The Pro\textsuperscript{33} Isoform of Integrin $\beta_3$ Enhances Outside-in Signaling in Human Platelets by Regulating the Activation of Serine/Threonine Phosphatases*

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K. Vinod Vijayan‡, Yan Liu, Wensheng Sun, Masaaki Ito§, and Paul F. Bray¶

From the \*Department of Medicine, Baylor College of Medicine, Houston, Texas 77030 and the §1st Department of Internal Medicine, Mie University School of Medicine, Mie, 514-8507 Japan

Integrin $\beta_3$ is polymorphic at residue 33 (Leu\textsuperscript{33} or Pro\textsuperscript{33}), and the Pro\textsuperscript{33}-positive platelets display enhanced aggregation, P-selectin secretion, and shorter bleeding times. Because outside-in signaling is critical for platelet function, we hypothesized that the Pro\textsuperscript{33} variant provides a more efficient signaling than the Leu\textsuperscript{33} isoform. When compared with Pro\textsuperscript{33}-negative platelets, Pro\textsuperscript{33}-positive platelets demonstrated significantly greater serine/threonine phosphorylation of extracellular signal-regulated kinase (ERK2) and myosin light chain (MLC) but not cytoplasmic phospholipase A\textsubscript{2} upon thrombin-induced aggregation. Tyrosine phosphorylation of integrin $\beta_3$ and the adaptor protein Shc was no different in the fibroblastogen-activated platelets from both genotypes. The addition of Integrin I (a$_{\text{ITAM}}$-fibrinogen blocker) or okadaic acid (serine/threonine phosphatase inhibitor) dramatically enhanced ERK2 and MLC phosphorylation in the Pro\textsuperscript{33}-negative platelets when compared with Pro\textsuperscript{33}-positive platelets, suggesting that integrin engagement during platelet aggregation activates serine/threonine phosphatases. The phosphatase activity of myosin phosphatase (MP) that dephosphorylates MLC is inactivated by phosphorylation of the myosin binding subunit of MP at Thr\textsuperscript{696} and aggregating Pro\textsuperscript{33}-positive platelets exhibited an increased Thr\textsuperscript{696} phosphorylation of MP. These studies highlight a role for the dephosphorylation events via the serine/threonine phosphatases during the integrin outside-in signaling mechanism, and the Leu\textsuperscript{33} $\rightarrow$ Pro polymorphism regulates this process. Furthermore, these findings support a mechanism whereby the reported enhanced $\alpha$ granule secretion in the Pro\textsuperscript{33}-positive platelets could be mediated by an increased phosphorylation of MLC, which in turn is caused by an increased phosphorylation and subsequent inactivation of myosin phosphatase.

Integrin $\alpha_{\text{ITAM}}$ mediates platelet adhesion and aggregation at the site of vascular injury and plays a pivotal role in hemostasis and thrombosis. The binding of platelet integrin $\alpha_{\text{ITAM}}$$\beta_3$ to ligands such as fibrinogen and von Willebrand factor during aggregation/adhesion transmits information into the platelets called outside-in signaling. These signals are critical for platelet physiology and contribute to platelet thrombus formation by promoting the secretion of internal granules, the secondary wave of aggregation, and the formation of membrane vesicles with procoagulant activities (1). Not surprisingly, Glanzmann thrombasthenic human platelets and mouse platelets expressing $\beta_3$ integrin in which the cytoplasmic tyrosines have been replaced with phenylalanines have defective outside-in signaling (2–4). All these lines of evidence indicate that hemostasis requires a coordinated interaction between integrin $\alpha_{\text{ITAM}}$$\beta_3$ and the signaling machinery in platelets. $\alpha_{\text{ITAM}}$$\beta_3$ outside-in signaling is initiated by integrin engagement via an organized interaction of tyrosine and serine/threonine kinases such as Src, Csk, Syk, and protein kinase C-$\beta$ with the integrin $\beta_3$ subunit (5, 6) and calcium and integrin-binding protein to the $\alpha$ subunit (7). The net result is the phosphorylation of tyrosine and serine/threonine residues of several proteins including extracellular signal-regulated kinases 2 (ERK2),\textsuperscript{1} myosin light chain (MLC), and cytoplasmic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}).

Phosphorylation of ERK, MLC, and cPLA\textsubscript{2} modulates various biological functions. For example, tyrosine/threonine phosphorylation of ERK1/ERK2 is involved in cell growth and proliferation (8), megakaryocyte differentiation and proplatelet formation (9, 10), and release of stored Ca\textsuperscript{2+} in platelets (11). Serine/threonine phosphorylation of MLC is essential for migration, cytoskeletal clustering of integrins (12), and shape change and secretion in platelets (13), whereas serine phosphorylation of cPLA\textsubscript{2} participates in the release of potent agonist arachidonic acid (14), platelet spreading on fibrinogen, and phosphorylation of PP1\textsuperscript{125} focal adhesion kinase in platelets (15). Since the net phosphorylation on any protein is regulated by both kinases and phosphatases, it is likely that phosphatases also play a role in the process of outside-in signaling. Indeed, integrin $\alpha_{\text{ITAM}}$$\beta_3$ engagement activates tyrosine phosphatases PTP1B by calpain cleavage (16), and we have recently shown that integrin $\alpha_{\text{ITAM}}$$\beta_3$ engagement can regulate the activity of $\alpha_{\text{ITAM}}$-associated protein phosphatase 1 PP1, a serine/threonine phosphatase (17). Phospho-ERK is dephosphorylated by dual-specific tyrosine/threonine phosphatases from the mitogen-activated protein kinase phosphatase family members (MKP-3, MKP-4) and serine/threonine phosphatases such as MKP-1, MKP-5, and MKP-6.

‡ Supported by a Scientist Development Grant 0435017N from the National American Heart Association. To whom correspondence may be addressed: Thrombosis Research Section, Baylor College of Medicine, One Baylor Plaza, BCM 286, N1319, Houston, TX 77030. Tel.: 713-798-3450; Fax: 713-798-3415; E-mail: vietvijay@bcm.tmc.edu.

¶ 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: pbray@bcm.tmc.edu.

The abbreviations used are: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MLC, myosin light chain; ppMLC, diphosphorylated MLC; cPLA\textsubscript{2}, cytoplasmic phospholipid A\textsubscript{2}; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; MP, myosin phosphatase; MBS, myo-in-binding subunit; CHO, Chinese hamster ovary; BSA, bovine serum albumin.

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21756

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protein phosphatase 2A PP2A (18, 19), whereas phospho-MLC is dephosphorylated by a serine/threonine phosphatase from the PP1 family such as myosin phosphatase (MP) (20). Platelet MP is composed of three subunits, a 36-kDa catalytic subunit of the type 1 protein phosphatase δ, a 130-kDa regulatory subunit called myosin-binding subunit (MBS) or myosin-targeting subunit, and a small 20-kDa subunit (20).

Integrin β₃ is polymorphic at residue 33 (Leu³³ or Pro³³, also known as PlA¹ or PlA², respectively). This polymorphism is not rare, and 25% of individuals of northern European descent express Pro³³ isoforms on their platelets (21). Platelets expressing the Pro³³ isofrom have shortened bleeding times and exhibit enhanced activation, α granule secretion, and aggregation (22, 23) and are associated with acute coronary syndromes in some studies (21). Furthermore, CHO and 293 cells expressing the Pro³³ isofrom demonstrate enhanced adhesion and migration (24, 25). However, an underlying mechanism for the increased platelet reactivity observed in some but not all functional assays with the Pro³³-positive platelets is still elusive. Although our previous study demonstrated an increased phosphorylation of ERK2 and MLC in CHO and 293 cells expressing the Pro³³ isofrom, outside-in signaling in human platelets expressing the Pro³³ isofrom has not been investigated. Because outside-in signaling is critical for platelet function, we hypothesized that the Pro³³ variant provides a more efficient signaling than the Leu³³ isofrom. We show here that when compared with the platelets lacking the Pro³³ isofrom, Pro³³-positive platelets demonstrate enhanced phosphorylation of the ERK2-MLC axis pathway. More importantly, this enhanced signaling in the Pro³³-positive platelets is in part due to an inefficient activation of a serine/threonine phosphatases following integrin α₃β₃ ligation and is independent of integrin β₃ tyrosine phosphorylation.

MATERIALS AND METHODS

Reagents—Human fibrinogen was from Enzyme Research Laboratories Inc. (South Bend, IN). Okadaic acid, bovine serum albumin (BSA), and phosphatase inhibitor mixture were from Sigma. Antibodies specific for the phosphorylated ERK1/2, phosphorylated cPLA₂, and total cPLA₂ were obtained from Cell Signaling Technology (Beverly, MA), whereas anti-ERK2/2 antibody was obtained from Promega (Madison, WI). Antibody specific for the dephosphorylated myosin light chain (ppMLC) was a generous gift from Dr. James Staddon, (Eisai London Research, London, UK). Anti-MLC and -Shc antibodies were from Upstate Biotechnology Inc. (Santa Cruz, CA). Antibodies that recognize tyrosine-phosphorylated integrin β₃ were purchased from BIOSOURCE International, Inc. (Camarillo, CA). Thrombin and eptifibatide were generous gifts from Drs. John Fenton (New York State Department of Health, Wadsworth Center, Albany, NY) and D. Phillips (Portala pharmaceuticals Inc., San Francisco, CA), respectively.

Platelet Aggregation and Adhesion—Blood was obtained in acid-citrate-dextrose anticoagulant from normal, healthy, and fasting donors of known PI₄ genotype. In this study, we used PlA¹A¹ homozygous platelets (designated Pro³³-negative platelets or Leu³³/³³) and PlA¹A² heterozygous or PlA²A² homozygous platelets (designated Pro³³-positive platelets). Washed platelets from all three genotypes were prepared (26), suspended in Tyrode’s buffer (136 mM NaCl, 5.3 mM KCl, 0.33 mM NaH₂PO₄, 0.44 mM KH₂PO₄, 5.5 mM glucose, pH 7.4), and allowed to recover for ~2 h at 37 °C. Aggregation was performed using varying concentrations of thrombin for 2 min using 2 × 10⁸ platelets in 225 µL of Tyrode’s in a BIO-DATA 4-channel platelet aggregometer. In some experiments, 25 µM Integrilin or 250 nM okadaic acid or control Me₆SO (0.1%) were added 3 min prior to initiating aggregation. After 2 min, the reaction was stopped by solubilization with 25 µL of 10× SDS sample buffer. For adhesion studies, 12.5 µg/ml fibrinogen or heat-treated BSA was immobilized in a 100-mm tissue culture plate. 3 ml containing 3 × 10⁸ platelets were added to each plate and incubated for 45 min at 37 °C in 5% CO₂. The fibrinogen bound platelets and the non-adherent platelets from the BSA-coated plate were lysed in 1 ml of ice-cold Triton X-100 lysis buffer, and the protein content was determined.

Immunoblotting—For these studies, 50 µg of protein obtained from the Triton-X 100 lysates or 90 µl of SDS protein lysates described above were separated by 10% reducing SDS-PAGE, transferred to nitrocellulose membrane, and blocked with 5% nonfat milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 1% Tween 20 for 1 h at 22 °C. The blots were incubated overnight with the following antibodies: anti-phospho ERK1/2 (Thr²⁰², Tyr²⁰⁵), anti-pp-MLC (Thr²⁰¹, Ser²⁰⁵), anti-phospho cPLA₂ (Ser⁴⁰⁵), anti-phospho integrin β₃ (Tyr³⁴⁷) and (Tyr³⁵⁸), anti-phospho Shc (Tyr³⁸⁵), and anti-phospho MBS (Tyr³⁸⁵). The blots were washed with Tris-buffered saline and then incubated with horseradish-peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat antibodies for 2 h, and the immunoreactive bands were visualized using an ECL (Amersham Biosciences) system. To confirm equal loading of proteins, the membrane was stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C and blocked with 5% nonfat milk. The blots were reprobed with antibodies to ERK1/2, MLC, cPLA₂, integrin β₃, Shc, and MBS as described above. The signals were scanned using Photoshop 6 software, and the densitometric quantitation was performed using NIH Image software from beta 4.0.2 of Scion Image, Scion Corp., Frederick, MD.

RESULTS

Enhanced Phosphorylation of ERK2 in Aggregating Pro³³-positive platelets—To determine whether the PlA² polymorphism of integrin β₃ regulates outside-in signaling to ERK2 in human platelets, we studied thrombin-induced aggregation using the Pro³³-positive and Pro³³-negative platelets. Significantly (p = 0.02) greater levels of phosphorylated ERK2 were detected in the Pro³³-positive platelets when compared with Pro³³-negative platelets at varying concentrations of thrombin (Fig. 1A). By densitometry, and when compared with the Pro³³-negative platelets, Pro³³-positive platelets exhibited a maximum of ~2.4-fold increase in ERK2 phosphorylation at 0.5 units/ml thrombin (Fig. 1B). The level of total ERK1/2 in each lane was equivalent and could not account for the signaling differences (Fig. 1A). Essentially similar results were obtained with the Pro³³ homozygous platelets (Fig. 1C).

To address whether the ERK2 phosphorylation was a result of direct thrombin signaling or α₁bβ₃-dependent platelet aggregation, we analyzed ERK2 phosphorylation in the absence of platelet aggregation. Inhibition of integrin-fibrinogen engagement with Integrilin blocked platelet aggregation and dramatically enhanced ERK2 phosphorylation in platelets lacking the Pro³³ form. (Fig. 1D). These results are consistent with a previous study that showed increased phosphorylation of ERK2 upon blocking integrin-fibrinogen interaction using RGDS peptide or AP-2 antibody (27). These studies indicate that ERK2 phosphorylation was caused by thrombin signaling, possibly via activation of protease-activated receptors, whereas integrin engagement with fibrinogen during aggregation (α₁bβ₃ outside-in signaling) results in a negative regulation (dephosphorylation) of ERK2.

Under similar conditions, Integrilin blocked the aggregation of the Pro³³-positive platelets and only modestly enhanced ERK2 phosphorylation (Fig. 1D). At a concentration of 0.5 units/ml thrombin, the addition of Integrilin enhanced a maximum of ~7-fold in ERK2 phosphorylation in the Pro³³-negative platelets and only ~2-fold ERK2 phosphorylation in the Pro³³-positive platelets. These studies indicated that the engagement of integrin with fibrinogen during aggregation resulted in the dephosphorylation of ERK2 in a more efficient manner in the Pro³³-negative platelets than in the Pro³³-positive platelets. Thrombin signaling alone cannot account for the Leu³³ → Pro differences in ERK2 signaling since ERK2 phosphorylation was not different in the Integrilin-treated Pro³³-positive and Pro³³-negative platelets. These studies suggest that a difference in the extent of an ERK2 dephosphorylation event upon integrin engagement in the Pro³³-positive platelets, when compared with the Pro³³-negative platelets, is likely responsible for the enhanced ERK2 phosphorylation in the Pro³³-positive platelets.
Enhanced Phosphorylation of MLC in Aggregating Pro\textsuperscript{33}-positive Platelets—We have previously reported an ~3-fold increase in α granule (P-selectin) secretion in response to thrombin in the Pro\textsuperscript{33}-positive platelets when compared with the Pro\textsuperscript{33}-negative platelets (26). Since Thr\textsuperscript{18}/Ser\textsuperscript{19} phosphorylation of MLC is critical for platelet secretion (13), we examined the phosphorylation status of MLC in platelets based on the Leu\textsuperscript{33} → Pro genotype. Substantially greater levels of diphosphorylated MLC were detected in the Pro\textsuperscript{33}-positive platelets when compared with the Pro\textsuperscript{33}-negative platelets at varying concentrations of thrombin (Fig. 2A). When compared with the platelets lacking the Pro\textsuperscript{33} form, Pro\textsuperscript{33}-positive platelets exhibited a maximum of ~2.5-fold increase in phosphorylated MLC at 0.5 units/ml thrombin concentrations (Fig. 2B). This signaling difference was not due to the difference in total MLC loaded (Fig. 2A). In a manner similar to the ERK2 studies, inhibition of platelet aggregation enhanced MLC diphosphorylation by ~5-fold in Pro\textsuperscript{33}-negative platelets and only by ~2.0-fold in Pro\textsuperscript{33}-fold in Pro\textsuperscript{33}-positive platelets at 0.5 units/ml thrombin concentrations (Fig. 2C). These studies indicate that a difference in the extent of MLC dephosphorylation in the Pro\textsuperscript{33}-positive platelets, when compared with the Pro\textsuperscript{33}-negative platelets, is likely responsible for an enhanced Pro\textsuperscript{33}-mediated MLC signaling. Under similar conditions, Ser\textsuperscript{205} phosphorylation of cPLA\textsubscript{2}, a substrate for ERK2 and P38 (29), was no different in thrombin-induced aggregating Pro\textsuperscript{33}-positive and Pro\textsuperscript{33}-negative platelets (Fig. 2D), indicating that the Pro\textsuperscript{33}-mediated enhanced signaling is specific to ERK2-MLC pathway.

Enhanced Signaling in the Pro\textsuperscript{33}-positive Platelets Is Independent of Tyrosine Phosphorylation of Integrin β\textsubscript{3} and Adaptor Protein Shc—Previous studies have shown an essential role for tyrosine phosphorylation of β\textsubscript{3} in the α\textsubscript{IIb}β\textsubscript{3} outside-in signaling process (4). To understand better whether the enhanced ERK2-MLC signaling observed in Pro\textsuperscript{33}-positive platelets was initiated from the integrin β\textsubscript{3} itself, we examined the tyrosine phosphorylation of β\textsubscript{3} from the Pro\textsuperscript{33}-positive and Pro\textsuperscript{33}-negative platelets either suspended over BSA substrate or adhered to fibrinogen. When compared with the platelets in suspension, platelets adhering to fibrinogen demonstrated an increased phosphorylation of Tyr\textsuperscript{747} and Tyr\textsuperscript{759} of integrin β\textsubscript{3} (Fig. 3A left panel). However, the level of Tyr\textsuperscript{747} and Tyr\textsuperscript{759} phosphorylation of integrin β\textsubscript{3} as determined by densitometry was no different between the fibrinogen adhered Pro\textsuperscript{33}-positive and the Pro\textsuperscript{33}-negative platelets (Fig. 3A right panel).

The adaptor protein Shc is tyrosine-phosphorylated during the process of α\textsubscript{IIb}β\textsubscript{3} outside-in signaling (30), and since phosphorylation at Tyr\textsuperscript{317} is essential for ERK2 activation in other cell types, we examined the status of Tyr\textsuperscript{317} phosphorylation in platelets based on the Pro\textsuperscript{33} genotype. Shc Tyr\textsuperscript{317} phosphorylation increased during aggregation, reaching a maximum at 20 s after the addition of thrombin, and decreased with further continued stirring (Fig. 3B). Similar patterns of Shc phosphorylation in response to thrombin-induced aggregation were reported previously (30). However, the extent of Tyr\textsuperscript{317} phosphorylation of Shc was no different between the aggregating Pro\textsuperscript{33}-positive and Pro\textsuperscript{33}-negative platelets (Fig. 3B). Nevertheless, the same blot reprobed for ERK2 revealed enhanced ERK2 phosphorylation in the Pro\textsuperscript{33}-positive platelets (not shown), suggesting that the ERK2 phosphorylation status in platelets was not dependent on Shc phosphorylation. Taken together, these results imply that the enhanced ERK2 and MLC signaling in the Pro\textsuperscript{33}-positive platelets are independent of Shc and β\textsubscript{3} tyrosine phosphorylation.
Differential Activation of Serine/Threonine Phosphatases in the Pro33-positive platelets—Since integrin engagement during platelet aggregation resulted in a dephosphorylation of ERK2 and MLC, we examined a possible role for serine/threonine phosphatases in this process. Because serine/threonine phosphatases PP2A and PP1 participate in dephosphorylating ERK2 and MLC, generic PP2A/PP1 inhibitor was employed. The addition of 250 nM okadaic acid, a concentration that inhibits PP2A and PP1 (31), efficiently enhanced ERK2 and MLC phosphorylation in platelets lacking the Pro33 form (Fig. 4, A and C). In contrast, under similar conditions, okadaic acid only had a modest increase in ERK2 and MLC phosphorylation in the Pro33-positive platelets (Fig. 4, B and D). These results are consistent with the observations in Figs. 1D and Fig. 2C, wherein Pro33-negative platelets but not Pro33-positive platelets exhibited an increased phosphorylation of ERK2 and MLC upon treatment with Integrin. Thus, the phosphorylation of ERK2 and MLC in the Pro33-negative platelets can be increased by blocking integrin-fibrinogen engagement at the platelet surface or by inactivating serine/threonine phosphatases inside the platelet. These studies indicate that integrin-fibrinogen engagement during aggregation negatively regulates ERK2/MLC signaling, possibly through an efficient activation of serine/threonine phosphatases in platelets lacking the Pro33 form. In contrast, integrin engagement in the Pro33-positive platelets only modestly activated these phosphatases.

Because okadaic acid failed to appreciably enhance MLC phosphorylation in the Pro33-positive platelets, we considered whether the PP1-myosin phosphatase is rendered inactive in these platelets following integrin engagement. The phosphatase activity of myosin phosphatase is regulated by phosphorylation of its regulatory subunit (MBS), such that phosphorylation at residue Thr505 (pMBS) inactivates the phosphatase (32). We therefore examined the phosphorylation status of MBS at Thr505 in the aggregating platelets based on the Leu33 → Pro genotype. A greater phosphorylation of MBS was detected in the Pro33-negative platelets when compared with the Pro33-positive platelets at 0.5 units/ml thrombin (Fig. 5A). By densitometry and when compared with Pro33-negative platelets, Pro33-positive platelets exhibited an ~2-fold increase in the level of phosphorylated MBS (Fig. 5B). This result suggests that the enhanced MLC phosphorylation observed in the Pro33-positive platelets upon integrin engagement may in part be due to the increased phosphorylation and thus inactivation of myosin phosphatase.

DISCUSSION

Platelet physiology is influenced by outside-in signals that are generated by the engagement of integrin αIIbβ3. In this study, we use platelet aggregation and adhesion studies to investigate the impact of the Leu33 → Pro polymorphism of integrin β3 on outside-in signaling. Several prior studies have considered a key role for phosphorylation events via kinases in the process of outside-in signaling, but the contribution of the dephosphorylation events via phosphatases during outside-in signaling is poorly understood. The results from our studies identify an enhanced outside-in signaling to ERK2-MLC axis in the Pro33 variant of integrin β3, which is caused in part by an inefficient activation of serine/threonine phosphatase(s) following integrin ligation. Furthermore, this study illustrates a novel role for the Pro33 polymorphism of integrin β3 in regulating the activation of myosin phosphatases by a mechanism involving a phosphorylation event of the myosin binding subunit.
Using platelets of known Leu33 Pro genotype, we examined the outside-in signaling to ERK2 and MLC during platelet aggregation. When compared with the platelets lacking the Pro33 form, the Pro33-positive platelets exhibited an enhanced phosphorylation of ERK2 and MLC (Figs. 1A and 2A). This observation is consistent with our previous study in which we identified a greater phosphorylation of ERK2 and MLC in Pro33 CHO and 293 cells adhered to fibrinogen (26).

ERK2 signaling in platelets is required for the release of stored Ca2+, for GPIb-IX-dependent activation of integrin β3, and platelet aggregation to low doses but not to high doses of thrombin, collagen, arachidonic acid, and U46619 (33, 34). ERK2 activation promotes cell adhesion and spreading (35), and dominant negative ERK2 inhibits these processes (36). We observed the Pro33 isofom in CHO cells to enhance actin polymerization, spreading, adhesion, and migration to fibrinogen (24, 25). Furthermore, mitogen-activated kinase (MAPK) kinase inhibition abolished the Pro33-mediated enhancement in cell adhesion (26) and migration (25) to fibrinogen. On the other hand, MLC phosphorylation promotes myosin ATPase activity and increases actinomyosin contractile responses that are involved in platelet shape change, secretion, and migration (37). Pro33-positive platelets exhibit an ~3-fold increase in α-granule secretion in response to thrombin (26). Moreover, MLC phosphorylation is required for aggregation and secretion in response to ADP (38, 39), and increased aggregation and secretion were reported in Pro33-positive platelets in response to ADP (22, 23).

FIG. 3. Tyrosine phosphorylation of integrin β3 and adaptor protein Shc in the fibrinogen engaged platelets. A, washed Pro33-negative (Pro33-neg) and Pro33-positive (Pro33-pos) platelets were allowed to adhere to fibrinogen (FGN) or maintained in suspension over a BSA matrix for 45 min, after which platelets were solubilized, and 50 μg of proteins were separated by 10% SDS-PAGE and blotted with anti-phospho β3 (pY747 β3, and pY759 β3) (left panel). Densitometric quantification of phosphorylated integrin β3 (ratio of phosphorylated β3 to total β3 in arbitrary units) at residues 747 and 759 in platelets that adhered to fibrinogen at 45 min. Results are mean ± S.E., n = 4 (right panel). B, washed platelets were aggregated using 0.25 units/ml thrombin over varying time points, and lysates were immunoblotted with an anti-phospho Shc at Tyr317 (pShc) or anti-Shc (Shc) antibodies. Results are representative of 2 experiments.

FIG. 5. Threonine 696 phosphorylation of MBS in platelets. A, washed Pro33-negative (Pro33-neg) and Pro33-positive (Pro33-pos) platelets were aggregated using thrombin and platelet lysates analyzed by using anti-phospho MBS at Thr696 (pMBS). The same blot was stripped and immunoblotted with antibody to total MBS (MBS). B, densitometric quantification of MBS phosphorylation (ratio of phosphorylated MBS to total MBS in arbitrary units) in 0.5 units/ml thrombin aggregating platelets. Results are mean ± S.E., n = 4, *p = 0.047.
Fig. 6. Proposed model for increased MLC phosphorylation in the Pro33-positive platelets. The addition of thrombin results in the phosphorylation of MLC via the protease-activated receptors (PAR), whereas platelet aggregation via αIIbβ3-fibrinogen interaction leads to dephosphorylation of MLC. Thus, the phosphorylation pattern of MLC during thrombin-induced platelet aggregation is a composite of both these signaling events. The dephosphorylation of MLC during aggregation can be blocked by either blocking αIIbβ3-fibrinogen interaction (Integrilin) or blocking myosin phosphatase using okadaic acid, suggesting that αIIbβ3-fibrinogen interaction activates a serine/threonine phosphatase. However, in the case of the Pro33-positive platelets, αIIbβ3-fibrinogen interaction also results in an increased phosphorylation of MP at Thr696. Phosphorylated MP is inactive, and therefore, when compared with the Pro33-negative platelets, integrin engagement in the Pro33-positive platelets does not effectively dephosphorylate MLC, resulting in an increased MLC phosphorylation.

Thus, our findings that the increased outside-in signaling to ERK2 and MLC in the Pro33-positive platelets correlate well with the enhanced functions of these signaling molecules in the physiology of Pro33-positive platelets.

Despite the increased phosphorylation of ERK2 in the Pro33-positive platelets, tyrosine phosphorylation of integrin β3 and adaptor protein Shc was not increased in the Pro33-positive platelets (Fig. 3). This observation is in contrast to the classical activation of ERK2 pathway in other cell types that primarily couple Shc to the Ras-Raf-MEK axis (8). Consistent with our observations, others have reported that activation of Ras (40) and Raf (41) is not sufficient to lead to ERK2 phosphorylation in platelets, although all of these signaling components are expressed in platelets. Outside-in signaling is initiated via several kinases that associate with the β3 tail (5), and phosphorylation of Shc and β3 could favor signaling via kinases. An enhanced signaling in the Pro33-positive platelets without a corresponding increase in β3 and Shc phosphorylation could imply little role for kinases coupled to β3 and Shc in the enhanced ERK2 outside-in signaling.

We pursued the possibility that a differential activation of phosphatases (or the extent of dephosphorylation events) could account for the Pro33-mediated increased phosphorylation events. Indeed, inhibition of Pro33-negative platelet aggregation resulted in a 7- and 5-fold enhanced phosphorylation of ERK2 and MLC, respectively, suggesting that αIIbβ3 engagement during aggregation activates serine/threonine phosphatases. A similar ~10-fold increase in the ERK2 phosphorylation at residue Thr183 upon blocking platelet aggregation with RGDS peptide was reported previously (42). More importantly, the increase in ERK2/MLC phosphorylation upon blocking integrin-fibrinogen interaction appeared to be lost when we studied the Pro33-positive platelets (Figs. 1D and 2C). These studies suggested that serine/threonine phosphatases are activated by integrin ligation during platelet aggregation, and the Pro33 polymorphism regulated this activation process. This idea is supported by the observations that serine/threonine phosphatase PP1/PP2A inhibitor okadaic acid enhanced ERK2 and MLC phosphorylation more efficiently in the platelets lacking the Pro33 form when compared with the Pro33-positive platelets (Fig. 4). Thus, our studies using platelets from both genotypes suggest that dephosphorylation events are as important as phosphorylation events and that changes in the extent of dephosphorylation events could alter the process of outside-in signaling.

How could the engagement of Pro33 isoform of integrin lead to inefficient activation of serine/threonine phosphatases such as MP? Dissociation of MP subunits by arachidonic acid (43), phosphorylation of MBS at Thr696 by Rho kinases (42), or phosphorylation of CPI-17, an inhibitory phosphoprotein of MP through protein kinase C (44), are different mechanisms that could contribute to the inactivation of MP. Indeed, we observed an ~2-fold increased phosphorylation of MBS at Thr696 in the Pro33-positive platelets. Thus, increased phosphorylation of regulatory subunits of myosin phosphatase and its subsequent inactivation could account for the increased MLC signaling in the Pro33-positive platelets. Rho kinase is activated by integrin ligation, and its activity regulates the stability of αIIbβ3 adhesion contacts under shear to von Willebrand factor (45). In addition, we observed an enhanced adhesion of Pro33-CHO cells to von Willebrand factor (28). It is therefore likely that the Pro33 isoform could induce a greater Rho activation upon integrin ligation, leading to a greater Thr696 phosphorylation of MBS. Our proposed model for an increased signaling in the Pro33-positive platelets is shown in Fig. 6.

In conclusion, we have shown that integrin αIIbβ3 engagement during platelet aggregation leads to serine/threonine phosphatase activation (as revealed by the dephosphorylation of ERK2 and MLC) and that the Leu33 → Pro polymorphism regulates the level of the myosin phosphatase activation via...
Thr966 phosphorylation events. Thus, studies using platelets from both genotypes have 1) shed light on the critical role for the dephosphorylation process in the outside in signaling process and 2) revealed that the Leu33 → Pro polymorphism regulates this dephosphorylation process. These studies provide further insight into the molecular mechanism underlying the increased function (secretion) and increased signaling (MLC phosphorylation) exhibited by a commonly inherited variation of integrin β3.

REFERENCES

1. Phillips, D. R., Nannizzi-Alaimo, L., and Prasad, K. S. (2001) Thromb. Haemostasis 86, 246–258
2. Chen, Y. P., O’Toole, T. E., Ylanne, J., Rosa, J. P., and Ginsberg, M. H. (1994) Blood 84, 1857–1865
3. Wang, R., Shattil, S. J., Ambruso, D. R., and Newman, P. J. (1997) Nature 381, 808–811
4. Buensuceso, C. S., Obergfell, A., Soriani, A., Eto, K., Kiosses, W. B., Arias-Salgado, E. G., Kawakami, T., and Shattil, S. J. (2002) J. Biol. Chem. 277, 644–653
5. Naik, U. P., and Naik, M. U. (2003) Blood 102, 1355–1362
6. Whalen, A. M., Galasinski, S. C., Shapiro, P. S., Nahreini, T. S., and Ahn, N. G. (1997) Mol. Biol. Cell. 8, 1947–1958
7. Jiang, P., Jia, Y., and Cohen, I. (2002) Blood 99, 3579–3584
8. Haimovich, B., Ji, P., Ginalis, E., Kramer, R., and Greco, R. (1999) Thromb. Haemostasis 81, 618–624
9. Enumi, Y., Takayama, H., and Okuma, M. (1995) J. Biol. Chem. 274, 1355–1362
10. Vijayan, K. V., Huang, T. C., Liu, Y., Bernardo, A. Dong, J. F., Goldschmidt-Clermont, P. J., Alevriadou, B. R., and Bray, P. F. (2003) FEBS Lett. 540, 41–46
11. Kamm, K. E., and Stull, J. T. (2001) J. Clin. Invest. 108, 11927–11934
12. Ezumi, Y., Takayama, H., and Okuma, M. (1995) J. Biol. Chem. 270, 22381–22384
13. Haimovich, B., Ji, P., Ginalis, E., Kramer, R., and Greco, R. (1999) Thromb. Haemostasis 81, 618–624
14. Haemovitch, B., Ji, P., Ginalis, E., Kramer, R., and Greco, R. (1999) Thromb. Haemostasis 81, 618–624
15. Hariharan, K. V., Liu, Y., Li, T. T., and Bray, P. F. (2004) J. Biol. Chem. 279, 33309–33304
16. Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U., and Arkinstall, S. (1998) Science 280, 1292–1295
17. Chambers, C., and Brautigam, D. L. (1999) Cell. Signal. 11, 575–580
18. Hendrix, C., Coleman, L., Hamlington, J., Barnard, M. R., Kickler, T., Christie, D. J., Kundra, S., and Bray, P. F. (2000) Circulation 101, 1013–1018
19. Feng, D., Lindpaintner, K., Larson, M. G., Rao, V. S., O’Donnell, C. J., Lipinska, I., Schmitz, C., Sutherland, P. A., Silbershatz, H., D’Agostino, R. B., Muller, J. E., Myers, R. H., Levy, D., and Towler, G. H. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 1142–1147
20. Vajery, K. V., Goldschmidt-Clermont, P. J., Roos, C., and Bray, P. F. (2000) J. Clin. Invest. 105, 795–802
21. Williams, M. S., and Bray, P. F. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 1984–1989
22. Vajery, K. V., Liu, Y., Dong, J. F., and Bray, P. F. (2003) J. Biol. Chem. 278, 3860–3867
23. Nadal, F., Levy-Toledano, S., Grelae, F., Caen, J. P., Rosa, J. P., and Bryckaert, M. (1997) J. Biol. Chem. 272, 22381–22384
24. Vajery, K. V., Huang, T. C., Liu, Y., Bernardo, A. Dong, J. F., Goldschmidt-Clermont, P. J., Alevriadou, B. R., and Bray, P. F. (2003) FEBS Lett. 540, 41–46
25. Kramer, R. M., Roberts, E. F., Um, S. L., Borsch-Haubold, A. G., Watson, S. P., Fisher, M. J., and Jakobowski, J. A. (1996) J. Biol. Chem. 271, 27723–27729
26. Cowan, K. J., Law, D. A., and Phillips, D. B. (2000) J. Biol. Chem. 275, 36423–36429
27. McCluskey, A., Sim, A. T., and Sakoff, J. A. (2002) J. Med. Chem. 45, 1151–1175
28. Ichikawa, K., Ito, M., and Hartshorne, D. J. (1996) J. Biol. Chem. 271, 4735–4749
29. Li, Z., Yi, X., and Xu, D. (2001) J. Biol. Chem. 276, 42226–42232
30. McNicol, A., Philpott, C. L., Shibou, T. S., and Israels, S. J. (1998) Biochim. Biophys. Acta 1355–1362
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K. Vinod Vijayan, Yan Liu, Wensheng Sun, Masaaki Ito and Paul F. Bray

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