Enhanced activation of mitogen activated protein kinase and myosin light chain kinase by the Pro33 polymorphism of integrin $\beta_3$.

K. Vinod Vijayan, Yan Liu, Jing-Fei Dong, and Paul F. Bray$^*$

Department of Medicine, Baylor College of Medicine, Houston, TX 77030, USA.

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Running title: Enhanced signaling of $\beta_3$ Pro33 through MAPK and MLCK.
Integrin $\beta_3$ is polymorphic at residue 33 (Leucine33Proline), and the Pro33 variant exhibits increased outside-in signaling to focal adhesion kinase (FAK) and greater actin reorganization. Since FAK activation and intact cytoskeleton are critical links for integrin-mediated signaling to mitogen activated protein kinase (MAPK), we explored the role for integrin $\alpha_{IIb}\beta_3$ in this signaling using CHO and human kidney 293 cell lines expressing either the Leu33 or Pro33 isoform of $\beta_3$. Compared to Leu33 cells, Pro33 cells demonstrated substantially greater activation of extracellular regulated signal kinase 2 (ERK2) but not the other MAPK family members, Jun-N kinase (JNK) or P38 upon adhesion to immobilized fibrinogen, (but not fibronectin), and upon integrin crosslinking. ERK2 activation was mediated through MAPK kinase (MAPKK) and required phosphoinositide 3-OH kinase signaling and an intact actin cytoskeleton. Human platelets and CHO cells that expressed the Pro33 isoform showed enhanced activation of the ERK2 substrate myosin light chain kinase (MLCK) upon adhering to fibrinogen. Furthermore, compared to platelets and cells expressing the Leu33 isoform, the Pro33 variant showed greater alpha granule release, clot retraction and adhesion to fibrinogen under shear stress, and these functional differences were abolished by MLCK and MAPKK inhibition. Post-integrin occupancy signaling through the MAPK and MLCK after $\alpha_{IIb}\beta_3$ crosslinking may in part explain the increased adhesive properties of the Pro33 variant of integrin $\beta_3$. 

ABSTRACT

Integrin $\beta_3$ is polymorphic at residue 33 (Leucine33Proline), and the Pro33 variant exhibits increased outside-in signaling to focal adhesion kinase (FAK) and greater actin reorganization. Since FAK activation and intact cytoskeleton are critical links for integrin-mediated signaling to mitogen activated protein kinase (MAPK), we explored the role for integrin $\alpha_{IIb}\beta_3$ in this signaling using CHO and human kidney 293 cell lines expressing either the Leu33 or Pro33 isoform of $\beta_3$. Compared to Leu33 cells, Pro33 cells demonstrated substantially greater activation of extracellular regulated signal kinase 2 (ERK2) but not the other MAPK family members, Jun-N kinase (JNK) or P38 upon adhesion to immobilized fibrinogen, (but not fibronectin), and upon integrin crosslinking. ERK2 activation was mediated through MAPK kinase (MAPKK) and required phosphoinositide 3-OH kinase signaling and an intact actin cytoskeleton. Human platelets and CHO cells that expressed the Pro33 isoform showed enhanced activation of the ERK2 substrate myosin light chain kinase (MLCK) upon adhering to fibrinogen. Furthermore, compared to platelets and cells expressing the Leu33 isoform, the Pro33 variant showed greater alpha granule release, clot retraction and adhesion to fibrinogen under shear stress, and these functional differences were abolished by MLCK and MAPKK inhibition. Post-integrin occupancy signaling through the MAPK and MLCK after $\alpha_{IIb}\beta_3$ crosslinking may in part explain the increased adhesive properties of the Pro33 variant of integrin $\beta_3$. 

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INTRODUCTION

During the formation of a platelet thrombus, the binding of fibrinogen and von Willebrand factor to integrin α_{IIb}β_{3} triggers outside-in signals. These signals promote secretion of alpha and dense granules, the secondary wave of aggregation, filamentous actin formation, cytoskeletal rearrangement, and the formation of platelet membrane vesicles with procoagulant activities (1). Defects in outside-in signaling through α_{IIb}β_{3} cause abnormal platelet-mediated clot retraction and aggregation, and excessive bleeding in the hereditary disorder Glanzmann thrombasthenia (2, 3) and in mice engineered to express β_{3} integrin in which the cytoplasmic tyrosines have been replaced with phenylalanine (4).

A number of molecules and pathways have been identified to be involved in the earliest integrin-mediated outside-in signaling events (5). Several downstream effects and mediators have also been identified; including mitogen activated protein kinases (MAPKs)\(^1\) and myosin light chain kinases (MLCKs)\(^1\). MAPKs are a family of serine-threonine kinases activated by diverse extracellular stimuli like growth factors, cytokines, hormones, and other stress factors (6). The MAPK\(^1\) cascade consists of a three kinase module; MAPK is the most distal, being activated by a MAPK/ERK kinase (MEK)\(^1\), which in turn is activated by a MEK kinase (MEKK). Activation of MAPK requires dual phosphorylation on threonine and tyrosine residue in a Thr-X-Tyr motif. There are four known members of the MAP kinase family: 1) the extracellular regulated signal kinase (p44 Kd ERK1 and p42 Kd ERK2)\(^1\), 2) c-Jun N-terminal kinase or Stress activated protein kinase (p46 Kd JNK1 and P55Kd JNK2)\(^1\), 3) the p38 kinase and 4) ERK5/big MAP kinase (BMK1)\(^1\). ERK1 and ERK2 are involved in cell growth, proliferation, and adhesion, while ERK5 is important for angiogenesis (6, 7). JNK and p38 have
a role in apoptosis (6). All the MAPK family members except BMK1 have been identified in human platelets, but their function is inadequately understood.

Myosin light chain kinase (MLCK) is a Ca\(^{2+}\)/calmodulin dependent enzyme that phosphorylates Thr\(^{18}\) and Ser\(^{19}\) on the regulatory light chain of myosin (8). MLCK contains multiple MAPK consensus phosphorylation sites (P-x-S[T]-P) (9) and is directly phosphorylated by ERK2 (10). Phosphorylation of myosin light chains (MLC) by MLCK is a critical regulatory step in myosin function and regulates cell migration, cytoskeletal clustering of integrins, and shape change and secretion in platelets (11).

Integrin \(\beta_3\) is polymorphic at residue 33 (Leu33 or Pro33, also known as PI\(^{A1}\) or PI\(^{A2}\), respectively) and the Pro33 form has been associated with an enhanced adhesive phenotype in cell lines and platelets (12, 13), and with acute coronary syndromes in some studies (14). This polymorphism is not rare, and 25% of individuals of northern European descent express Pro33 isoforms on their platelets. Compared to Leu33, the Pro33 cells exhibit enhanced integrin \(\alpha_{\text{IIb}}\beta_3\) mediated outside-in signaling to focal adhesion kinase (FAK)\(^1\) and cytoskeletal dependent cellular functions, like fibrin clot retraction and cell adhesion (12). This raises the possibility that compared to Leu33 cells, Pro33 cells can provide more efficient \(\alpha_{\text{IIb}}\beta_3\) outside-in signaling. Evidence supporting a role for FAK and the cytoskeleton in integrin-mediated MAPK activation (15, 16), led us to explore whether the Leu-Pro substitution at amino acid 33 could modulate \(\alpha_{\text{IIb}}\beta_3\) signaling through MAPK. Using chinese hamster ovary cells (CHO)\(^1\) and 293 cell lines over expressing equivalent levels of the two isoforms of \(\alpha_{\text{IIb}}\beta_3\), and human platelets we found that compared to the Leu33 isoform, the Pro33 variant of \(\beta_3\) induced greater outside-in activation of ERK2 and/or MLCK. Inhibition of MLCK and ERK2 activation abolished the increased
adhesion to fibrinogen and clot retraction associated with Pro33 cells and MLCK inhibition abolished the increased P-selectin secretion in Pro33 platelets.
METHODS

Reagents - Human fibronectin was obtained from Life Technology Inc Gaithersburg, Maryland, USA. Human Fibrinogen was from Enzyme Research laboratories Inc; South Bend, Indiana USA. Cytochalasin, Wortmannin, Bovine Serum Albumin (BSA), phosphatase inhibitor cocktail, PMA\(^1\) (Phorbol 12-myristate 13-acetate) and sorbitol were from Sigma Chemical Co. St Louis, Missouri, USA. Anti-\(\alpha_{\text{IIb}\beta_3}\) (P2) and anti P-selectin FITC antibodies were from Immunotech, Marseilles, France. Anti-mouse FITC labeled antibody was from Pierce Chemical Co., Rockford, Illinois, USA. Anti-\(\alpha_{\text{V}\beta_3}\) LM609 antibody was from Chemicon International Inc; Temecula, California, USA. Antibodies specific for the phosphorylated forms of ERK, JNK, and P38; anti-ERK1/2; and inhibitors PD98059 (2’-Amino-3’-methoxyflavone) and U0126 were obtained from Promega Madison, WI. Antibody specific for the diphosphorylated myosin light chain (ppMLC) was a generous gift from Dr. James Staddon, (Eisai London Research, London, U.K). Antibodies to JNK and MLC were from Santa Cruz Biotechnology, Inc; California, USA and anti-P38 antibody was obtained from Cell Signaling Technology, Beverly MA, USA. Anti-LIBS6 was a gift from Dr. Mark Ginsberg (Scripps Research Institute, San Diego, CA) and 10E5 and c7E3 antibodies were gifts from Dr. Barry Coller (Rockefeller University, NY, NY).

Cell lines and flow cytometric analysis - Stable cell lines overexpressing \(\alpha_{\text{Iib}\beta_3}\) were generated by flow cytometric sorting using monoclonal antibodies specific for \(\alpha_{\text{Iib}\beta_3}\) as previously described (12). These included the “vector only” control CHO cells (designated LK), and CHO cells over expressing the Leu33 and Pro33 isoforms of \(\alpha_{\text{Iib}\beta_3}\) (designated Leu33 and Pro33, respectively). To address concerns about clonal variation that may have occurred in the CHO cell lines, a second set of cell lines was also generated in the 293 human embryonal kidney
cell line: line PC/Z, vector only control; line 293Leu33, stably expressing the Leu33 isoform of α_{IIb}β_{3}; and line 293Pro33, stably expressing the Pro33 isoform of α_{IIb}β_{3}. Cell surface expression of α_{IIb}β_{3} on CHO and 293 cells were analyzed by flow cytometry using P2 antibody followed by an anti-mouse FITC labeled antibody by flow cytometry (12). The mean channel number that corresponds to cell fluorescence intensity was used as a measure of α_{IIb}β_{3} surface expression. Assessment of α_{IIb}β_{3} expression levels was performed within 24 hours of each experiment to assure equivalent expression between the Leu33 and Pro33 cell lines.

**Adhesion to immobilized ligands and crosslinking of α_{IIb}β_{3} receptors** - Cells were grown to 70-80% confluence and detached using 0.05% trypsin. After neutralization the cells were suspended in Tyrode’s buffer (138 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 0.36 mM Na2HPO4, 5.5 mM glucose, pH 7.4) containing 1.8 mM CaCl2 and 0.49 mM MgCl2 for adhesion studies with fibrinogen. For studies with fibronectin, cells were suspended in Hanks balanced salt solution (HBSS) (136 mM NaCl, 5.3 mM KCl, 0.33 mM Na2HPO4, 0.44 mM KH2PO4, 5.5 mM glucose, pH 7.4) with 1.8 mM CaCl2 and 0.49 mM MgCl2. Twenty four well tissue culture plates were coated with either 12.5 µg/ml fibrinogen, 12.5 µg/ml fibronectin, 10 µg/ml of anti-α_{IIb}β_{3} P2 antibody, 10 µg/ml anti-α_{v}β_{3} LM609 antibody or 2.5 mg/ml heat treated BSA. Two hundred microliters of 5 x 10^5 cells/ml were added to each well and incubated for various time points (2.5, 5, 10 minutes) at 37°C in 5% CO2. In some experiments cells were incubated for 30 minutes with either 10 µM cytochalasin D, 100 nM wortmannin, 20 µM PD98059, 10 µM U0216 or DMSO (control) prior to the adhesion experiments. The unbound cells were removed by washing and the cells bound to fibrinogen or fibronectin were lysed in ice cold lysis buffer (CLB) (15 mM HEPES, pH 7.0, 145 mM NaCl, 0.1 mM MgCl2, 10 mM EGTA, 1% Triton X-100, 2 mM Na3VO4, 250 µg/ml 4-2-aminoethyl-benzene sulfonylfluoride, 15 µg/ml of protease
inhibitors chymostatin, antipain, pepstatin and Leupeptin and a phosphatase inhibitor cocktail (Sigma) as previously described (12). The non adherent cells from the BSA coated wells were collected, diluted 1:1 in Tyrodes, centrifuged at 100 g for 5 minutes and the pellet solubilized in lysis buffer. The lysates were incubated for 30 minutes on ice, clarified by centrifugation at 3750 g for 30 minutes and the protein concentration determined using the Bio-Rad protein assay kit. Cells incubated with 0.5 M sorbitol or 100 nm PMA served as a positive control for MAPK activation. In some experiments MAPK activation was assessed after monoclonal antibody clustering of the αIIbβ3 receptor: 5 x 10^5 cells suspended in 50 µl of Tyrode’s buffer containing 1.8 mM CaCl_2 and 0.49 mM MgCl_2, pH 7.4 were first incubated with 10 µg/ml of F(ab’)_2 fragment of 10E5 antibody or c7E3 antibody for 30 minutes at 4°C followed by 1:200 dilution of Fab specific goat antimouse IgG for 20 mins at 37°C as described in other systems (17). The cells were washed once with 200 µl of Tyrode’s buffer and lysed.

**Adhesion of platelets to immobilized fibrinogen** - Experiments performed on the blood of normal donors had been approved by the Human Subject Review Committee of Baylor College of Medicine. Blood was obtained in acid-citrate-dextrose from normal, healthy and fasting donors of known PI^A genotype. Washed platelets were prepared (13), suspended in Tyrode’s buffer, and allowed to recover for 2 hours at 37°C. Fibrinogen (12.5 µg/ml) or heat denatured BSA was immobilized on six well plates as described above. One milliliter containing 1 x 10^8 platelets was added to each well and incubated for 15 minutes at 37°C in 5% CO_2. The fibrinogen bound platelets and the non-adherent platelets from the BSA well were lysed in CLB and the protein content determined.

**MAP kinase and MLC kinase activation** - MAP kinase activation was assessed by immunoblotting using monoclonal antibodies specific for the active (dual tyrosine and threonine
phosphorylated) forms of activated P44/42, P38 or JNK. MLC activation was determined by immunoblotting using (ppMLC) antibody specific for the diphosphorylated (Thr$^{18}$, Ser$^{19}$) on myosin light chain. For these studies 20-50 µg of protein obtained from lysates described above were separated by 7-10% reducing SDS-PAGE, transferred to nitrocellulose membrane, blocked with 5% non fat milk in Tris buffered saline (TBS-T) (20 mM Tris-HCL, pH 7.6, 150 mM NaCl) containing 1% tween 20 overnight at 4°C, and incubated with an anti-phospho ERK1/2 antibody (1:5000 dilution), anti-phospho JNK antibody, anti-phospho P38 antibody (1:1000), or anti-ppMLC antibody (1:500) for 2 ½ hours at room temperature. The blots were washed with TBS-T, incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000) for 1 hour, and the immunoreactive bands visualized using an ECL (Amersham Life Science, Piscataway, New Jersey, USA) system. To confirm equal loading of the MAP kinase, the membrane was stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 minutes at 50°C, blocked with 5% non fat milk. The blots were re-probed with antibodies to ERK1/2 (1:5000) or JNK (1:3000) or P38 (1:1000) or MLC (1:1000) as described above. The signals were scanned using photoshop 5.5 software and the densitometric quantitation performed using NIH Image software from beta 4.0.2 of Scion Image, Scion Corporation, Frederick, Maryland, USA. (http://www.scioncorp.com).

Adhesion using parallel plate flow chamber - Glass cover slips were coated with 200 µl of 12.5 µg/ml fibrinogen and incubated for 3 hours in a humidified chamber. Before using each cover slip, excess fibrinogen was rinsed with Tyrode’s buffer and then the parallel plate flow chamber was assembled with the cover slip forming the base of the chamber. Measurements of cell adhesion under flow conditions were as we have previously described (18). During the experiments, the parallel plate flow chamber was mounted on an inverted-stage microscope.
(Nikon eclipse TE300, Nikon instruments, Melville NY, USA) equipped with a X20 phase objective and a high-speed camera (Quantix photometric, Photometric Ltd, Tucson, Arizona, USA) connected to a computer terminal. In most experiments 5 x 10^5 cells were perfused through the chamber for 5 minutes at a constant flow rate that produces a constant wall shear stress of 25 sec^{-1}. In some cases, cells were pretreated for 30 minutes with 20 µM MAPK inhibitor PD98059 or 10 µg/ml ML-7 and control DMSO before perfusion. Four fixed fields of observation were identified and the number of cells adhering to these regions in each experiment counted using the Metamorph imaging system.

**Clot retraction assay** - Clot retraction was measured as we have previously done (12). Briefly, CHO cells (4 x 10^6) were pretreated with either DMSO or 20 µM PD98059 for 1 hour. Cells were washed once in Tyrode’s buffer, resuspended in 300 µl of αMEM containing 28 mM CaCl_2 and 25 mM HEPES, pH 7.4, and mixed with 200 µl of fibronectin depleted plasma, 250 µg of fibrinogen, and 5 µg of aprotinin. Clot formation was initiated by the addition of 2.5 U thrombin. After incubation at 37° C for varying time periods, the volume of liquid not incorporated into the clot was measured.

**Platelet secretion studies** – Secretion of α granule was studied by assaying for P-selectin expression using a modification of our previously described whole blood flow cytometry assay (13). Briefly, 10 µl of washed platelets (2 x 10^6) of known PlA genotype were incubated with 10 µl of 0.2 mg/ml of anti P-selectin FITC antibody in a total of 40 µl Tyrode’s containing 10 mM HEPES and activated using varying concentrations of thrombin for 2 minutes. Samples were fixed with 0.5% paraformaldehyde and analyzed for P-selectin expression by flow cytometry. In some experiments, platelets were incubated with DMSO or 10 µg/ml ML-7 before thrombin stimulation.
RESULTS

Immobilized fibrinogen induces outside-in signaling to ERK2 but not to JNK and P38 -

To determine whether integrin \( \alpha_{\text{IIb}}\beta_3 \) can induce outside-in signaling to MAP kinase, CHO cells overexpressing \( \alpha_{\text{IIb}}\beta_3 \) were allowed to adhere to immobilized fibrinogen or maintained in suspension over a BSA substrate. Substantially greater levels of phosphorylated ERK2 were detected in adherent Pro33 cells compared to Leu33 cells at 2.5, 5 and 10 minutes (Fig. 1A, lanes 3 versus lanes 2). Compared to Leu33 cells, Pro33 cells exhibited a ~10 fold increase in phosphorylated ERK2 at 2.5 minutes, and a ~5 fold increase at 5 and 10 minutes (Fig. 1B). The level of total ERK in Leu33 and Pro33 cells was equivalent and could not account for the signaling differences (Fig. 1A). ERK2 signaling was dependent upon \( \alpha_{\text{IIb}}\beta_3 \) and fibrinogen since 1) no phosphorylated ERK2 was detected in the vector control “LK” cells not expressing \( \alpha_{\text{IIb}}\beta_3 \) (Fig1A, lane 1), 2) cells maintained in suspension over the BSA substrate did not trigger activation of ERK2 (Fig. 1A, Lanes 4-6), and 3) adhesion to fibrinogen under these conditions is completely inhibited with integrin and \( \alpha_{\text{IIb}}\beta_3 \) specific function blocking antibody 10E5 (12). A 2.5 fold increase in ERK2 phosphorylation was observed in Pro33 cells compared to Leu33 cells in response to soluble fibrinogen binding (data not shown).

Although CHO cell lines were generated by cell sorting and should not have been subject to clonal variation, we generated a second set of 293 cell lines to confirm the effects of the substitution of Leu to Pro at amino acid 33. Essentially the same results were obtained with the 293 cells as with the CHO cells (Fig. 1C and 1D). Densitometry revealed that compared to 293Leu33 cells, 293Pro33 cells demonstrated 7 fold greater phosphorylation of ERK2 at 2.5 minutes (not shown). Relative to CHO cells, in 293 cells the Pro33-dependent difference in
ERK2 activation appeared to lessen over time (not shown), perhaps reflecting cell type specificities. Nevertheless at early time points in both cell lines, the \( \beta_3 \) Pro33 substitution exhibited greater activation of ERK2. For the data shown in the rest of this report we show studies with CHO cells, although similar results were obtained in 293 cells.

Under similar conditions, adhesion of Leu33 and Pro33 cells to fibronectin did not cause activation of ERK2 (Fig. 2A) indicating that the differential signaling due to the Pro33 polymorphism of integrin \( \beta_3 \) is ligand specific. The cells used in these experiments were fully capable of activating ERK2 since PMA and sorbitol caused robust activation in all three cell lines (Fig. 2B, lane 1-6). The absence of ERK signaling on fibronectin might reflect trans dominant inhibition of integrin signaling, perhaps due to the cross talk between the overexpressed \( \alpha_{\text{IIb}}\beta_3 \) and the endogenous \( \alpha_5\beta_1 \) integrins.

To determine whether the enhanced phosphorylation of ERK2 was mediated primarily through \( \alpha_{\text{IIb}}\beta_3 \), ERK2 activation was examined in cells adhering to wells coated with anti-\( \alpha_{\text{IIb}}\beta_3 \) antibody P2, anti-\( \alpha_v\beta_3 \) antibody LM609 or BSA. There was a \( \sim10 \) fold greater phosphorylation of ERK2 in Pro33 cells compared to Leu33 cells that adhered to P2 antibody (Fig. 3A and 3B). No ERK2 activation was detected in LK cells (Fig. 3A, lane 4) and in cells maintained in suspension over BSA substrate (Fig. 3A, lanes 7-9). Adhesion to LM609 coated wells did not trigger activation of ERK2 in either Leu33 or Pro33 cells (Fig. 3A), indicating that low levels of chimeric hamster-human \( \alpha_v\beta_3 \) expressed in these cell lines did not contribute to ERK2 signaling.

We next examined whether other related MAP kinase members are activated by the integrin \( \alpha_{\text{IIb}}\beta_3 \)-fibrinogen interaction. In contrast to ERK2 activation, no phosphorylated JNK or phosphorylated P38 were observed in response to cell adhesion to fibrinogen at 2.5, 5 and 10 minutes (Fig. 4A and 4B). However JNK and P38 in these cells were fully activated in response
to 0.5 M sorbitol treatment (Fig. 4A and 4B, lanes 7-9). These studies indicated that 1) integrin \( \alpha_{\text{IIb}}\beta_3 \) is capable of inducing outside-in signaling to ERK2 but not to JNK or P38, and 2) compared to Leu33 isoform, the Pro33 variant of \( \alpha_{\text{IIb}}\beta_3 \) confers early and efficient ERK2 signaling.

**Crosslinking \( \alpha_{\text{IIb}}\beta_3 \) activates ERK2** – Adhesive processes often involve multivalent receptor-ligand interaction and the clustering of integrins. We used antibody-mediated crosslinking to further assess the role of this mechanism in the Pro33 signaling effect. Antibody crosslinking of \( \alpha_{\text{IIb}}\beta_3 \) resulted in greater activation of ERK2 in Pro33 cells compared to Leu33 cells (Fig. 5A, lane 3 versus 2), while incubation with either 10E5, or the secondary antibody alone did not cause activation in either cell line (Fig. 5A, lanes 4-9). Similar results were obtained in both CHO (Fig. 5B, lane 3 versus lane 2) and 293 cells (not shown) using the anti-\( \beta_3 \) antibody c7E3. Crosslinking with the c7E3 antibody showed a small basal level of ERK2 phosphorylation even in vector only (LK) cells (Fig. 5B), perhaps due to the ability of Fab fragment from c7E3 to bind other endogenous integrins on CHO cells. In these crosslinking studies, densitometry showed 1.5-3.1 fold greater ERK2 phosphorylation in Pro33 cells compared to Leu33 cells (not shown).

**ERK2 activation is mediated by MEK and requires post ligand binding events** – Sequential activation of the Ras GTPase and the kinase Raf and MEK is the best-characterized pathway for activation of the ERKs (15). To determine whether the activation of ERK2 in CHO cells adherent to fibrinogen was mediated through MEK, we pre-incubated cells in the presence and absence of MEK inhibitors PD98059 or U0216 and studied cell interactions with fibrinogen. Adhesion of DMSO treated (control) cells to immobilized fibrinogen caused greater activation of ERK2 in Pro33 than Leu33 cells (Fig. 6A, lane 3 versus lane 2) or (Fig. 6B, lane 5 versus 3). In
contrast, treatment with PD98059 (Fig. 6A) or U0216 (Fig. 6B) completely prevented the induction of ERK2 activity in both Leu33 and Pro33 cells. Since PD98059 binds to inactive MEK and prevents Raf from phosphorylating MEK, these data demonstrate that activation of ERK in Leu33 and Pro33 cells is mediated through the upstream MEK/Raf.

To examine whether cytoskeleton assembly has a role in ERK2 activation, cells were pre-incubated with cytochalasin-D. Cytochalasin-D completely prevented the induction of ERK2 activity in both Leu33 and Pro33 cells (Fig. 6C, lanes 2 and 3 versus lanes 5 and 6). Cytoskeletal reorganization in response to integrin activation is activated by lipid kinases like PI 3-Kinase (19), and we used the PI 3-kinase inhibitor wortmannin to examine the possible effect of PI 3-kinase in the αIIbβ3 mediated activation of ERK2. Induction of ERK2 activity in both Leu33 and Pro33 cells were ablated by wortmannin (Fig. 6C, lanes 2 and 3 versus lanes 8 and 9). These results indicate that the activation of ERK2 is dependent on post ligand binding events such as actin polymerization and PI3-K signaling.

Outside-in signaling in CHO and platelets to myosin light chain (MLC) - Compared to Leu33-expressing CHO cells, Pro33 cells show a dramatically increased reorganization of the actin cytoskeleton when bound to immobilized fibrinogen (12). Because myosin and MLCK are critical for reorganization of the actin cytoskeleton and MLCK is a cytoplasmic substrate of ERK2 (10), we examined outside-in signaling in CHO cells and human platelets to MLCK. MLCK phosphorylates Thr18/Ser19 on myosin light chain (MLC), and diphosphorylated MLC can be detected by a specific antibody (20). Adhesion to immobilized fibrinogen caused greater phosphorylation of MLC in Pro33 cells than in Leu33 cells (Fig. 7A, lane 3 versus lane 2). Compared to Leu33 cells, Pro33 cells exhibits ~1.8 fold increase in the levels of diphosphorylated MLC. Since MLCK activation is required for MLC phosphorylation at
Thr^{18}/Ser^{19}, we interpret the data in Fig. 7 to mean that MLCK has been differentially activated in the Pro33 and Leu33 cell lines. The difference in MLCK activation between Leu33 and Pro33 CHO cells was largely transient and appeared to lessen at later time point (data not shown).

The above studies were conducted exclusively in α_{III}β_{3}-expression CHO or 293 cells. We next assessed MLCK activity in human platelets expressing the Pro33 isoform of β_{3}. Compared to platelets lacking the Pro33 form, adhesion of Pro33-positive platelets to fibrinogen resulted in an enhanced diphosphorylation of MLC (Fig. 7B, lane 1 versus lane 3). No phosphorylation of MLC was detected on platelets maintained in suspension over BSA substrate. Compared to Pro33-negative platelets, adhesion of Pro33-positive platelets to fibrinogen revealed a 3.5 fold increase in the levels of diphosphorylated MLC (Fig. 7C).

Inhibition of ERK2 and/or MLCK activation abolishes enhanced functional effects of the Pro33 expressing platelets and cells - Fibrin clot retraction is a classic α_{III}β_{3}-mediated outside-in signaling function. Compared to the Leu33 cells, Pro33 variant exhibited a small but significant increase in fibrin clot retraction at varying time points (P=0.02) and this increase was abolished by the MEK inhibitor, PD98059 (P=0.63) (Fig. 8A and B). PD98059 was also able to abolish the greater adhesion of Pro33 cells to fibrinogen under shear stress in a parallel plate flow chamber (data not shown).

Because MLCK signaling modulates platelet secretion (11), we examined the functional consequence of an enhanced MLCK signaling in Pro33 platelets. Compared to the Leu33 platelets, Pro33 positive platelets exhibited ~3 fold increase in α granule secretion (as reflected in P-selectin expression) in response to 0.5 U of thrombin concentration (P=0.04) and this increase was abolished by the MLCK inhibitor, ML-7 (P=0.35) (Fig. 9A and B). We also tested the effect of MLCK inhibition on cell adhesion under fluid shear stress. Compared to Leu33
cells, significantly more Pro33 cells adhered to fibrinogen under shear stress \((P=0.005)\) (Fig. 9C), and inhibiting MLCK abolished the difference in adhesion between Leu33 and Pro33 \((P=0.713 \text{ in the presence of ML-7})\). The enhanced adhesion of 293A2 cells to fibrinogen could also be abolished by treatment with ML-7 (Fig. 9D). These data indicate that enhanced ERK2 and/or MLC signaling through the Pro33 isoform of integrin \(\beta_3\) regulates cellular functions of \(\alpha_{\text{IIb}}\beta_3\).

**DISCUSSION**

Outside-in signaling is crucial to link integrin ligation with numerous cellular processes, including adhesion, spreading, migration and clot retraction. In this study, we used CHO and 293 cells and human platelets to evaluate the impact of the Leu33Pro polymorphism on outside-in \(\alpha_{\text{IIb}}\beta_3\) signaling. The major findings of this study demonstrate that Pro33 form of \(\alpha_{\text{IIb}}\beta_3\) can induce enhanced outside-in signaling via MAPK and MLCK, and that these signaling pathways are indispensable for the hyperfunctional responses shown by Pro33 platelets and cells. These studies identify a novel means for regulating integrin function through post receptor occupancy; i.e., through ERK2 and MLCK signaling, and provide further insights regarding the molecular mechanisms responsible for the prothrombotic phenotype of a common inherited variation in integrin \(\beta_3\).

*Integrin-mediated ERK2 activation.* Several different approaches and two different sets of \(\alpha_{\text{IIb}}\beta_3\)-expression cell lines were used to address the impact of the Leu33-Pro substitution of integrin \(\beta_3\) on post-receptor occupancy signaling. Compared to Leu33 cells, Pro33 cells demonstrated substantially greater activation of ERK2 when cells bound to immobilized
fibrinogen (Fig. 1 and 6). This enhanced ERK2 phosphorylation was substrate-specific (Fig. 2) and dependent upon $\alpha_{\text{IIb}}\beta_3$ (Fig. 3), an intact actin cytoskeleton and signaling through MAPKK and PI3-kinase (Fig. 6). What are the consequences of this enhanced ERK2 phosphorylation? Integrin-mediated ERK2 activation has been most intensively studied as a regulator of gene expression and cell proliferation, but this pathway also regulates haptotactic and chemotactic cell migration (21). Cell adhesion and spreading are inhibited by dominant negative ERK (22), and promoted by ERK activation (23). Inhibition of ERK activation blocks the formation of peripheral actin microspikes (24), and active ERK is targeted to newly forming focal adhesions after integrin engagement (25). We observed the Pro33 isoform to enhance actin polymerization, spreading adhesion and clot retraction on integrin engagement (12) and have observed a Pro33-mediated increase in haptotactic migration (26). MAPKK inhibition had little effect on the Leu33 cell adhesion to immobilized fibrinogen (data not shown) or clot retraction (Fig. 8), but MAPKK inhibition abolished the Pro33 enhancement of these cell functions. Thus, the enhanced ERK2 activation seen in cells expressing the Pro33 variant of integrin $\alpha_{\text{IIb}}\beta_3$ would be predicted to have greater in vivo adhesive and migratory properties.

**Integrin-mediated MLCK activation.** Human platelets and CHO cells expressing the Pro33 variant exhibited enhanced activation of MLCK compared to Leu33 expressing cells upon adhering to immobilized fibrinogen (Fig. 7). Phosphorylation of MLCK is a critical regulatory step in myosin function, promoting myosin ATPase activity and increasing an actinomyosin contractile response that is involved in platelet shape change and secretion, regulation of cell migration and polymerization of actin cables (10). This is consistent with our previous studies, in which we identified 1) greater actin polymerization, adhesion and migration of Pro33 cells on fibrinogen compared to Leu33 cells (12, 26), and 2) a lower threshold for platelet activation, $\alpha$
granule release and fibrinogen binding in Pro33 homozygous platelets compared to Leu33 expressing platelets (13). Because MLCK is a substrate for ERK2 the results with PD98059 and ML-7 (Fig. 8 and Fig. 9) strongly suggest that outside-in signaling via ERK and MLCK controls the enhanced functions of adhesion and clot retraction and secretion in Pro33 cells and platelets. It is therefore conceivable that the enhanced ERK2-MLCK signaling in Pro33 cells following the ligation of $\alpha_{IIb}\beta_3$, leads to a greater cytoskeletal change and favors stronger and sustained adhesion compared to Leu33 cells. Our findings indicate that platelet physiology and signaling will be altered between Leu33 and Pro33 form of $\alpha_{IIb}\beta_3$, and the potential prothrombotic consequences apply to a large number of individuals.

*Integrin crosslinking enhances ERK activation in Pro33 cells.* Our studies showed that crosslinking integrin $\alpha_{IIb}\beta_3$ enhanced activation of ERK2 in Pro33 cells (Fig. 5) in a manner similar to cell adhesion to immobilized fibrinogen (Fig. 1). This suggests that clustering of integrin $\alpha_{IIb}\beta_3$ following adhesion to fibrinogen may underlie the enhanced activation of ERK2 in Pro33 cells. This idea is supported by the rapid remodeling of cytoskeletal machinery in Pro33 cells (12), and observations that identify the cytoskeletal apparatus as a key component in the process of integrin clustering (27). The requirement of intact cytoskeletal structures for ERK2 activation (Fig. 6C) is also consistent with earlier observations that cytochalasin-D blocks integrin-mediated MAP kinase and Raf activation (16). It is likely that disruption of actin structure preludes the formation of a highly ordered cytoskeletal system that is essential for the recruitment of signaling molecules vital for the activation of ERK2. Indeed, PI3 kinase may be one such intermediate signaling molecule, since wortmannin completely abolished ERK2 activation (Fig. 6C). These results suggest that the enhanced signaling to ERK2 in Pro33 cells is
dependent on post fibrinogen binding events, involving clustering of \( \alpha_{IIb}\beta_3 \) with subsequent actin rearrangement and signaling through PI3 kinase.

**Extracellular structure and intracellular signaling.** Integrin cytoplasmic domains have been shown to play important role in integrin signaling (28), and this study illustrates that residues in the extracellular region of integrin \( \beta_3 \) can also contribute to signal transduction. Similar regulation of intracellular signals by the extracellular domain of integrin \( \beta_1 \) has been reported (29). How could a conformational change in the extracellular domain (30) of \( \beta_3 \) alter intracellular signaling? We are currently pursuing two major possibilities wherein the altered extracellular conformation in the Pro33 isoform might physically 1) alter the cytoplasmic domain for a more efficient juxtaposition of \( \beta_3 \) tails with proximal signaling molecules like src and syk, and/or 2) induce or inhibit associations with transmembrane signaling molecules (e.g., platelet endothelial cell adhesion molecule [PECAM], FcyRIIA, Junctional adhesion molecule [JAM1], etc.) that either enhance or repress, respectively, intracellular signaling. In either case, ERK2 activation could be modulated through FAK dependent or independent pathways.

**ERK and MLCK signaling in platelets.** ERK signaling is important for megakaryocyte differentiation and proplatelet formation (31, 32), for the GPIb-IX dependent activation of platelet integrin \( \alpha_{IIb}\beta_3 \) (33), and for regulating the release of stored \([\text{Ca}^{2+}]\) (34). The effect of ERK signaling in platelet aggregation and secretion appears to depend on the concentration of agonists: inhibition of ERK activation blocks aggregation to low doses of collagen, arachidonic acid, U46619 and thrombin (33, 35), but high concentrations of agonists can induce aggregation despite ERK inhibition (36, 37). Signaling through ERK also regulates cell spreading (31, 38, 39). Perhaps the major effect of ERK signaling in platelets is to regulate this post-receptor occupancy process that may be distinct from agonist-induced inside out signaling. This would
be quite consistent with previous data showing no difference in fibrinogen binding to the Pro33 and Leu33 isoforms of β₃ (13, 40), but consistent differences in outside-in processes such as bleeding times, spreading, actin reorganization, and clot retraction (12, 41). Moreover, signaling through MLCK is required for platelet aggregation and secretion in response to ADP (42, 43). Pro33 expressing platelets show greater activation of MLCK (Fig 7) and this correlates with the increased aggregation and greater secretion of P-selectin in Pro33 positive platelets in response to ADP (13, 44). Importantly, in this study we demonstrate that the enhanced MLCK signaling in Pro33 platelets is required for its increased platelet reactivity, as determined by α granule secretion (Fig 9A and 9B). Finally, ERK and P38 phosphorylates and activates phospholipase C (PLA2) in platelets (45-47), which would release more arachidonic acid (AA) in Pro33-positive individuals during platelet aggregation. This hypothesis is supported by data from our laboratory and others where Pro33-expressing platelets and cell lines exhibit a greater dependency on cyclooxygenase than do cells expressing only Leu33 (13, 41, 48, 49).

In conclusion, we show that integrin α₁₁bβ₃ engagement to immobilized fibrinogen induces outside-in signaling to ERK2 and MLCK and that the Leu33Pro polymorphism regulates the extent of this signaling. Taken in the context of known functions of ERK and MLCK, our findings support a mechanism whereby the Pro33 variant has little effect on direct agonist-induced integrin activation, but rather enhances signaling and cell adhesive functions after α₁₁bβ₃ has been engaged or crosslinked by fibrinogen. This prothrombotic phenotype may partially explain the reported arterial thrombotic risk of Pro33-positive individuals in some clinical epidemiology studies.
REFERENCES

1. Phillips, D. R., Nannizzi-Alaimo, L., and Prasad, K. S. (2001) *Thromb.Haemost.* **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) *J.Clin.Invest* **100**, 2393-2403

4. Law, D. A., DeGuzman, F. R., Heiser, P., Ministri-Madrid, K., Killeen, N., and Phillips, D. R. (1999) *Nature* **401**, 808-811

5. Shattil, S. J. (1999) *Thromb Haemost* **82**, 318-325

6. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) *Adv.Cancer Res.* **74**, 49-139

7. Regan, C. P., Li, W., Boucher, D. M., Spatz, S., Su, M. S., and Kuida, K. (2002) *Proc.Natl.Acad.Sci.U.S.A* **99**, 9248-9253

8. Ikebe, M. and Hartshorne, D. J. (1985) *J.Biol.Chem.* **260**, 10027-10031

9. Clark-Lewis, I., Sanghera, J. S., and Pelech, S. L. (1991) *J.Biol.Chem.* **266**, 15180-15184

10. Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P., and Cheresh, D. A. (1997) *J.Cell Biol.* **137**, 481-492

11. Kamm, K. E. and Stull, J. T. (2001) *J.Biol.Chem.* **276**, 4527-4530
12. Vijayan, K. V., Goldschmidt-Clermont, P. J., Roos, C., and Bray, P. F. (2000) *J.Clin.Invest* **105**, 793-802

13. Michelson, A. D., Furman, M. I., Goldschmidt-Clermont, P., Mascelli, M. A., Hendrix, C., Coleman, L., Hamlington, J., Barnard, M. R., Kickler, T., Christie, D. J., Kundu, S., and Bray, P. F. (2000) *Circulation* **101**, 1013-1018

14. Williams, M. S. and Bray, P. F. (2001) *Exp.Biol.Med.(Maywood.)* **226**, 409-419

15. Howe, A., Aplin, A. E., Alahari, S. K., and Juliano, R. L. (1998) *Curr.Opin.Cell Biol.** 10*, 220-231

16. Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K., and Juliano, R. L. (1994) *J.Biol.Chem.* **269**, 26602-26605

17. Hu, Y., Kiely, J. M., Szente, B. E., Rosenzweig, A., and Gimbrone, M. A., Jr. (2000) *J.Immunol.* **165**, 2142-2148

18. Fredrickson, B. J., Dong, J. F., McIntire, L. V., and Lopez, J. A. (1998) *Blood* **92**, 3684-3693

19. Rittenhouse, S. E. (1996) *Blood* **88**, 4401-4414

20. Ratcliffe, M. J., Smales, C., and Staddon, J. M. (1999) *Biochem.J.* **338**, 471-478

21. Stockton, R. A. and Jacobson, B. S. (2001) *Mol.Biol.Cell* **12**, 1937-1956

22. Lai, C. F., Chaudhary, L., Fausto, A., Halstead, L. R., Ory, D. S., Avioli, L. V., and Cheng, S. L. (2001) *J.Biol.Chem.* **276**, 14443-14450
23. Zhu, X. and Assoian, R. K. (1995) *Mol.Biol.Cell* **6**, 273-282

24. Brunton, V. G., Fincham, V. J., McLean, G. W., Winder, S. J., Paraskeva, C., Marshall, J. F., and Frame, M. C. (2001) *Neoplasia* **3**, 215-226

25. Fincham, V. J., James, M., Frame, M. C., and Winder, S. J. (2000) *EMBO J.* **19**, 2911-2923

26. Sajid, M., Vijayan, K. V, Souza, S., and Bray, P. F. (2001) *Arterioscler Thromb Vasc Biol.* In press.

27. Zhou, X., Li, J., and Kucik, D. F. (2001) *J.Biol.Chem.* **276**, 44762-44769

28. Dedhar, S. and Hannigan, G. E. (1996) *Curr.Opin.Cell Biol.* **8**, 657-669

29. Miao, H., Li, S., Hu, Y. L., Yuan, S., Zhao, Y., Chen, B. P., Puzon-McLaughlin, W., Tarui, T., Shyy, J. Y., Takada, Y., Usami, S., and Chien, S. (2002) *J.Cell Sci.* **115**, 2199-2206

30. Valentin, N. and Newman, P. J. (1994) *Curr.Opin.Hematol.* **1**, 381-387

31. Whalen, A. M., Galasinski, S. C., Shapiro, P. S., Nahreini, T. S., and Ahn, N. G. (1997) *Mol.Cell Biol.* **17**, 1947-1958

32. Jiang, F., Jia, Y., and Cohen, I. (2002) *Blood* **99**, 3579-3584

33. Li, Z., Xi, X., and Du, X. (2001) *J.Biol.Chem.* **276**, 42226-42232

34. Rosado, J. A. and Sage, S. O. (2000) *J.Biol.Chem.* **275**, 9110-9113

35. McNicol, A., Philpott, C. L., Shibou, T. S., and Israels, S. J. (1998) *Biochem Pharmacol* **55**, 1759-1767
36. Borsch-Haubold, A. G., Kramer, R. M., and Watson, S. P. (1996) *Biochem.J.* **318**, 207-212

37. Borsch-Haubold, A. G., Pasquet, S., and Watson, S. P. (1998) *J.Biol.Chem.* **273**, 28766-28772

38. Dorsey, J. F., Cunnick, J. M., Mane, S. M., and Wu, J. (2002) *Blood* **99**, 1388-1397

39. Gu, J., Tamura, M., and Yamada, K. M. (1998) *J.Cell Biol.* **143**, 1375-1383

40. Bennett, J. S., Catella-Lawson, F., Rut, A. R., Vilaire, G., Qi, W., Kapoor, S. C., Murphy, S., and FitzGerald, G. A. (2001) *Blood* **97**, 3093-3099

41. Undas, A., Sanak, M., Musial, J., and Szczeklik, A. (1999) *Lancet* **353**, 982-983

42. Hashimoto, Y., Sasaki, H., Togo, M., Tsukamoto, K., Horie, Y., Fukata, H., Watanabe, T., and Kurokawa, K. (1994) *Biochim.Biophys.Acta* **1223**, 163-169

43. Wilde, J. I., Retzer, M., Siess, W., and Watson, S. P. (2000) *Platelets.* **11**, 286-295

44. 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 Tofler, G. H. (1999) *Arterioscler.Thromb Vasc.Biol.* **19**, 1142-1147

45. Kramer, R. M., Roberts, E. F., Strifler, B. A., and Johnstone, E. M. (1995) *J.Biol.Chem.* **270**, 27395-27398

46. Sato, T., Kageura, T., Hashizume, T., Hayama, M., Kitatani, K., and Akiba, S. (1999) *J.Biochem.(Tokyo)* **125**, 96-102
47. Borsch-Haubold, A. G., Kramer, R. M., and Watson, S. P. (1997) *Eur.J.Biochem.* **245**, 751-759

48. Cooke, G. E., Bray, P. F., Hamlington, J. D., Pham, D. M., and Goldschmidt-Clermont, P. J. (1998) *Lancet* **351**, 1253

49. Vijayan, K. V., Goldschmidt-Clermont, P. J., Roos, C. M., and Bray, P. F. (1998) *Blood* **92**, 1410-1410. (Abstr.)
FOOTNOTES

Abbreviations used in this paper:

MAPK, Mitogen-activated protein kinase; ERK, Extracellular regulated signal kinase; JNK, Jun
N-terminal kinase; FAK, Focal adhesion kinase; CHO, Chinese hamster ovary; PMA, Phorbol
12-myristate 13-acetate; MEK, Mitogen activated protein kinase kinase; MLCK, Myosin light
chain kinase.

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**FIGURE LEGENDS**

**Fig. 1. Activation of ERK2 in CHO and 293 cells adhering to immobilized fibrinogen.** A) CHO cell lines LK (vector only parental), Leu33 (designated A1) and Pro33 (designated A2) cells were allowed to adhere to fibrinogen (lane 1-3) or maintained in suspension over BSA matrix (lane 4-6) for 2.5, 5 and 10 minutes, after which cells were solubilized and 20 µg of protein was separated by 10% SDS-PAGE, blotted with an anti-phospho ERK antibody (panels designated pERK2). ERK2 migrated in the expected molecular weight of 42 kD as determined with molecular weight markers. The same blot was stripped and re-probed with anti-ERK (panels designated ERK1/2) to assess equivalency of total ERK1/2. This blot is representative of 4 different experiments. B) Densitometric quantification of the ERK2 activation (ratio of phosphorylated ERK2 to total ERK2 in arbitrary units) from cells that adhered to fibrinogen at 2.5, 5 and 10 minutes. The enhanced ERK2 activation of Pro33 over Leu33 was significant at (P=0.01) by repeated measure ANOVA. C) Activation of ERK2 in 293 cells adhering to immobilized fibrinogen. Vector only 293 cells (lanes 1 and 4), 293A1 (lanes 2 and 5) and 293A2 cells (lanes 3 and 6) were studied as described above in panel A. The 2.5 minute time point is shown. This blot is representative of 3 different experiments. D) Mean fluorescence intensity of P2 antibody (αIIbβ3 specific) binding to the three CHO cell lines and three 293 cell lines used in these experiments performed within 24 hours of the adhesion experiments.

**Fig. 2. Activation of ERK2 in CHO cells adhering to immobilized fibronectin.** A) Immunoblot of phospho ERK2 and total ERK1/2. CHO cells were allowed to adhere to fibronectin (lane 2-4) or maintained in suspension over a BSA substrate (lane 5-7) for 2.5, 5 and
10 minutes and processed as in Fig. 1. Lane 1 contains lysates of LK cells treated with 100 nM PMA as positive control for the phospho ERK blotting. The same blot was stripped and re-probed with anti-ERK (ERK1/2). B) Immunoblot showing ERK1/2 activation in all three cell lines treated with 100 nM PMA or 0.5 M sorbitol for 10 minutes. The blot was probed with anti-phospho ERK antibody (upper panel) or anti-ERK antibody (lower panel). This blot is representative of two different experiments. Surface expression of $\alpha_{IIb}\beta_3$ was not detectably different between the Leu33 (A1) and Pro33 (A2) cell lines (not shown).

**Fig. 3. Activation of ERK2 in CHO cells adhering to immobilized antibodies.** A) Immunoblot showing phosphorylated ERK2 from the 3 CHO cell lines that adhered to LM609 antibody (lane 1-3), P2 antibody (lane 4-6) or maintained in suspension over BSA matrix (lane 7-9) for 5 minutes. The blot was probed with anti-phospho ERK antibody (upper panel) or anti-ERK antibody (lower panel). B) Densitometric quantification of the ERK2 activation from cells that adhered to P2 antibody at 5 minutes in 3 different experiments. The increased ERK2 activation of Pro33 (A2) over Leu33 (A1) cells was significant at ($P=0.03$) by repeated measure ANOVA. C) Mean fluorescence intensity of P2 antibody binding to the 3 CHO cell lines by flow cytometry. These signaling differences were not due to either differences in total ERK levels (Fig. 3A, lower panel) or differences in the surface expression of $\alpha_{IIb}\beta_3$ (Fig. 3C).

**Fig. 4. Activation of JNK and P38 in CHO cells adhering to immobilized fibrinogen.** CHO cells were studied and lysates processed as in Fig. 1, and immunoblotted for active and total JNK (A) and P38 (B). A) Immunoblot probed with an anti-phospho JNK antibody that recognizes activated JNK1 (P46 KDa) and JNK2 (P54 kDa). The same blot was stripped and re-probed
with anti-JNK1. Cells treated with 0.5 M sorbitol (lane 7-9) was included in every experiment to confirm the ability to detect phospho JNK and to demonstrate that JNK can be activated in these cells. Data shown are representative of three experiments. B) Immunoblots probed with an anti-phospho P38 antibody and with an anti-P38 antibody. Lane 7-9 shows cells treated with 0.5 M sorbitol (lane 7-9).

**Fig. 5. Activation of ERK2 in CHO cells using antibody mediated crosslinking of integrin β3.** Integrin αIIbβ3 on CHO cells was crosslinked using either 10E5 (A) or c7E3 (B) followed by goat anti-mouse (GAM) antibody. Total ERK levels were not different in each lane (Fig. 5A lower panel). These blots are representative of 3 different experiments. C) Mean fluorescence intensity of P2 antibody to the 3 CHO cell lines by flow cytometry. Surface expression of αIIbβ3 was not detectably different between the Leu33 (A1) and Pro33 (A2) cells and could not account for the signaling difference.

**Fig. 6. Activation of ERK2 in CHO cells is mediated through upstream MEK and requires post ligand binding events.** Cells were incubated with DMSO or inhibitors, allowed to adhere to fibrinogen for 5 minutes, and immunoblotted as in the above experiments. MEK inhibitors were 20 µg/ml PD98059 (A), 10 µg/ml U0126 (B). Panel C contains cells treated with 10 µg/ml cytochalasin D (cyto-D) or 100 nm wortmannin (wortman) prior to adhesion to immobilized fibrinogen. This data is representative of 2 different experiments. ERK2 was not phosphorylated in any experiment where cells were maintained in suspension over a BSA matrix (not shown). Surface expression of αIIbβ3 was not detectably different between the Leu33 and Pro33 cell lines in these experiments (not shown).
Fig. 7. Activation of MLCK in CHO and human platelets adhering to immobilized fibrinogen. A) CHO cell lines LK, Leu33 (A1) and Pro33 (A2) cells were allowed to adhere to fibrinogen (lane 1-3) or maintained in suspension over BSA matrix (lane 4-6) for 2.5 minutes, after which cells were solubilized and 50 µg of protein was separated by 7% SDS-PAGE, blotted with an anti-phospho MLC antibody (upper panel) or anti-MLC antibody (lower panel). Similar results were obtained in two other experiments. The level of total MLC in Leu33 and Pro33 cells was equivalent (Fig. 7A, lower panel). Cells maintained over BSA substrate showed little to no diphosphorylation of MLC. B) Immunoblot showing diphosphorylated MLC from washed human platelets that were allowed to adhere to fibrinogen for 15 minutes or maintained in suspension over BSA matrix. The blot was probed with anti-phospho MLC antibody (upper panel) or anti-MLC antibody (lower panel). C) Densitometric quantification of the MLCK activation from 4 PlA1,A1 (Pro33 negative) subjects and 5 PlA1,A2 (Pro33 positive) subjects. Compared to PlA1,A1 platelets, PlA1,A2 platelets demonstrated 3.5 fold greater MLCK activation upon binding to fibrinogen.

Fig. 8. Effect of inhibiting ERK2 activation on the β3 Pro33-mediated increase in clot retraction. CHO Cells were treated with DMSO or 20 µg/ml MEK inhibitor PD98059 for 30 minutes, washed and thrombin induced clot retraction performed at time points indicated. Clot retraction of LK (●), Leu33 (○), or Pro33 (△) in the presence of DMSO (A) or 20 µM PD98059 (B). Pro33 cells showed significantly greater fibrin clot retraction compared to Leu33 cells (P=0.02), and this difference was abolished by the MEK inhibitor PD98059 (P=0.633, repeated measures ANOVA). The results are expressed as SEM of 3 independent experiments.
expression of $\alpha_{\text{IIb}}\beta_3$ was not detectably different between the Leu33 and Pro33 on both cell lines (not shown).

**Fig. 9. Effect of inhibiting MLCK activation on the $\beta_3$ Pro33-mediated increased secretion in platelets and increased adhesion in cells.** Washed platelets were incubated with varying thrombin concentrations for 2 minutes in the presence of DMSO (A) or 10 $\mu$g/ml MLCK inhibitor ML-7 (B) and evaluated for P-selectin expression. Results are expressed as SEM from 7 Pl$^{A1,A1}$ (Pro33 negative) subjects and 7 Pl$^{A1,A2}$ (Pro33 positive) subjects. †$P=0.04$, *$P=0.03$, ‡$P=0.01$ for Pro33 neg vs. Pro33 pos. ††$P=0.06$, ‡‡$P=0.35$, ‡‡‡$P=0.1$ for Pro33 neg vs. Pro33 pos in the presence of ML-7. CHO (C) or 293 (D) cells were treated with DMSO or ML-7 (10 $\mu$g/ml) for 30 minutes, washed and perfused over immobilized fibrinogen in a parallel flow chamber at a flow rate of 25 sec$^{-1}$. The number of cells was scored using a camera linked to Metamorph Imaging system. C) Compared to Leu33 cells, Pro33 cells demonstrated 2-fold greater adhesion to fibrinogen ($P=0.005$) and ML-7 abolished this difference in adhesion ($P=0.792$). The results are expressed as SEM of 3 independent experiments. D) Compared to 293A1 cells, 293A2 cells demonstrated 3-fold greater adhesion to fibrinogen ($P=0.001$) and ML-7 abolished this difference in adhesion ($P=0.695$). The results are expressed as SEM of 2 independent experiments. Surface expression of $\alpha_{\text{IIb}}\beta_3$ was not detectably different between the Leu33 and Pro33 receptors on either the CHO or 293 cell lines (not shown).
**Fig. 1**

A. Western blot analysis of FGN and BSA samples showing phosphorylated ERK (pERK2) and total ERK (ERK1/2) levels for LK, A1, and A2 samples. The time points are 0.0, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 minutes.

B. Graph showing the densitometry of pERK2/ERK2 for LK, A1, and A2 samples at time points 2.5, 5, and 10.0 minutes.

C. Western blot analysis of CHO and 293 samples showing pERK2 and ERK1/2 levels for Pc/Z, 293A1, and 293A2 samples.

D. Bar graph comparing the mean fluorescence intensity of CHO and 293 samples for LK, A1, A2, Pc/Z, 293A1, and 293A2 samples.
Fig. 2
**Fig. 3**

Panel A: Western blot analysis showing the expression levels of pERK2 and ERK1/2. The blots were probed with LM609, P2, and BSA. Samples are designated as LK A1 A2 and LK A1 A2.

Panel B: Bar graph illustrating the ratio of pERK2 to ERK2 across LK, A1, and A2 conditions.

Panel C: Column chart depicting the mean fluorescence intensity for LK, A1, and A2 samples.

Legend:
- LK: Control
- A1: Treatment 1
- A2: Treatment 2

The graph data points are labeled as follows:
- 0.0: LK
- 0.1: A1
- 0.2: A2
- 0.3: LK
- 0.4: A1
- 0.5: LK
- 0.6: A1
- 0.7: LK
- 0.8: A1
- 0.9: LK

Mean fluorescence intensity ranges from 0 to 350.
Fig. 5
Fig. 6
### A

|        | FGN | BSA |
|--------|-----|-----|
| ppMLC  | ![Image](ppMLC_FGN.png) | ![Image](ppMLC_BSA.png) |
| MLC    | ![Image](MLC_FGN.png)    | ![Image](MLC_BSA.png)    |
|        | 1   | 2   |
|        | 3   | 4   |
|        | 5   | 6   |

### B

|        | Pro33 neg | Pro33 pos |
|--------|-----------|-----------|
| ppMLC  | ![Image](ppMLC_FGN_neg.png) | ![Image](ppMLC_FGN_pos.png) | ![Image](ppMLC_BSA_neg.png) | ![Image](ppMLC_BSA_pos.png) |
| MLC    | ![Image](MLC_FGN_neg.png)    | ![Image](MLC_FGN_pos.png)    | ![Image](MLC_BSA_neg.png)    | ![Image](MLC_BSA_pos.png)    |
|        | 1         | 2         | 3         | 4         |

### C

![Graph](Graph.png)

**pMLC/MLC densitometry**

- Pro33 neg
- Pro33 pos

**Fig. 7**
Fig. 8
Fig. 9
Enhanced activation of mitogen activated protein kinase and myosin light chain kinase by the Pro33 polymorphism of integrin β3
K. Vinod Vijayan, Yan Liu, Jing-Fei Dong and Paul F. Bray

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