acα, and a non-specific control siRNA pool were purchased from Dharmacon (Thermo Fisher Scientific, Lafayette, CO). 293 αIIbβ3 cells were transfected with 100 nM siRNA oligonucleotides using siLimporter according to the manufacturer’s instruction. In some experiments, these cells were transiently transfected using Lipofectamine with CDNA for HA-tagged PP2Acα or the control vector (gift from Dr. A. Verin, University of Chicago, Chicago, IL) After 48–72 h, the cells were used for either Western blotting, immunoprecipitation or cell adhesion experiments. For ERK1/2 immunoblotting studies, cell lysate was prepared following the treatment with 10 μM Src inactive control (PP3) or 5–10 μM Src inhibitor (PP2) for 30 min. For adhesion experiments, 1 × 10⁵ siRNA-treated cells in Tyrode’s buffer, were incubated for 15 min with 5% BSA or 12.5 μg/ml fibrinogen-coated 96-well plate as we have described previously (4). In certain experiments, cells were pretreated with either control DMSO, 5–10 μM PP2 (Src inhibitor), or 10 μM PP3 (inactive analog of PP2). Unbound cells were washed, and the adhered cell was quantified by assaying for acid phosphatase activity at 405 nm. In certain experiments, megakaryocytes were generated from the bone marrow cultures of the BALB/c mice and transfected with control or mouse PP2Acα siRNAs using Mirus transfecting reagent as we have described previously (4).

Src Kinase Activity Assay—Src activity was assayed using HTScan® Src kinase assay kit with the modification protocol suggested by the manufacturer. Lysates were generated from 293 αIIbβ3—deleted 293 αIIbβ3 cells, PP2Acα overexpressing 293 αIIbβ3 cells or platelets treated with DMSO or 50 nm okadaic acid. Src was immunoprecipitated from the lysate by incubating overnight at 4 °C with rabbit anti-Src antibody and protein A beads. After rinsing with kinase buffer, the immunoprecipitate was resuspended with 50 μl of IX kinase buffer containing 1.5 μM biotinylated Src substrate peptide, 20 μM ATP and 1.25 μM DTT for 30 min and mixed with equal volume of 50 μM EDTA to stop the reaction. 25 μl of the reaction solution was transferred to a 96-well streptavidin plates containing 75 μl of water and incubated at room temperature for 60 min. The wells were rinsed three times with 0.1% Triton X-100 in TBS and incubated with anti-phosphotyrosine antibody pY-100 (1:1000 dilution) for 120 min at 37 °C. Following washing, the wells were incubated with 100 μl of HRP-linked goat anti-mouse IgG antibody (1:4000 dilution) and incubated for 30 min. After rinsing, 100 μl of 1 mg/ml TMB (3,3′,5,5′-tetramethylbenzidine dihydrochloride) was added to the each well and incubated for 10 min at 37 °C. The reaction was stopped by adding 100 μl of stop solution, and the resulting absorbance at 450 nm was noted.

PTP-1B Activity Assay—Lysate obtained from the control and PP2Acα siRNA-treated 293 αIIbβ3 cells were immunoprecipitated with anti-PTP-1B antibody or mouse IgG. These immunoprecipitates were then assayed for the PTP-1B activity by evaluating the dephosphorylation of a phosphopeptide (RRLIEDAEpYAARG) using a Malachite green assay...
with 50 nM OA or DMSO for 30 min. Platelets, 293 α\textsubscript{Ib}β\textsubscript{3} (PP2Acα depleted and PP2Acα overexpressing) were lysed with cell lysis buffer containing 1% Triton X-100. Lysate were immunoprecipitated with anti-phosphoserine, anti-Src, anti-PP2Ac, or rabbit and mouse IgG antibodies using protein A/G-Sepharose beads. Beads were washed three times, and the proteins were separated by SDS-PAGE and immunoblotted with anti-Src, anti-phosphoserine, and anti-PP2Ac antibodies. The cDNA for PP2Acα in pcMV vector was amplified by PCR and subcloned into a glutathione S-transferase (GST) vector pGEX 4T-1 using BamH1 and Sal1 restriction enzymes. GST or GST-tagged PP2Acα proteins were expressed in Escherichia coli following induction with isopropyl-β-d-thiogalactopyranoside and purified using glutathione beads. Purified GST or GST-PP2Acα were pre-coupled with glutathione beads and mixed with 100 μg of lyte from 293 α\textsubscript{Ib}β\textsubscript{3} for 3 h at 4 °C. Beads were washed three times, and the PP2Acα-interacting proteins separated by SDS-PAGE and immunoblotted with Src antibody. 40 μg of protein lysate from siRNA-treated cells were separated by a 10% reducing SDS-PAGE and immunoblotted with anti-phospho Src Tyr-529, anti-phospho Src Tyr-418 and total Src antibodies using ECL. The signals on the films were scanned and the densitometric quantification performed using Image J software from NIH.

**Statistics**—Statistical significance of the data was analyzed by using a paired Student’s t test. Data are expressed as mean ± S.E.

**RESULTS**

Source Interacts with PP2Ac in 293α\textsubscript{Ib}β\textsubscript{3} Cells and Platelets—To gain mechanistic insights by which PP2Acα might negatively regulate α\textsubscript{Ib}β\textsubscript{3} signaling, we sought to identify PP2Acα-interacting proteins in 293 α\textsubscript{Ib}β\textsubscript{3} cells, using GST pull-down assays. PP2Acα was expressed as a GST fusion protein in E. coli (Fig. 1A). GST-PP2Acα protein in the pull-down assays detected the scaffolding A subunit of PP2A (PP2Aα), a known binding partner of PP2Ac (5) (Fig. 1B). This indicates that the GST-PP2Acα fusion protein is func-

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**FIGURE 2. Depletion of PP2Acα but not PP1c potentiates Src activity in 293 α\textsubscript{Ib}β\textsubscript{3} cells.** A, lysates from control and PP2Acα siRNA-treated cells were immunoblotted with anti-PP2Ac antibody (recognizes α and β isoforms of PP2Ac), anti-PP1c, anti-phospho Src Tyr-529, and actin (to demonstrate loading). Blots are representative of four experiments. B and C, effect of PP2Acα knockdown on Src Tyr-529 and Src Tyr-418 phosphorylation. Lysates were immunoblotted with anti-phospho Src Tyr-529 and Src Tyr-418 antibodies. The blot was reprobed with total Src antibody to demonstrate loading. Densitometric analysis of phospho Src Tyr-529 and Src Tyr-418 from 3–4 experiments is expressed in arbitrary units. The decreased Src Tyr-529 in PP2Acα-depleted cells were significant *, p = 0.01, while the increased Src Tyr-418 was significant at fp = 0.03. D, Src was immunoprecipitated from control and PP2Acα siRNA-treated cells and immunoblotted with anti-Src antibody. E, Src kinase activity from control and PP2Acα-depleted cells. The increased Src kinase activity in PP2Acα-depleted cells were significant at *, p = 0.03. n = 3. F, PP1c expression in control and PP1c siRNA-treated cells. The blot was reprobed with anti-PP2Ac and actin antibodies. G, knockdown of PP1c does not alter Src Tyr-529 or Src Tyr-418 phosphorylation. Results are representative of two experiments.

**Immunoprecipitation, GST Pull-down and Immunoblotting**—Blood was drawn in an acid/citrate/dextrose anticoagulant from normal donors. These donors signed informed consent approved by the Institutional Review Board of Baylor College of Medicine, Houston, TX. Washed platelets were prepared as we previously described (12). Washed platelets were incubated
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tional in protein interaction studies. More importantly, Src was identified to interact with GST-PP2Acα but not GST alone (Fig. 1C). To verify these findings, co-immunoprecipitation studies were also performed. Immunoblots of PP2Ac but not control IgG immunoprecipitates from 293 αinβ3 cells and resting human platelets detected Src (Fig. 1D). These studies indicate that Src is a part of the protein complex that interacts with PP2Ac in 293 αinβ3 cells and platelets.

**PP2Acα Negatively Regulates Src Activation**—The close proximity of PP2Ac and Src prompted us to test whether PP2Acα plays a role in Src activation. Therefore, we analyzed Src activation in 293 αinβ3 cells transfected with control siRNAs and four independent siRNAs targeting PP2Acα. Fig. 2A revealed specific knockdown of PP2Ac because the levels of a PP2Ac-related phosphatase, PP1c (catalytic subunit of all protein phosphatase 1 isoforms), and actin were comparable between the control and PP2Acα siRNA-treated cells. Phosphorylation of Src Tyr-529 promotes the intramolecular interaction of the C-terminal domain of Src with the SH2 domain and inhibits Src kinase activity (13). Src Tyr-529 phosphorylation was markedly (*p* = 0.01) decreased in response to the depletion of PP2Acα (Fig. 2B). Conversely, phosphorylation of Src Tyr-418 within the kinase activation domain, which promotes Src activity (13), was increased (*p* = 0.03) in PP2Acα-depleted cells (Fig. 2C). Total Src levels in the control and PP2Acα siRNA-treated cells were comparable (Fig. 2, B and C, lower panels). Next, we directly tested whether the depletion of PP2Acα potentiated Src kinase activity. Src was immunoprecipitated from the control and PP2Acα siRNA-treated 293 αinβ3 cells (Fig. 2D) and Src kinase activity analyzed as previously described (14). Consistent with the immunoblotting data obtained with the Src phosphospecific antibodies, the kinase activity of Src isolated from the PP2Acα-depleted cells were increased (*p* = 0.03) compared with the control cells (Fig. 2E).

To ascertain whether the Src activation was specific to PP2Acα inhibition, we examined Src Tyr-529 and Src Tyr-418 phosphorylation in 293 αinβ3 cells that were depleted for the catalytic subunits of α, β, and γ isoforms of PP1 (PP1c). Fig. 2F reveals that siRNA treatment reduced the expression of PP1c. This knockdown was specific to PP1c because the levels of PP2Ac and actin were comparable between the control and PP1c siRNA-treated cells (Fig. 2F, lower two panels). However, Src Tyr-529 and Src Tyr-418 phosphorylation levels were comparable between the control and PP1c-depleted cells (Fig. 2G). These observations indicate that the Src activation was specific to PP2Acα inhibition.

To further confirm the findings from the siRNA studies, we took a complementary approach and overexpressed a HA-tagged PP2Acα in 293 αinβ3 cells. Immunoblotting with anti-HA and anti-PP2Ac antibodies confirmed the overexpression of PP2Acα (Fig. 3A). Src was immunoprecipitated from these cells (Fig. 3B) and evaluated for Src kinase activity. Compared with the vector-treated cells (control), PP2Acα-overexpressing cells revealed a modest, but significantly lower (by ~22%; *p* = 0.005) basal Src activity (Fig. 3C). Collectively, these studies suggest that Src activity in 293 αinβ3 cells can be regulated by PP2Acα.

**To further establish that the depletion of PP2Acα activates Src in primary cells,** we studied Src activation in a bone marrow-derived murine megakaryocytes, an additional model system that has direct relevance to platelet biology. siRNA treatment resulted in the reduction in PP2Ac protein in megakaryocytes (Fig. 4A). Depletion of PP2Acα in megakaryocytes resulted in the dephosphorylation of Src Tyr-529 along with a concomitant increase in the Src Tyr-418 phosphorylation (Fig. 4B). Finally, to examine the role of PP2Ac in a more physiological context, we examined Src activation in human platelets treated with PP2Ac selective inhibitor-OA. OA at concentrations of 10–100 nM is required to inhibit PP2Ac in intact cells, while 10 μM can inhibit cellular PP1c activity (15). Compared with the DMSO-treated platelets, phosphorylation of Src Tyr-529 was distinctly reduced (*p* = 0.009), whereas the level of Src Tyr-418 phosphorylation was enhanced (*p* = 0.05) in response to 50 nM OA treatment (Fig. 4, C and D). Similar results were obtained with 100 nM OA (not shown). Total Src level in these studies was not altered (Fig. 4, C and D, lower panel). Consistent with the immunoblotting data obtained with the Src phosphospecific antibodies, the kinase activity of Src isolated from OA-treated platelets were increased (*p* = 0.05) (Fig. 4E). Taken together, these studies indicate that PP2Acα negatively regulates Src activity.

**Depletion of PP2Acα Enhances PTP-1B but Not Src Serine Phosphorylation**—Besides phosphorylation on Tyr residues, Src is also phosphorylated by serine/threonine (Ser/Thr) kinases at the N terminus, including the phosphorylation of Src Ser-12 that associate with an increased Src kinase activity (16, 17). Because Src is one of the proteins that complex with PP2Acα, we considered whether the loss of PP2Acα modulated the overall Ser phosphorylation of Src. Lysates from the control and PP2Acα siRNA-treated cells were immunoprecipitated with control rabbit IgG and phosphoserine antibodies, and the resulting immunoprecipitates were immunoblotted with anti-Src antibody. Src was detected in phosphoserine but not in control IgG immunoprecipitates (Fig. 5A, upper panel). In a reciprocal study, Src immunoprecipitates from the control and
PP2Ac siRNA-treated cells revealed comparable immunostaining with anti-phosphoserine antibody (Fig. 5A, lower panel). Densitometric studies revealed comparable levels of the ratio of phospho Src/total Src in arbitrary units (1.9 ± 0.5 versus 2.01 ± 0.4) in control and PP2Acα-depleted cells. Although these studies cannot identify phosphorylation of any specific Ser-50 phosphorylation that was accompanied by enhanced PTP-1B activity. Because the depletion of PP2Ac enhanced PTP-1B activity and also activated Src, we examined if there exist protein complexes between Src and PTP-1B. Src was detected in the PTP-1B immunoprecipitate from the PP2Acα-depleted cells but not in the control siRNA-treated cells (Fig.

FIGURE 4. Knockdown of PP2Acα in murine megakaryocytes and pharmacological inhibition of PP2A in human platelets enhances Src activation. A, PP2Acα expression in control and PP2Acα siRNA transfected murine megakaryocytes. The membrane was reprobed for actin to demonstrate equal loading. B, effect of PP2Acα knockdown on Src Tyr-529 and Src Tyr-418 phosphorylation. Results are representative of two experiments. C and D, Src Tyr-529 and Src Tyr-418 phosphorylation in human platelets treated with OA and DMSO. Lysates were immunoblotted with anti-phospho (Src Tyr-529 and Src Tyr-418) antibodies. The blot was reprobed with anti-Src antibody. Densitometric analysis of phospho Src/total Src from four experiments is expressed in arbitrary units. The decreased Src Tyr-529 in PP2Acα-depleted cells were significant at *, p = 0.009, while the increased Src Tyr-418 was significant at †, p = 0.05. E, Src kinase activity from DMSO- and OA-treated platelets. The increased Src kinase activity in OA-treated platelets were significant at *, p = 0.05. n = 3.

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Ser residues on Src, they imply that the loss of PP2Acα does not significantly alter the overall Ser phosphorylation of Src.

Because Src is also activated by protein tyrosine phosphatases that dephosphorylate Src Tyr-529, we focused our attention on protein tyrosine phosphatases. Although there are several candidate tyrosine phosphatases that can activate Src, we considered protein tyrosine phosphatase 1B (PTP-1B) for the following reasons. 1) Phosphorylation of PTP-1B at Ser-50 by Ser/Thr kinases enhances PTP-1B activity in vivo (18). 2) PTP-1B has a prominent role in regulating outside-in integrin signaling (3).

We considered whether the loss of PP2Acα enhanced PTP-1B Ser-50 phosphorylation. PTP-1B immunoprecipitate from the lysates of PP2Acα siRNA-treated 293 IIb3 cells demonstrated moderately enhanced Ser-50 phosphorylation compared with the control cells (Fig. 5B). In a complementary approach, direct immunoblotting studies with anti-PTP-1B Ser-50 antibody revealed ~2-fold increased (p = 0.02) PTP-1B Ser-50 phosphorylation in PP2Acα siRNA-treated 293 αIIbβ3 cells (Fig. 5C). We ascertained whether the hyperphosphorylation of PTP-1B Ser-50 in PP2Acα-depleted cells affected its enzymatic activity. Compared with the control siRNA-treated cells, loss of PP2Acα resulted in a 1.6-fold (p = 0.009) enhanced PTP-1B activity (Fig. 5D). PTP-1B recovery in the immunoprecipitates from the control, and PP2Acα-depleted cells were comparable (Fig. 5E, lower panel). PTP-1B enzymatic activity was specific because the mouse IgG immunoprecipitates only detected the baseline phosphate levels. Collectively, these studies indicated that the loss of PP2Acα in 293 αIIbβ3 cells resulted in an increased PTP-1B activity.
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**A**

**IP**

Phosphoserine

Con PP2A Con PP2A siRNA

**kDa**

Con PP2A Con PP2A

50 50

**IP**

Rabbit IgG

Ssrc

**kDa**

Con PP2A Con PP2A

50 50

50 50

**B**

**IP**

IgG IP PTP-1B IP

Con PP2A Con PP2A PTP-1B pSer50

**kDa**

Con PP2A PTP-1B

50 50

**E**

**IP**

PTP-1B IP

Control PP2Ac

**kDa**

Control PP2Ac

50 50

50 50

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Although the in vivo association of Src with the protein complex containing PTP-1B appears modest, the influence of such an association on Src signaling is relatively profound (see below in Fig. 6, B and C).

**PTP-1B Mediates Src Activation in PP2Ac-depleted Cells**—We examined whether the activation of Src in PP2Ac-depleted 293 αIIbβ3 cells was facilitated by PTP-1B. Src kinase activity in 293 αIIbβ3 cells correlated well with the Src phosphospecific immunoblotting data (Fig. 2, B–E). Therefore, Src Tyr-529 and Src Tyr-418 phosphorylation was compared in cells treated with the following siRNAs: PP2Acα, dual PP2Acα and PTP-1B, PTP-1B or control. Fig. 6A shows that compared with the cells treated with control siRNA, dual treatment of PTP-1B and PP2Acα siRNAs decreased the expression of both PP2Ac and PTP-1B proteins. More importantly, PP2Acα but not PTP-1B-depleted cells showed dephosphorylation of Src Tyr-529. Src Tyr-529 dephosphorylation observed in PP2Acα-depleted cells was abolished following the dual knockdown of PTP-1B and PP2Acα (Fig. 6B). Conversely, PP2Acα but not PTP-1B-depleted cells showed phosphorylation of Src Tyr-418. Src Tyr-418 phosphorylation observed in PP2Acα-depleted cells was reduced in dual PTP-1B and PP2Acα knockdown cells (Fig. 6C). These studies indicate that the PTP-1B mediated the dephosphorylation of Src Tyr-529 and contributes to the increased Src Tyr-418 phosphorylation observed in PP2Acα-depleted cells.

**Src Activation in PP2Acα-depleted 293 αIIbβ3 Cells Has Functional Consequences for Integrin αIIbβ3**—We have previously shown that the knockdown of PP2Acα in 293 αIIbβ3 cells enhanced their ability to adhere to fibrinogen and increased the activation of extracellular signal-regulated kinase (ERK1/2) (4). Importantly, ERK1/2 signaling was linked to αIIbβ3 adhesion in PP2Acα-depleted cells because blockade of ERK1/2 activation abolished their increased adhesiveness (4). Although the regulation of Src activity by PP2Acα is integrin αIIbβ3 independent, we evaluated whether the increased Src activity in PP2Acα-depleted 293 αIIbβ3 cells can regulate signaling and adhesive function.
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PP2Ac significantly \( (p < 0.0001) \) enhanced the adhesion of 293 \( \alpha_{IIb} \beta_3 \) cells to immobilized fibrinogen (Fig. 7C). The inability of parental (plain) 293 cells to adhere to fibrinogen and the inhibition of 293 \( \alpha_{IIb} \beta_3 \) cell adhesion to fibrinogen in the presence of 10E5 (\( \alpha_{IIb} \beta_3 \) specific blocking antibody) demonstrates that the adhesion is \( \alpha_{IIb} \beta_3 \) dependent. Importantly, the enhanced adhesion associated with the PP2Ac-depleted 293 \( \alpha_{IIb} \beta_3 \) cells to fibrinogen was abolished \( (p = 0.07) \) with 10 \( \mu M \) PP2. In contrast, 10 \( \mu M \) of PP3 did not alter \( (p < 0.0001) \) the increased adhesiveness of PP2Ac-depleted cells (Fig. 7C).

To validate the findings from the Src pharmacological inhibitor studies, adhesion assays were also performed with cells treated with dual PP2A- and Src-directed siRNAs. Compared with the cells treated with only control siRNA, dual treatment of PP2Ac and Src siRNAs decreased the expression of PP2Ac and Src proteins (Fig. 8A). The enhanced adhesive phenotype displayed by the PP2Acα-depleted 293 \( \alpha_{IIb} \beta_3 \) cells was abrogated by siRNA-mediated knockdown of Src (Fig. 8B). Because Src activation in the PP2Acα-depleted cells is dependent on PTP-1B, we examined the role of PTP-1B in the adhesion of PP2Acα-depleted cells. Fig. 8A shows decreased protein expression of PTP-1B and PP2Acα in PTP-1B and PP2Acα siRNA-treated cells. Dual knockdown of PTP-1B and PP2Acα abolished the increased adhesion exhibited by the PP2Acα-depleted cells (Fig. 8B). Knockdown of only Src or PTP-1B in 293 \( \alpha_{IIb} \beta_3 \) cells did not alter the adhesion. Collectively, these studies indicate that the enhanced Src and PTP-1B activation in PP2Acα-depleted 293 \( \alpha_{IIb} \beta_3 \) cells contribute to the increased \( \alpha_{IIb} \beta_3 \)-mediated adhesion to fibrinogen.

**DISCUSSION**

It is widely accepted that integrin \( \alpha_{IIb} \beta_3 \) function is regulated by cellular signaling that is generated by the opposing actions of protein kinases and protein phosphatases. However, the contribution of Ser/Thr protein kinases but not Ser/Thr phosphatases in integrin \( \alpha_{IIb} \beta_3 \) function has been extensively investigated. Here, using 293 \( \alpha_{IIb} \beta_3 \) cells, primary murine megakaryocytes and human platelets, we established that PP2Acα negatively regulates Src activity. Mechanistically, loss of PP2Acα leads to the hyperphosphorylation of PTP-1B Ser-50 along with an increased PTP-1B activity.
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![Diagram](image)

**FIGURE 7.** Pharmacological inhibition of Src by PP2 but not by an inactive control (PP3) blocks the enhanced ERK1/2 signaling and abrogates the increased adhesiveness of PP2Ac-α-depleted cells. A, lysates was prepared from either control or PP2Ac-α-depleted cells followed by treatment with PP3 (inactive Src inhibitor) or PP2 (Src inhibitor). They were separated by 10% SDS-PAGE and ERK activation was assessed by immunoblotting using antibodies specific for the active p44/42 ERK (pERK1/2) or total ERK. B, densitometric quantification of pERK/total ERK from three experiments in arbitrary units. C, effect of Src inhibitor (PP2) or inactive control for PP2 (PP3) on the increased adhesion of PP2Ac-α-depleted cells to fibrinogen. n = 4–5, *, p < 0.0001. Error bars are too narrow to be seen with 10E5 inhibition and plain 293 cell adhesion studies.

v-Src activity (20). However, it is unclear as to which subunit of the PP2A participated in the regulation of v-Src activity. Using a genetic approach in 293 αIIbβ3 cells, our data indicate that the inhibition of catalytic subunit of PP2A (PP2Acα) but not catalytic subunit of PP1 (PP1c) can result in Src activation (Fig. 2). Similar observations were noted in PP2Acα-depleted murine megakaryocytes and in human platelets treated with nanomolar concentrations of OA (Fig. 4). Despite these similarities, differences in the level of Src activation were noted between the model system and platelets. Densitometric quantification studies revealed that the depletion of PP2Acα in 293 αIIbβ3 cells decreased Src Tyr-529 phosphorylation by ~75%, increased Src Tyr-418 phosphorylation by ~45% and produced a ~30% increase in Src kinase activity (Fig. 2, B–E). In platelets, OA decreased Src Tyr-529 phosphorylation by ~95%, increased the Src Tyr-418 phosphorylation by ~300% and produced a ~35% increased Src activity (Fig. 4, C–E). The extent of increase in Src activity in OA-treated platelets was not as substantial as the data from the phosphospecific antibodies would have predicted. These observations suggest that potential differences in the mechanisms of Src activation between the PP2Acα-depleted 293 αIIbβ3 cells and the OA-treated platelets may exist. Thus, findings from the model system should be cautiously interpreted in the context of platelets. Additional studies using platelets from a megakaryocytic lineage specific PP2Acα-null mouse, as and when available in the future, should be considered to validate these findings.

Activated Src family kinases like c-Src and Ick phosphorylates PP2Ac at Tyr-307 and inhibits the activity of PP2A (21). Such an interrelationship between Src and PP2A implies that a pool of PP2A and Src could be in close proximity to each other. Indeed, our findings in resting platelets and 293 αIIbβ3 cells reveal a complex of PP2Ac and Src (Fig. 1). It is likely that protein complex containing Src and PP2A might be a part of the multiprotein complex anchored by integrin αIIbβ3 in platelet and/or 293 αIIbβ3 cells. Association of c-Src with the β3 subunit (2) and the interaction of PP2Ac with the αIIb subunit (4) support this notion. Indeed, previous studies have identified a multiprotein complex containing the SV40 small t-antigen and polyoma virus middle T-antigen to contain PP2A and Src (22, 23).

The data in this manuscript raise two interesting questions. First, how can inhibition of a Ser/Thr phosphatase (PP2Acα) activate a tyrosine kinase (Src)? While the occurrence of such a cross-talk has been widely accepted (24), the underpinning mechanisms are not clear. Inhibition of PP2Ac is likely to alter the Ser/Thr phosphorylation of multiple cellular proteins, including some that have the potential to regulate Src activity. Indeed, Ser phosphorylation of the N terminus of Src by okadaic acid is reported to stimulate c-Src kinase activity (16, 17). Ser phosphorylation of PTP-1B by okadaic acid (25) and the specific phosphorylation of PTP-1B Ser-50 by Ser/Thr kinases are reported to stimulate ~3 fold basal PTP-1B activity (18) or mildly (~25%) impair the dephosphorylation of insulin receptor substrate following insulin stimulation (26). Our studies show that the depletion of PP2Acα resulted in the hyperphosphorylation of PTP-1B Ser-50 with a concomitant increase in PTP-1B activity (Fig. 5). Importantly, Src activation in PP2Acα-depleted cells was largely mediated by PTP-1B because the dephosphorylation of Src Tyr-529 and the phosphorylation of Src Tyr-418 were not evident in cells with dual
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binding to $\alpha_{\text{IIb}}\beta_3$ (2, 3). Interestingly, Src activation by the inhibition of PP2Aco in our studies is independent of integrin activation. Nevertheless, the regulation of Src by PP2Aco could also occur in the milieu of integrin-associated protein complexes. For example, in resting platelets, the presence of PP2Ac in the $\alpha_{\text{IIb}}$ protein complex could contribute to maintain $\beta_3$-bound c-Src in an inactive conformation. Fibrinogen binding to the activated $\alpha_{\text{IIb}}\beta_3$ suppresses integrin $\alpha_{\text{IIb}}$-bound PP2Aco activity, which in turn could facilitate $\beta_3$-associated c-Src activation via PTP-1B activity within the integrin complex. Finally, activated Src could phosphorylate PP2Ac Tyr-307 to further inhibit PP2Aco activity and thereby maintain Src activation. In line with this argument, we have previously shown that PP2Aco associates with integrin $\alpha_{\text{IIb}}\beta_3$ complex and fibrinogen binding suppresses $\alpha_{\text{IIb}}\beta_3$-associated PP2Aco activity (4).

Studies in human platelets (2, 27) and in mouse models (28, 29) have revealed an essential role for Src and/or Src-$\alpha_{\text{IIb}}\beta_3$ interaction in outside-in $\alpha_{\text{IIb}}\beta_3$ integrin signaling dependent functions like adhesion, and spreading on fibrinogen. We observed that PP2Aco-depleted cells exhibited Src activation and increased adhesion to fibrinogen. siRNA-mediated knockdown of Src or pharmacological inhibition of Src with PP2 but not an inactive analog PP3 selectively abolished the increased adhesion of PP2Aco-depleted 293 $\alpha_{\text{IIb}}\beta_3$ cells. Consistent with the role of PTP-1B in Src activation, knockdown of PTP-1B abrogated the increased adhesion of PP2Aco-depleted 293 $\alpha_{\text{IIb}}\beta_3$ cells (Fig. 8). It is likely that ERK1/2 in PP2Aco-depleted cells could be one of the downstream effectors of Src in regulating $\alpha_{\text{IIb}}\beta_3$ adhesion. Such a notion is supported by the following observations: (a) ERK1/2 activation was noted in PP2Aco-depleted cells (4), (b) Src inhibitor partially ablates the increased ERK activation in PP2Aco-depleted cells (Fig. 7A), and (c) the ability of ERK and Src inhibitors to block the increased adhesiveness of PP2Aco cells to fibrinogen (4). Other investigators have reported that inhibition of Src activity blocked the increased migratory property of the endothelial and carcinoma cells treated with generic PP2A inhibitor (30, 31).

In summary, our studies indicate that the inhibition of PP2Aco leads to the hyperphosphorylation of PTP-1B Ser-50 along with increased PTP-1B activity, which dephosphorylates Src Tyr-529. Increased Src activity due to the depletion of PP2Aco contributes to the enhanced ERK1/2 signaling and the increased $\alpha_{\text{IIb}}\beta_3$ adhesiveness to fibrinogen. In a broader context, regulation of Src activity by a cross-talk between the Ser/Thr and Tyr phosphatases may provide additional mode of regulatory mechanisms, during processes like cell differentiation, proliferation and the pathological conditions associated with tumor growth and progression.

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knockdown of PP2Aco and PTP-1B (Fig. 6). Second, how can loss of PP2Aco lead to an increased PTP-1B Ser-50 phosphorylation? It is currently unclear whether PTP-1B Ser-50 is a direct substrate of PP2Aco. However, our preliminary studies with GST-PP2Aco pull-down assays and PP2Aco co-immunoprecipitation studies failed to detect a complex between PP2Aco and PTP-1B (not shown). Whether loss of PP2Aco leads to the activation of Ser/Thr kinases that phosphorylates PTP-1B Ser-50 is currently being investigated.

PTP-1B is recruited to the multiprotein complex anchored by the integrin $\alpha_{\text{IIb}}\beta_3$, including c-Src protein. On the other hand, C terminus Src kinase (CSK) that phosphorylates Src Tyr-529 dissociates from the $\alpha_{\text{IIb}}\beta_3$ protein complex. Such dynamic interactions constitutes an early mechanism that leads to integrin-dependent c-Src activation following fibrinogen

FIGURE 8. siRNA-mediated knockdown of Src or PTP-1B abrogates the increased adhesiveness of PP2Aco-depleted cells. A, expression of PP2Aco in 293 $\alpha_{\text{IIb}}\beta_3$ cells transfected with the following siRNAs: control, PP2Aco, PP2Aco, and PTP-1B, PP2Aco, and Src, PTP-1B, Src. The membrane was reprobed with anti-PTP-1B, anti-Src, and anti-actin antibodies. B, adhesion to immobilized fibrinogen by293 $\alpha_{\text{IIb}}\beta_3$ cells treated with control, PP2Aco, PP2Aco, and PTP-1B, PP2Aco, and Src, PTP-1B, Src-directed siRNAs. n = 4, *= p < 0.02.
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