Pro32Pro33 Mutations in the Integrin β_3 PSI Domain Result in $\alpha\text{IIb}\beta_3$ Priming and Enhanced Adhesion: Reversal of the Hypercoagulability Phenotype by the Src Inhibitor SKI-606[§]

Kendra H. Oliver, Tammy Jessen, Emily L. Crawford, Chang Y. Chung, James S. Sutcliffe, and Ana M. Carneiro

Departments of Pharmacology (K.H.O., T.J., C.Y.C., A.M.C.) and Psychiatry, Molecular Physiology, and Biophysics (E.L.C., J.S.S.), Vanderbilt University Medical Center, Nashville, Tennessee

Received January 14, 2014; accepted April 2, 2014

ABSTRACT

The plasma-membrane integrin $\alpha\text{IIb}\beta_3$ (CD41/CD61, GPIIb/IIIa) is a major functional receptor in platelets during clotting. A common isoform of integrin β_3 , Leu33Pro is associated with enhanced platelet function and increased risk for coronary thrombosis and stroke, although these findings remain controversial. To better understand the molecular mechanisms by which this sequence variation modifies platelet function, we produced transgenic knockin mice expressing a Pro32Pro33 integrin β_3 . Consistent with reports utilizing human platelets, we found significantly reduced bleeding and clotting times, as well as increased in vivo thrombosis, in Pro32Pro33 homozygous mice. These alterations paralleled increases in platelet attachment and spreading onto fibrinogen resulting from enhanced integrin $\alpha\text{IIb}\beta_3$ function. Activation with protease-activated receptor 4-activating peptide, the main thrombin signaling receptor in mice, showed no

significant difference in activation of Pro32Pro33 mice as compared with controls, suggesting that inside-out signaling remains intact. However, under unstimulated conditions, the Pro32Pro33 mutation led to elevated Src phosphorylation, facilitated by increased talin interactions with the β_3 cytoplasmic domain, indicating that the $\alpha\text{IIb}\beta_3$ intracellular domains are primed for activation while the ligand-binding domain remains unchanged. Acute dosing of animals with a Src inhibitor was sufficient to rescue the clotting phenotype in knockin mice to wild-type levels. Together, our data establish that the Pro32Pro33 structural alteration modifies the function of integrin $\alpha\text{IIb}\beta_3$, priming the integrin for outside-in signaling, ultimately leading to hypercoagulability. Furthermore, our data may support a novel approach to antiplatelet therapy by Src inhibition where hemostasis is maintained while reducing risk for cardiovascular disease.

Introduction

Platelet hyperaggregability is a critical factor influencing risk for arterial thrombosis (Lippi et al., 2011). The platelet integrin $\alpha\text{IIb}\beta_3$ (glycoprotein IIb/IIIa), the functional receptor for fibrinogen, mediates platelet aggregation through fibrinogen-dependent platelet cross-linking, a critical step in thrombus formation (Calvete, 1994; Ruggeri, 2002). While several polymorphisms in the integrin β_3 subunit (*ITGB3* gene) have been associated with impaired platelet function (Wang et al., 1993; Wang and Newman, 1998), the presence of one allele for the β_3 alloantigen $\text{Pl}^{\text{A}2}$ has been reported in some studies to be associated with increased risk for coronary events, atherosclerotic plaque rupture, and myocardial infarction (Kunicki and

Nugent, 2002; Knowles et al., 2007). The $\text{Pl}^{\text{A}2}$ antigen corresponds to a missense substitution of a leucine to proline at residue 33 of the mature integrin β_3 , located in a hydrophobic pocket of the β_3 extracellular PSI (plexin-semaphorin-integrin) domain (Leu33Pro; rs5918, also known as the HPA-1 or Z_w system) (Newman et al., 1989). While the number of studies assessing the influence of the Pro33 allele on platelet function is large, findings are inconsistent due to the small number of homozygous Pro33 subjects studied or possibly due to population stratification (Michelson et al., 2000b; Undas et al., 2001; Vijayan et al., 2003b; Angiolillo et al., 2004; Dropinski et al., 2005; Lev et al., 2007). Therefore, other in vivo models must be developed to determine the contributions of structural modifications in the PSI domain to platelet aggregation and thrombosis risk.

Structurally, the Leu33Pro substitution generates a Pro32-Pro33 sequence, which may increase the flexibility of integrin $\alpha\text{IIb}\beta_3$ extracellular domains (Xiong et al., 2004; Jallu et al., 2012). Several studies suggest that the increased platelet function in Pro33 carriers may result from a facilitation of

This work was supported, in part, by an Autism Speaks pilot grant; the National Institutes of Health National Institute of Mental Health [Grant R01-MH090256]; and the National Institutes of Health National Institute of Neurological Disorders and Stroke [Grant R01-NS049261].
dx.doi.org/10.1124/mol.114.091736.

[§] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: ANOVA, analysis of variance; BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FITC, fluorescein isothiocyanate; KI, knockin; KRH, Krebs-Ringer-HEPES; PAR, protease-activated receptor; PAR4-AP, PAR4-activating peptide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PSI, plexin-semaphorin-integrin; RGD, arginine-glycine-aspartic acid; SKI-606, 4-(2,4-dichloro-5-methoxyanilino)-6-methoxy-7-[3-(4-methylpiperazin-1-yl) propoxy]quinoline-3-carbonitrile; WT, wild-type.

integrin-mediated intracellular signaling (Goodall et al., 1999; Vijayan et al., 2003a,b, 2005). To achieve platelet activation, integrin $\alpha\text{IIb}\beta_3$ undergoes conformational changes that involve disruption of $\alpha\text{IIb}\beta_3$ interactions and extension of the cytoplasmic domain of integrin β_3 (Yang et al., 2009). This extension can be achieved by extracellular matrix binding under high flow conditions (outside-in activation) or by agonist-dependent translocation of talin or kindlin 3 to the plasma membrane and binding to the β_3 subunit (inside-out activation) (Vinogradova et al., 2000; Tadokoro et al., 2003; Wegener et al., 2007; Moser et al., 2008). These events trigger phosphorylation of tyrosine residues in the β_3 tail and expose domains necessary for the interaction of focal adhesion kinase (FAK), Src, and Hic-5 (Osada et al., 2001; Nieswandt et al., 2007; Kim-Kaneyama et al., 2012). Alternatively, $\text{G}\alpha_{13}$ downstream of thrombin [protease-activated receptor (PAR) 1/4] receptors can directly bind to the β_3 cytoplasmic domain and activate Src (Gong et al., 2010). Upon $\alpha\text{IIb}\beta_3$ separation, the αIIb cytoplasmic tail also can interact with signaling proteins, such as the calcium- and integrin-binding protein and the serine/threonine protein phosphatase PP1 (Vijayan et al., 2003b, 2004). Although several studies suggest inside-out-dependent increases in integrin-dependent signaling in cells expressing Pro33 integrin $\alpha\text{IIb}\beta_3$, the mechanism by which this extracellular PSI domain mutation influences integrin outside-in signaling remains unknown.

In the present study, we generated a new knockin (KI) transgenic mouse model where the Pro32Pro33 isoform is expressed from the endogenous integrin β_3 locus and examined the effects of this sequence variation on platelet function, integrin $\alpha\text{IIb}\beta_3$ activation, and outside-in signaling. In these mice, we demonstrate decreased clotting time, enhanced fibrinogen-mediated platelet adhesion, and elevated basal outside-in signaling without full $\alpha\text{IIb}\beta_3$ integrin activation. Importantly, we show that early signaling events linked to Src activation dictate the proaggregatory phenotype in the KI mice.

Materials and Methods

Thrombin and equine tendon type I fibrillar collagen were purchased from Chronolog (Havertown, PA). Sterile saline, ADP, fibrinogen, SKI-606 [4-(2,4-dichloro-5-methoxyanilino)-6-methoxy-7-[3-(4-methylpiperazin-1-yl) propoxy]quinoline-3-carbonitrile] (Golas et al., 2003), and epinephrine were purchased from Sigma-Aldrich (St. Louis, MO). PAR4-activating peptide (PAR4-AP; AYPGKF) was purchased from GL Biochem (Shanghai, China). Flow cytometry antibodies [conjugated to phycoerythrin (PE) or fluorescein isothiocyanate (FITC)] to integrin αIIb (CD41-PE) and integrin β_3 (CD61-FITC) were purchased from BioLegend (San Diego, CA) and anti-activated $\alpha\text{IIb}\beta_3$ (JON/A-FITC) and anti-P-selectin-PE antibodies from EMFRET Analytics & Co. (KG, Würzburg, Germany). Western and immunocytochemistry antibodies—mouse anti- αIIb , anti- β_3 , anti-Src, anti-pSrc⁴¹⁶, anti-FAK, anti-pFAK³⁹⁷, anti-extracellular signal-regulated kinase (ERK), and anti-pERK—were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Mouse anti-actin and mouse anti-talin were purchased from Sigma-Aldrich. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX) and phalloidin-Cy5 (Molecular Probes, Eugene, OR) were purchased from Life Technologies Corporation (Grand Island, NY). Secondary antibodies (goat anti-mouse-Cy2; goat anti-rabbit-Cy3; and mouse anti-rabbit and goat anti-mouse, both conjugated to horseradish peroxidase) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

KI Mouse Line. The construct used to target the mouse *Itgb3* had the Ser23Gln33 mutated to Pro32Pro33. Two LoxP sites flanked the Neo-Cre cassette containing the neomycin gene, an angiotensin-converting enzyme 161 testis-specific promoter, and Cre open reading frame. Arms for homologous recombination were obtained by polymerase chain reaction (PCR) and verified by complete sequencing (Gene Dynamics LLC, Tigard, OR). The construct was injected onto C57BL/6J embryonic cells and implanted onto C57BL/6J blastocysts (inGenious Targeting Laboratory, Inc., Ronkonkoma, NY). Screening of clones was tested by two complementary PCR/restriction fragment length polymorphism approaches (Fig. 1). In PCR1 (Fig. 1C), primer A (5'-GCTAACGTCGCTGGTC-3') and primer B (3'-CACTTGGTCGTGGCAGCCCGGACC-3') generated an 8.5-kb band in KI allele only. In PCR2 (Fig. 1D), primer C (5'-AGCCAGCTCATTCTTGGCTCTTA-3') and primer D (5'-AAACGCTCTACCACACAGCTCACT-3') generated a 4121-bp band. The digestion of the 4121 bp with *MspI* generated two fragments (879 and 4121 bp) in wild-type (WT) and three fragments (4121, 608, and 271 bp) in KI allele. Mice were genotyped by PCR (Fig. 1E) using genomic DNA extracted from tails

Fig. 1. Generation of mice bearing the *Pro32Pro33 mutation (KI mice). (A) Sequence alignment of mouse and human mature β_3 integrin. The Pro32Pro33 mutation introduced in the KI mouse model is shown below the alignment. (B) Targeting strategy to generate the KI mice, where exon 3 contains the S32P, Q33P substitution. The self-excising Cre/Neo cassette, flanked by LoxP sites, is located 5' of exon 3. Two complementary PCR strategies were used to screen embryonic stem (ES) cells. (C) PCR1 using primers A and B showing presence of the Cre/Neo cassette in recombinant ES cells. (D) PCR2 using primers C and D followed by *MspI* digestion showing successful targeting of the KI allele in clone 2. (E) Genotyping by PCR confirms excision of the Cre/Neo cassette, and *EcoRI* digestion reveals fragments measuring 330 and 270 bp identifying KI mice.

and primer A and primer F (5'-AAGGGGAAAAGTCACCCCTTG-3') followed by digestion with EcoRI.

Animals and Housing. All mice were group housed in temperature- and humidity-controlled conditions under a 12-hour light/dark cycle with food and water available ad libitum. All studies were performed in accordance with humane guidelines established by the Vanderbilt Institutional Animal Care and Use Committee under an approved protocol (M/11/065). Age- and sex-matched mice were used in all experiments (8–20 weeks of age). The colony manager determined experimental cohorts, and experimenters were blinded to the genotypes. All experiments were run with either wt/wt (WT) or ki/ki (KI) homozygous mice of both sexes.

Blood Collection. Cardiac puncture was performed in euthanized mice using a 25-gauge needle/1-ml syringe containing sodium citrate. For platelet isolation, whole blood was layered onto 1.5 ml of Fico/Lite Platelets (Atlanta Biologicals, Inc., Lawrenceville, GA) and spun for 15 minutes at 700g. Platelets were washed in 1 ml modified Tyrodes-HEPES buffer (10 mM HEPES, 11.9 mM NaHCO₃, 127.2 mM NaCl, 5 mM KCl, 0.4 mM NaH₂PO₄, 1 mM MgCl₂, 5 mM glucose; pH 7.4), collected by centrifugation at 5000g for 5 minutes, and counted in a Coulter counter (Beckman Coulter, Brea, CA).

Whole-Blood Flow Cytometry. Briefly, 250 μ l of whole blood was mixed with 750 ml of Tyrodes-HEPES buffer and added to a tube containing buffer or PAR4-AP. Antibodies (2.5 μ l) were added to tubes, and activation was stopped by addition of 500 μ l of 2% paraformaldehyde in phosphate-buffered saline (PBS) (0.138 M NaCl, 0.0027 M KCl; pH 7.4) 15 minutes after activation. Samples were analyzed at the Nashville Veterans Affairs Medical Center Flow Cytometry Resource Center (Nashville, TN) (Michelson et al., 2000a).

Tail Bleed. Mice were maintained under anesthesia (2% isoflurane and 1 l/min oxygen; JD Medical Distributing Co., Inc., Phoenix, AZ), and a transverse incision was made with a scalpel over a lateral vein. The tail was immersed in normal saline (37°C) in a hand-held test tube. The time from the incision to the cessation of bleeding was recorded as the bleeding time.

Whole-Blood Clotting Time. Whole blood (90 μ l) was added to a single well containing a small metal bead in the presence of 10 μ l of CaCl₂ (16.4 mM), and the number of seconds to interruption of the small magnetic bead movement was recorded (Diagnostics Stago, Parsippany, NJ).

Nonlethal Thromboembolism. The *nonlethal* systemic thrombosis method was chosen, as it may reveal increases in platelet aggregation (Smyth et al., 2001). Mice were kept under anesthesia with 2% isoflurane and the right jugular vein exposed by a lateral neck incision for collection of 100 μ l of whole blood in sodium citrate. The left jugular vein was exposed to inject a coagulation solution containing 100 μ g/ml ADP, 200 μ g/ml collagen, and 200 μ g/ml epinephrine in sterile saline at a dose of 5 μ l/g during 10 seconds. One minute after the injection a sample of blood was collected into sodium citrate. Six minutes after injection mice were euthanized by rapid decapitation.

Whole-Blood Aggregation. Electrical impedance was determined using a multiplate analyzer (Dynabyte GmbH, Munich, Germany) by adding 175 μ l of 37°C 2 $\times$ CaCl₂ to 175 μ l of citrated whole blood, following agonist (200 μ M PAR4-AP) addition. Aggregation and the velocity of aggregation were determined over a 6-minute period.

Aggregation in Washed Platelets. Blood was spun for 10 minutes at 500g, and 500 μ l of platelet-rich plasma was collected from the top layer of the supernatant. Pelleted platelets were suspended in Tyrodes-HEPES buffer and adjusted to a concentration of 3×10^8 platelets/ml. The change in light transmission was monitored with an aggregometer in the presence of 0.05 U/ml thrombin.

Platelet Attachment and In-Cell Westerns. In-cell Westerns were performed as described previously (Chen et al., 2005; Carneiro et al., 2008). Whole blood was diluted 1:8 in Krebs-Ringer-HEPES (KRH) buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM

MgSO₄, 1.2 mM KH₂PO₄, 1.8 g/l glucose, 10 mM HEPES; pH 7.4) or PBS and seeded onto 25 μ g/ml fibrinogen-coated 96-well plates. After adding 45 μ l diluted blood/well, MnCl₂ (0.2 mM), 200 μ M PAR4-AP, or buffer was added to all wells and incubated at 37°C for 15 minutes. Wells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton for 10 minutes at room temperature. After blocking in 1% bovine serum albumin (BSA) and 5% normal goat serum for 30 minutes, primary antibodies were added at a 1:1000 dilution overnight at 4°C. Wells were washed three times with PBS, and secondary antibodies (IRDye 800CW donkey anti-rabbit IgG and IRDye 680RD donkey anti-mouse IgG; LI-COR Biosciences, Lincoln, NE) were added at a 1:250 dilution for 1 hour at room temperature. Plates were washed three times with PBS and scanned in an Odyssey Infrared Imaging system (LI-COR Biosciences).

Platelet Spreading. Washed platelets were resuspended in KRH (10¹⁰/ml) and seeded onto 12-well (5-mm-diameter) glass printed slides (Thermo Scientific Cel-Line Specialty Printed Microscope Slides; SSG Braunschweig, Germany) previously coated with 25 μ g/ml fibrinogen and blocked with 1% BSA. Each well received 4 μ l of platelets and 1 μ l of 1 mM PAR4-AP, 1 mM PAR4-AP + 0.5 μ M SKI-606, or KRH buffer. Slides were incubated at 37°C for 15 minutes, washed once with 1 $\times$ PBS, and fixed with 4% paraformaldehyde. Platelets were permeabilized with 0.2% Triton X-100 in PBS and blocked in 1% BSA and 5% normal goat serum. Slides were incubated with primary antibodies at 1:1000 dilution overnight at 4°C. Slides were washed with PBS and incubated with secondary antibodies and phalloidin-Cy5 at 1:200 dilution in 1% BSA for 1 hour at room temperature. Slides were washed and mounted in Acqua Poly Mount (Polysciences, Inc., Warrington, PA). Images were captured with a Zeiss LSM510 META Inverted Confocal Microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) located at the Vanderbilt Cell Imaging Shared Resource. Images were obtained with a 63 $\times$ /1.40 Plan-Apochromat oil lens using Zeiss Image Browser. Platelet number and area were quantified by a blinded experimenter using ImageJ analysis software in the talin channel (Cy2).

Western Blotting and Coimmunoprecipitations. Washed platelets were resuspended in KRH, incubated at room temperature for 30 minutes, lysed by addition of 1 volume of 1% Triton X-100 in PBS (containing protease inhibitor; Roche, Indianapolis, IN), and clarified by centrifugation at 13,000g for 10 minutes at 4°C. Lysates were collected for input (10 μ g), and 200 μ g of protein extract was incubated with 30 μ l integrin β_3 antibody (2C9.G2 hamster anti- β_3 ; BioLegend) covalently attached to protein A magnetic beads (Dynabeads; Life Technologies/Invitrogen, Grand Island, NY) for 1 hour at 4°C. Beads were isolated magnetically and washed with 1 ml 0.5% Triton X-100 in PBS. Coimmunoprecipitated proteins were eluted with 1 $\times$ NuPAGE lithium dodecyl sulfate sample buffer (Life Technologies/Invitrogen) and Western blot analysis performed. Proteins were detected by chemiluminescence and exposed to Hyperfilm though multiple exposures to ensure linear distribution of signal. Films were scanned, and band densities were established using ImageJ software (NIH, Bethesda, MD).

Src In Vivo Inhibition. A 10 mM stock of SKI-606 (Sigma-Aldrich) in dimethylsulfoxide was diluted in sterile saline (0.9% NaCl) to 0.1 mg/ml immediately prior to administration. Mice were maintained under anesthesia at 2% isoflurane during the whole procedure. The jugular vein was exposed for collection of 300 μ l of blood in sodium citrate for a pre-SKI-606 clotting time measurement. After this initial blood draw, SKI-606 was administered intraperitoneally at 1 mg/kg. After 30 minutes, cardiac puncture was performed for collection of blood samples (post-SKI-606). Blood samples were used to perform clotting time experiments and in-cell Westerns.

Data Analysis and Statistics. All data were analyzed in Prism 4.0c (GraphPad Software, Inc., La Jolla, CA) using Student's *t* tests or two-way analysis of variance (ANOVA) with Bonferroni post-tests where appropriate. Welch's correction parameters were used in samples with unequal variances (indicated in *Results*). Nonparametric *t* tests were used when each WT/KI pair was normalized to the WT

data (all WT = 100). Two-way ANOVA with drug and genotype as factors with Bonferroni-corrected post-tests were used for multiple comparisons ($\alpha = 0.0125$). A P value of <0.05 was considered statistically significant. All data are shown as mean $\pm$ S.E.M., represented by error bars.

Results

Introduction of the Pro32Pro33 Residues in the Mouse Integrin β_3 . Alignment of the mature human and mouse integrin β_3 protein sequences reveals a lack of conservation at residues 32 and 33 (Fig. 1A), corresponding to residues 58 and 59 in the immature protein, respectively. (Full sequence alignment can be found in Supplemental Fig. 1.) The human sequence contains a Pro32 and Leu33, modified to Pro32Pro33 by the Leu33Pro polymorphism, which introduces a structural flexibility that may lead to functional changes observed in platelets (Jallu et al., 2012). Therefore, we designed the targeting construct to change Ser32Gln33 to Pro32Pro33 (Fig. 1B; details on the targeting construct can be found in *Materials and Methods*). C57BL/6 embryonic stem cells were screened for homologous recombination by two complementary PCRs (Fig. 1, C and D), and correct targeting was confirmed by Southern blotting. Germ-line transmission of the Pro32Pro33 allele and excision of the Cre/Neo cassette was confirmed by PCR (Fig. 1E) and sequencing of the final targeted locus (KI). Pro32Pro33 KI mice were born at Mendelian ratios, independently of the genotype of the parents, and were fertile with no obvious developmental or behavioral effects.

Enhanced Clot Formation and Aggregation in KI Mice. Mice expressing the Pro32Pro33 integrin β_3 had normal platelet production and blood cell count (Supplemental Table 1). To establish the physiologic consequences of the Pro32Pro33 integrin β_3 substitution, we measured platelet function using in vivo and ex vivo paradigms. Clotting time was significantly decreased in KI mice when measured by tail bleed (Fig. 2A) or whole-blood clot formation (Fig. 2B). To test whether the increased clotting could influence thrombosis in vivo, we implemented a model of in vivo nonfatal thromboembolism. In this model we injected a solution containing weak agonists (0.5 mg/kg ADP, 100 μ g/kg epinephrine, and

1 mg/kg collagen) to prevent a ceiling fatal effect, which would prevent us from detecting increases in thromboembolism in KI mice. We collected blood from mice before and 1 minute after the injection of agonists and counted the number of platelets in each sample. Statistical analysis using repeated-measures ANOVA revealed a significant reduction in the number of circulating platelets in KI mice as compared with wild-type mice, indicating increased thrombosis in KI mice following stimulation in vivo (Fig. 2C). To examine whether the enhanced clotting phenotype resulted from increased platelet function, we measured ex vivo platelet aggregation. Whole-blood aggregation in the presence of PAR4-AP (PAR4 stimulation) led to a significant increase in the velocity of clot formation in KI mice compared with WT controls (Fig. 2, D and E). These changes were also recapitulated in aggregation experiments using washed platelets, demonstrating that the proaggregatory phenotype derives from enhanced platelet function (Fig. 2F).

Enhanced Adhesion and Spreading in KI Platelets.

To examine the consequences of the Pro32Pro33 mutation on integrin α IIb β_3 function, we examined platelet adhesion ex vivo. Platelet adhesion depends on both integrin affinity (determined by ligand binding) and avidity (determined by integrin cross-linking), which can be assessed by adhesion to immobilized fibrinogen. Although basal binding to fibrinogen (Mn^{2+} -free; Supplemental Fig. 2) was not significantly different between genotypes, homozygous KI platelets had increased adhesion to fibrinogen in the presence of 0.2 mM $MnCl_2$ (Fig. 3A). Binding of KI platelets to fibrinogen was increased as compared with wild-type platelets at low fibrinogen levels, suggesting increased downstream integrin platelet activation leading to increased adhesion. We then measured platelet adhesion to arginine-glycine-aspartic acid (RGD) peptides, which do not induce clustering of the receptor. We observed similar levels of platelet attachment to wells coated with RGD (Fig. 3B), suggesting that the Pro32Pro33 mutation does not alter the affinity of α IIb β_3 of the ligand-binding domain to RGD.

Adhesion comprises two integrin-initiated events, attachment and spreading (Arias-Salgado et al., 2005; Lawson and Schlaepfer, 2012). We used confocal microscopy to determine platelet number and surface area after adhesion to 25 μ g/ml

Fig. 2. Enhanced clot formation and aggregation in KI mice. (A) Tail bleed time is significantly decreased in KI mice (Student's t test: $**P = 0.0049$; WT = 15; KI = 20). (B) Clotting time is significantly decreased in KI mice (Student's t test: $*P = 0.0164$; WT = 14; KI = 20). (C) Thromboembolism experiment showing equivalent initial number of circulating platelets between WT and KI samples (Pre). After administration of agonist in vivo (Post), the number of circulating platelets significantly decreases in KI mice but not in WT control mice (two-way ANOVA agonist effect: $P = 0.0319$; Bonferroni post-test, KI_{Pre} versus KI_{Post} : $*P < 0.05$; WT = 10; KI = 11). (D) Whole-blood aggregation stimulated with 200 μ M PAR4-AP. (E) Aggregation velocity [arbitrary units (A.U.)/ μ min] is significantly increased in KI mice (unpaired t test with Welch's correction: $*P = 0.0267$; WT = 6; KI = 6). (F) Aggregation in washed platelets. Representative plot showing increased KI aggregation in platelets stimulated by 0.05 U/ml thrombin. Similar results were observed in six independent experiments.

Fig. 3. Increased spreading and adhesion mediated by outside-in signaling in KI platelets. Platelet adhesion was monitored by in-cell Western blot of β -actin on platelets bound to increased concentrations of immobilized fibrinogen (A) or RGD peptides (B) in the presence of 0.2 mM MnCl_2 [(A) two-way ANOVA, fibrinogen: $P < 0.0001$; genotype: $P = 0.0070$; Bonferroni post-test, KI versus WT at 25 $\mu\text{g/ml}$: $*P < 0.05$; (B) two-way ANOVA, RGD: $P < 0.0001$]. (C) Platelets were allowed to adhere to fibrinogen-coated (25 $\mu\text{g/ml}$) coverslips (incubation time, 15 minutes). Representative confocal images of platelets stained with talin are shown. Scale bar, 10 μm . (D) Number of platelets attached per image was quantified in WT and KI samples (unpaired t test with Welch's correction: $**P = 0.0091$; number of images: WT = 8; KI = 6). (E) Platelet spreading was assessed by quantification of platelet area (in arbitrary units) in WT and KI samples (unpaired t test with Welch's correction: $***P < 0.0001$; number of platelets: WT = 86; KI = 111). Image acquisition and analysis is described in *Materials and Methods*. (F) Platelets were allowed to adhere to 25 $\mu\text{g/ml}$ fibrinogen for 15 minutes, washed, and fixed with 4% paraformaldehyde to assess kinase phosphorylation by in-cell Western blot. In-cell Western data were normalized for each WT/KI pair (pSrc/Src, nonparametric t test: $*P = 0.0304$; WT = 10; KI = 10).

fibrinogen (Fig. 3C). We found that talin staining better represented the spreading of cells onto fibrinogen-coated slides compared with phalloidin (Supplemental Fig. 3) and observed significant increases in the number and mean area of attached KI platelets compared with WT platelets (Fig. 3D: platelet number; Fig. 3E: platelet area). The significant increases in spreading led us to examine proximal intracellular signaling cascades, including Src and FAK, in the context of platelet adhesion to fibrinogen. In-cell Western analyses revealed increases in Src(Tyr⁴¹⁶) but not FAK (Tyr³⁹⁷) or ERK phosphorylation in adhered KI platelets (Fig. 3F). Confocal imaging of pSrc(Tyr⁴¹⁶) staining in platelets adhered onto fibrinogen demonstrates that Src phosphorylation occurs at specific locations adjacent to the plasma membrane of attached platelets (Supplemental Fig. 4). The significant increases in attachment and spreading in KI platelets are indicative of enhanced integrin $\alpha\text{IIb}\beta_3$ function, likely due to enhanced integrin clustering and ligand-induced propagation of intracellular signals.

Enhanced Basal Talin Binding to Integrin $\alpha\text{IIb}\beta_3$ Is Independent from Conformational Changes in the Ligand-Binding Domain. The integrin β_3 Pro32Pro33 mutation may influence $\alpha\text{IIb}\beta_3$ function by altering receptor expression at the plasma membrane, by facilitating changes in the ligand-binding domain and consequent integrin activation, or by facilitating changes in the conformation of the transmembrane and intracellular domains of $\alpha\text{IIb}\beta_3$ and,

thus, facilitating outside-in signaling. Flow cytometry using extracellular epitope antibodies revealed comparable plasma membrane expression levels of both αIIb and β_3 subunits between genotypes (Fig. 4, A and B, respectively). Changes in the conformation of the integrin $\alpha\text{IIb}\beta_3$ ligand-binding domain were assayed using an antibody that recognizes the active conformation of $\alpha\text{IIb}\beta_3$ [JON/A (Bergmeier et al., 2002)]. No changes were observed in JON/A binding to WT, KI, and *Itgb3*^{-/-} platelets, indicating the absence of fully activated integrin $\alpha\text{IIb}\beta_3$ in resting platelets (Fig. 4C). Western blot analysis of unstimulated platelets also revealed no significant alterations in the expression levels of integrin $\alpha\text{IIb}\beta_3$ or any proximal signaling proteins, such as talin, Src, and FAK (Fig. 4D). We then tested the hypothesis that the Pro32Pro33 mutation influences outside-in signaling by altering the availability of the intracellular domain of β_3 to interact with intracellular proteins. Because talin binding is the first step involved in integrin activation, we examined coimmunoprecipitation of talin and $\alpha\text{IIb}\beta_3$ with an anti-integrin β_3 antibody. Whereas talin was not found to bind to β_3 in WT platelets, we observed a significant increase in talin associations with integrin β_3 in KI platelets (Fig. 4E). We then examined whether downstream signaling pathways, including Src, are also altered under unstimulated conditions. We observed a significant increase in Src(Tyr⁴¹⁶) phosphorylation with a concomitant decrease in FAK(Tyr³⁹⁷) phosphorylation in KI platelets, while no significant differences in ERK

Fig. 4. Increased talin and pSrc binding to Pro32Pro33 integrin β 3 in unstimulated platelets. Platelets were isolated from WT and KI mice, and baseline plasma membrane expression of α IIb (A) and β 3 (B) were determined by flow cytometry. (C) JON/A (active integrin α IIb β 3 antibody) binding was assessed by flow cytometry. Overlapping traces of analyses for platelets isolated from WT, KI, and *Itgb3*^{-/-} are shown. (D) Western blot analysis of isolated platelets found no change in total expression of integrin α IIb β 3 or downstream signaling molecules (talin, Src, FAK, and ERK) between WT and KI samples. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (E) Coimmunoprecipitation (Co-IP) of talin and α IIb with anti-integrin β 3 antibodies. Platelet lysates were incubated with beads covalently coupled to anti-integrin β 3 antibody and bound proteins eluted with lithium dodecyl sulfate (LDS) buffer as described in *Materials and Methods*. A representative fraction (10%) of proteins in the lysate is shown in the input blot. Talin Co-IP is significantly increased in KI samples (unpaired *t* test with Welch's correction: **P* = 0.0331; WT = 10; KI = 10). (F) Signaling pathways immediately downstream of α IIb β 3 are significantly altered in KI platelets. Results are shown as phosphokinase/kinase ratios, normalized to the mean of WT (unpaired *t* test with Welch's correction, pSrc/Src: **P* = 0.0378; WT = 8; KI = 8; Student's *t* test, pFAK/FAK: **P* = 0.0372; WT = 9; KI = 9). (G) Co-IP of integrin α IIb β 3 and Src reveals that Src bound to integrin β 3 is more likely to be phosphorylated in KI platelets (unpaired *t* test: ***P* = 0.0037; WT = 6; KI = 6). Unstimulated platelets were lysed and incubated with anti-integrin β 3 antibody-coupled beads. Immunoprecipitated complexes were eluted with LDS buffer. Data shown here are representative images from 6–10 independent experiments. Image acquisition and analysis is described in *Materials and Methods*.

phosphorylation were observed (Fig. 4F). We then examined whether the increases in pSrc levels in KI platelets result from enhanced Src associations with the β 3 tail. We observed that Src/ β 3 associations were comparable between genotypes. However, there was an increase in pSrc associated with the Pro32Pro33 β 3 (Fig. 4G). Together, these data demonstrate that the integrin β 3 Pro32Pro33 mutations increased talin binding to the intracellular domains of α IIb β 3, concomitant with increased pSrc associations in unstimulated platelets. These changes result in altered Src and FAK phosphorylation levels, which occur without observed activation of the ligand-binding domain of α IIb β 3.

Activation of Inside-Out Signaling Normalizes Some, but Not All, Genotype-Specific Differences between KI and WT Platelets. Several of the phenotypes presented in Fig. 2 were examined in the context of protease-activated receptor activation by thrombin or PAR4-AP. Whereas our data revealed altered outside-in signaling in unstimulated platelets, several studies have provided evidence for agonist-induced

enhancement of FAK and ERK signaling in platelets and cells expressing the human Pro33 integrin β 3 (Vijayan et al., 2003b, 2005; Carneiro et al., 2008). We then examined whether PAR4 activation modifies the cellular phenotypes observed in KI platelets, focusing on Src activation. Flow cytometry experiments revealed comparable levels of JON/A antibody binding to "active" α IIb β 3 in both WT and KI platelets upon PAR4 stimulation (Fig. 5A). PAR4-stimulated P-selectin plasma membrane expression was not different in KI and WT mice, indicating similar platelet activation upon strong agonist stimulation in both WT and KI platelets (Fig. 5B). PAR4 activation did not significantly increase Src(Tyr⁴¹⁶) phosphorylation in WT platelets in suspension (Fig. 5C). However, upon adhesion, PAR4 stimulation significantly increased Src phosphorylation in WT but not in KI platelets (Fig. 5D; Supplemental Fig. 5). Because baseline Src phosphorylation in the KI platelets may have reached ceiling levels, activation of inside-out signaling does not further increase Src phosphorylation. These data, along with the findings in Fig. 4, demonstrate that

Fig. 5. Activation of PAR4 signaling partially normalizes WT and KI genotype differences in Src phosphorylation. (A) Integrin $\alpha\text{IIb}\beta_3$ activation by PAR4 was determined by flow cytometry. (B) P-selectin plasma membrane levels are elevated by PAR4 activation in both WT and KI samples. Traces show data from WT, KI, and *Itgb3*^{-/-} platelets. (C) Platelets in suspension were stimulated with PAR4-activating peptide followed by Western blot analysis of Src phosphorylation. PAR4 activation does not significantly increase Src phosphorylation in either genotype (nonparametric *t* tests for pSrc/Src ratios, WT_{vehicle} versus KI_{vehicle}: $^{**}P < 0.01$; WT_{vehicle} $N = 14$, WT_{PAR4} $N = 7$, KI_{vehicle} $N = 14$, KI_{PAR4} $N = 8$). (D) PAR4 activation in attached platelets differentially influences Src phosphorylation. PAR4 stimulation significantly increases pSrc levels in WT platelets (nonparametric *t* tests, WT_{vehicle} versus KI_{vehicle}: $^{*}P < 0.05$; WT_{vehicle} versus WT_{PAR4}: $^{*}P < 0.05$; WT = 6, KI = 6). MFI, mean fluorescence intensity.

structural changes elicited by the Pro32Pro33 mutation are independent of inside-out signaling.

Src Activation Is the Dominant Step Driving the Increased Clotting in KI Platelets. To test whether Src activation is necessary for the enhanced adhesion and clotting phenotypes observed in KI mice, we exposed platelets to the dual Src/Abl tyrosine kinase inhibitor SKI-606 (bosutinib). SKI-606 is an orally available inhibitor of Src, Fgr, and Lyn (Boschelli et al., 2001; Remsing Rix et al., 2009). SKI-606 significantly reduced the number of KI platelets attached to fibrinogen while significantly increasing WT attachment (Fig. 6B). With platelet activation via both PAR4 and fibrinogen, SKI-606 (0.1 μM) fully inhibited Src phosphorylation (Supplemental Fig. 5) and had a very complex influence on platelet attachment. However, in the presence of PAR4 stimulation, KI attachment was comparable to WT samples, suggesting that PAR4 activates signaling pathways that may counteract the effects of the Pro32Pro33 mutation in β_3 . Possibly PAR4-mediated stimulation of RhoA potentiates focal adhesion formation despite enhanced Src phosphorylation. In the presence of SKI-606, however, platelet adhesion was comparable to levels observed in vehicle-treated platelets. These data indicate that multiple signaling pathways downstream from PAR4 activation, beyond Src, modify platelet attachment in the context of the Pro32Pro33 mutation.

The role of Src-family tyrosine kinases in platelet spreading is well established (Schoenwaelder et al., 1994; Oberfell et al., 2002; Arias-Salgado et al., 2005; Vielreicher et al., 2007; Séverin et al., 2012). Consistent with previous studies, Src inactivation by SKI-606 significantly reduced KI platelet spreading onto fibrinogen-coated slides (Fig. 6C). PAR4 activation significantly enhanced spreading in WT platelets and induced a small reduction in spreading in KI samples. In

the context of PAR4 activation, SKI-606 reduced platelet spreading in both genotypes, although significant genotype differences were still observed. These data suggest that Src signaling in KI platelets leads to enhanced platelet spreading and that inhibition of Src is sufficient to normalize platelet spreading to wild-type levels.

We then examined whether in vivo administration of SKI-606 can normalize KI clotting ex vivo. Mice were anesthetized and blood collected before and after intraperitoneal administration of 1 mg/kg SKI-606. In-cell Western analysis confirmed that SKI-606 significantly reduced adhesion-dependent Src (Tyr⁴¹⁶) phosphorylation in the presence of PAR4-AP (Fig. 6D). At this concentration SKI-606 significantly increased clotting time in KI mice, whereas no significant differences were observed in WT (Fig. 6E). This differential effect was concentration-dependent, as we observed increased clotting times after injection of higher concentrations of SKI-606, albeit with larger increases in KI (Supplemental Fig. 6). Taken together, these data demonstrate that the integrin β_3 Pro32Pro33 mutation is sufficient to induce increased talin association to $\alpha\text{IIb}\beta_3$ and enhanced basal Src phosphorylation, which are responsible for facilitated platelet spreading and a prothrombotic phenotype in KI mice.

Discussion

Platelet aggregation is a tightly controlled event, essential for the maintenance of thrombosis and hemostasis. Here, we focused on the study of a common integrin β_3 coding polymorphism, Leu33Pro within the $\alpha\text{IIb}\beta_3$ integrin (Newman et al., 1989). Flow cytometry studies utilizing anti-Leu33 β_3 antisera from Pro33 thrombocytopenia patients reveal conformational changes in an epitope located between residues 9 and 50, likely due to the formation of a diproline sequence in

Fig. 6. Inhibition of Src rescues the spreading and clotting phenotypes observed in KI mice. (A–D) Ex vivo inhibition of Src. Platelets were resuspended in KRH, seeded onto fibrinogen-coated slides (25 μ g/ml), and incubated with different agonists for quantification of cell adhesion. (A) Representative confocal images of talin staining. (Left to right) Vehicle, Src/Abl tyrosine kinase inhibitor SKI-606, PAR4-AP, PAR4-AP + SKI-606. (Top) WT platelets; (bottom) KI platelets. Scale bar, 10 μ m. (B) Platelet attachment. SKI-606 elicits opposing effects in WT and KI platelets, increasing adhesion in WT while decreasing adhesion in KI samples. PAR4-AP activation does not significantly enhance platelet attachment. PAR4-AP activation normalizes the effects of SKI-606 on platelet attachment in both genotypes (two-way ANOVA, interaction $F_{(34,3)} = 22.51$; $P < 0.0001$; genotype $F_{(34,1)} = 11.80$; $P = 0.0016$; Bonferroni post-tests: WT_{vehicle} versus KI_{vehicle}: $***P < 0.001$; WT_{SKI-606} versus KI_{SKI-606}: $***P < 0.001$; WT_{PAR4-AP+SKI-606} versus KI_{PAR4-AP+SKI-606}: $***P < 0.001$; WT_{vehicle} versus WT_{SKI-606}: $###P < 0.001$; KI_{vehicle} versus KI_{SKI-606}: $###P < 0.001$; WT_{SKI-606} versus WT_{PAR4-AP}: $^*P < 0.05$; KI_{SKI-606} versus KI_{PAR4-AP}: $^^P < 0.01$; WT_{SKI-606} versus WT_{PAR4-AP+SKI-606}: $^^P < 0.01$; KI_{SKI-606} versus KI_{PAR4-AP+SKI-606}: $^^P < 0.001$; number of images: vehicle: WT = 9, KI = 6; SKI-606: WT = 3, KI = 4; PAR4-AP: WT = 5, KI = 5; PAR4-AP + SKI-606: WT = 5, KI = 5). (C) Platelet spreading. SKI-606 alone normalized platelet spreading of KI platelets to WT levels. PAR4-AP activation significantly enhanced spreading in WT platelets, while reducing KI platelet spreading. Additionally, SKI-606 significantly reduced PAR4-AP-stimulated platelet spreading in both genotypes (two-way ANOVA, interaction $F_{(1516, 3)} = 39.72$; $P < 0.0001$; genotype $F_{(1516, 1)} = 110.5$; $P < 0.0001$; treatment $F_{(1516, 3)} = 99.10$; $P < 0.0001$; Bonferroni post-tests: WT_{vehicle} versus KI_{vehicle}: $***P < 0.001$; WT_{PAR4-AP} versus KI_{PAR4-AP}: $***P < 0.001$; WT_{PAR4-AP+SKI-606} versus KI_{PAR4-AP+SKI-606}: $***P < 0.001$; KI_{vehicle} versus KI_{SKI-606}: $###P < 0.001$; WT_{vehicle} versus WT_{PAR4-AP}: $###P < 0.001$; WT_{PAR4-AP} versus WT_{PAR4-AP+SKI-606}: $###P < 0.001$; WT_{SKI-606} versus WT_{PAR4-AP}: $^^P < 0.001$; KI_{SKI-606} versus KI_{PAR4-AP}: $^^P < 0.001$; KI_{SKI-606} versus KI_{PAR4-AP+SKI-606}: $^^P < 0.001$; KI_{vehicle} versus KI_{PAR4-AP}: $###P < 0.001$; KI_{vehicle} versus KI_{PAR4-AP+SKI-606}: $###P < 0.001$; KI_{PAR4-AP} versus KI_{PAR4-AP+SKI-606}: $###P < 0.001$; number of platelets: vehicle: WT = 159, KI = 480; SKI-606: WT = 146, KI = 51; PAR4-AP: WT = 104, KI = 198; PAR4-AP + SKI-606: WT = 101, KI = 265). Image acquisition and analysis is described in *Materials and Methods*. (D and E) In vivo inhibition of Src. Blood was collected before (Pre) and after (Post) administration of 1 mg/kg SKI-606 to anesthetized mice. Blood was diluted 1:20 and seeded onto fibrinogen-coated (25 μ g/ml) 96-well plates. (D) In-cell Western of platelets adhered to fibrinogen. Src phosphorylation was assessed by staining platelets with total Src or pSrc at Tyr⁴¹⁶. As there was variability in the volume of blood collected before and after SKI-606 administration, data were normalized to vehicle (dotted line in right panel) to assess PAR4-induced Src phosphorylation. SKI-606 significantly decreases PAR4-AP-induced Src phosphorylation in both KI and WT samples (repeated-measures ANOVA, SKI-606: $^*P = 0.0228$; WT = 6, KI = 6). (E) Clotting time is significantly increased in KI mice treated with SKI-606 (repeated-measures ANOVA, SKI-606: $^*P = 0.0103$; Bonferroni post-test, KI_{vehicle} versus KI_{SKI-606}: $^*P < 0.05$; WT = 6, KI = 6).

the PSI domain (Barron-Casella et al., 1999; Bougie et al., 2012). To mimic the structural changes induced by the Leu33Pro mutation in humans, we introduced the Pro32-Pro33 sequence in the mouse *Itgb3* locus, replacing the

endogenous Ser32Gln33. Pro32Pro33 mice have altered adhesion and increased velocity in aggregation, resulting in a proaggregatory phenotype observed in tail bleeding and nonfatal thromboembolism. Therefore, our data demonstrate

that the Pro32Pro33 KI mouse model, despite not being a fully humanized allele, replicates the phenotypes observed in the human Pro33 platelets.

In accordance with data obtained from Pro33 human samples, no changes in plasma membrane integrin $\alpha\text{IIb}\beta_3$ expression were observed in KI mice (Goodall et al., 1999). JON/A binding to resting platelets indicates that the murine Pro32Pro33 $\alpha\text{IIb}\beta_3$ is not in an open conformation. This finding, however, does not exclude the possibility that other conformational changes have taken place or that the presence of two successive proline residues increases the flexibility of the PSI/hybrid domains, thus facilitating extension and opening of the $\alpha\text{IIb}\beta_3$ heads. In fact, our findings are consistent with the Pro32Pro33 mutation maintaining the ligand-binding domain in the closed conformation while modifying the transmembrane domains, thus enhancing talin association with the β_3 intracellular carboxy-terminal tail and initiating outside-in signaling (Fig. 7). This unique phenotype confers a gain of function to the receptor without exerting a dramatic deleterious effect, as observed in other gain-of-function integrin β_3 polymorphisms (Ruiz et al., 2001; Mor-Cohen et al., 2007; Ghevaert et al., 2008). The D723H mutation results in constitutive activation of integrin $\alpha\text{IIb}\beta_3$, increased cell adhesion, with no effects on platelet aggregation in vitro. This mutation has dramatic effects on platelet size and is found in Glanzmann thrombasthenia patients, suggesting that constitutive activation of $\alpha\text{IIb}\beta_3$ is deleterious for clotting (Ghevaert et al., 2008). Other mutations disrupting disulfide bridges in the extracellular domains lead to high-affinity binding to soluble fibrinogen and thus result in a loss of platelet aggregation in vitro (Fang et al., 2013). These polymorphisms differ from our KI model because they display increased fibrinogen binding/affinity and represent a constitutively active receptor. The Pro32Pro33 integrin β_3 , though displaying enhanced priming and basal Src signaling, remains sensitive to modulation by inside-out signaling and does not present enhanced JON/A binding or adhesion onto RGD peptides, and thus represents a “facilitated” receptor, but not one that is constitutively active.

Regardless of the molecular mechanism, KI platelets exhibit specific alterations in intracellular Src and FAK signaling (Fig. 7). These signaling changes drive clotting and spreading phenotypes in KI platelets, as both were normalized to wild-type levels in the presence of the Src inhibitor SKI-606. The role of Src (c-Src specifically) in platelet spreading has been established in several studies (Oberfell et al., 2002; Arias-Salgado et al., 2003). Deletion of Src in mouse platelets reduces spreading on fibrinogen independently of other Src-family kinases, but Src is not necessary for the aggregation of platelets under flow conditions (Séverin et al., 2012). Src activation through Tyr⁴¹⁶ phosphorylation can be achieved by several mechanisms, although the data presented here revealed enhanced interactions with the β_3 cytoplasmic domain (Arias-Salgado et al., 2003, 2005; Xiao et al., 2013). c-Src interacts with the last three residues in the intracellular carboxy-terminal tail of integrin β_3 , typically after activation of $\alpha\text{IIb}\beta_3$ and talin binding. This interaction, albeit a low-affinity one, is sufficient to disrupt the interaction between c-Src SH3 domain and its kinase domain, promoting c-Src activation (Xiao et al., 2013). Our data indicate enhanced talin binding, which may “free” the Src-interacting RGT tail of β_3 , thus facilitating Src activation. Future studies

Fig. 7. Mechanism of enhanced outside-in signaling in KI mice. (A) In wild-type mice, $\alpha\text{IIb}\beta_3$ integrin is activated by talin binding upon activation of inside-out signaling, represented here by PAR4 activation. Talin binding separates the intracellular domains of αIIb and β_3 , resulting in the opening of the extracellular domains of $\alpha\text{IIb}\beta_3$ and exposing the ligand-binding domain, recognized by the antibody JON/A. These conformational changes initiated by talin binding also provide a platform for protein associations. While Src binding to β_3 is independent of its phosphorylation state, Src/ β_3 interactions are sufficient to trigger Src autophosphorylation. (B) In KI mice, a mutation within the PSI domain of β_3 allows talin binding in nonstimulated conditions. The increased talin binding likely enhances the probability of Src associations and phosphorylation, thus enhancing platelet activation downstream of fibrinogen binding. These molecular changes may have reached “ceiling levels” in KI platelets, as activation of PAR4 signaling does not further enhance Src phosphorylation.

will reveal the specific Src kinase involved in the activation of KI platelets. A second mechanism worthy of consideration is that Src activation, through its association with FAK, can inhibit RhoA and increase spreading (Panetti, 2002; Serrels and Frame, 2012). Although possible, we feel that this explanation is unlikely to be the mechanism driving the KI phenotypes, as we detected reduced FAK(Tyr³⁷⁹) phosphorylation under basal conditions, a necessary step during the formation of the FAK/Src/integrin $\alpha\text{IIb}\beta_3$ complex (Chan et al., 1994; Cobb et al., 1994; Schaller et al., 1994; Xing et al., 1994). Finally, Src could also be engaged through $\text{G}\alpha_{13}$ activation (Gong et al., 2010). Platelets lacking $\text{G}\alpha_{13}$ have no adhesion-dependent Src(Tyr⁴¹⁶) phosphorylation and reduced spreading. It is possible that $\text{G}\alpha_{13}$ activation through PAR4 stimulation is involved in the spreading of WT samples and could dampen constitutively activated Src in KI mice, as suggested by the significant statistical drug by genotype interactions in the presence of PAR4-AP.

Although several other signaling events may be altered in KI platelets, we capitalized on a Food and Drug Administration–approved Src kinase inhibitor to reverse the Pro32Pro33-induced hypercoagulable state. SKI-606 (bosutinib) is an orally available tyrosine kinase inhibitor approved for the treatment of chronic myelogenous leukemia with low bleeding risk

(Quintas-Cardama et al., 2009; Remsing Rix et al., 2009). Orally available antiplatelet therapies include warfarin, aspirin, clopidogrel, and ticagrelor (Varon and Spectre, 2009). Most of these pharmacotherapies present multiple drug–drug interactions and increased risk for bleeding (Phillips et al., 2005; Varon and Spectre, 2009). Additionally, in the context of stent placement, the human Pro33 allele remains a risk factor for thrombosis and death, even in the presence of dual antiplatelet therapy (Goldschmidt-Clermont et al., 2000; Motovska et al., 2009). Our study may be an important step toward identifying patient-specific, safe, and efficacious therapies as we were able to normalize, but not dramatically reduce, clotting times specifically in Pro32Pro33 platelets. In these studies, SKI-606 normalized Pro32Pro33 platelet response to platelet spreading with fibrinogen and PAR4-AP activation, partially representative of thrombus formation. Further studies will likely reveal more specific pharmacotherapies for patients expressing this polymorphism.

In conclusion, our data 1) demonstrate a functional link between the Pro32Pro33 structural modification in the extracellular PSI domain and cytoplasmic alterations that result in the activation of Src signaling underlying platelet spreading, clotting, and thrombus formation in vivo; and 2) suggest a novel, tailored therapeutic strategy targeting Src to reduce thrombotic risk.

Acknowledgments

The authors thank Randy Blakely, Roy Zent, and Ambra Pozzi for critical reading of the manuscript. The authors thank Christa Gaskill, Heewon Kim, Matt Duvernay, and Jonathan Schoenecker for technical assistance. The authors thank Michael Dohn from the Carneiro laboratory for assistance in editing the manuscript.

Authorship Contributions

Participated in research design: Oliver, Jessen, Sutcliffe, Carneiro.
Conducted experiments: Oliver, Jessen, Crawford, Carneiro.
Contributed new reagents or analytic tools: Sutcliffe, Carneiro.
Performed data analysis: Oliver, Chung, Carneiro.
Wrote or contributed to the writing of the manuscript: Oliver, Sutcliffe, Carneiro.

References

- Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Sabaté M, Fernández C, Stranieri C, Trabetti E, Pignatti PF, and Macaya C (2004) PIA polymorphism and platelet reactivity following clopidogrel loading dose in patients undergoing coronary stent implantation. *Blood Coagul Fibrinolysis* 15:89–93.
- Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, and Shattil SJ (2003) Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci USA* 100:13298–13302.
- Arias-Salgado EG, Lizano S, Shattil SJ, and Ginsberg MH (2005) Specification of the direction of adhesive signaling by the integrin beta cytoplasmic domain. *J Biol Chem* 280:29699–29707.
- Barron-Casella EA, Nebbia G, Rogers OC, King KE, Kickler TS, and Casella JF (1999) Construction of a human platelet alloantigen-1a epitope(s) within murine glycoprotein IIIa: identification of residues critical to the conformation of the antibody binding site(s). *Blood* 93:2959–2967.
- Bergmeier W, Schulte V, Brockhoff G, Bier U, Zirngibl H, and Nieswandt B (2002) Flow cytometric detection of activated mouse integrin alphaIIb beta3 with a novel monoclonal antibody. *Cytometry* 48:80–86.
- Boschelli DH, Wang YD, Ye F, Wu B, Zhang N, Dutia M, Powell DW, Wissner A, Arndt K, and Weber JM et al. (2001) Synthesis and Src kinase inhibitory activity of a series of 4-phenylamino-3-quinolinecarboxitriles. *J Med Chem* 44:822–833.
- Bougie DW, Rasmussen M, Zhu J, and Aster RH (2012) Antibodies causing thrombocytopenia in patients treated with RGD-mimetic platelet inhibitors recognize ligand-specific conformers of alphaIIb beta3 integrin. *Blood* 119:6317–6325.
- Calvete JJ (1994) Clues for understanding the structure and function of a prototypic human integrin: the platelet glycoprotein IIb/IIIa complex. *Thromb Haemost* 72:1–15.
- Carneiro AM, Cook EH, Murphy DL, and Blakely RD (2008) Interactions between integrin alphaIIb beta3 and the serotonin transporter regulate serotonin transport and platelet aggregation in mice and humans. *J Clin Invest* 118:1544–1552.
- Chan PY, Kanner SB, Whitney G, and Aruffo A (1994) A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *J Biol Chem* 269:20567–20574.
- Chen H, Kovar J, Sissons S, Cox K, Matter W, Chadwell F, Luan P, Vlahos CJ, Schutz-Geschwender A, and Olive DM (2005) A cell-based immunocytochemical assay for monitoring kinase signaling pathways and drug efficacy. *Anal Biochem* 338:136–142.
- Cobb BS, Schaller MD, Leu TH, and Parsons JT (1994) Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. *Mol Cell Biol* 14:147–155.
- Dropinski J, Musial J, Jakiel B, Wegrzyn W, Sanak M, and Szczeklik A (2005) Anti-thrombotic action of clopidogrel and P1(A1/A2) polymorphism of beta3 integrin in patients with coronary artery disease not being treated with aspirin. *Thromb Haemost* 94:1300–1305.
- Fang J, Nurden P, North P, Nurden AT, Du LM, Valentin N, and Wilcox DA (2013) C560R beta3 caused platelet integrin alpha II b beta3 to bind fibrinogen continuously, but resulted in a severe bleeding syndrome and increased murine mortality. *J Thromb Haemost* 11:1163–1171.
- Ghevaert C, Salsmann A, Watkins NA, Schaffner-Reckinger E, Rankin A, Garner SF, Stephens J, Smith GA, Debili N, and Vainchenker W et al. (2008) A nonsynonymous SNP in the ITGB3 gene disrupts the conserved membrane-proximal cytoplasmic salt bridge in the alphaIIb beta3 integrin and cosegregates dominantly with abnormal proplatelet formation and macrothrombocytopenia. *Blood* 111:3407–3414.
- Golas JM, Arndt K, Etienne C, Lucas J, Nardin D, Gibbons J, Frost P, Ye F, Boschelli DH, and Boschelli F (2003) SKI-606, a 4-anilino-3-quinolinecarboxitrile dual inhibitor of Src and Abl kinases, is a potent antiproliferative agent against chronic myelogenous leukemia cells in culture and causes regression of K562 xenografts in nude mice. *Cancer Res* 63:375–381.
- Goldschmidt-Clermont PJ, Cooke GE, Eaton GM, and Binkley PF (2000) PIA2, a variant of GPIIbA implicated in coronary thromboembolic complications. *J Am Coll Cardiol* 36:90–93.
- Gong H, Shen B, Flevaris P, Chow C, Lam SC, Voyno-Yasenetskaya TA, Kozasa T, and Du X (2010) G protein subunit Galpha13 binds to integrin alphaIIb beta3 and mediates integrin “outside-in” signaling. *Science* 327:340–343.
- Goodall AH, Curzen N, Panesar M, Hurd C, Knight CJ, Ouwehand WH, and Fox KM (1999) Increased binding of fibrinogen to glycoprotein IIIa-proline33 (HPA-1b, PIA2, Zwb) positive platelets in patients with cardiovascular disease. *Eur Heart J* 20:742–747.
- Jallu V, Poulain P, Fuchs PF, Kaplan C, and de Brevern AG (2012) Modeling and molecular dynamics of HPA-1a and -1b polymorphisms: effects on the structure of the beta3 subunit of the alphaIIb beta3 integrin. *PLoS One* 7:e47304.
- Kim-Kaneyama JR, Miyauchi A, Lei XF, Arita S, Mino T, Takeda N, Kou K, Eto K, Yoshida T, and Miyazaki T et al. (2012) Identification of Hic-5 as a novel regulatory factor for integrin alphaIIb beta3 activation and platelet aggregation in mice. *J Thromb Haemost* 10:1867–1874.
- Knowles JW, Wang H, Itakura H, Southwick A, Myers RM, Iribarren C, Fortmann SP, Go AS, Quertermous T, and Hlatky MA (2007) Association of polymorphisms in platelet and hemostasis system genes with acute myocardial infarction. *Am Heart J* 154:1052–1058.
- Kunicki TJ and Nugent DJ (2002) The influence of platelet glycoprotein polymorphisms on receptor function and risk for thrombosis. *Vox Sang* 83 (Suppl 1): 85–90.
- Lawson C and Schlaepfer DD (2012) Integrin adhesions: who's on first? What's on second? Connections between FAK and talin. *Cell Adhes Migr* 6:302–306.
- Lev EI, Patel RT, Guthikonda S, Lopez D, Bray PF, and Kleiman NS (2007) Genetic polymorphisms of the platelet receptors P2Y(12), P2Y(1) and GP IIIa and response to aspirin and clopidogrel. *Thromb Res* 119:355–360.
- Lippi G, Franchini M, and Targher G (2011) Arterial thrombus formation in cardiovascular disease. *Nat Rev Cardiol* 8:502–512.
- Michelson AD, Barnard MR, Krueger LA, Frelinger AL, 3rd, and Furman MI (2000a) Evaluation of platelet function by flow cytometry. *Methods* 21:259–270.
- Michelson AD, Furman MI, Goldschmidt-Clermont P, Mascelli MA, Hendrix C, Coleman L, Hamlington J, Barnard MR, Kickler T, and Christie DJ et al. (2000b) Platelet GP IIIa (PIA) polymorphisms display different sensitivities to agonists. *Circulation* 101:1013–1018.
- Mor-Cohen R, Rosenberg N, Peretz H, Landau M, Collier BS, Awidi A, and Seligsohn U (2007) Disulfide bond disruption by a beta 3-Cys549Arg mutation in six Jordanian families with Glanzmann thrombasthenia causes diminished production of constitutively active alpha IIb beta 3. *Thromb Haemost* 98:1257–1265.
- Moser M, Nieswandt B, Ussar S, Pozgajova M, and Fässler R (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med* 14:325–330.
- Motovska Z, Widimsky P, Kvasnicka J, Petr R, Bilkova D, Hajkova J, Marinov I, Simek S, and Kala P; PRAGUE-8 study investigators (2009). High loading dose of clopidogrel is unable to satisfactorily inhibit platelet reactivity in patients with glycoprotein IIIa gene polymorphism: a genetic substudy of PRAGUE-8 trial. *Blood Coagul Fibrinolysis* 20:257–262.
- Newman PJ, Derbes RS, and Aster RH (1989) The human platelet alloantigens, PIA1 and PIA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J Clin Invest* 83:1778–1781.
- Nieswandt B, Moser M, Pleines I, Varga-Szabo D, Monkley S, Critchley D, and Fässler R (2007) Loss of talin1 in platelets abrogates integrin activation, platelet aggregation, and thrombus formation in vitro and in vivo. *J Exp Med* 204:3113–3118.
- Obergfell A, Eto K, Mocsai A, Buensuceso C, Moores SL, Brugge JS, Lowell CA, and Shattil SJ (2002) Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. *J Cell Biol* 157:265–275.
- Osada M, Ohmori T, Yatomi Y, Satoh K, Hosogaya S, and Ozaki Y (2001) Involvement of Hic-5 in platelet activation: integrin alphaIIb beta3-dependent tyrosine phosphorylation and association with proline-rich tyrosine kinase 2. *Biochem J* 355:691–697.
- Panetti TS (2002) Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration. *Front Biosci* 7:d143–d150.

- Phillips DR, Conley PB, Sinha U, and Andre P (2005) Therapeutic approaches in arterial thrombosis. *J Thromb Haemost* **3**:1577–1589.
- Quintás-Cardama A, Han X, Kantarjian H, and Cortes J (2009) Tyrosine kinase inhibitor-induced platelet dysfunction in patients with chronic myeloid leukemia. *Blood* **114**:261–263.
- Remsing Rix LL, Rix U, Colinge J, Hantschel O, Bennett KL, Stranzl T, Müller A, Baumgartner C, Valent P, and Augustin M et al. (2009) Global target profile of the kinase inhibitor bosutinib in primary chronic myeloid leukemia cells. *Leukemia* **23**:477–485.
- Ruggeri ZM (2002) Platelets in atherothrombosis. *Nat Med* **8**:1227–1234.
- Ruiz C, Liu CY, Sun QH, Sigaud-Fiks M, Fressinaud E, Muller JY, Nurden P, Nurden AT, Newman PJ, and Valentin N (2001) A point mutation in the cysteine-rich domain of glycoprotein (GP) IIIa results in the expression of a GPIIb-IIIa (alphaIIb-beta3) integrin receptor locked in a high-affinity state and a Glanzmann thrombasthenia-like phenotype. *Blood* **98**:2432–2441.
- Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, and Parsons JT (1994) Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* **14**:1680–1688.
- Schoenwaelder SM, Jackson SP, Yuan Y, Teasdale MS, Salem HH, and Mitchell CA (1994) Tyrosine kinases regulate the cytoskeletal attachment of integrin alpha IIb beta 3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin polymers. *J Biol Chem* **269**:32479–32487.
- Serrels B and Frame MC (2012) FAK and talin: who is taking whom to the integrin engagement party? *J Cell Biol* **196**:185–187.
- Séverin S, Nash CA, Mori J, Zhao Y, Abram C, Lowell CA, Senis YA, and Watson SP (2012) Distinct and overlapping functional roles of Src family kinases in mouse platelets. *J Thromb Haemost* **10**:1631–1645.
- Smyth SS, Reis ED, Väänänen H, Zhang W, and Collier BS (2001) Variable protection of beta 3-integrin—deficient mice from thrombosis initiated by different mechanisms. *Blood* **98**:1055–1062.
- Tadokoro S, Shattil SJ, Eto K, Tai V, Liddington RC, de Pereda JM, Ginsberg MH, and Calderwood DA (2003) Talin binding to integrin beta tails: a final common step in integrin activation. *Science* **302**:103–106.
- Undas A, Brummel K, Musial J, Mann KG, and Szczeklik A (2001) PI(A2) polymorphism of beta(3) integrins is associated with enhanced thrombin generation and impaired antithrombotic action of aspirin at the site of microvascular injury. *Circulation* **104**:2666–2672.
- Varon D, and Spectre G (2009) Antiplatelet agents. *Hematology Am Soc Hematol Educ Program* 2009:267–272.
- Vielreicher M, Harms G, Butt E, Walter U, and Obergfell A (2007) Dynamic interaction between Src and C-terminal Src kinase in integrin alphaIIb beta3-mediated signaling to the cytoskeleton. *J Biol Chem* **282**:33623–33631.
- Vijayan KV, Huang TC, Liu Y, Bernardo A, Dong JF, Goldschmidt-Clermont PJ, Alevriadou BR, and Bray PF (2003a) Shear stress augments the enhanced adhesive phenotype of cells expressing the Pro33 isoform of integrin beta3. *FEBS Lett* **540**:41–46.
- Vijayan KV, Liu Y, Dong JF, and Bray PF (2003b) Enhanced activation of mitogen-activated protein kinase and myosin light chain kinase by the Pro33 polymorphism of integrin beta 3. *J Biol Chem* **278**:3860–3867.
- Vijayan KV, Liu Y, Li TT, and Bray PF (2004) Protein phosphatase 1 associates with the integrin alphaIIb subunit and regulates signaling. *J Biol Chem* **279**:33039–33042.
- Vijayan KV, Liu Y, Sun W, Ito M, and Bray PF (2005) The Pro33 isoform of integrin beta3 enhances outside-in signaling in human platelets by regulating the activation of serine/threonine phosphatases. *J Biol Chem* **280**:21756–21762.
- Vinogradova O, Haas T, Plow EF, and Qin J (2000) A structural basis for integrin activation by the cytoplasmic tail of the alpha IIb-subunit. *Proc Natl Acad Sci USA* **97**:1450–1455.
- Wang R, McFarland JG, Kekomaki R, and Newman PJ (1993) Amino acid 489 is encoded by a mutational “hot spot” on the beta 3 integrin chain: the CA/TU human platelet alloantigen system. *Blood* **82**:3386–3391.
- Wang R and Newman PJ (1998) Adhesive and signaling properties of a naturally occurring allele of glycoprotein IIIa with an amino acid substitution within the ligand binding domain—the Pena/Penb platelet alloantigenic epitopes. *Blood* **92**:3260–3267.
- Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH, and Campbell ID (2007) Structural basis of integrin activation by talin. *Cell* **128**:171–182.
- Xiao R, Xi XD, Chen Z, Chen SJ, and Meng G (2013) Structural framework of c-Src activation by integrin β_3 . *Blood* **121**:700–706.
- Xing Z, Chen HC, Nowlen JK, Taylor SJ, Shalloway D, and Guan JL (1994) Direct interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. *Mol Biol Cell* **5**:413–421.
- Xiong JP, Stehle T, Goodman SL, and Arnaout MA (2004) A novel adaptation of the integrin PSI domain revealed from its crystal structure. *J Biol Chem* **279**:40252–40254.
- Yang J, Ma YQ, Page RC, Misra S, Plow EF, and Qin J (2009) Structure of an integrin alphaIIb beta3 transmembrane-cytoplasmic heterocomplex provides insight into integrin activation. *Proc Natl Acad Sci USA* **106**:17729–17734.

Address correspondence to: Dr. Ana M. Carneiro, Vanderbilt University Medical Center, 461B Preston Research Building, 23rd Avenue South at Pierce, Nashville, TN 37232-6600. E-mail: ana.carneiro@vanderbilt.edu