A Novel Interaction of the Catalytic Subunit of Protein Phosphatase 2A with the Adaptor Protein CIN85 Suppresses Phosphatase Activity and Facilitates Platelet Outside-in α1β3 Integrin Signaling

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The transduction of signals generated by protein kinases and phosphatases are critical for the ability of integrin α1β3 to support stable platelet adhesion and thrombus formation. Unlike kinases, it remains unclear how serine/threonine phosphatases engage the signaling networks that are initiated following integrin ligation. Because protein-protein interactions form the backbone of signal transduction, we searched for proteins that interact with the catalytic subunit of protein phosphatase 2A (PP2Ac). In a yeast two-hybrid study, we identified a novel interaction between PP2Ac and an adaptor protein CIN85 (Cbl-interacting protein of 85 kDa). Truncation and alanine mutagenesis studies revealed that PP2Ac binds to the P3 block (396PAIPPKKPRP405) of the proline-rich region in CIN85. The interaction of purified PP2Ac with CIN85 suppressed phosphatase activity. Human embryonal kidney 293 α1β3 cells overexpressing a CIN85 P3 mutant, which cannot support PP2Ac binding, displayed decreased adhesion to immobilized fibrinogen. Platelets contain the ~85 kDa CIN85 protein along with the PP2Ac-CIN85 complex. A myristylated cell-permeable peptide derived from residues 395–407 of CIN85 protein (P3 peptide) disrupted the platelet PP2Ac-CIN85 complex and decreased α1β3 signaling dependent functions such as platelet spreading on fibrinogen and thrombin-mediated fibrin clot retraction. In a phospho-profiling study P3 peptide treated platelets also displayed decreased phosphorylation of several signaling proteins including Src and GSK3β. Taken together, these data support a role for the novel PP2Ac-CIN85 complex in supporting integrin-dependent platelet function by dampening the phosphatase activity.

An ischemic vascular event ensues when a blood vessel is occluded by platelet-rich thrombi at the site of ruptured atherosclerotic plaque. Exposure of platelets to collagen and/or von Willebrand factor under high shear stress within the milieu of plaque initiates the inside-out signaling processes that activate integrin α1β3 (1). The activated integrin can engage fibrinogen and generate outside-in signals that facilitate cytoskeletal reorganization and support a cascade of functional changes, including platelet spreading, stable adhesion, granule secretion, and clot retraction; all processes that augment the size of the platelet thrombi (2, 3).

The coordinated signaling networks that are initiated by the engagement of agonists and integrin receptors on platelets are essential to the development of thrombi (4, 5). Spatial and temporal assembly of multiprotein signaling complexes by adaptor proteins relay signals following the engagement of integrin. The reversible phosphorylation of proteins on tyrosine, serine, and threonine residues, under the control of kinases and phosphatases, is an essential component of signaling. Because the outside-in signaling process greatly amplifies the size of platelet thrombi, there is a desire to selectively target this signaling process in a new class of antithrombotic agents (6). Although several tyrosine kinases are considered important for outside-in signaling, the role of Ser/Thr phosphatases in this signaling process is less explored.

Protein phosphatase 2A (PP2A)3 is a Ser/Thr phosphatase that consist of a catalytic subunit (PP2Ac) and a structural subunit (PP2Aa), which associates with a range of regulatory B subunits to form PP2A heterotrimers. The subcellular localization, substrate specificity, and catalytic activity of PP2A is regulated by the B subunits (7). The tumor suppressing property of PP2A has garnered considerable therapeutic interest for activating this phosphatase in hematological cells associated with malignancies (8, 9). Previous studies have noticed an inhibition of platelet function following treatment with okadaic acid and/or calyculin A at concentrations that block protein phosphatase 1 (PP1) and PP2A activities (10–13). The paucity of

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isoform-specific pharmacological inhibitors and knock-out mice models has limited our ability to understand the role of PP2A in platelets. In model cells overexpressing platelet integrin \( \alpha_{IIb}\beta_{3} \), we noticed that siRNA-mediated depletion of PP2A\(\alpha\) promoted activation of the tyrosine kinase Src and enhanced \( \alpha_{IIb}\beta_{3} \)-mediated adhesion to immobilized fibrinogen (14, 15). These studies suggest that suppression of PP2A activity facilitates integrin \( \alpha_{IIb}\beta_{3} \) function, but how PP2A activity is regulated in platelets remains unclear.

Because protein-protein interactions form the foundation of signaling networks, in this study we sought to identify proteins that interact with PP2A\(\alpha\). Using a yeast two-hybrid screening, we identified an adaptor protein, CIN85 (Cbl-interacting protein of 85 kDa) as bait and screened the human bone marrow library. Among the 19 interacting clones, we identified SH3KBP1 (Src homology 3-domain kinase-binding protein 1) that encodes for an adaptor protein called Cbl-interacting protein of 85 kDa (CIN85) (16). CIN85 is also referred to as regulator of ubiquitous kinase (Ruk) (17) or SH3 domain containing gene expressed in tumorigenic astrocytes (SETA) (18). CIN85 can interact with a variety of proteins to assemble multiprotein complexes that can coordinate signaling and regulate a range of cellular processes, including T and B cell receptor signaling, receptor-tyrosine kinase endocytosis, and cell adhesion (19, 20).

Because PP2A\(\alpha\) has not been identified in the CIN85 interactome, we evaluated this interaction in epitope-transfected HEK 293 cells. PP2A\(\alpha\) co-immunoprecipitated with CIN85 from the cell lysate containing HA-tagged PP2A\(\alpha\) and FLAG-tagged CIN85. In contrast, an unrelated dual specificity phosphatase 7 (DUSP7) did not co-immunoprecipitate with CIN85 from the cell lysate containing HA-tagged DUSP7 and FLAG-tagged CIN85 (Fig. 1A). To identify the nature of the PP2A\(\alpha\)-CIN85 interaction, we expressed and purified CIN85 as a GST fusion protein in \( Escherichia coli \) (Fig. 1B). In a GST pulldown assay, CIN85-GST fusion protein but not GST protein interacted with PP2A\(\alpha\) (Fig. 1C). Under similar conditions, the recombinant von Willebrand factor (VWF) A1A2A3 domain protein did not interact with the CIN85-GST protein (Fig. 1C). Taken together, the pulldown and co-immunoprecipitation assays suggest that CIN85 can interact directly and specifically with PP2A\(\alpha\). Additional characterization studies revealed that the half-maximal binding (apparent \( K_{d} \)) of CIN85-GST to PP2A\(\alpha\) was 6.7 ± 1.3 nM (Fig. 1D). We next sought to investigate if the interaction of PP2A\(\alpha\) with CIN85 affected PP2A\(\alpha\) activity. CIN85-GST moderately decreased PP2A\(\alpha\) activity in a dose-dependent fashion with a maximal inhibition of ~25%, whereas GST alone did not affect the activity (Fig. 1E), suggesting that the PP2A\(\alpha\)-CIN85 complex can suppress PP2A\(\alpha\) activity \textit{in vitro}. Collectively, these studies identify CIN85 as a novel PP2A\(\alpha\) interacting protein that has the potential to restrain its phosphatase activity.

**Results**

**CIN85 Is a Novel PP2A\(\alpha\) Interacting Protein**—To identify PP2A\(\alpha\) effectors, we utilized a yeast two-hybrid system with full-length \( PPP2CA \) (encodes PP2A\(\alpha\)) as bait and screened the human bone marrow library. Among the 19 interacting clones, we identified SH3KBP1 (Src homology 3-domain kinase-binding protein 1) that encodes for an adaptor protein called Cbl-interacting protein of 85 kDa (CIN85) (16). CIN85 is also referred to as regulator of ubiquitous kinase (Ruk) (17) or SH3 domain containing gene expressed in tumorigenic astrocytes (SETA) (18). CIN85 can interact with a variety of proteins to assemble multiprotein complexes that can coordinate signaling and regulate a range of cellular processes, including T and B cell receptor signaling, receptor-tyrosine kinase endocytosis, and cell adhesion (19, 20).

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**PP2A\(\alpha\)-CIN85 Complex Controls Platelet Signaling and Function**

Platelets Express CIN85 and the Endogenous Complex of PP2A\(\alpha\)-CIN85—Multiple variants of CIN85 are expressed and tissue-specific enrichment of certain isoforms of CIN85, in part, due to alternative splicing and different promoter usage have also been reported (22–24). RNA and protein expression profiling studies have revealed the presence of SH3KBP1/CIN85 in human and mouse platelets (25–27). Consistent with these reports, immunoblotting of human platelet lysates reveal the presence of ~85 kDa CIN85 (Fig. 3A). To examine if the formation of the PP2A\(\alpha\)-CIN85 complex in platelets is dependent on activation, human platelets were layered on BSA substrate, where they remained in suspension, or were allowed to adhere to immobilized fibrinogen. The platelet lysates were then immunoprecipitated with an anti-PP2A\(\alpha\) antibody. Immunoblots of PP2A\(\alpha\) immunoprecipitates detected the
CIN85 protein from platelets in suspension but not after adhesion to fibrinogen (Fig. 3B). Similar to platelets, the PP2Ac-CIN85 complex was also noticed in HEK 293 cells expressing α₁bβ₃ suspended over BSA, but not in integrin-engaged cells (Fig. 3C). In a time kinetics study, we noticed that the adhesion of platelets to immobilized fibrinogen needed 45 to 60 min to

**FIGURE 1. CIN85 is a novel PP2Ac interacting protein.** A, lysate from HEK 293 cells transfected with either EV, HA-tagged PP2Ac and FLAG-tagged CIN85 or HA-tagged DUSP7 and FLAG-tagged CIN85 were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted (IB) with anti-HA and FLAG antibodies. Lysate panel shows the expression of PP2Ac (~36 kDa), DUSP7 (~44 kDa), and CIN85 (~85 kDa). Blots are representative of 3 experiments. B, characterization of GST proteins by Coomassie Blue staining. C, CIN85-GST or GST was coupled to glutathione beads and used for a pulldown assays with purified PP2Ac and recombinant VWF A1A2A3 domain protein. PP2Ac and VWF A1A2A3 domain protein used in pulldown assays are depicted as input. Blots are representative of 3 experiments. D, increasing concentrations of CIN85-GST were added to immobilized PP2Ac; the CIN85-GST protein bound to PP2Ac was detected using ELISA. *, p < 0.05, n = 3. E, increasing concentrations of CIN85-GST and GST proteins were incubated with immobilized PP2Ac and the resultant phosphatase activity was assayed using a PP2A phosphatase activity kit. n = 3.
dissociate the PP2Ac-CIN85 complex (not shown). These studies indicate that in platelets and nucleated cells the PP2Ac-CIN85 complexes are responsive to integrin engagement.

**Forced Disruption of the PP2Ac-CIN85 Complex in Platelets**

Previous studies have demonstrated the feasibility of myristoylated (Myr) synthetic/PP2Ac-CIN85 complexes were assessed in platelets treated with a Myr P3 peptide (Myr-VPAIPPKKPRPPK), which was derived from residues 395–407 in the P3 region of CIN85 or with a scrambled control peptide (Myr-PAKPVRPIRPKPP). CIN85 protein was detected in PP2Ac immunoprecipitates obtained from the control DMSO and scrambled peptide-treated platelets. Treatment of platelets with the P3 peptide disrupted the interaction of PP2Ac with CIN85 (Fig. 4A). In an alternate GST pulldown assay, PP2Ac interacted with the CIN85-GST fusion protein in lysates from scrambled peptide-treated platelets. Disintegration of the PP2Ac-CIN85 complex was evident when lysate from the P3 peptide-treated platelets was used (Fig. 4B). These studies indicated that myristoylated P3 peptide disrupt the platelet PP2Ac-CIN85 complex.

We sought to understand the relevance of the PP2Ac-CIN85 complex in the function of IIb/IIIa expressing HEK 293 cells and platelets. Compared with cells transfected with PP2Ac and wild type CIN85, adhesion of cells overexpressing PP2Ac and the CIN85 P3 mutant (that cannot engage PP2Ac; Fig. 2D) to immobilized fibrinogen was significantly reduced at 15 and 30 min but not at 60 min (Fig. 5A). The effects of the P3 peptide on platelet functions, which are initiated by the engagement of integrin IIb/IIIa, were then assessed. Treatment of platelets with the P3 peptide showed decreased platelet spreading on immobilized fibrinogen, whereas the scrambled peptide or DMSO-treated platelets did not (Fig. 5, B and C). Moreover, fibrin clot retraction, an independent function that relies on IIb/IIIa outside-in signaling to mediate changes in the actin cytoskeleton, was also reduced by the P3 peptide, compared with the scrambled peptide and the control DMSO (Fig. 5, D and E).

We assessed the signaling milieu in platelets following treatment with P3 peptide. Because platelet adhesion also disrupts CIN85-PP2Ac complexes, our initial phosphoprofiling screen was set up in platelets to assess basal changes in phosphorylation caused by the forced disruption of CIN85-PP2Ac.
Compared with the DMSO and scrambled peptide, P3 peptide-treated platelets displayed altered basal site-specific phosphorylation of tyrosine and/or serine/threonine kinases (supplemental Table S1). To test if fibrinogen-engaged platelets pretreated with P3 peptide display altered phosphorylation, we focused on Src and GSK3β because these kinases participate in signaling initiated by the engagement of platelet integrin αIIbβ3. Phosphorylation of Src Tyr418 (Fig. 6, A and B) and GSK3β Ser9 (Fig. 6, D and E) was decreased in P3 peptide-pretreated fibrinogen-adhered platelets when compared with fibrinogen-ad-
handed platelets treated with DMSO or the scrambled peptide. Taken together, these studies reveal that disruption of the PP2Ac-CIN85 complex in HEK 293IIb/H9251IIIb/H9252IIIb cells and platelets lead to decreased function with a concomitant reduction in signaling.

Discussion

Adaptor proteins interact with numerous other proteins via their distinct structural domains to form multiprotein complexes. Thus, by acting as platforms, these proteins have the ability to fine-tune the transmission of signals in response to receptor engagement. Indeed, CIN85 interacts with structural proteins, cell surface receptors, protein kinases, inositol 5’-phosphatase, E3 ubiquitin ligase, GTPase activating proteins (GAP), guanine nucleotide exchange factors (GEF), and other adaptor proteins via its SH3, Pro-rich and coiled-coil domains (19, 20). This enables CIN85 to participate in T and B cell receptor signaling, endocytosis of receptor-tyrosine kinases, and cell adhesion (19, 20). Although many proteins associate with CIN85, an interaction with PP2Ac has not yet been described in any cell type and CIN85 has not been investigated in platelets. In a yeast two-hybrid study with PPP2CA as bait, we identified the SH3KBP1 clone from the human bone marrow library and validated these observations in epitope-transfected cells, HEK 293 αIIbβ3 cells, and human platelets. The P3 region within the proline-rich domain of CIN85 mediates its interaction with PP2Ac (Figs. 1 and 2).

PP2A regulates cellular functions ranging from cell proliferation, apoptosis, differentiation, cell adhesion, and migration and therefore its activity is thought to be tightly controlled by the presence of several inhibitory PP2A complexes. Besides the prototypic heterotrimers, “atypical” PP2A complexes consisting of PP2Ac and α4 protein exist wherein, PP2A is maintained in an inactive state (30, 31). Our identification of the PP2Ac-CIN85 complex and the in vitro finding that CIN85 restrains PP2A activity (Fig. 1D) bears some similarity to the atypical PP2A-α4 complex. Besides these multiprotein complexes, the activity of PP2A is also regulated by cellular PP2A inhibitors. PP2A inhibitory proteins (PP2A inhibitor 1 (I1), PP2A inhibitor 2 (I2/SET), and two A inhibitor protein (TIP)) bind directly to PP2Ac and suppress PP2A activity (32, 33).

What is the likely function of the PP2Ac-CIN85 complex in platelets? It is possible that the recruitment of PP2Ac by the adaptor protein CIN85 provides a local control on the phosphatase activity. This will also enable signaling via protein kinases to proceed. Forced disruption of the PP2Ac-CIN85 complex by the Myr P3 peptide or dissociation of this complex following integrin engagement in a delayed temporal manner may provide a switch to turn on the phosphatase activity and thereby
suppress phosphorylation events (Fig. 7). In line with this argument, disruption of the PP2Ac-CIN85 complex led to site-specific dephosphorylation of several tyrosine and serine/threonine kinases. The ability of the P3 peptide to dampen the signaling of multiple kinases including Src, which has a prominent role in integrin signaling, correlates with the reduction in platelet spreading on immobilized fibrinogen (Fig. 5). Although PP2A is a Ser/Thr phosphatase, the P3 peptide altered tyrosine phosphorylation of Src kinase. This observation is consistent with our previous study, wherein, blocking PP2A activity in platelets and HEK 293/αβ3 cells led to an increase in c-Src Tyr418 phosphorylation and enhanced αβ3 adhesive function (14). Collectively, these studies argue that the PP2Ac-CIN85 complex facilitates platelet integrin-dependent function in part by dampening the phosphatase activity.

Because PP2A is involved in several cellular processes, direct targeting of PP2Ac to either inhibit or activate the enzyme could have adverse effects. Instead, disruption of the PP2Ac-CIN85 complex by the cell permeable peptide COG112 or immunosuppressant FTY720 (8, 36, 37).

In summary, we report for the first time that PP2Ac can interact with CIN85 via the P3 region and this complex participates in platelet physiology by dampening phosphatase activity. Given the ubiquitous expression of PP2Ac and CIN85, it is likely that the spatial and temporal regulation of PP2A activity by the adaptor protein CIN85 could emerge as a common feature in diverse signaling contexts in many cell types beyond platelets.

Materials and Methods

Yeast Two-hybrid Screening—Yeast two-hybrid screening was performed using the Mate and Plate Human Bone Marrow Library (Clontech, Palo Alto, CA), which was constructed using homologous recombination-mediated cloning in the Y187 yeast strain. The bait vectors, pGBK7-PP2A (1–330 aa) were generated through PCR using primers 5′-GCCGAATTCTAG-GACGAGAAGGTGTTCAC-3′ (forward) and 5′-CCGGGAT-CCCTACAGGAAATGCTGGGT-3′ (reverse). They were then transformed into the AH109 yeast strain using a lithium acetate-based method of transformation. For analyzing the interactions, AH109 yeasts containing the bait plasmid were mated with Y187 yeasts containing the respective cDNA library prey. The mated yeasts were plated on selection plates lacking leucine (Leu) and tryptophan (Trp) and incubated at 30 °C until colonies appeared. To rule out false-positive colonies, the expression of the β-galactosidase (β-gal) marker gene was qualitatively analyzed using nitrocellulose filter lift assays. Briefly, colonies were transferred to 3-mm filter paper, permeated by brief immersion in liquid nitrogen, and incubated on filter paper saturated with Z-buffer containing 1 mg/ml of X-Gal at 30 °C for 0.5–8 h. The His- and β-gal positive transformants were further screened for Ade2 reporter gene activity, by plating onto synthetic medium supplemented with histidine but lacking adenine, leucine, and tryptophan (-ALWH). Plasmids from positive colonies were recovered in DH5α E. coli cells selected for ampicillin and kanamycin resistance. Colonies that showed resistance for both markers were analyzed by DNA sequencing using T7 primers. Approximately 5 × 10⁶ transformants were screened. Plasmids recovered from yeast were retransformed into native AH109 yeast with the bait construct or a nonspecific control selected on high stringency plates with SD-/Ade-/His-/Leu-/Trp/X-β-galactosidase to confirm the interaction.

Human Platelet Isolation—Blood was drawn and mixed with acid citrate dextrose anticoagulant from healthy fasting individuals. All donors signed the informed consent document approved by the Institutional Review Board of Baylor College of Medicine. Blood was centrifuged at 189 g for 15 min at room temperature to obtain platelet-rich plasma (PRP). In some experiments, PRP supplemented with 75 mM prostacyclin E1 was centrifuged at 524 g for 10 min. The resultant platelet pellets were resuspended in Tyrode’s buffer (138 mM NaCl, 5.3 mM KCl, 0.33 mM NaHPO₄, 0.44 mM KH₂PO₄, 5.5 mM glucose, pH 7.4) and centrifuged. Washed platelets were then suspended in Tyrode’s buffer and adjusted to a final concentration of 2.5 × 10⁸ platelets/ml.

Transfection, Co-immunoprecipitation Assays, and Immuno blotting—HEK 293 cells were co-transfected with HA-tagged PP2Ac and FLAG-tagged full-length CIN85 using Lipo-
fectamine 2000 supplemented with plus reagent (Life Technologies). In some experiments, FLAG-tagged CIN85 mutants lacking distinct domains or alanine mutants in the proline-rich region of CIN85 were used as described before (21). Cells transfected with empty FLAG and HA vectors (EV), HA-tagged dual-specificity phosphatase 7 (DUSP7) (gift from Igor Astsaturov; Addgene plasmid #27976), and FLAG-tagged CIN85 served as controls. After 48 h, the cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA cell lysis buffer (Cell Signaling) supplemented with 1 mM Na3VO4 and 1 μg/ml of leupeptin. In some experiments, washed platelets were allowed to adhere to a culture dish coated with 100 μg/ml of immobilized fibrinogen (Enzyme Research), or suspended over a BSA substrate for 45 min at 37°C. Both the adhered and suspended platelets were lysed with RIPA buffer. In other experiments, washed platelets were treated with 0.2% DMSO, 200 μM Myr-PAKPVRIPRKPP (control scrambled peptide), or 200 μM Myr-399VPAPPPKRPPK407 (P3) peptide (Selleck Chemicals, Houston, TX) for 30 min at 37°C before lysis. Transfected cell lysates were immunoprecipitated with an anti-FLAG antibody (Sigma) and the immune complexes were captured using Protein G-Sepharose beads (GE Healthcare). The complexes were washed three times with RIPA buffer, after which the proteins were released from the beads by boiling with 2× SDS loading buffer. A similar procedure was performed with platelet lysate except the lysate was subjected to immunoprecipitation with an anti-control mouse IgG or anti-PP2Ac antibody (EMD Millipore). Immunoprecipitated proteins or peptide-treated platelet lysates were separated on 10% SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with antibodies against HA, FLAG (Santa Cruz Inc.), CIN85 (EMD Millipore Inc.), phospho-Src Tyr-418, phospho-GSK3β Ser-9, Scc, or actin (Cell Signaling Technologies).

**GST Pulldown Assays—**CIN85 cDNA was subcloned into the GST pGEX-4T1 vector between the BamHI and Xhol restriction sites. GST and CIN85-GST fusion vectors were expressed in BL21 E. coli cells following induction with isopropyl β-D-thiogalactopyranoside (Sigma) and then purified using glutathione beads. Purified GST or CIN85-GST (2.5 μg) pre-coupled with glutathione beads were mixed with 0.5 μg of purified PP2A (EMD Millipore), 0.5 μg of recombinant von Willebrand factor (VWF) A1A2A3 domain protein overnight at 4°C. Beads were washed three times and the interacting proteins were separated by SDS-PAGE and immunoblotted with anti-PP2Ac antibody. The blot was stripped and reblotted with anti-VWF (Dako) antibody.

**Binding Assays—**The half-maximal binding of CIN85-GST to PP2Ac was determined using ELISA. Briefly, 0.5 μg/ml of PP2Ac or BSA were immobilized to wellrock Maxisorp plates (Nunc, Denmark) and blocked with 3% BSA (Calbiochem) in PBS. Increasing concentrations of CIN85-GST were diluted in PBS and added into the wells and incubated for 1 h at 37°C. Following washing, bound CIN85-GST was detected with an anti-GST antibody (Santa Cruz Biotechnolgoy, Santa Cruz, CA) and anti-mouse IgG/HRP (Thermo Fisher). 3,3’, 5,5’Tetramethylbenzidine (Sigma) was then added to develop color for 5 min at room temperature and the reaction was stopped by adding an equal volume of 1 M HCl. Plates were read at 450 nm. Binding of GST-CIN85 to PP2Ac were deduced by subtracting the GST-CIN85 binding to immobilized BSA at the corresponding concentration. The software KaleidaGraph 4.5 was used for curve-fitting and determining the approximate half-maximal binding values using the equation $y = B_{max} \times x/(K_{d} + x)$ and reported as the mean ± S.E.

**PP2A Phosphatase Assays—**Plates were coated with 0.5 μg/ml of PP2Ac in TBS and blocked with 3% BSA. Increasing concentrations of GST and CIN85-GST proteins were added to the wells and incubated for 2 h at 37°C. After washing, the wells were incubated with phosphatase assay buffer (from the assay kit) and the phosphopeptide K-Rp-I-R-R, a PP2A-specific substrate, for 20 min at 30°C. The phosphates released following the dephosphorylation of the phosphopeptides were detected by malachite green and measured at 620 nm using a PP2A phosphatase assay kit (EMD Millipore). The absorbance value was compared with the standard curve generated using the phosphate standard solution from the assay kit and expressed as picomoles of phosphate/min.

**Adhesion, Spreading and Clot Retraction Assays—**The stable HEK 293 α1β3 cells generated and characterized previously (39) were transfected with PP2Ac and CIN85 constructs and analyzed for adhesion to immobilized fibrinogen (100 μg/ml) as described before (15). Washed platelets treated with DMSO, scrambled, or P3 peptide (200 μM) were incubated with fibrinogen (100 μg/ml)-coated coverslips for 30 min at 37°C. Adhered platelets were fixed with 3.7% paraformaldehyde and permeabilized with 0.01% Triton X-100. Spreading was visualized by staining actin with rhodamine phalloidin and visualized using a fluorescence microscope. Quantification of the surface area was performed using NIH Image J software. For the fibrin clot retraction assays, PRP treated with DMSO, scrambled, or P3 peptide was supplemented with 3 mM CaCl2 and challenged with 1 unit/ml of thrombin (Hematologic Technologies Inc.). The amount of liquid not incorporated into the clot was subtracted from the initial volume to determine the volume of clot. It is expressed as percentage of the initial volume.

**Phosphoprofiling Studies—**A human Phosphokinase Array Kit (BD Bioscience) was utilized for these studies as described before (38) with minor modifications. Washed platelets pretreated with DMSO, scrambled, or P3 peptides were lysed and diluted with the buffer included in the kit. A nitrocellulose membrane spotted with phosphokinase antibodies was incubated with 500 μg of lysate. The membrane was washed and incubated with a biotinylated antibody mixture, which was followed by streptavidin-HRP and detected by ECL.

**Statistics—**Data are expressed as mean ± S.E. The statistical significance of the data analyzed were found by using paired Student’s t test or unpaired Student’s t test where indicated.

**Author Contributions—**T. K., S. P., Q. D., and T. S. generated and analyzed the data. V. L. B. provided critical reagents. M. A. C. designed study. K. V. V. conceived, designed, and interpreted the study and wrote the paper.

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