MINIREVIEW

Molecular Mechanisms of Prothrombotic Risk Due to Genetic Variations in Platelet Genes: Enhanced Outside-In Signaling Through the Pro^{33} Variant of Integrin β_3

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In recent years inherited variations in platelet proteins have emerged as potential risk factors that could predispose individuals to arterial thrombosis. Although many studies have examined the association of platelet gene polymorphisms with particular disease states, the underlying mechanisms by which most of these polymorphisms contribute to the pathophysiology of thrombosis have remained largely unexplored. This review will focus on the cellular and molecular features by which these genetic changes affect platelet physiology. Although many genes have been investigated in this regard, only the genes encoding integrins β_3 and α_2, and the platelet Fc receptor, $Fc_\gamma RIIA$, have been studied in any depth. In some cases (such as integrin α_2), evidence supports a quantitative trait locus. For other genes, nonsynonymous nucleotide substitutions lead to structural and functional consequences. A large portion of this review will focus on the widely studied Leu33Pro (PI^A) polymorphism of integrin β_3, and will consider the potential mechanisms by which the Pro^{33} polymorphism could induce a prothrombotic risk. A detailed understanding of how polymorphisms modulate platelet physiology will be important for understanding individual differences in response to antiplatelet therapy. Exp Biol Med 231:505–513, 2006

Key words: platelets; genes; polymorphism; integrin; thrombosis; signaling; adhesion; aggregation

Introduction

Platelets actively participate in vascular thrombosis, resulting in myocardial infarction and stroke, and there is an established genetic component in the pathophysiology of these multifactorial diseases (1–3). Over the past decade much effort has been invested in the study of genetic variations that may modify these disease processes, and several polymorphisms in platelet receptors have been associated with the clinical phenotype. Most experts agree that the genetic risk for the majority of patients with cardiovascular disease amounts to the sum of small effects from numerous different genes (4). Considering the high prevalence and life-threatening potential of cardiovascular disease, the overall significance of even modest effects of prothrombotic platelet polymorphisms may be substantial. Furthermore, the pharmacogenetic interaction between antiplatelet therapies and platelet polymorphisms (5, 6) emphasizes the importance of understanding the effect these inherited variations have on platelet physiology. The clinical aspects of platelet receptor polymorphisms have been reviewed in detail elsewhere (7, 8) and will not be the focus of this review. Rather, we will consider the mechanisms by which polymorphisms may enhance platelet functions, with an emphasis on the nonsynonymous Leu33Pro (PI^A) polymorphism of integrin β_3.

Genetic Mechanisms by Which Polymorphisms Affect Platelet Function. Polymorphisms are stable genetic variations represented in more than 1% of the chromosomes in a given population. Variations in the human genetic sequence occur approximately once every 1,000 base pairs, and there are 200,000 amino acid–altering single nucleotide polymorphisms (SNPs), indicating that genetic variations are a common feature of the human

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Table 1. Platelet Gene Polymorphism with Evidence for Altered Platelet Function

Quantitative trait loci	Structure/function	Unknown
Integrin α_2 807 T/C	Integrin β_3 Leu33Pro	α_2AR long/short (11)
Integrin α_2 −52 T/C (12)	Fc_γRIIA	P_2Y_{12} (13)
Integrin α_2 −92 T/C (14)	GPIbα Ko (15, 16)	Serotonin receptor T102C (17); 44-base pair insertion/deletion (18)
GPVI (19)	GPIbα VNTR (16)	c-mpl 550 C/A (20)
GPIbα −5T/C (21)	α_2AR Asn251Lys (22)	PAR1 IVS-14A/T (23)
	GNB3 (24)	
	Integrin α_2 Thr799Met (25)	

genome (9, 10). Table 1 summarizes those genes and polymorphisms for which there is evidence for a functional consequence of the genetic alteration. There are two general mechanisms by which DNA polymorphisms might affect platelet function: either by affecting the quantity or the quality of the protein product. The polymorphisms in Table 1 are grouped according to one of these mechanisms, or if the mechanism is unclear it is listed as "unknown." Only three polymorphisms (one in each of three genes) have been characterized in detail: integrins α_2 and β_3, and Fc_γRIIA. For the remainder, only one or two publications have addressed the physiologic effects of the polymorphism, and the key references are listed in Table 1. It should also be appreciated that in some cases it may not be clear whether the polymorphism is itself causative or is in strong linkage disequilibrium (LD) with the causative genetic change.

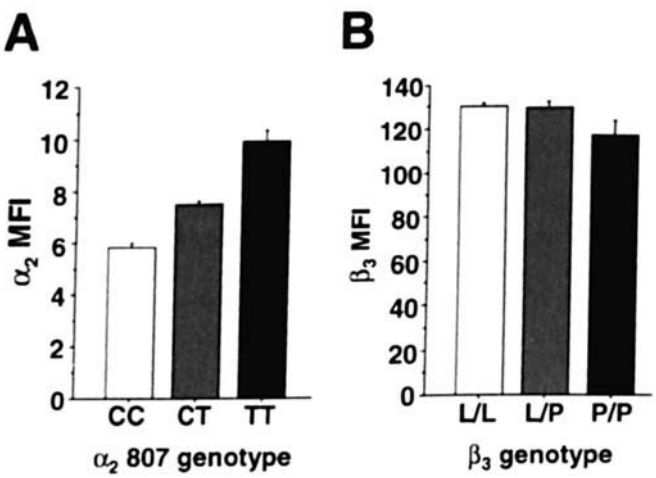

Figure 1. Platelet surface integrin α_2 and β_3 levels by genotype. Levels of integrins $\alpha_2\beta_1$ (A) and $\alpha_{IIb}\beta_3$ (B) were determined on resting platelets in whole blood from 385 healthy donors using flow cytometry and monoclonal antibodies specific for α_2 and β_3. There were significant differences in α_2 expression between subjects carrying the CC and CT genotype ($P < 0.001$), CC and TT genotype ($P < 0.001$), and CT and TT genotype ($P < 0.001$). The numbers of subjects with CC, CT, and TT genotypes were 141, 169, and 48, respectively. In contrast, no significant differences in β_3 expression between Leu^{33}/Leu^{33} (L/L) and Leu^{33}/Pro^{33} (L/P) platelets ($P = 0.78$), Leu^{33}/Leu^{33} (L/L) and Pro^{33}/Pro^{33} (P/P) platelets ($P = 0.08$), or Leu^{33}/Pro^{33} (L/P) and Pro^{33}/Pro^{33} (P/P) platelets ($P = 0.11$) were noted. There were 305 Leu^{33}/Leu^{33} subjects, 72 Leu^{33}/Pro^{33} subjects, and 8 Pro^{33}/Pro^{33} subjects. Data are mean ± SE of the mean fluorescence intensity.

Quantitative Trail Loci. Perhaps the simplest means by which a polymorphism would affect platelet function is by altering the quantity of the protein that is expressed by enhancing either transcription or translation. This could occur with polymorphisms in noncoding sequences of a gene. Integrin $\alpha_2\beta_1$ (gene *ITGA2*) is one of the platelet collagen receptors. The laboratory of T.J. Kunicki and coworkers was the first to show that platelets from subjects with the 807T allele have higher levels of $\alpha_2\beta_1$, and this has been confirmed by other groups (26–29). Higher levels of $\alpha_2\beta_1$ expression correlates with a greater rate of platelet attachment to surfaces coated with collagen Type I under shear stress (27). Thus, carriage of the 807T allele would be predicted to yield a prothrombotic platelet phenotype. The 807T/C SNP is synonymous and likely not responsible for the differences in receptor levels. This SNP is in linkage disequilibrium with several other polymorphisms in the *ITGA2* gene (30), including an SNP at −52, which may influence the density of $\alpha_2\beta_1$ receptors (12). Thus, it is possible that the 807T polymorphism tracks with the causative SNP at −52, which is responsible for altered *ITGA2* transcription and thus, enhanced platelet adhesion to collagen surfaces.

Although the T→C nucleotide substitution at position 1565 in exon 2 of the β_3 gene would not be expected to alter gene expression or protein stability, this has not been formally examined. We have quantified integrin $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ expression on platelets from 385 healthy human subjects (Fig. 1). We confirmed the association between 807T/C and $\alpha_2\beta_1$ expression, but observed no significant difference in $\alpha_{IIb}\beta_3$ expression according to the Leu33Pro genotype. In addition, we have made a number of different cell lines with each isoform and have no evidence that the Leu33Pro substitution affects protein stability or expression (unpublished data). Thus, it is unlikely that altered $\alpha_{IIb}\beta_3$ levels in the Pro^{33}-positive platelets could explain a prothrombotic phenotype for these platelets.

Amino Acid Substitutions Altering Function. If polymorphisms occur in the coding sequence of the gene and alter an amino acid, a change in the structure, antigenicity, or function of the encoded protein (or a combination of these) is possible.

Table 2. Association of the Leu33Pro Polymorphism with Platelet Aggregation[a]

	Leu^{33}/Leu^{33} (n = 1017)	Pro^{33}/Leu^{33} (n = 369)	Pro^{33}/Pro^{33} (n = 36)	P value[b]
Epinephrine (μM)	0.9 (0.9–1.0)	0.7 (0.7–0.9)	0.6 (0.4–1.0)	0.007
ADP (μM)	3.1 (3.0–3.2)	3.0 (2.9–3.2)	2.8 (2.4–3.3)	0.190

[a] Data represent the mean minimal agonist concentration producing irreversible platelet aggregation.
[b] P values obtained using analysis of variance, after adjusting for potential confounding factors such as age, sex, body mass index, diabetics, triglyceride, total and high-density lipoprotein cholesterol, cardiovascular disease, menopausal status, and estrogen replacement therapy. Reproduced with permission following modification (45).

$Fc_\gamma RIIA$ His131Arg. Platelets have a single Fc receptor, $Fc_\gamma RIIA$. This receptor is also expressed on T lymphocytes, where it has variable ability to induce mitogenesis after binding mouse IgG_1 (31), depending upon whether residue 131 is a histidine or an arginine (32). Compared with the Arg131 isoform, the His131 form binds murine IgG_1 with lower affinity than does the Arg131 form; the Arg131 form binds human IgG_2 poorly (33). We have shown that this polymorphism of *FCGR2A* does not affect intrinsic platelet reactivity, but that some activation-dependent platelet monoclonal antibodies bind preferentially to Arg131-positive platelets, cross-link $Fc_\gamma RIIA$, and activate platelets (34). Thus, assays of cellular responsivity using monoclonal antibodies and cells expressing $Fc_\gamma RIIA$ must take caution to use Fab fragments, block the $Fc_\gamma RIIA$, or otherwise consider the His131Arg genotype in the data analysis (34).

Integrin β_3 Leu33Pro (PI^A). The Leu33Pro polymorphism of integrin (glycoprotein IIIa) was originally identified because of its prominence in causing alloimmune thrombocytopenias (35). Newman *et al.* (36) identified the genetic basis for this polymorphism, which induces a Leu33Pro substitution, and which alters integrin β_3 antigenicity. This study also demonstrated that Leu33 was responsible for the PI^{A1} epitope, and Pro33 for the PI^{A2} epitope. The prevalence of the PI^{A1} and PI^{A2} alleles varies by ethnic background and geographical distribution. The common allele is PI^{A1} with reported frequencies of 0.84–0.89 in whites, 0.92 in blacks, and >0.99 in Asians. The PI^{A2} allele is not rare in certain populations, occurring with a frequency of 0.11–0.15 in whites and 0.08 in blacks. However, the PI^{A2} allele is nearly absent in Asians (<0.01). This polymorphism was also the first platelet variation linked to myocardial infarction (37), findings that have led to a plethora of subsequent genetic epidemiology studies in this area. Meta-analysis studies concluded that the Pro^{33} polymorphism offers a mild risk for myocardial infarction but not stroke, especially for younger subjects and those undergoing revascularization procedures (38, 39). As will be explained, the weight of the evidence suggests this polymorphism affects integrin β_3 function.

Linkage Disequilibrium with a Causative Gene. Linkage disequilibrium refers to the phenomenon wherein a combination of alleles occurs more frequently than what would be predicted by a chance. LD between alleles occurs because of infrequent chromosomal crossover between genes during meiosis. For any polymorphism that has been associated with a clinical or physiologic phenotype, it should always be considered that it is merely in LD with (i.e., a "marker" for) the causative variation, such as those described above. For example, it is conceivable that the mere presence of C at 1565 on β_3 is not directly responsible for the observed prothrombotic phenotype in the Pro^{33}-positive platelets, but rather the β_3 gene containing 1565 C may be in LD with a different causative variation in the same or different gene. To date, there is no evidence to support the possibility of this polymorphism.

Physiologic Consequences of the Leu33Pro Polymorphism of Integrin β_3. There is strong immunologic evidence that the Leu33Pro substitution alters protein structure, because each antigen can induce an immune response in individuals lacking that antigen. The initial evidence suggesting that this structural change may affect platelet function came from observations that most anti-Leu^{33} antibodies block fibrinogen binding and inhibit aggregation of Leu^{33}/Leu^{33} platelets, and retard or partially inhibit aggregation of Pro^{33}/Leu^{33} platelets (40), despite the linear distance between position 33 and the putative fibrinogen binding sites (amino acids 109–171 and 211–222) on integrin β_3 (41, 42). This issue has been examined formally in subsequent studies with platelets and stably transfected cell lines.

Studies with Platelets. *In vivo* studies of platelet function found Pro^{33}-positive subjects have significantly shorter bleeding times, and enhanced thrombin generation and prothrombin consumption than Pro^{33}-negative subjects (5, 43, 44). The largest study of *ex vivo* platelet function was the Framingham Offspring Study, which included 1422 subjects (45), and which considered confounders in their analyses. This study found that significantly less epinephrine was required to induce platelet aggregation in the Pro^{33}-positive than in Pro^{33}-negative platelets, and this effect was PI^{A2} allele dose dependent (i.e., a dominant effect) (Table 2). A similar trend was observed when platelets were stimulated with ADP. We have found that compared with Pro^{33}-negative platelets, Pro^{33}-positive platelets exhibit increased α granule secretion in response to ADP and thrombin (5, 46). Other studies using small numbers of subjects have obtained inconsistent results, perhaps due to differences in the assays, study design, or variation in donor

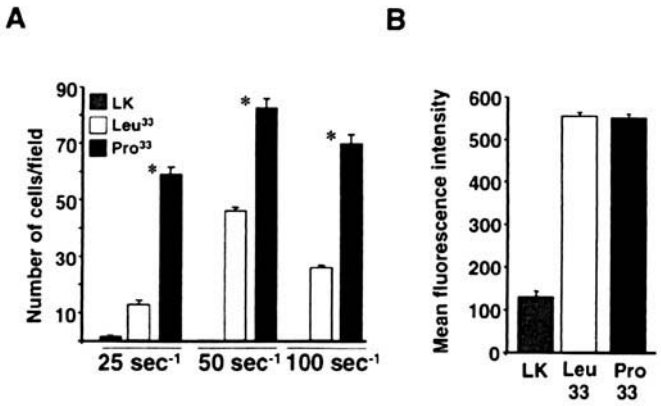

Figure 2. Adhesion of Leu^{33} and Pro^{33} CHO cells on fibrinogen under fluid shear stress. (A) Cells were perfused over 100 μg/ml fibrinogen in a parallel plate flow chamber and the number of adherent cells quantified. LK is the control CHO cells transfected with LK444 parental vector. Compared with Leu^{33} cells, Pro^{33} cells show 4.5-fold increased adhesion at 25 $secs^{-1}$, 1.7-fold increased adhesion at 50 $secs^{-1}$, and 2.6-fold increased adhesion at 100 $secs^{-1}$. The increased adhesion of Pro^{33} over Leu^{33} was significant at $^*P < 0.001$. (B) Mean fluorescence intensity of integrin $\alpha_{IIb}\beta_3$ was detected using P2 monoclonal antibody to the three cell lines; $n = 3$. Reproduced with permission following modification (54).

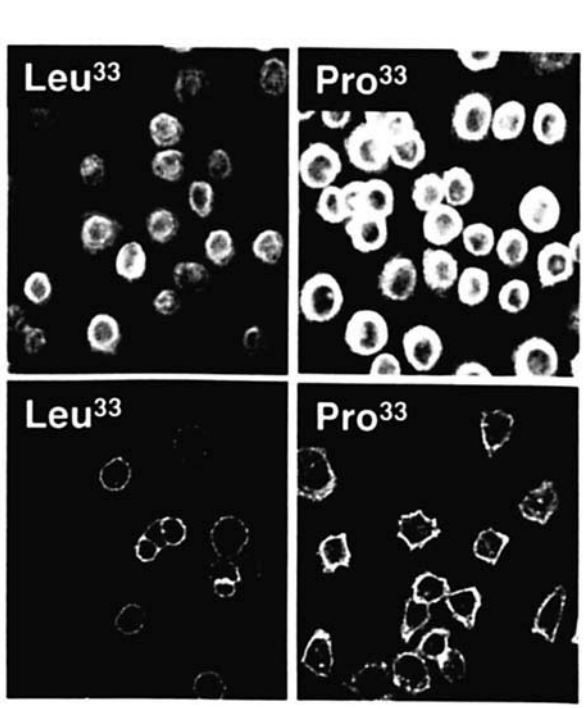

Figure 3. Confocal microscopy (A) following rhodamine phallodin staining of CHO cells that adhered to a fibrinogen-coated coverslip for 5 mins. Compared with the Leu^{33} cells an increased F-actin staining was observed for the Pro^{33} cells. (B) Following 15 mins of incubation on fibrinogen, greater spreading of the Pro^{33} cells was observed. The morphometric analysis of 35–50 cells revealed a significant increase ($P < 0.001$ and $P = 0.01$) for F actin and spreading by the Pro^{33} cells, respectively. Reproduced with permission following modification (52).

characteristics that might affect platelet function (6, 47–51). Considering the marked variation in platelet function between individuals, it is difficult to interpret the results of small studies that do not consider confounding variables such as age, gender, etc. But taken together, the bulk of the available evidence suggests that the Pro^{33} polymorphism of integrin β_3 can alter human platelet aggregation, adhesion, and secretion.

Studies with Heterologous Cells. To overcome the limitation caused by heterogeneity of human platelets, model systems including Chinese Hamster Ovary (CHO) and human kidney embryonal 293 cell lines overexpressing either the Leu^{33} or Pro^{33} isoforms of $\alpha_{IIb}\beta_3$ have been utilized (52). These cells were used to study integrin outside-in signaling functions such as adhesion, spreading, migration, and clot retraction. Compared with the Leu^{33} cells, a significant 30%–60% increase in $\alpha_{IIb}\beta_3$-mediated adhesion and haptotactic migration of the Pro^{33} cells to immobilized fibrinogen under static conditions was observed (52, 53). Pro^{33} cells also displayed increased fibrin clot retraction. We performed these assays only when the Leu^{33} and Pro^{33} cell lines had equivalent levels of $\alpha_{IIb}\beta_3$ expression. Another group found no effect of the polymorphism on static adhesion to fibrinogen using a lymphocyte cell line, GM1500, following stimulation with exogenous phorbol myristate acetate (51). Figure 2 shows how compared with Leu^{33} cells, fluid shear stress augmented the difference in adhesiveness of Pro^{33} cells to fibrinogen by 1.7-fold to 4.5-fold (54). Adhesion under shear was integrin $\alpha_{IIb}\beta_3$-mediated and substrate specific, because the increased Pro^{33} adhesiveness was observed with von Willebrand factor (VWF) and cryoprecipitate (composed of VWF and fibrinogen) but not fibronectin (54). Confocal microscopy revealed a greater spreading and extensive actin polymerization of the Pro^{33} cells compared with the Leu^{33} cells on fibrinogen substrate (Fig. 3) (52). In addition, compared with the Leu^{33} cells, Pro^{33}-expressing $\alpha_v\beta_3$ cells had an enhanced haptotactic migratory response to vitronectin and osteopontin but not to fibrinogen, suggesting that the α subunit influences the substrate specificity of this genetic effect (53). In summary, studies with human platelets and heterologous cells demonstrate that the $\alpha_{IIb}\beta_3$-dependent functions of aggregation, secretion, adhesion, spreading, migration, and clot retraction are increased by the Pro^{33} isoform (Fig. 4).

Potential Cellular and Molecular Mechanisms for the Prothrombotic Phenotype Associated with the Pro^{33} Polymorphism. How could the structural alteration of the Pro^{33} isoform confer increased $\alpha_{IIb}\beta_3$ function? In this section we will consider an approach to evaluating the potential mechanisms by which any nonsynonymous polymorphism might induce a prothrombotic platelet phenotype, using the Pro^{33} isoform of integrin β_3 as a prototype. A few factors that are likely to play a critical role are discussed below.

Increased Affinity for Fibrinogen by the Pro^{33} Isoform of $\alpha_{IIb}\beta_3$. It is conceivable that the conformational change induced by the Pro^{33} isoform of integrin $\alpha_{IIb}\beta_3$ could increase the affinity of the receptor for its ligands. However,

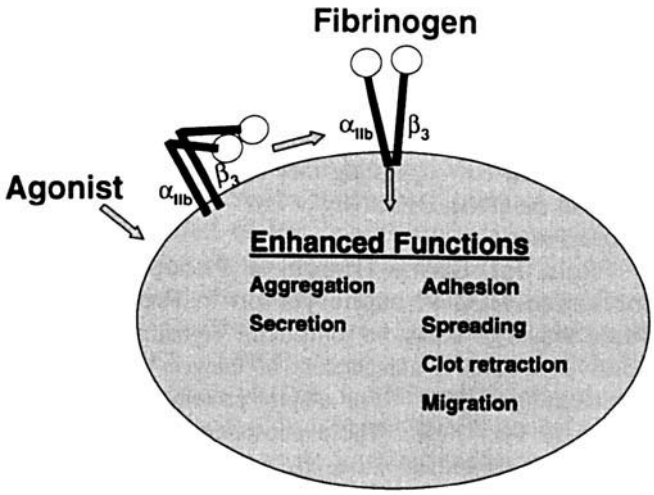

Figure 4. Functional consequences of the Pro^{33} isoform of integrin β_3. Fibrinogen binding by the Pro^{33} isoform of integrin β_3 results in an increased $\alpha_{IIb}\beta_3$-mediated function in platelets and heterologous cells.

Bennett *et al.* (51) observed no significant difference in the affinity of fibrinogen binding or in maximal fibrinogen binding between the Pro^{33}-positive and the Pro^{33}-negative platelets. The concentration of fibrinogen producing half-maximal binding was not significantly different for the Pro^{33}-positive and the Pro^{33}-negative CHO cells, suggesting that the binding kinetic of soluble fibrinogen was not altered by the Pro^{33} isoform (52). Thus, these two studies found no evidence that the Pro^{33} isoform alters the affinity of the $\alpha_{IIb}\beta_3$ for fibrinogen. It is somewhat difficult to reconcile this conclusion with the enhanced agonist-induced platelet aggregation observed in the Framingham study (45). Furthermore, when we purified $\alpha_{IIb}\beta_3$ from human platelets of known Leu33Pro genotype we found that purified human Pro^{33}-positive integrins exhibited an enhanced binding to immobilized fibrinogen and prothrombin,[1] suggesting an intrinsic difference in the Pro^{33} isoform that favors fibrinogen binding. Thus, the possibility that the Pro^{33} isoform alters affinity or otherwise enhances inside-out signaling to $\alpha_{IIb}\beta_3$ remains unclear.

Increased $\alpha_{IIb}\beta_3$ Avidity by the Pro^{33} Isoform. Li *et al.* (55) have shown that integrins cluster after activation, and the loop of integrin β_3 containing residue 33 is extended to a position that would allow it to participate in receptor oligomerization (56). Thus, it is possible that the Pro^{33} isoform could alter the avidity of the $\alpha_{IIb}\beta_3$ *via* increased clustering. To date, no studies have examined whether the Leu→Pro 33 change affects the avidity of $\alpha_{IIb}\beta_3$. However, the distribution patterns of integrin $\alpha_{IIb}\beta_3$ as detected by fluorescence microscopy were similar in Leu^{33} and Pro^{33} CHO cells that adhered to fibrinogen (54). This observation may indirectly suggest that the enhanced adhesion of the Pro^{33} cells to fibrinogen is not caused by extensive integrin clustering.

Differential Association of Transmembrane Signaling Molecules by the Pro^{33} Isoform of $\alpha_{IIb}\beta_3$. Integrin $\alpha_{IIb}\beta_3$ has been shown to associate with transmembrane molecules, including some that transduce signals, such as $Fc_\gamma RIIa$, which contains an immunoreceptor tyrosine-based activation motif; platelet-derived growth factor receptor β; and integrin-associated protein and tetraspannin family members CD9 and CD63 (57–60). The conformational change in the extracellular region of integrin β_3 in the Pro^{33}-positive platelets could alter its interaction with some of these transmembrane signaling molecules. None of the published studies on the Pro^{33} polymorphism have addressed this possibility. In unpublished studies we could not detect an association between $\alpha_{IIb}\beta_3$ and platelet endothelial cell adhesion molecule-1 (which contains an immunoreceptor tyrosine-based inhibitor motif) that was dependent upon the Leu33Pro genotype. Additional studies are needed to clarify whether the Pro^{33} isoform modulates the interaction of transmembrane signaling and adhesion molecules.

Increased $\alpha_{IIb}\beta_3$ Outside-In Signaling by the Pro^{33} Isoform. Ligand binding to $\alpha_{IIb}\beta_3$ induces outside-in signaling, which results in rearrangement of the platelet actin cytoskeleton and activation of signaling molecules (61). Rearrangement of the platelet cytoskeleton is essential for platelet shape change, spatial regulation of receptors, creation of membrane microdomains, and recruitment of several kinases and phosphatases. Integrin outside-in signaling results in the phosphorylation of multiple proteins including the focal adhesion kinase (FAK), extracellular-signal regulated kinase 2 (ERK2), and myosin light chain (MLC). These functions collectively integrate into increased platelet reactivity.

Signaling to FAK, ERK2, and MLC. Compared with Leu^{33} cells, adhesion of Pro^{33} CHO or 293 cells (or both cell types) to immobilized fibrinogen shows increased phosphorylation of FAK at Tyr^{125}, ERK2 at Thr^{183} and Tyr^{185}, and MLC at Thr^{18} and Ser^{19} (52, 62). The increased adhesiveness and migration of the Pro^{33} CHO cells is abolished by inhibiting actin cytoskeleton rearrangement with cytochalasin-D (52–54). The $\alpha_{IIb}\beta_3$ outside-in signaling changes exhibited by the Pro^{33} isoform are also observed with human platelets after adhesion to fibrinogen and thrombin-induced aggregation (46, 62). The enhanced cellular function (adhesion, migration, and clot retraction) exhibited by the Pro^{33} CHO cells are abolished by inhibiting ERK2 phosphorylation with mitogen-activated protein kinase kinase (MEK) inhibitors PD98059 and U0126 (46, 53). These results are consistent with the established role of ERK2 signaling in regulating cell adhesion, spreading, and haptotactic and chemotactic cell migration, (63–66).

MLC phosphorylation promotes myosin ATPase activity and increases the actinomyosin contractile response that is involved in platelet shape change, secretion, ADP-

[1] Vijayan KV, Bray PF. Unpublished data.

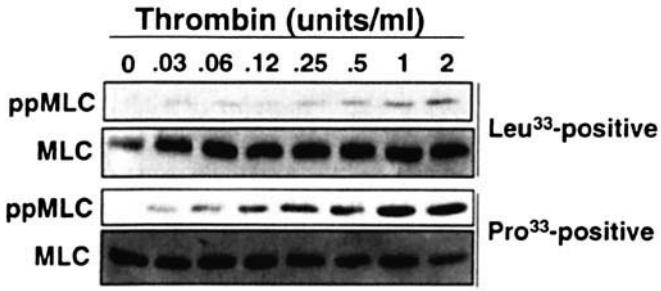

Figure 5. Increased MLC phosphorylation in Pro^{33}-positive platelets. Leu^{33}-positive or Pro^{33}-positive platelets were aggregated using varying concentrations of thrombin and blotted with antiphospho MLC (ppMLC) or total MLC. Reproduced with permission following modification (62).

induced aggregation, and migration (67, 68). Pro^{33}-positive platelets exhibit increased platelet MLC phosphorylation and enhanced α granule (P-selectin) secretion. Blocking MLC phosphorylation abolishes the increased adhesiveness of the Pro^{33} cells and increased secretion of the Pro^{33}-positive platelets, suggesting that signaling through MLC mediates the enhanced activity of Pro^{33}-expressing cells and platelets (46, 62).

Role for Serine-Threonine Phosphatases in Increased MLC Phosphorylation in Pro^{33}-Positive Platelets. The enhanced outside-in signaling in Pro^{33}-positive platelets is independent of integrin β_3 phosphorylation at Tyr^{747}, Tyr^{759}, or adaptor protein Shc phosphorylation at Tyr^{317} (62). These phosphorylation events are believed to be critical in $\alpha_{IIb}\beta_3$ outside-in signaling and ERK2 phosphorylation (69, 70). The phosphorylation state of platelet signaling molecules at any time is a composite of

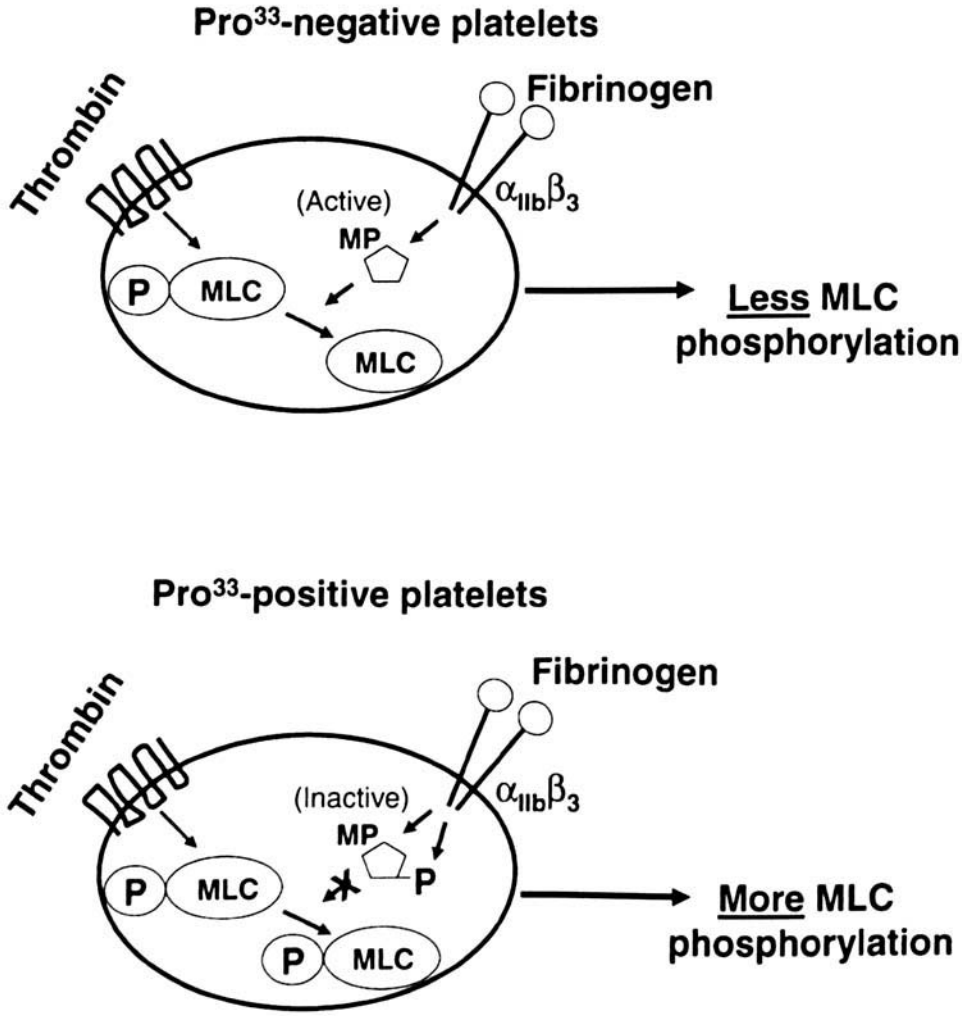

Figure 6. Model for increased MLC phosphorylation in Pro^{33}-positive platelets. Platelet agonists such as thrombin induce robust phosphorylation of MLC. In contrast, $\alpha_{IIb}\beta_3$-fibrinogen interaction leads to dephosphorylation of MLC. Thus, the phosphorylation pattern of MLC during thrombin-induced platelet aggregation is a composite of both these signaling events. Blocking $\alpha_{IIb}\beta_3$-fibrinogen interaction or inhibiting myosin phosphatase (MP) blocks the dephosphorylation of MLC during aggregation, suggesting that $\alpha_{IIb}\beta_3$-fibrinogen interaction activates a serine/threonine phosphatase. However, in case of the Pro^{33}-positive platelets, $\alpha_{IIb}\beta_3$-fibrinogen interaction also results in an increased phosphorylation of MP at Thr^{696}. Phosphorylated MP is inactive, and therefore, compared with the Pro^{33}-negative platelets, integrin engagement in the Pro^{33}-positive platelets does not effectively dephosphorylate MLC, resulting in an increased MLC phosphorylation. Reproduced with permission of the publisher following modification (62).

agonist-mediated phosphorylation and outside-in integrin-mediated dephosphorylation. Figure 5 shows that when platelets are stimulated with thrombin, MLC shows much greater phosphorylation in the Pro^{33}-positive platelets than in the Pro^{33}-negative platelets (62). However, when the $\alpha_{IIb}\beta_3$-fibrinogen interaction is blocked, MLC phosphorylation dramatically increases in the Pro^{33}-negative platelets, with a minor effect on Pro^{33}-positive platelets, suggesting that integrin engagement negatively regulates MLC phosphorylation, and this process is modified by the Pro^{33} isoform. The Ser/Thr phosphatase inhibitor okadaic acid enhances ERK2 and MLC phosphorylation more efficiently in the Pro^{33}-negative platelets, indicating that a phosphatase is involved in the Pro^{33}-dependent effects on MLC and ERK2. Taken together, these signaling studies indicate: (i) $\alpha_{IIb}\beta_3$ engagement activates Ser/Thr phosphatases, and (ii) residue 33 of integrin β_3 regulates the activation of Ser/Thr phosphatases. Furthermore, Pro^{33}-positive platelets exhibit an increased phosphorylation of the PP1-myosin phosphatase (MP) that dephosphorylates MLC (62). This phosphorylation occurs at Thr^{696} in the myosin binding subunit of MP, thus blocking the MP activity. Thus, compared with the Leu^{33}-positive platelet MP activity is inactivated in aggregating Pro^{33}-positive platelets. The model shown in Figure 6 indicates how the increased MLC phosphorylation observed during the aggregation of Pro^{33}-positive platelet is due in part to inefficient activation of a serine/threonine phosphatase *via* increased Thr^{696} phosphorylation.

Clues from $\alpha_{IIb}\beta_3$ Crystal Structure. The extracellular domains of recombinant $\alpha_{IIb}\beta_3$ have now been crystallized. Can we glean any clues from this structure information that might help us understand the functional and signaling effects regulated by the leucine or proline at residue 33 of integrin β_3? Xiao *et al.* (56) have identified three conformations of extracellular $\alpha_{IIb}\beta_3$: (i) a low-affinity, bent conformation; (ii) an extended form of intermediate affinity with the two N-terminal headpieces in a closed conformation; and (iii) a ligand-bound conformation with α_{IIb} and β_3 extended, but with the headpieces open and in a high-affinity state. The headpiece of β_3 consists of the βI, hybrid, and plexin-semaphorin-integrin (PSI) domains. PSI domains are conserved in other species and known to bind other proteins, and there is evidence to indicate that the PSI domain participates in integrin activation (71). The Leu^{33} residue of β_3 is in the long and flexible loop of the PSI domain. Xiao *et al.* proposed that after ligand binding the rigid nature of the hybrid and PSI domains causes a widening of the angle between the α and β legs that favors leg extension and separation. The substitution of a helix-favoring residue Leu with a helix-disrupting residue Pro is likely to alter the local conformation of integrin β_3—particularly because there is a Pro at position 36. Assuming the Pro^{33} isoform of β_3 causes no major structural alterations, a PSI domain containing a Pro^{33} might be more rigid, which could favor leg separation, or provide greater stability to the open-head conformation after ligand binding than would the Leu^{33} isoform (or both). Such changes might favor outside-in signaling.

It is also noteworthy that the Leu^{33} residue is contained within three disulfide loops: Cys13-Cys435, Cys16-Cys38, and Cys26-Cys49 (56). Because disulfide bonds form in the endoplasmic reticulum during synthesis of the nascent peptide, it is not certain that a Pro^{33} isoform would fold and assemble disulfides such as Leu^{33}. So despite invaluable information derived from the crystal structure of Leu^{33} β_3, until a Pro^{33} structure is solved, we can only speculate about its structure and how this might affect function.

Summary

There is abundant evidence that inherited variations in platelet genes affect platelet physiology by quantitative or qualitative effects on important platelet adhesive and signaling molecules. It may be expected that individuals who inherit multiple prothrombotic genetic effects—even if each individual variant has a relatively small effect on platelet physiology—will be at increased risk for clinical thrombotic events. The extensively studied Pro^{33} polymorphism of integrin β_3 has been associated with adverse cardiovascular events in many studies. Extensive experimentation using cell lines and platelets demonstrates that the Pro^{33} polymorphism imparts a prothrombotic platelet phenotype, with (i) enhanced platelet aggregation and secretion; (ii) increased cell adhesion, spreading, migration, and clot retraction; and (iii) greater $\alpha_{IIb}\beta_3$ outside-in signaling. On the basis of available data it appears that compared with the Leu^{33} isoform, postreceptor occupancy with the Pro^{33} isoform results in a rapid remodeling into a highly ordered cytoskeletal structure that supports the recruitment and activation of signaling molecules such as FAK, ERK2, and MLC, thereby modulating integrin function. Investigation into the molecular mechanisms has revealed that inefficient activation of Ser/Thr phosphatases is responsible in part for the efficient $\alpha_{IIb}\beta_3$ outside-in signaling in the Pro^{33}-positive platelets. Thus, although the effect of the Pro^{33} polymorphism on inside-out signaling and $\alpha_{IIb}\beta_3$ affinity is still unclear, an emerging concept is that this common genetic variation contributes to increased signaling and cytoskeletal changes, thereby reinforcing platelet responses to postactivation events, and stabilizing platelet-platelet or platelet-extracellular matrix interactions.

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