Regulation of integrin activation occurs by specific interactions among cytoplasmic proteins and integrin α and β cytoplasmic tails. We report that the catalytic subunit of protein phosphatase 1 (PP1c) constitutively associates with the prototypic integrin αIIbβ3 in platelets and in cell lines overexpressing the integrin. PP1c binds directly to the cytoplasmic domain of integrin αIIb, subunit containing a conserved PP1c binding motif K/RRVG. Anchored PP1c is inactive; while thrombin-induced platelet aggregation or fibrinogen-αIIbβ3 engagement caused PP1c dissociation and concomitant activation as revealed by dephosphorylation of PP1c substrate, myosin light chain. Inhibition of ligand binding to activated αIIbβ3 blocks PP1c dissociation and represses PP1c activation. These studies reveal a previously unrecognized role for integrins whereby the α subunit cytoplasmic tail localizes the machinery for initiating and temporally maintaining the regulatory signaling activity of a phosphatase.

Integrin signaling mediated by reversible phosphorylation of proteins controls fundamental cellular processes like adhesion and migration. The prototypic integrin, platelet αIIbβ3, undergoes affinity/avidity modulation for its ligands through the process of inside-out signaling, resulting in firm adhesion and platelet aggregation upon vascular injury. Subsequent binding of fibrinogen to integrin αIIbβ3 generates outside-in signaling (1). Both αIIbβ3 inside-out and outside-in signaling result in tyrosine or serine/threonine phosphorylation of multiple signaling proteins, serving to initiate and amplify signaling cascades that support the formation of stable platelet-platelet and platelet-extracellular matrix interactions. Cytoskeletal proteins like talin (2), and non-receptor tyrosine and serine/threonine kinases, including Src, Csk, Syk (3), protein kinase C (4), integrin-linked kinase (5), and calcium- and integrin-binding protein (6), associate with integrin β and α cytoplasmic tails and mediate these integrin-dependent signaling events.

The net tyrosine or serine/threonine phosphorylation of a protein substrate is regulated by the activities of both protein kinases and phosphatases. While kinases and phosphorylation events during integrin mediated signaling have been clearly demonstrated, the role for phosphatases is poorly understood. Phosphatases play an essential role in dampening platelet phosphorylation events, and serine/threonine dephosphorylation of cytoskeletal proteins like cofilin, talin, and vasodilator-stimulated phosphoprotein correlates with platelet activation (7–9). Unlike the situation with kinases, only serine/threonine protein phosphatase PP2A has been reported to associate with integrin β1 (10), while only tyrosine phosphatase SHP-2 is known to associate with β3 (11). We are unaware of any reports of a phosphatase associating with an integrin α subunit.

PP1c is the best characterized and major serine/threonine protein phosphatase. The catalytic subunit of PP1 (PP1c) does not exist freely in the cell but associates with a host of regulatory polypeptides to form distinct multimeric holoenzymes. This association targets PP1c to a specific subcellular location and affects its activity and substrate specificity via allosteric modulation (12). The different isoforms of PP1 (PP1α, PP1β, PP1γ, and PP1y2) exhibit ~90% similarity in amino acid sequence and have similar catalytic rates, substrate specificities, and inhibition profiles in vitro, suggesting a predominant role for the regulatory subunit in determining the specificity and the diversity of PP1 function. PP1c binds to its regulatory polypeptide via a conserved PP1c binding motif K/RVXF, although interactions independent of this motif have also been reported (12).

We have studied the prototypic integrin (αIIbβ3), and our data demonstrate for the first time the specific association of PP1c with integrin αIIb and that ligand occupancy can initiate transient dephosphorylation events.

MATERIALS AND METHODS

Platelet Aggregation and Adhesion—Blood was drawn in an acid/citrate/dextrose anticoagulant from normal, healthy, and fasting donors and washed platelets prepared as described (13). 200-μl aliquots from 2 × 10^5 platelets/ml were allowed to aggregate using 0.5 and 1 unit/ml thrombin in a Biodata aggregation profiler as described (14). In some experiments 10 nM integrin, 250 nM okadaic acid, or 1% MeSO was added prior to thrombin addition. The reaction was stopped after 2 min by adding SDS sample buffer or 1% Triton X-100 and samples analyzed for myosin light chain (MLC) phosphorylation or immunoprecipitation as described. In some experiments, fibrinogen or BSA-coated dishes were incubated with 2 × 10^5 platelets for 45 min at 37 °C. The fibrinogen-bound platelets and the nonadherent platelets from BSA-coated plates were lysed using Triton X-100.

Immunoprecipitation and Western Blotting—800 μg/ml platelet lysates from the above experiments or 1% Triton X-100 platelet lysates obtained from ~3 × 10^8 resting platelets were immunoprecipitated using 3 μg of anti-αIIb (SEW-8) (gift from Dr. Newman, Blood Research Institute, Milwaukee, WI) or anti-PP1c (Fl-18) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit IgG (Pierce) and protein G-Sepharose. Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose, probed with monoclonal antibodies to αIIb (I32.1) or PP1c (E-9, Santa Cruz Biotechnology, Inc.) or GpIIb IIIa (WM23), and developed using the ECL system (Amersham Biosciences). The signals were scanned using Photoshop Version 6 software, and densitometric quantification was performed using NIH Image software (Scion Image Beta Version 4.02, Scion Corp., Frederick, MD).

The abbreviations used are: PP1, protein phosphatase 1; PP1c, catalytic subunit of PP1; MLC, myosin light chain; ppMLC, diphosphorylated MLC; BSA, bovine serum albumin.

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‡ To whom correspondence may be addressed: Thrombosis Research Section, Baylor College of Medicine, One Baylor Plaza, BCM 286, N1319, Houston, TX 77030. Tel.: 713-798-3480; Fax: 713-798-3415; E-mail: phray@bcm.tmc.edu (for P. F. B.) or E-mail: vvijayan@bcm.tmc.edu (for K. V. V.).
Interaction of PP1c with Integrin αIIbβ3—Integrin αIIbβ3 was purified from outdated human platelets as described by Shock and Parise (15). Silver staining and Western blotting with anti-αIIb (132.1) and anti-β3 (AP3) monoclonal antibodies confirmed the purity of the sample. Five biotinylated 11-amino acid peptides corresponding to residues 985–995 of integrin αIIb were synthesized for these studies: wild type αIIb, (LAMWKRVAGPFKM), a control with scrambled sequence (LWKVAGPFKRM), and peptides with alanine substitutions at 989, 990, and 992 (Protein Sequencing Core Facility, Baylor College of Medicine, Houston, TX). 25–50 µg/ml peptide was mixed with 1–2 µg/ml purified PP1c or 750 µg/ml platelet lysates and precipitated using streptavidin-agarose beads. The beads were washed three times and bound proteins eluted using SDS sample buffer. Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose, probed with anti PP1c (E-9), and signals detected using ECL.

Phosphatase Activity of αIIb-bound PP1c—Varying concentrations of αIIb peptide (25, 5, and 1 µg/ml) or control peptide (25 µg/ml) were incubated with purified and active 0.1 unit of PP1c (Upstate Biotechnology, Lake Placid, NY) for 16 h at 4 °C. MLC in lysates obtained from integrin-blocked and thrombin-stimulated 3 × 10⁸ platelets served as a substrate for PP1. Phosphatase reaction was carried out by mixing the peptide/PP1c mixture with the platelet lysate at 30 °C for 2 h. Following termination of the reaction, lysates were separated by SDS-PAGE and immunoblotted with ppMLC antibody (gift from Dr. James Staddon, Eisai London Research, London). The same blot was stripped and reprobed with anti-MLC antibody to assess equivalency of total MLC (Santa Cruz Biotechnology, Inc.).

RESULTS AND DISCUSSION

Constitutive Association of PP1c with the Integrin αIIbβ3—We were struck by the KVGF992 sequence in the cytoplasmic nature and intrinsic to integrin αIIbβ3. To ascertain whether there was a direct interaction of PP1c with integrin αIIbβ3, we examined the binding of a purified polyhistidine-tagged PP1c protein with the biotinylated integrin cytoplasmic peptides. Purified PP1c and PP1c from the resting platelet lysates bound specifically to a biotinylated αIIb peptide containing the KVGF motif but not to a control peptide with scrambled sequence (Fig. 1C). Compared with PP1c from platelet lysates, purified recombinant PP1c migrated at a slightly larger than 36 kDa due to the polyhistidine modification. Mutating Lys989, Val990, and Phe992 to alanine abolished the ability of the αIIb peptide to support PP1c binding (Fig. 1D). Thus, the PP1c binding KVXF motif appears to mediate the direct interaction of PP1c with the integrin complex. Although over 50 polypeptides are known to bind directly or indirectly to PP1c, this is the first example describing an interaction of PP1c with any cell adhesion receptor.

αIIbβ3-Fibrinogen Interaction Results in PP1c Dissociation—We next asked whether the association of αIIbβ3 with PP1c is altered upon integrin activation and ligand engagement. Platelet aggregation serves as a convenient test system for this issue, since agonist stimulation activates the integrin, enabling it to bind fibrinogen. Thrombin-induced platelet aggregation caused dissociation of PP1c from the integrin complex (Fig. 2A). Blockade of integrin-fibrinogen engagement with integrin repressed the dissociation of PP1c from the fibrinogen-αIIbβ3-mediated event. Compared with platelets that were maintained in suspension over the BSA substrate, platelets that adhered to fibrinogen exhibited a dissociation of PP1c from the integrin, it is conceivable that thrombin signaling itself might have some role. We therefore examined PP1c association with the integrin following platelet adhesion to immobilized fibrinogen, an αIIbβ3-mediated event. Although the association and dissociation results may appear modest, its consequence on signaling is not (see below in Fig. 3). While these results suggest that the αIIbβ3-fibrinogen interaction regulates the dissociation of PP1c from the integrin, it is conceivable that thrombin signaling itself might have some role. We therefore examined PP1c association with the fibrinogen-αIIbβ3-mediated event. Compared with platelets that were maintained in suspension over the BSA substrate, platelets that adhered to fibrinogen exhibited a dissociation of PP1c from the integrin αIIbβ3 (Fig. 2C). Collectively, these results suggest that fibrinogen binding to the integrin αIIbβ3 and not direct thrombin signaling is sufficient for the dissociation of PP1c from αIIbβ3.

Dissociated PP1c Is Active and Participates in Integrin-mediated Dephosphorylation Events—To address whether PP1c dissociation from αIIbβ3 modulates integrin signaling via subse-
and immunoblotted with antibodies to PP1c and integrin with either PP1c or integrin antibodies. Compared with lysates of resting platelets, a slight reduction of immunoprecipitated αIIbβ3 was observed in the lysates of thrombin-treated platelets, perhaps due to some redistribution of the αIIbβ3 to the detergent-insoluble cytoskeletal fraction. A, resting and 0.5 unit/ml thrombin-treated platelets for 2 min in the presence and absence of integrin were lysed and immunoprecipitated with αIIb antibody. Immunoprecipitates were immunoblotted with antibodies to PP1c and αIIb. C, platelets were either allowed to adhere to fibrinogen (FGN) or maintained in suspension over BSA substrate. Lysates were prepared and immunoprecipitated with an αIIb antibody and immunoblotted with anti-αIIb or anti-PP1c antibodies. Results are representative of two to three experiments in all the panels.

Next, we probed the phosphorylation state of MLC, a PP1c substrate during platelet aggregation. When the dissociation of PP1c from αIIbβ3 is prevented using integrin, robust phosphorylation of MLC was observed (Fig. 3A, left panel). In contrast, when fibrinogen binding to αIIbβ3 is allowed to proceed and PP1c dissociates from the integrin, MLC becomes dephosphorylated (Fig. 3A, right panel), implying activation of a PP1 phosphatase. Indeed, application of the PP1/PP2A phosphatase inhibitor, okadaic acid, enhanced MLC phosphorylation during platelet aggregation (Fig. 3B). Thus, dissociation of PP1c from the integrin correlated with an increase in PP1 activity (decreased MLC phosphorylation). In contrast, blocking the dissociation of PP1c from the integrin with integrin resulted in a decreased PP1 activity (increased MLC phosphorylation).

These results suggested that the dissociation of PP1c from the integrin with integrin or free PP1c, integrin-bound PP1c has less activity toward MLC. We tested this possibility by studying the in vitro phosphatase activity of purified and active PP1c in the presence of either the wild type αIIb cytoplasmic domain peptide or the control scrambled peptide. The PP1c substrate was MLC in lysates obtained from integrin-blocked and thrombin-stimulated platelets. Addition of the αIIb peptide to PP1c resulted in a dose-dependent inhibition of PP1 activity, while the control scrambled peptide was inactive (Fig. 3C). These results indicate that the association of PP1c with αIIbβ3 negatively modulates its activity to MLC but nevertheless provides a local high concentration of phosphatase for tonic dephosphorylation events upon integrin engagement.

Engagement of signaling inhibitory receptors on platelets like platelet endothelial cell adhesion molecule-1 is known to dampen phosphorylation events via recruitment and activation of protein-tyrosine phosphatases SHP-1 and SHP-2 (16). Additionally, integrin αIIbβ3 engagement also activates tyrosine phosphatase PTP1B by calpain cleavage and dephosphorylates tyrosine residues (17). Our results support a model wherein PP1c is anchored to the platelet integrin αIIbβ3 under resting conditions. Addition of agonist results in integrin activation and protein phosphorylation, while binding of fibrinogen leads to PP1c release, activation, and transient dephosphorylation events. A similar concept has been previously described wherein PP1c interacts with the vitamin D receptor in a ligand-independent manner, while PP1c activity increases in a ligand-dependent manner leading to dephosphorylation of p70 S6 kinase (18).

What is the significance of the PP1c-integrin association? PP1c-integrin association may ensure a localized concentration of phosphatase near the integrin tail that can be utilized for transient dephosphorylation events upon receptor engagement. We have yet to formally assess the stoichiometry of this association, but preliminary studies suggest that the majority of the αIIbβ3 molecules are not bound to PP1c. Nevertheless, our data show that this association regulates MLC phosphorylation, and others (19, 20) have reported serine/threonine de-
phosphorylation of extracellular signal-regulated kinases ERK (19) and Jun-N kinase JNK (20) upon fibrinogen binding. More importantly, these transient dephosphorylation events are essential for platelet physiology because phosphatase inhibitors impair platelet aggregation, adhesion, and spreading to fibrinogen (21, 22). Furthermore, serine/threonine dephosphorylation of cytoskeletal proteins like cofilin, talin, and vasodilator-stimulated phosphoprotein correlates with platelet activation (26). Besides the well documented role of KVGF or deletions in the GFFKR region result in constitutive integrin activation (27). Mutations in the GFFKR region also support this idea (25).

It is conceivable that PP1c could dephosphorylate (and thus activate) actin-depolymerizing protein like cofilin and promote efficient cytoskeletal reorganization that favors stronger α<sub>IIb</sub>β<sub>3</sub> adhesion by avidity modulation. Indeed in B lymphocytes, PP1c dephosphorylation events are proposed to be required for high avidity α<sub>4</sub>β<sub>2</sub> binding to intercellular adhesion molecule 1 and α<sub>4</sub> to vascular cell adhesion molecule (23, 24). The rapid dephosphorylation of cofilin upon platelet activation and the association of PP1/PP2A with cofilin in human T lymphocytes also support this idea (25).

Perhaps, in a resting integrin the physical proximity of integrin α and β tails may assist the α<sub>IIb</sub>-associated PP1c to maintain the serine/threonine residues on integrin β<sub>3</sub> in a dephosphorylated state. PP1c bound to the regulatory peptide has a distinct substrate specificity such that the α<sub>IIb</sub> anchored PP1c may retain its activity against β<sub>3</sub> but not MLC. Mutations or deletions in the GFFKR region result in constitutive integrin activation (26). Besides the well documented role of KVGF sequences in forming a salt bridge between the α and β subunits, it is tempting to speculate that the PP1c anchorage via this motif may help maintain integrin α<sub>IIb</sub>β<sub>3</sub> in the resting state. Indeed, competition of the native PP1c binding site on this motif may help maintain integrin sequences in forming a salt bridge between the activation (26). Besides the well documented role of KVGF or deletions in the GFFKR region result in constitutive integrin activation (27). Mutations in the GFFKR region also support this idea (25).

In summary, we report for the first time that PP1 associates with and is regulated by α<sub>IIb</sub>β<sub>3</sub> engagement in platelets. This functional relationship may extend to other family members of serine/threonine phosphatases and other integrins like α<sub>1</sub>, α<sub>4</sub>, and α<sub>5</sub> with the similar phosphatase binding motif, thereby regulating a variety of biological events.

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Protein Phosphatase 1 Associates with the Integrin $\alpha_{IIb}$ Subunit and Regulates Signaling
K. Vinod Vijayan, Yan Liu, Tong-Tong Li and Paul F. Bray

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