Decreased Thrombotic Tendency in Mouse Models of the Bernard-Soulier Syndrome


Objective—The platelet glycoprotein (GP)Ib-V-IX complex is a receptor required for normal hemostasis deficient in the Bernard-Soulier bleeding disorder. To evaluate the consequences of GPIb-V-IX deficiency in thrombosis we generated mouse models of the disease by targeting the GPIbβ subunit.

Methods and Results—Complete deletion (GPIbβ−/−) or an intracellular truncation (GPIbβΔIC−/−) reproduced typical and variant forms of Bernard-Soulier, with absent and partial (20%) expression of the complex on the platelet surface. Both strains exhibited thrombocytopenia and enlarged platelets with abnormal microtubular structures but normal granule composition. They exhibited prolonged tail bleeding times, which were less pronounced in GPIbβΔIC−/−. Decreased thrombus formation was observed after blood perfusion over a collagen coated surface at high shear. Resistance to vascular occlusion and an abnormal thrombus composition were observed in a model of FeCl3-induced lesion of carotid arteries. In a model of laser-induced lesion of mesenteric arterioles, thrombosis was strongly reduced in GPIbβ−/− mice, while a more modest effect was observed in GPIbβΔIC−/− animals. Finally, the two strains were protected against death in a model of systemic thromboembolism.

Conclusions—This study provides in vivo evidence of a decreased thrombotic tendency linked to defective platelet GPIb-V-IX in mouse models of Bernard-Soulier syndrome. (Arterioscler Thromb Vasc Biol. 2007;27:241-247.)

Key Words: GPIb-V-IX complex ■ von Willebrand factor ■ knockout ■ thrombosis models ■ hemostasis

The glycoprotein (GP)Ib-V-IX complex is a specialized multi subunit receptor abundantly expressed at the surface of platelets which has a key role in normal hemostasis by ensuring reversible adhesion of platelets to exposed subendothelial collagen via von Willebrand factor (vWF). The GPIb-V-IX complex has also been shown to be responsible for intracellular signal transduction via interaction of intracellular domains with signaling molecules. An important role in the platelet morphology completes its main functions. These properties are clearly illustrated by the existence of a severe bleeding tendency in patients with the rare Bernard-Soulier syndrome who carry genetic defects of GPIb-V-IX. These patients also have decreased numbers of platelets of enlarged size owing to still unresolved mechanisms.

The extreme rarity of Bernard-Soulier has prevented correct assessment of the incidence of thrombotic events in this disease. One report described the occurrence of unstable angina in a Bernard-Soulier patient, suggesting that the absence of the complex does not fully protect against thrombosis. Indications of decreased thrombosis have also come from assays involving perfusion of Bernard-Soulier blood over thrombogenic surfaces such as exteriorized blood vessels or collagen. Mice deleted of the GPIbα or GPIbβ subunit have been reported to reproduce the bleeding and platelet morphological defects found in Bernard-Soulier patients, but there is as yet no information concerning their thrombotic tendency. Recently, transgenic mice missing the GPIbα extracellular domain, which display a partial Bernard Soulier phenotype, without the additional abnormalities in platelet morphology and count, have been reported with decreased arterial thrombosis.

To evaluate the degree of protection against thrombosis in Bernard-Soulier syndrome we generated two mouse strains by genetic recombination: a knockout strain lacking receptor expression by inactivating the GPIbβ gene (GPIbβ−/−) and a knock-in strain by interrupting the GPIbβ intracellular domain (GPIbβΔIC−/−). Thrombosis was studied in vitro in collagen perfusion assays and in several in vivo artery injury and thromboembolism models.

Materials and Methods

Generation of GPIbβ−/− and GPIbβΔIC−/− Animals

A knockout construct (GPIbβ−/−) was generated by replacing the coding sequence with a neo cassette. A knock-in construct (GPIbβΔIC−/−) was obtained by introducing a stop codon after the transmembrane domain. The targeting vectors were electroporated into ES cells followed by injection into blastocysts and implantation into pseudopregnant females. GPIbβ−/−, GPIbβΔIC−/−, and GPIbβΔIC−/− mouse colonies were established by breeding heterozy-
Platelet Preparation, Flow Cytometry, Western Blotting, and Electron Microscopy

Please see the supplemental materials for details.

Bleeding Time Assays

The bleeding time was measured by severing a 3-mm segment from the distal end of the tail of 6- to 8-week-old mice. Please see the supplemental materials for details.

In Vitro Model of Thrombosis on Immobilized Collagen in a Flow System

Platelet adhesion under flow was studied as described previously.11 Whole blood anticoagulated with hirudin (100 U/mL) was perfused at 1500 s⁻¹ or 3000 s⁻¹ through a collagen-coated glass capillary and surface coverage was evaluated by off-line analysis. Some samples were prepared for scanning electron microscopy. Please see the supplemental materials for details.

FeCl₃-Induced Carotid Artery Thrombosis

FeCl₃-induced arterial injury was performed according to published procedures.12,13 The right common carotid artery was exposed to arteries with 2.5% glutaraldehyde. Please see the supplemental materials for details.

Transmission electron microscopy (TEM) were prepared by fixing the hyde solution, and embedded in paraffin. Samples for light and transmission electron microscopy (TEM) were prepared by fixing the arteries with 2.5% glutaraldehyde. Please see the supplemental materials for details.

Laser-Induced Mesenteric Artery Thrombosis

Laser-induced arterial thrombosis was studied as described by Nonne et al.14 Reproducible superficial lesions inducing reversible parietal thrombi were produced by adjusting the firing time and laser intensity. Please see the supplemental materials for details.

Thromboembolism Model

The model of acute systemic vascular thromboembolism by infusion of a mixture of collagen (0.3 mg/kg) and adrenaline (60 µg/kg) has been described previously.14 Please see the supplemental materials for details.

Statistical Analyses

Non parametric Mann–Whitney tests, area under curves, and Logrank tests were performed using GraphPad PrisM version 3.00 for Windows (GraphPad Software; *** P<0.0001, ** P<0.01, *P<0.05; ns P>0.05).

Results

Generation of Mouse Models of Bernard-Soulier Syndrome Targeting the GPIbβ Subunit

Two targeting vectors were designed for inactivation of the GPIbβ gene (GPIbβ⁻/⁻) and deletion of the GPIbβ intracellular domain (GPIbβΔIC⁻/⁻; supplemental Figure I). GPIbβ⁻/⁻ was obtained by inserting a neo cassette into the second exon, thereby eliminating most of the coding sequence. GPIbβΔIC⁻/⁻ was generated by inserting a stop codon into the native sequence three amino acids after the transmembrane domain. After electroporation and karyotype analysis, one KO clone (194 ES) and two KI clones (59 ES and 22 ES) were selected for injection into blastocysts. Animals with germine transmission were obtained and the offspring from crossing of heterozygotes were screened for the +/-, +/-, and +/-/- genotypes. A Mendelian distribution and no overt developmental or morphological abnormalities were observed for the two mutations.

The lack of expression of GPIbβ in platelets from GPIbβ⁻/⁻ mice was confirmed by Western blotting using the mAb RAM.1 against the extracellular domain of mouse GPIbβ (supplemental Figure I). In GPIbβΔIC⁻/⁻ cells, a GPIbβ reactive band with a lower molecular weight (18 kDa) was revealed by RAM.1, confirming the deletion of this domain.

Hematologic Parameters, Platelet Properties and Bleeding Tendencies in GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ Mice

Analysis of the hematologic parameters of GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ revealed the characteristic decreased platelet counts and enlarged platelets of the Bernard-Soulier syndrome but normal leukocyte and erythrocyte counts (Figure 1A). Heterozygotes from both strains had normal platelet counts and morphology (data not shown).

Transmission electronic microscopy (TEM) analysis revealed discoid platelets with an enlarged diameter (6±1 for GPIbβ⁻/⁻ and 6±2 µm for GPIbβΔIC⁻/⁻) compared with control GPIbβ⁺/⁺ (2±1 µm; Figure 1B). Intracellularly, α-granules were present at a normal density and size but a 2-fold increase in the number of microtubules per platelet was observed as compared with control platelets with however a normal equatorial localization (Figure 1C).

Flow cytometry analysis showed that GPIbβ⁻/⁻ platelets lacked GPIbβ expression and expressed little GPIbα (3% of GPIbβ⁺/⁺ cells). By contrast, in the GPIbβΔIC⁻/⁻ mice, GPIbβ, and GPIbα represented close to 20% of normal levels (Figure 1D). Expression of integrin αIIbβ3 in GPIbβ⁺/⁺ and GPIbβΔIC⁻/⁻ represented 170% as compared with GPIbβ⁺/⁻ attributable to increased platelet size (data not shown).

Platelet counts were reduced by 73% and 71% in GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ mice, respectively, compared with wild-type littermates (Figure 1A). Heterozygotes had normal platelet counts and morphology (data not shown).

In wild-type animals, 82% had bleeding times shorter than 12 minutes, whereas 76% of the GPIbβ⁻/⁻ mice bled for more than 20 minutes (Figure 2). GPIbβΔIC⁻/⁻ mice had a decreased bleeding tendency than GPIbβ⁻/⁻ with 52% of the mice bleeding after 20 minutes. Heterozygotes from the GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ strains had bleeding times similar to those of their respective +/+ genotypes (data not shown).

Decreased Thrombus Formation During Perfusion of GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ Blood Over a Collagen Surface at High Shear Rates

The thrombotic tendency was first explored in blood perfusion assays over immobilized collagen (Figure 3). In GPIbβ⁺/⁺, single adherent platelets progressively formed aggregates which were individually larger at 3000 s⁻¹ than at 1500 s⁻¹ after 2 minutes (Figure 3A and 3D), with a slightly
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their wild-type littermates. B and C, GPIb counts were on average three times lower as compared with Platelets were counted manually and white blood cell (WBC) The analysis in the number of microtubules showed an increase similar in GPIb (3% of GPIb RAM.6, respectively. GPIb /H11001 and GPIb /H11002 IC /H9004 and GPIb /H11002 platelets were studied by transmission electron microscopy. B, Representative GPIb/H9252 platelets of normal size. GPIb/ H9252 mice. A, Analysis of the hematologic parameters and platelet phenotypes in GPIbβ−/− and GPIbβΔIC−/− mice. A, Analysis of the hematologic parameters of GPIbβ−/−, GPIbβ−/− and GPIbβΔIC−/− mice. Platelets were counted manually and white blood cell (WBC) and red blood cell (RBC) using an automatic counter. Platelet counts were on average three times lower as compared with their wild-type littermates. B and C, GPIbβ−/−, GPIbβ−/− and GPIbβΔIC−/− washed platelets were studied by transmission electron microscopy. B, Representative GPIbβ−/− discoid platelets of normal size. GPIbβ−/− and GPIbβΔIC−/− platelets displayed an enlarged size with an elliptic shape. C, High magnification illustrating an increase in the number of microtubules in GPIbβ−/− and GPIbβΔIC−/−. D, The surface of α-granules was similar in GPIbβ−/− and GPIbβΔIC−/− as compared with control. The analysis in the number of microtubules showed an increase of two fold in platelets of GPIbβ−/− and GPIbβΔIC−/− compared with control platelets. E, fluorescence-activated cell sort (FACS) analysis of GPIbβ and GPIbα platelet surface expression with fluorescein-isothiocyanate (FITC)-tagged RAM.1 and RAM.6, respectively. GPIbβ was absent from GPIbβ−/− platelets and expressed at 20% of normal levels in GPIbβΔIC−/− mice. GPIbα expression was strongly decreased in GPIbβ−/− platelets (3% of GPIbβ−/−) and found at 20% of normal levels in GPIbβΔIC−/− mice.

increased total surface coverage at 3000 s−1 (Figure 3B and 3E). A major defect was observed in GPIbβ−/− and GPIbβΔIC−/− blood at 3000 s−1, where platelet adhesion was nearly abolished with respective decreases of 98 and 83% in the total surface coverage and no evidence of aggregate formation at 2 minutes (Figure 3B). Electron microscopy analysis revealed the rare attachment of individual GPIbβ−/− platelets to the collagen fibers and attachment of a few GPIbβΔIC−/− platelets as strings along the fibers (Figure 3C). These cells were however almost incapable of attracting circulating platelets to form an aggregate. At 1500 s−1 some adhesion and aggregates were observed in GPIbβ−/− blood which were greatly decreased in size as compared with the wild-type (Figure 3F). At this shear rate, adhesion and aggregate formation occurred in GPIbβΔIC−/− blood and was approximately 50 to 60% of that in wild-type blood (Figure 3E). Therefore, the absence of the GPIb-IX complex or a decrease in surface expression, following truncation of its intracellular domain, resulted in a decreased tendency to thrombus formation in vitro under high shear conditions.

## Decreased Arterial Thrombosis in GPIbβ−/− and GPIbβΔIC−/− Mice

Thrombotic tendency was then evaluated in vivo in two arterial models. The first was a carotid artery model where injury was induced by application of FeCl3 (Figure 4). In wild-type (n=8), stable occlusion occurred in 75% of the arteries, with permanent cessation of flow (Figure 4A). In GPIbβ−/− (n=8), stable occlusion occurred in only 12.5% of the arteries, whereas 25% exhibited unstable occlusion and 62.5% did not occlude. A decrease in stable occlusion was also observed in GPIbβΔIC−/− (n=8) animals where 62.5% of the arteries were patent (unstable or no occlusion) at the end of the 30 minutes period (Figure 4A). The time to first occlusion, either stable or transient, was increased in GPIbβ−/− (1646±84 s) or GPIbβΔIC−/− (1242±169 s) as

**Figure 1.** Hematologic parameters and platelet phenotypes in GPIbβ−/− and GPIbβΔIC−/− mice.

<table>
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<tr>
<td>RBC</td>
<td>9(0.3)</td>
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**Figure 2.** Tail bleeding times in GPIbβ−/− and GPIbβΔIC−/− mice. Bleeding times were measured in GPIbβ−/− and GPIbβΔIC−/− mice and their wild-type littermates (GPIbβ−/−) by tail transection as described in the Methods. Each point corresponds to the time at which bleeding had ceased in individual mice or to a 20 minutes time when bleeding did not stop. In GPIbβ−/− mice 76% of the animals still bled after 20 minutes compared with 18% in GPIbβ−/−. GPIbβΔIC−/− mice had a lower bleeding tendency than GPIbβ−/− mice with 52% of them still bleeding after 20 minutes. Statistical analyses were performed using a Log-rank test.

**Figure 3.** Aggregate formation of GPIbβ−/− and GPIbβΔIC−/− platelets in vitro under high shear conditions. A, GPIbβ−/− platelets in strings along the fibers (Figure 3C). These cells were however almost incapable of attracting circulating platelets to form an aggregate. At 1500 s−1 some adhesion and aggregates were observed in GPIbβ−/− blood which were greatly decreased in size as compared with the wild-type (Figure 3F). At this shear rate, adhesion and aggregate formation occurred in GPIbβΔIC−/− blood and was approximately 50 to 60% of that in wild-type blood (Figure 3E). Therefore, the absence of the GPIb-IX complex or a decrease in surface expression, following truncation of its intracellular domain, resulted in a decreased tendency to thrombus formation in vitro under high shear conditions.

**Figure 4.** Decreased arterial thrombosis in GPIbβ−/− and GPIbβΔIC−/− mice. Thrombotic tendency was then evaluated in vivo in two arterial models. The first was a carotid artery model where injury was induced by application of FeCl3 (Figure 4). In wild-type (n=8), stable occlusion occurred in 75% of the arteries, with permanent cessation of flow (Figure 4A). In GPIbβ−/− (n=8), stable occlusion occurred in only 12.5% of the arteries, whereas 25% exhibited unstable occlusion and 62.5% did not occlude. A decrease in stable occlusion was also observed in GPIbβΔIC−/− (n=8) animals where 62.5% of the arteries were patent (unstable or no occlusion) at the end of the 30 minutes period (Figure 4A). The time to first occlusion, either stable or transient, was increased in GPIbβ−/− (1646±84 s) or GPIbβΔIC−/− (1242±169 s) as
In a second model, lesions of mesenteric arterioles were induced in a model of platelet-dependent intravascular thrombosis with 90% mortality 5 minutes after injection. GPIbβ−/− and GPIbβΔIC−/− mice had a lower death rate, with 40% and 60% of the mice being still alive 30 minutes after collagen-adrenaline injection.

**Discussion**

The central objective of this study was to determine the thrombotic tendency in mouse models of the Bernard-Soulier syndrome. A knockout strain deleted of the GPIbβ gene reproduced a typical Bernard-Soulier phenotype and most of
the defects observed in a previously described GPIbβ deficient strain,⁹ including a tripling of the platelet size, a 75% decrease in platelet number, a profound decrease in GPIb expression, and an increased bleeding tendency. A difference was noted at the ultrastructural level with normal density and size of platelet granules. This contrasted with the report of reduced level of GPIb-IX on the platelet surface. This discrepancy is difficult to explain but does not appear to be attributable to the targeting strategy which is very similar in the two studies with insertion of a Neo cassette at the same 5′-restriction site, and only differed in the length of the 3′ untranslated region.

In the present study, a novel finding was the observation of a doubling in the number of microtubule rings in the knock-out strain which retained an equatorial location and the capacity to maintain a discoid shape despite the enlarged platelet size. From this we infer that the defects observed in the previously described GPIbβ deficient strain,⁹ including a tripling of the platelet size, a 75% decrease in platelet number, a profound decrease in GPIb expression, and an increased bleeding tendency. A difference was noted at the ultrastructural level with normal density and size of platelet granules. This contrasted with the report of reduced level of GPIb-IX on the platelet surface. This discrepancy is difficult to explain but does not appear to be attributable to the targeting strategy which is very similar in the two studies with insertion of a Neo cassette at the same 5′-restriction site, and only differed in the length of the 3′ untranslated region.

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even with the added intracellular mutation of GPIbβ, is sufficient to support minimal hemostasis.

The GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ models allowed us to assess the thrombotic tendency associated with Bernard-Soulier. Defective thrombus formation was documented in collagen flow assays at high shear rates known to depend on GPIb/VWF interaction. These defects are in line with those observed in earlier studies of Bernard-Soulier patients after blood perfusion over collagen or vascular matrices. More recently a similar defect in collagen flow studies has been reported in transgenic mice engineered on a GPIb/H9004/H11002 background. Other receptors involved in platelet-collagen interaction have been evaluated in similar ex vivo perfusion systems, such as integrin α2β1 and GPVI, which are the major receptors contributing to platelet adhesion and activation after direct interaction with collagen. GPVI-FcRγ deficiency induced by immunodepletion or after genetic ablation have established a critical role for GPVI in such perfusion assays over collagen whereas α2β1 deficiency resulted in normal thrombus formation. GPIV-deficient platelets tethered normally, but failed to spread and extend aggregates, in contrast with the deficient initial tethering in GPIb-deficient mice. In the experiments presented here, platelet tethering and aggregation nevertheless occurred in GPIb-deficient mice under conditions of intermediate shear (1500 s⁻¹), which in human blood have been described as being GPIb-dependent. Platelet capture and activation by α2β1 and GPVI can probably take place at these shears in mouse blood.

In vivo results in a FeCl₃ carotid thrombosis model were consistent with the flow experiments demonstrating a lower incidence of vessel occlusion in both GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ mice. Histology and ultrastructural analyses of the carotids showed that thrombus formation was not completely prevented but that thrombi consisted of less extensive loose aggregates. This suggested a defective propagation of activation through the platelet layers, resulting in decreased thrombus growth. Transgenic mice lacking the GPIbβ extracellular domain evaluated in a similar carotid model appeared to be similarly protected against vessel occlusion. No information was presented on the presence and histology of the thrombi. Thrombosis was also severely impaired after laser-induced arterial injuries, especially in the GPIbβ⁻/⁻ strain. A more modest protection of GPIbβΔIC⁻/⁻ contrasted with the carotid FeCl₃ model where both strains were similarly protected. Such variable responses in the same mouse strain depending on the nature of the vessel injury have now been observed in several mouse strains. For example, FcRγ/GPVI deficiency has been reported to induce full or only minor protection against arterial thrombosis in different studies depending on the type of injury and exposure to collagen and thrombin. The degree of collagen exposure and thrombin activation have been reported to vary depending on the concentration of FeCl₃ applied to arteries and the sensitivity to thrombin blockade can change with the extent of laser injury in mesenteric arteries. In spite of these differences, which await standardization between laboratories, this study and the recent work by Konstantinides et al establish that the GPIb-VWF interaction is, contrary to the GPVI-collagen axis, critical to arterial thrombosis independently of the nature of the lesion.

Abnormal laser-induced arterial thrombosis in the GPIbβ-deficient strain presented some similarities with results in vWF-deficient mice in a FeCl₃ mesenteric artery injury model. In the absence of this GPIb ligand a defect was also observed at the early stages of adhesion and thrombus growth. Interestingly, thrombosis was still observed in vWF-deficient mice similar to the present detection of parietal thrombi in the carotid artery model. This analogous response further supports the hypothesis that mechanisms in addition to GPIb-vWF-dependent responses can support arterial thrombus formation.

Less expectedly, GPIbγ-deficient mice were also protected against systemic thromboembolism, after intra venous collagen-adrenaline injection. These results suggest that this model, which is widely thought to reflect platelet responses to soluble agonists, could also depend on GPIb/VWF-dependent responses. The mechanisms of platelet activation in this model are not entirely understood, but vWF-dependent activation could theoretically occur in the lung microcirculation where high shear conditions are encountered.

GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻ present abnormalities of platelet morphology and count in addition to vWF-binding deficiencies. These additional defects could also contribute to decrease thrombus formation. Enlarged platelets are potentially more susceptible to stress and would have harder time to adhere. However this parameter is probably not sufficient to explain the whole defect as different thrombotic tendencies were observed in GPIbβ⁻/⁻ and GPIbβΔIC⁻/⁻, despite a similar platelet size increase. Low platelet counts have also been linked to decreased thrombus formation in perfusion models. To evaluate the influence of thrombocytopenia in our models, GPIbβΔIC⁻/⁻ mice were injected with TPO to increase platelet numbers. Despite doubling of the platelet count, comparable defects in thrombus formation were ob-
served in collagen flow assays (data not shown). Similarly, doubling of the platelet count in splenectomized GP Ibβ/ICAM−/− mice did not correct the defect in superficial laser-induced lesions (data not shown).

In conclusion, this study has provided in vivo demonstration of a decreased thrombotic tendency in mouse models of the Bernard-Soulier bleeding disorder. This antithrombotic protection linked to a GP Ib defect is in line with in vitro and in vivo thrombosis studies using GP Ib/VCAM blocking agents23–27 and recent results in GP Ib β transgenic mice.30 The finding is still difficult to extrapolate to man, especially in the face of a report of an acute coronary syndrome in a BSS patient.3 However, the GP Ibβ−/− model should permit further evaluation of the role of this receptor in thrombosis under atherosclerotic conditions, particularly after crossing with APOE- or LDLR-deficient mice.

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Disclosures

None.

References

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SUPPLEMENTARY DATA

Detailed Methods:

Isolation of the mouse GPIbβ gene and construction of the targeting vectors

A PCR probe corresponding to mouse GPIbβ cDNA (Genbank AB001419) was used to clone the gene from a 129/Sv genomic library. The knockout construct (GPIbβ−/−) was generated in the pBX vector by replacing the coding sequence with a neo cassette (Supplementary figure A). The knock-in construct (GPIbβΔIC−/−) was obtained by introducing a stop codon after the transmembrane domain (Supplementary figure B). Primers used for these constructs were (Supplementary figure):     (1) 5'-GATAGGAAATCCCTGGTACCCCTGTCTGGTC-3; (2) 5'-TTGGCGCTACCGGTGGATGTGGAATGTGTGC-3'; (3) 5'-GTTCATAGTTGGATATCATAATTTAAAACAA-3' ; (4) 5'-CACCTCAAGTTGGGGTCTAGTGATGGTTTC-3' ; (5) 5'-GCAGCTTTCTAGGAAGAGCCA-3' ; (6) 5'-CAATAGCAGAGCGTCTAGCAG-3'; (7) 5'-GCAGCTTTCTAGGAAGAGCCA-3' ; (8) 5'-CAATAGCAGCGCGTGTAGCAG-3'.

Generation of GPIbβ−/− and GPIbβΔIC−/− animals

The targeting vectors were electroporated into the P1 embryonic stem (ES) cell line and positive clones were screened by PCR and Southern blotting. In the knock-in cells, the PGK-neo-loxP cassette was excised with Cre. Two ES cell lines were chosen for each construct (194, 113 and 59, 22) for injection into blastocytes and implantation into pseudopregnant females. Chimeras from the cell lines demonstrated germ line transmission.

Mouse strains

GPIbβ−/−, GPIbβΔIC−/− and GPIbβ+/+ mouse colonies were established at the animal facilities of the Etablissement Français du Sang-Alsace by breeding heterozygotes initially obtained by mating chimeric male mice (129/Sv) with C57Bl/6 females. Genotyping was performed on mouse tail DNA by PCR using primers 5'-GCAGCTTTCTAGGAAGAGCCA-3’ and 5'-AGCAGGGCTAGCAACAGAAGCAG-3’ for GPIbβ−/− and primers 5’-GCAGCTTTCTAGGAAGAGCCA-3’ and GTCAGCACCAATTCGGTGGTGCG for GPIbβΔIC−/−. All animal studies were approved by the EFS-Alsace review board on animal research.
**Platelet preparation**

Washed platelets were prepared from ACD-anticoagulated blood by Ficoll separation from PRP and sequential centrifugation\(^1\). The cells were resuspended at a density of 3 x 10^5/µL in Tyrode’s buffer containing 0.35% HSA and 2 µL/mL potato apyrase.

**Western blotting**

Proteins from Triton X-100 platelet lysates were separated by SDS-PAGE, transferred to PVDF membranes and revealed with a primary antibody followed by an HRP-conjugated secondary antibody and ECL. RAM.1 is a rat mAb directed against mouse and human GPIb\(^\beta\)\(^2\) and L41 is a rabbit polyclonal antibody directed against mouse GPIb\(^\beta\) intracellular domain. This antibody was manufactured by Eurogentec (Eurogentec, Belgium).

**Flow cytometry**

EDTA-anticoagulated blood was incubated with a primary mAb which was revealed with a FITC-conjugated secondary antibody. Samples were then incubated with PE-labeled anti-GPIbIIIa (αIIbβ3) and analyzed on a FACScalibur flow cytometer. RAM.6, a rat MoAb directed against mouse extracellular domain of GPIbα is produced in our laboratory.

**Electron microscopy**

Platelet suspensions were fixed by adding 1 volume of washed platelets to 9 volumes of fixative solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2% sucrose, 305 mOsm/L, pH 7.3) previously warmed to 37°C for 1 hour. Fixed cells were washed, post-fixed for 1 hour at 4°C with 1% osmium tetroxide in cacodylate buffer, washed in the same buffer, dehydrated in graded ethanol solutions and embedded in Epon. Ultrathin sections (100 nm) were stained with lead citrate and uranyl acetate and examined under a Philips CM 120 Bio Twin (Eindhoven, The Netherlands) transmission electron microscope (120 kV). The surface area of each α-granule within an individual platelet section was calculated for 55 (GPIb\(^\beta\)\(^{+/+}\)), 48 (GPIb\(^\beta\)\(^{+/-}\)) and 71 (GPIb\(^\beta\)ΔIC\(^{-/-}\)) randomly selected platelet sections using Metamorph software (Universal Imaging, Paris, France). The number of microtubules within an individual platelets was numbered manually for 15 (GPIb\(^\beta\)\(^{+/+}\)) and 16 (GPIb\(^\beta\)\(^{-/-}\), GPIb\(^\beta\)ΔIC\(^{-/-}\)) randomly selected platelets.
Bleeding time assays

The bleeding time was measured by severing a 3 mm segment from the distal end of the tail of 6 to 8 week-old mice. The amputated tail was maintained in contact with air at room temperature (~20 °C) and the blood drops were gently blotted every 15 s on a Whatman filter paper (Whatman plc, Middlesex, UK). Care was taken not to exert any pressure on the tail tip. The bleeding time was defined as the interval from severing until no blood appeared on the filter for two successive times. If bleeding did not cease within 20 min, the tail was cauterized and 1,200 s was noted as the bleeding time.

In vitro model of thrombosis on immobilized collagen in a flow system

Whole blood anticoagulated with hirudin (100 U/mL) was perfused at 1,500 s⁻¹ or 3,000 s⁻¹ through a collagen-coated glass capillary (VitroCom, Mountain Lakes, NJ). The interaction of DIOC₆-labeled platelets was visualized in real time under an inverted fluorescence microscope (Leica DMIRB; Leica Microsystems SA, Westlar, Germany) (40x objective/0.55) coupled to a video camera (DAGE MTI, Michigan City, MI) and images were recorded on DVD-RAM tapes (WDR 200; Matsushita Electric Industrial, Osaka, Japan). Offline analysis of the surface coverage was performed on single frames obtained from digital photographs of the capillary surface and processed with Methamorph™ software (Version 5; Universal Imaging Corporation, Downingtown, PA). Some samples were prepared for scanning electron microscopy (SEM) by rinsing the capillary with buffer after a 120 s perfusion time and fixing the surface with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The microcapillary was sectioned longitudinally, washed, dehydrated in graded ethanol solutions and air-dried with hexadimethyldisilazane. Samples were mounted with carbon paint, sputter-coated with gold-palladium and examined under a Sirion scanning electron microscope at 5 kV (FEI, Eindhoven, The Netherlands).

FeCl₃-induced carotid artery thrombosis

The right common carotid artery was exposed by dissection and a miniature Doppler flow probe (Model 0.5VB; Transonic Systems, Ithaca, NY) was positioned around the artery. A 1 x 2 mm² strip of 1M Whatman filter paper (Whatman International) soaked in 20% FeCl₃ was applied to the adventitial surface of the artery for 3 min. The area was then rinsed with saline and the blood flow was continuously monitored for 30 min. The time to first occlusion (stable or unstable) and the numbers of arteries respectively patent and occluded at 30 min were recorded. Occlusion was defined as the cessation of blood flow for over 30 min,
confirmed by visualization of the thrombus. After 30 min, the mice were euthanized and the carotids were processed for histology or microscopy.

Histological samples were prepared by perfusing the left ventricle with 3 mL of 4% paraformaldehyde in PBS, after which the artery was removed, incubated overnight in 4% paraformaldehyde solution and embedded in paraffin. Longitudinal sections (5 µm thick) mounted on slides were deparaffinized, rinsed, rehydrated and stained for elastin and nuclei with Elastica van Gieson’s solutions (Merck). The slides were then dehydrated in graded ethanol and Histosol (Sigma) solutions and coverslipped. After washing the carotids, samples for light and transmission electron microscopy (TEM) were prepared by fixing the arteries with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (305 mOsm/L, pH 7.3) for 1 h at RT. The carotids were then treated with 1% osmium tetroxide for 1 h, dehydrated in graded ethanol solutions and embedded in Epon (Ladd Research Industries, Euromedex, France). Cross-sections (300 nm thick) were stained with toluidine blue for light microscopy and viewed under an inverted microscope (20x objective/0.4) (Leica Microsystems). Thin sections (100 nm thick) were stained with lead citrate and uranyl acetate for TEM and examined under a CM120 microscope at 120 kV (Philips, Eindhoven, The Netherlands).

**Laser-induced mesenteric artery thrombosis**

Localized injury of the luminal surface of mesenteric arterioles was induced in mice weighing 15 g using a pulsed nitrogen dye laser (440 nm) applied through the microscope objective (x 63/0.70) of a DMIRB microscope (Leica Microsystems) with a Micropoint laser system (Photonics Instruments, St Charles, IL). Reproducible superficial lesions inducing a reversible parietal or more extensive almost occlusive thrombus were produced by adjusting the firing time and laser intensity. For each type of lesion, 5 to 26 arterioles were targeted over a period of 1 h with only one injury per vessel. To precisely delimit the contour of the thrombi and measure their surface area, DIOC6 (0.5 µmol/g of body weight) was injected into the jugular vein prior to injury. Images were acquired sequentially with wide field and fluorescent light using a SensiCam CCD camera (2 x 2 binning; Cooke, Auburn Hill, MI) controlled by Slidebook software (Intelligent Imaging Innovations, Denver, Co, USA).

**Thromboembolism model**

Mice weighing 20-25 g were anesthetized and the jugular veins were exposed surgically. A mixture of collagen (0.3 mg/kg) and adrenaline (60 µg/kg) was infused for 3-4 s
into the right jugular vein. Mice were observed for 30 min and the mice that recovered were killed thereafter.

REFERENCES


Supplementary Figure I

Legend to the supplementary figure:

Generation of mouse models of Bernard-Soulier syndrome targeting the GPIbβ subunit

Two targeting vectors were designed for knockout of the GPIbβ gene (GPIbβ^{−/−}) (A) and deletion of the GPIbβ intracellular domain (GPIbβΔIC^{−/−}) (B). GPIbβ^{−/−} was obtained by inserting a neo cassette into the second exon, thereby eliminating most of the coding sequence (residues 646 to 1142, leaving the first three codons in exon 1 and 502 codons at the end of exon 2). GPIbβΔIC^{−/−} was generated by inserting a stop codon into the native sequence three amino acids after the transmembrane domain and a neo cassette into the intron, flanked by Lox sites to allow its excision in ES cells. After electroporation, 8 and 6 positive ES clones were obtained for the KO and KI strains respectively. Southern blot analyses were performed on two ES clones positive for the knockout or knock-in sequence (before treatment with Cre), to confirm single insertion, using the neo cassette as a 750 nt probe. For the clones tested for the KO and KI mice a band at 7.5 kb and 25 kb was observed respectively (supplementary figure I, panels A and B). Following karyotype analysis one KO clone (194 ES) and two KI clones (59 ES and 22 ES) were selected for injection into blastocytes. Respectively 11, 20 and 7 animals with germline transmission were obtained and the offspring from crossing of heterozygotes were screened for the +/+ , +/- and -/- genotypes. A Mendelian distribution and no overt developmental or morphological abnormalities were observed for the two mutations.

The lack of expression of GPIbβ in platelets from GPIbβ^{−/−} mice was confirmed by western blotting using the mAb RAM.1 against the extracellular domain of mouse GPIbβ (A). In GPIbβ^{+/−} and GPIbβ^{+/+} platelets a single band was observed at the expected size for mouse GPIbβ (25 kDa). In GPIbβΔIC^{−/−} cells, a GPIbβ reactive band with a lower molecular weight (18 kDa) was revealed by RAM.1 (B) but not by L41 against the intracellular region of mouse GPIbβ (data not shown), confirming the deletion of this domain. GPIbβΔIC^{+/−} platelets contained GPIbβ of both normal and decreased sizes. These results confirmed that correct targeted disruption and point mutagenesis had been achieved in the KO and KI strains respectively.
Supplementary Figure I

A

Western Blot

SpeI

25 Kb

10

Southern Blot

SpeI/EcoRI

194 113

β

βΔIC

B

Western Blot

SpeI

25 Kb

10

Southern Blot

SpeI

194 113

β