Targeting Platelet GPIbβ Reduces Platelet Adhesion, GPIb Signaling and Thrombin Generation and Prevents Arterial Thrombosis

Eric Maurer,* Chaojun Tang,* Mathieu Schaff, Catherine Bourdon, Nicolas Receiver, Catherine Ravanat, Anita Eckly, Béatrice Hechler, Christian Gachet, François Lanza, Pierre H. Mangin

Objective—The glycoprotein (GP) Ib-V-IX complex regulates the adhesion, activation, and procoagulant activity of platelets. We previously reported that RAM.1, a rat monoclonal antibody directed against the extracellular domain of mouse GPIbβ, diminished adhesion of platelets and Chinese hamster ovary cells transfected with the human GPIb-IX complex to von Willebrand factor under flow conditions. Here, we further evaluated the functional importance of GPIbβ by studying the impact of RAM.1 on GPIb-mediated platelet responses and in vitro and in vivo thrombus formation.

Approach and Results—We show that RAM.1 dramatically reduced GPIb-mediated filopodia extension of Chinese hamster ovary GPIb-IX cells after adhesion to von Willebrand factor. RAM.1 also reduced filopodia extension and GPIb-mediated Ca²⁺ signaling after adhesion of mouse platelets to von Willebrand factor. RAM.1 inhibited thrombin generation in platelet-rich plasma without impairing phosphatidylserine exposure. In addition, RAM.1 reduced thrombus formation after perfusion of mouse whole blood over collagen in a shear-dependent manner. This effect was confirmed in vivo, because injection of F(ab)² fragments of RAM.1 diminished thrombus formation induced by laser beam injury of mesenteric arterioles and forceps injury of the abdominal aorta. In contrast, RAM.1 F(ab)² did not prolong the tail-bleeding time or increase the volume of blood lost.

Conclusions—These findings are the first evidence that targeting a subunit other than GPIbα can lead to an antithrombotic effect via the GPIb-IX complex. This could represent an alternative way to reduce thrombus formation with a minor impact on hemostasis. (Arterioscler Thromb Vasc Biol. 2013;33:1221-1229.)

Key Words: hemostasis ■ platelets ■ thrombosis ■ von Willebrand factor

Platelets adhere and aggregate at sites of vascular injury to form a plug, which stops blood loss. Under pathological conditions, excessive platelet accumulation after atherosclerotic plaque rupture can induce occlusive thrombus formation, leading to life-threatening ischemic complications. At the elevated blood flows found in arteries, the glycoprotein (GP) Ib-V-IX receptor ensures the first step of platelet recruitment to the damaged vessel wall through its binding to von Willebrand factor (VWF) present in the subendothelium. GPIb-IX also participates in thrombus growth by interacting with the VWF exposed at the surface of activated platelets located at the edge of the thrombus. Besides, the GPIb-V-IX complex contributes to the platelet procoagulant activity, which is responsible for thrombin generation, a key factor of thrombus formation. Numerous ex vivo and in vivo studies have shown that targeting the GPIbα/VWF axis with different blockers, including antibodies, nanobodies, aptamers, or proteins isolated from snake venom, efficiently reduces thrombus growth in various experimental models. These studies suggest that targeting the GPIb-V-IX complex could represent an interesting antithrombotic strategy.

GPIb-V-IX belongs to the leucine-rich repeat protein family and is composed of 4 type I transmembrane glycoproteins: GPIbβ is disulfide linked to 2 GPIbα subunits to form GPIb, which noncovalently associates with GPIIX and GPV. GPIbα interacts through its intracellular domain with filamin-1, which is linked to the actin cytoskeleton. This interaction plays an important role in the surface expression of the GPIb-V-IX complex and in its anchorage within the plasma membrane. GPIbα also binds to 6 members of the 14-3-3 family, which participate in integrin αIIbβ₃ activation. The intracellular domains of GPIbβ and GPV have been reported to interact with calmodulin, but the functional significance of this interaction remains to be established. All of the known extracellular ligands of the GPIb-IX complex (VWF, thrombin, Mac-1, P-selectin, thrombospondin-1, factor XI and factor XII, and high-molecular weight kininogen) bind to the 45-kDa globular N-terminal extracellular domain of GPIbα.
whereas no docking site for any other molecule has been identified on GPIbβ or GPIX. The binding of VWF to GPIbα induces an intracellular signal involving a member of the Src-kinase family, which activates phospholipase Cγ2 and leads to intracellular Ca2+ mobilization and filopodia extension.28 Studies in Chinese hamster ovary (CHO) cell lines expressing mutated and deleted forms of the GPIb-IX complex have pointed to the intracellular region of GPIbβ rather than GPIbα as being critical for VWF-induced signaling.29 However, the in vivo importance of GPIb-V-IX signaling still remains unknown.

We previously reported that the rat monoclonal antibody RAM1 directed against the mouse GPIbβ extracellular domain inhibited VWF binding to platelets, and the adhesion of platelets and CHO cells transfected with the human GPIb-IX complex (hGPIb-IX CHO) to immobilized VWF under flow conditions.30,31 Although the precise mechanisms of these effects remain unclear, RAM.1 does not seem to prevent VWF binding to GPIbα through a steric hindrance notably supported by the fact that a Fab fragment of this antibody had a similar effect.32 It has recently been reported that RAM.1 did not modify VWF binding to GPIb-IX reconstituted in phospholipid bilayer nanodiscs, further suggesting that the effect of RAM.1 may not be attributable to direct interference with the VWF–GPIbα interaction.32

Here, we observed that RAM.1 induced a dramatic decrease in filopodia extension of hGPIb-IX CHO cells adhering to VWF, suggesting that it could also affect GPIb-mediated signals. To establish this in platelets and evaluate its impact on thrombosis, we performed in vitro and in vivo studies in mice. We observed that RAM.1 inhibited GPIb-induced filopodia extension, in vitro and ex vivo, and GPIb-mediated intracellular Ca2+ mobilization after adhesion of mouse platelets to VWF. Interestingly, RAM.1 reduced collagen and tissue factor (TF)-induced thrombin generation in mouse platelet-rich plasma without impairing surface exposure of phosphatidylserine. RAM.1 also prevented thrombus formation in an in vitro flow system and in 2 in vivo murine thrombosis models, without affecting the tail-bleeding time. Altogether, these results suggest that targeting GPIbβ could strongly reduce GPIb-mediated signaling, thrombin generation and thrombus growth, whereas having a minor impact on hemostasis.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

RAM.1 Inhibits GPIb-Mediated Signaling

The GPIb-V-IX complex supports adhesion of platelets to VWF, a process initiating an intracellular signal. Because RAM.1 was found to inhibit flow-dependent adhesion of hGPIb-IX CHO cells, we also examined its effect on GPIb-mediated filopodia extension, which reflects signaling in these cells.26 RAM.1 (10 μg/mL) did not significantly modify the number of hGPIb-IX CHO cells, adhering to VWF under static conditions (Figure 1B), but reduced by 73±1% the number of cells extending filopodia (n=3; P=0.001) clearly indicating impaired signaling (Figure 1A and 1C). Because RAM.1 is targeted against mouse GPIbβ, we next examined its effects on murine platelets. RAM.1 reduced the morphological changes of platelets with almost no cells extending ≥5 filopodia as compared with 42±3% in control (n=3; P=0.007), in the absence of a significant effect on adhesion (Figure 1D–1F). These results converge to establish that RAM.1 is an inhibitor of the GPIb-mediated signals leading to cytoskeletal reorganization. A salient feature of GPIb-mediated signals is the activation of phospholipase Cγ2, which generates inositol 1,4,5-trisphosphate through phosphatidylinositol 4,5-bisphosphate hydrolysis and ultimately leads to the mobilization of intracellular Ca2+ stores.28 Real-time confocal microscopy showed that RAM.1 strongly inhibited the intracellular Ca2+ spikes of individual VWF-adherent platelets, an effect which was concomitant with the inhibition of filopodia extension (Figure 1G–1I). The mean increase in intracellular Ca2+ concentration was reduced by 58±7% in the presence of RAM.1 as compared with the control (n=3; P=0.0001; Figure 1H). Altogether these results support the view that RAM.1 efficiently inhibits GPIb-mediated signaling. This effect seems to be specific because RAM.1 did not affect platelet morphological changes, or aggregation induced by soluble agonists (adenosine 5′-diphosphate, thrombin, or the thromboxane A2 mimetic U46619) or other immobilized proteins (collagen, fibrinogen; data not shown).

RAM.1 Reduces TF- and Collagen-Induced Thrombin Generation

The GPIb-V-IX complex is known to regulate the procoagulant activity of platelets and its defect, such as in patients with Bernard-Soulier syndrome results in a reduced prothrombin consumption.51 In agreement with these observations, we recently reported that mouse platelets lacking GPIb have a profound defect in thrombin generation.7 The ability of RAM.1 to decrease thrombin generation was measured in a calibrated automated thrombin assay using mouse-citrated platelet-rich plasma. RAM.1 reduced by 41±10% and 70±7%, the total amount of thrombin generated (endogenous thrombin potential) in response to TF (208±33 nmol/L, versus 350±37 nmol/L thrombin×minutes; n=3; P=0.002) and collagen (173±38 nmol/L versus 568±107 nmol/L thrombin×minutes; n=3; P=0.029), respectively (Figure 2A–2C). RAM.1 also decreased by 39±6% and 61±7%, the maximal concentration of thrombin generated (Peak) after stimulation with TF (n=3; P=0.003) or collagen (n=3; P=0.046), respectively (Figure 2D). As a control, we showed that Xia.B2, a function-blocking antibody binding to GPIbα, did not significantly decrease TF-induced thrombin generation in this assay (Figure 1 in the online-only Data Supplement). Surprisingly, a flow cytometry analysis revealed that RAM.1 did not reduce the surface exposure of phosphatidylserine, because annexin-V binding was unchanged after stimulation with collagen-related peptide, thrombin, a mixture of collagen-related peptide and thrombin, or the Ca2+ ionophore A23187 (n=7; P>0.05; Figure 2E). These results indicate that RAM.1 markedly impairs thrombin generation through a mechanism that seems independent of phosphatidylserine exposure.
RAM.1 Inhibits Thrombus Formation in a Flow-Based Assay

To address the role of RAM.1 in thrombus formation in vitro, anticoagulated mouse whole blood labeled with 3,3′-dihexyloxacarbocyanine iodide was perfused over immobilized collagen. The 3D reconstructed images obtained by confocal microscopy indicated that RAM.1 (20 μg/mL) efficiently reduced the thrombus volume at a shear rate of 3000 s⁻¹ (Figure 3A). Interestingly, the effect of RAM.1 was shear dependent with no inhibition at a shear rate of 300 s⁻¹ and a progressive 28±9% (n=3; P=0.054), 47±10% (n=3; P=0.019), and 54±3% (n=3; P=0.001) reduction in the thrombus volume at 750 s⁻¹, 1500 s⁻¹, and 3000 s⁻¹, respectively (Figure 3B–3E).

RAM.1 Inhibits GPIb-Mediated Filopodia Extension Ex Vivo

To study the effects of RAM.1 in vivo, we generated F(ab)² fragments to prevent Fc-dependent platelet activation and subsequent removal from the circulation. We first confirmed that RAM.1 F(ab)² inhibited filopodia extension after adhesion of mouse platelets to VWF to a similar extent as RAM.1 immunoglobulin G (Figure II in the online-only Data Supplement). We next verified that after intravenous injection of 1, 2, or 3 mg/kg of RAM.1 F(ab)², this antibody bound to circulating platelets and did not reduce the platelet count nor the expression level of GPIbα (Figure 4A–4C). Finally, we observed that platelets isolated 30 minutes after injection of RAM.1 F(ab)² fragments (3 mg/kg) exhibited a clear reduction in their ability...
to extend filopodia on a VWF surface compared with untreated mice (n=5; P=0.007; Figure 4D and 4E).

**RAM.1 Inhibits Thrombus Formation In Vivo**

The impact of RAM.1 on thrombus formation was evaluated in 2 models of localized arterial injury. In the well-characterized model of laser injury of mesenteric arterioles, 20 minutes after injection of RAM.1 F(ab)2 fragments (3 mg/kg) into C57Bl/6 mice, the thrombus area was reduced by 57±9% as compared with the control (1–5 vessels per mouse: control: n=5 [13 vessels]; RAM.1: n=5 [11 vessels]; P=0.040; Figure 5A and 5B). To confirm this result, we used a second model of arterial thrombosis in which the aorta is mechanically injured with a forceps.35 Similarly as in the laser injury model, injection of RAM.1 F(ab)2 fragments (3 mg/kg) strongly decreased thrombus growth (area under the curve: 48±10% reduction; control: n=9 mice; RAM.1: n=11 mice; P=0.031; Figure 5C and 5D). Overall these data indicate that targeting GPIbβ with RAM.1 inhibits thrombus growth in vivo.

**RAM.1 Does Not Prolong the Tail-Bleeding Time and the Volume of Blood Loss**

The effect of RAM.1 on hemostasis was investigated in a tail-bleeding assay. No prolongation of the tail-bleeding time was observed 20 minutes after injection of RAM.1 F(ab)2 (3 mg/kg) as compared with control F(ab)2 fragments (n=10; P>0.05; Figure 6A). Moreover, RAM.1 did not increase the volume of blood lost over a 30-minute period of time (4.9±2.1 μL) as compared with the control (26.7±12.6 μL; n=10; P>0.05; Figure 6B). These results suggest that treatment with RAM.1 does not modify hemostatic functions in mice.

**Discussion**

We originally reported that the rat antimouse GPIbβ antibody, RAM.1, decreased binding of VWF to the human GPIb-V-IX complex and impaired human platelet adhesion to VWF under flow conditions, and we wanted to further characterize the properties of this antibody especially in vivo.30 This study extends our previous findings by providing evidence that targeting GPIbβ with RAM.1 inhibits VWF-induced GPIb signaling both in a human and in a mouse system, as shown by decreases in filopodia extension and intracellular Ca²⁺ fluxes. Interestingly, RAM.1 also impacts on TF- and collagen-induced thrombin generation. Moreover, we found that RAM.1 inhibits in vitro thrombus growth after perfusion of anticoagulated mouse whole blood over collagen. This observation was in accordance with the defective thrombus formation occurring in vivo in 2 murine models based, respectively, on laser-induced injury of mesenteric arterioles and mechanical injury of the aorta. Finally, we showed that RAM.1 seems to have a minor impact on hemostasis, because it did not prolong the time to first arrest bleeding nor increased the volume of blood lost in a tail-bleeding assay.

One major finding of our previous work was the inhibition of platelet adhesion to VWF under flow conditions.30 In
In the present study, we found that neither mouse platelets nor hGPIb-IX CHO cells displayed a significant decrease in adhesion to VWF in a static assay. These apparently contradictory results can probably be related to the fact that the inhibitory effect of RAM.1 on GPIbα/VWF interactions would be more evident under flow than under static conditions. One possibility is that receptor/ligand interactions being less stable under flow, they become more sensitive to an inhibitory effect. It is also possible that the use of botrocetin in the static assay, which potently increases the adhesiveness of the VWF A1 domain for GPIbα, could overcome the inhibitory effect of RAM.1.

We previously reported that binding of VWF to GPIbα activates phospholipase Cγ2 through one or more members of the Src-kinase family, leading to the mobilization of intracellular Ca²⁺ stores and filopodia extension.28 This signaling cascade was inhibited by blocking the VWF binding site of GPIbα. We now provide evidence that targeting GPIbβ with RAM.1 also inhibits GPIb signaling. This points to a role of GPIbβ in VWF-induced platelet activation, in agreement with another previous study, where we found that the intracellular region of GPIbβ, rather than that of GPIbα, participates in GPIb signaling leading to filopodia extension.29 The mechanism is still unknown, but because its intracellular domain is devoid of catalytic activity, one may hypothesize that it acts through binding partners. So far, only 14-3-3ζ and calmodulin have been described to interact with GPIbβ. Although calmodulin has been reported to play a role in platelet shape change, its pharmacological inhibition did not affect GPIb-mediated filopodia formation after adhesion of platelets to VWF (P. Mangin and F. Lanza, unpublished data, 2003). We and others found that the GPIbα/14-3-3ζ interaction participates in GPIb-mediated integrin αIIbβ3 activation, but not in the signaling cascade leading to filopodia extension.25,26 However, it was recently proposed that binding of 14-3-3ζ to the S609 residue of GPIbα regulates Src kinase and protein kinase C activation, leading to an increase in intracellular Ca²⁺ concentrations.36 Whether other as yet unidentified binding partners of GPIbβ participate in GPIb signaling remains to be determined.

The mechanism through which RAM.1 inhibits GPIb signaling remains unclear. Its binding to GPIbβ could alter the organization of the GPIb-IX complex within the plasma membrane and in turn reduce the binding properties of GPIbα, which would result in the transmission of a weaker signal.30 Alternatively, a change in conformation could disturb interaction of GPIb-IX with intracellular partners involved in GPIb-mediated signaling. This hypothesis could be supported by observation that RAM.1 promotes dephosphorylation of S166 in the intracytoplasmic domain of GPIbβ, a site implicated in 14-3-3 binding.30,31
Based on reduced prothrombin consumption in patients with Bernard-Soulier syndrome, the GPIb-IX complex is known to participate in the process of thrombin generation. We provide evidence that RAM.1 inhibits thrombin generation in mouse platelet-rich plasma. Because RAM.1 did not reduce annexin-V binding in response to strong agonists, such as collagen-related peptide, thrombin, or A23187, the mechanism involved seems to be distinct from the exposure of phosphatidylserine.

Because GPIbα binds several coagulation factors, including factor XII and factor XI, high-molecular weight kininogen, and thrombin, one could hypothesize that RAM.1 reduces their binding and thereby lowers the ability of platelets to promote thrombin generation. However, we recently showed that the procoagulant function of the GPIb-V-IX complex seems to be independent of the N-terminal domain of GPIbα, which contains all known ligand-binding sites.7 Future studies are needed to precisely define the mechanism by which RAM.1 impairs thrombin generation.

We observed that RAM.1 inhibits in vitro thrombus formation during perfusion of mouse whole blood over collagen. The effect of RAM.1 was modest at low-shear rates, in agreement with the fact that other receptors, such as integrins αIIBβ3, participate in the initial phase of thrombus formation and can overcome blockade or absence...
β as playing a role in GPIb signaling and are identified a region between R164 and P170 in the intracellular domain of GPIb, evaluating its importance is challenging. We recently demonstrated in 2 distinct thrombosis models, suggesting that pharmacological targeting of GPIbβ could reduce thrombus growth in vivo. Because RAM.1 inhibits platelet adhesion to VWF and thrombin generation, its antithrombotic effect is likely to result from these GPIb-IX–mediated functions. Whether the effect of RAM.1 on dampening GPIb signals also participates in this process is speculative. So far, the role of GPIb signaling in thrombus growth remains elusive, and because no specific agent against human GPIbβ is needed to investigate this in future studies.

Importantly, the inhibitory effect of RAM.1 was demonstrated. Although the role of GPIb binding to VWF, transiently prolonged the cutaneous bleeding time in healthy volunteers and increased the bleeding risk. This is in agreement with the fact that the aptamer ARC1789, which blocks GPIb binding to VWF, transiently prolonged the cutaneous bleeding time in healthy volunteers and increased the bleeding risk of patients undergoing carotid endarterectomy with one of them presenting a serious adverse event of intraoperative bleeding.40-42 Our data indicated that RAM.1 does not prolong the mouse tail-bleeding time, suggesting no major impact on hemostasis. From these observations, we propose that targeting GPIbβ could prevent excessive thrombus growth with a potentially minor effect on the risk of bleeding.

In conclusion, this study provides evidence that a ligand of the mouse GPIbβ extracellular domain can blunt VWF-induced GPIb signaling and strongly reduce thrombin generation. This effect combined with its capacity to inhibit platelet adhesion to VWF under flow could contribute to the strong reduction in thrombus formation, suggesting that targeting the extracellular domain of GPIbβ may prevent thrombosis.

Acknowledgments

We thank Stéphanie Magnenat, Sylvie Moog, Simone Schuhler, and Jean-Yves Rinckel for technical assistance; Monique Freund for animal care; and Juliette Mulvihill for reviewing the English of the article.

Sponsors of Funding

This work was supported by ARMESA (Association de Recherche et Développement en Médecine et Santé Publique) and by a grant from the Fondation de France (2011-00020448). E. Maurer was supported by a postdoctoral fellowship from the FFCSA (Fondation Franco-Chinoise pour la Science et ses Applications) and the CSC (China Scholarship Council).

References

Bernard-Soulier syndrome and VWF disease, respectively. Therefore, pharmacological targeting of the GPIbβ/VWF axis might result in an increased bleeding risk. This is in agreement with the fact that the aptamer ARC1789, which blocks GPIb binding to VWF, transiently prolonged the cutaneous bleeding time in healthy volunteers and increased the bleeding risk of patients undergoing carotid endarterectomy with one of them presenting a serious adverse event of intraoperative bleeding.40-42 Our data indicated that RAM.1 does not prolong the mouse tail-bleeding time, suggesting no major impact on hemostasis. From these observations, we propose that targeting GPIbβ could prevent excessive thrombus growth with a potentially minor effect on the risk of bleeding.

In conclusion, this study provides evidence that a ligand of the mouse GPIbβ extracellular domain can blunt VWF-induced GPIb signaling and strongly reduce thrombin generation. This effect combined with its capacity to inhibit platelet adhesion to VWF under flow could contribute to the strong reduction in thrombus formation, suggesting that targeting the extracellular domain of GPIbβ may prevent thrombosis.

**Figure 5.** RAM.1 F(ab)2 fragments inhibit thrombus growth in 2 in vivo thrombosis models. Control or RAM.1 F(ab)2 fragments (3 mg/kg) and 3,3′-dihexyloxacarbocyanine iodide (5 μL of a 100 μmol/L solution/g of body weight) were injected into the jugular vein of C57Bl/6 mice. A and B, Laser-induced lesions were generated in mesenteric arterioles. A, Tracings represent the mean surface area±SEM of the thrombus developing over time. B, Bar graphs represent the area of the thrombus during 100 s and are expressed as the area under curve (AUC). Control: n=13 vessels in 5 mice; RAM.1 F(ab)2: n=11 vessels in 5 mice (*P<0.05). C and D, Forceps were used to produce a mechanical injury of the aorta of mice. C, Tracings represent the mean surface area±SEM of the thrombi developing over time. D, Bar graphs represent the area of the thrombus during 30 minutes and are expressed as the AUC. Control: n=9 mice; RAM.1 F(ab)2: n=11 mice (*P<0.05; **P<0.01).

**Figure 6.** RAM.1 F(ab)2 fragments do not modify the tail-bleeding time or the volume of blood lost. Control or RAM.1 F(ab)2 fragments (3 mg/kg) were injected into C57Bl/6 mice. The tail-bleeding times and volumes of blood lost over a 30-minute period were determined in 10 mice for each group. The symbols represent individual mice, and the mean value±SEM for each population is indicated.


Significance

Blood platelets are critical in hemostasis, which stops blood loss. They also play a key role in arterial thrombosis responsible for ischemic diseases, such as myocardial infarction or stroke, which are the leading cause of mortality worldwide. The glycoprotein (GP) Ib-V-IX complex plays an important role in platelet adhesion, activation, aggregation, and procoagulant activity and has been proposed as an interesting antithrombotic target. However, a defect in either GPIbα or its main ligand, von Willebrand factor, leads to hemorrhagic disorders, suggesting that a pharmacological targeting of the GPIbα/von Willebrand factor axis could result in an increased bleeding risk. In this study, we show that targeting GPIbβ with the monoclonal antibody RAM.1 inhibits GPIb-mediated platelet activation, procoagulant activity, and thrombus formation in vitro and in vivo. Interestingly, RAM.1 does not prolong the tail-bleeding time of wild-type mice, suggesting that targeting GPIbβ could represent an antithrombotic strategy with a potentially low-bleeding risk.
Targeting Platelet GPIbβ Reduces Platelet Adhesion, GPIb Signaling and Thrombin Generation and Prevents Arterial Thrombosis

Eric Maurer, Chaojun Tang, Mathieu Schaff, Catherine Bourdon, Nicolas Receveur, Catherine Ravanat, Anita Eckly, Béatrice Hechler, Christian Gachet, Francois Lanza and Pierre H. Mangin

Arterioscler Thromb Vasc Biol. 2013;33:1221-1229; originally published online April 4, 2013; doi: 10.1161/ATVBAHA.112.301013

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/6/1221

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/04/04/ATVBAHA.112.301013.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
MATERIALS AND METHODS

Materials

Paraformaldehyde (PFA) and glutaraldehyde were from Electron Microscopy Sciences (Hatfield, PA). Human VWF was isolated from factor VIII concentrates (EFS-Alsace, Strasbourg, France) according to a previously described method. Botrocetin was purified from snake venom (Latoxan, Valence, France) as described previously. Acid citrate dextrose (ACD) solution was from Bioluz (St-Jean-de-Luz, France) and hirudin from Transgene (Illkirch-Graffenstaden, France). The monoclonal antibody RAM.1 was produced in our laboratory and the rat IgG isotype control was obtained from Becton Dickinson (Le Pont de Claix, France). The anti-GPIbα antibody, Xia.B2, was from Emfret Analytics (Eibelstadt, Germany). Acid-soluble type I fibrillar collagen (ASC) solution was prepared from bovine Achilles tendon as previously described. Fatty acid-free human serum albumin (HSA), TRITC-phalloidin, fibrinogen, bovine serum albumin (BSA), U46619, adenosine 5’-diphosphate (ADP) and bovine thrombin were from Sigma-Aldrich (Lyon, France). The GPIIb-IIIa antagonist eptifibatide (Integrilin®) was obtained from Millennium Pharma (San Francisco, CA). 3,3’-dihexyloxacarbocyanine iodide (DIOC₆), Oregon Green 488 BAPTA-AM-1 and Calcein red-orange-AM were from Molecular Probes (Eugene, OR). Xylasine (Rompun®) and ketamine (Imalgen®) were provided by Bayer (Puteaux, France) and Merial (Lyon, France), respectively. Calibrated automated thrombin (CAT) reagents were from STAGO (Asnières, France) and the fluorogenic substrate Z-GGR-AMC was from Bachem (Bubendorf, Switzerland). Fluoresceinisothiocyanate (FITC)–conjugated annexin V was from Roche Diagnostics (Meylan, France) and recombinant human tissue factor (Innovin) was from Dade Behring. Cross-linked collagen-related peptide (CRP) was obtained from Dr. R.W. Farndale (University of Cambridge, Cambridge, UK). Ca²⁺ ionophore A23187 was from Calbiochem (La Jolla, California). CHO cells transfected with human GPIb-IX complex were kindly provided by Dr J. Lopez (Houston, TX).
Preparation of F(ab')2 fragments

F(ab')2 fragments were prepared as described with minor modifications.5 Monoclonal antibody (MoAb) RAM.1 was dialyzed overnight against a 0.1 mol/L sodium acetate buffer (pH 4). The antibody (2.4 mg/mL) was digested by incubation with pepsin (Sigma-Aldrich (Lyon, France); 1 part pepsin to 50 parts MoAb) for 2.5 hours at 37°C. Digestion was stopped by adding 1 volume of a 2 mol/L Tris buffer (pH 9). Digested IgG were dialysed against 20 mmol/L Tris buffer (pH8) and F(ab')2 fragments were further purified on a monoQ column.

Platelet count

Whole blood was collected into EDTA (6 mmol/L) after severing the tail of an anesthetized mouse. The platelet count was determined in a Scil Vet ABC automatic cell counter (Scil Animal Care Company, Holtzheim, France) set to murine parameters.

Static adhesion assay

Glass coverslips were coated for 2 h with human VWF (10 µg/mL) and the surface was blocked with PBS-1% HSA for 1 h. Adhesion of CHO cells transfected with the GPIb-IX complex to a VWF surface was studied as previously described.6 Briefly, 105 cells were allowed to adhere to VWF in the presence of botrocetin (1 µg/mL) and EDTA (5 mmol/L) at 37°C for 30 min. After a washing step, the adherent cells were fixed with 4% PFA, stained with TRITC-phalloidin (2 µg/mL) and viewed by epifluorescence microscopy. The number of adherent cells and the percentage of cells extending at least one filopod were quantified. Adhesion of platelets to VWF was investigated as described elsewhere.7 Briefly, mouse platelets in Tyrode's buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO3, 0.4 mmol/L NaH2PO4, 1 mmol/L MgCl2, 2 mmol/L CaCl2, 5 mmol/L Hapes, 3.5 mg/mL HSA, 5.5
mmol/L glucose, pH 7.3), preincubated for 15 min with RAM.1 or an isotype control, were allowed to adhere to VWF (9 x 10^6 cells/coverslip) in the presence of botrocetin (1 µg/mL) and Integrilin (40 µg/mL) at 37°C. After 15 min, non-adherent platelets were removed by washing and adherent platelets were fixed with 4% PFA, stained with TRITC-phalloidin (2 µg/mL) and viewed by epifluorescence microscopy. The number of adherent platelets and of those extending filopodia were determined. Alternatively, the adherent platelets were fixed with 2.5% glutaraldehyde and processed for scanning electron microscopy as previously described.7

**Measurement of cytosolic Ca^{2+} concentrations**

Intracellular Ca^{2+} concentrations were monitored using a quantitative dual-dye ratiometric assay as previously described.8 Briefly, mouse platelets resuspended at 5 x 10^5/mL after a first washing step were simultaneously loaded with the membrane-permeating non ratiometric Ca^{2+} indicator dye Oregon Green 488 BAPTA-AM-1 (5 µmol/L) and the morphological dye Calcein red-orange-AM (4 µmol/L) for 30 min at 37°C. The dye-loaded platelets were washed a second time and finally resuspended in Tyrode's buffer containing apyrase (0.02 U/mL). The increases in platelet and Ca^{2+}-dependent fluorescence intensity upon adhesion of the cells were measured in the ranges 572-700 nm and 495-535 nm, respectively, by confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany) (1.85 frames/s for 10 min). The ratio of the signal intensities in the two dye channels was converted into intracellular Ca^{2+} concentrations (Leica TCS SP5 LASAF software).

**Measurement of annexin V binding**

Measurement of annexin V binding was performed as previously described.9 Briefly, washed mouse platelets in Tyrode's buffer were activated with CRP (10 µg/mL) or thrombin (1 U/mL) alone or together, or with A23187 (0.5 mmol/L) for 10 min at 37°C. A 2 µL aliquot of activated or resting platelets was then incubated with FITC-conjugated annexin V for 20 min and
analyzed by flow cytometry. A forward scatter/FL1 dot plot was performed to determine the ratio of activated to non-activated platelets. Activated annexin V-positive platelets were quantified as the percentage of total platelets.

Calibrated automated thrombin generation analyses

Calibrated automated thrombin (CAT) analyses were performed by the thrombogram method as previously described. Briefly, 20 μL of tissue factor (0.5 pmol/L Innovin 1/12 000 final dilution) or collagen (30 μg/mL) were added in triplicate to 80 μL of citrated (3.15%) mouse platelet-rich plasma (PRP) (1.3 × 10⁸ platelets/mL final concentration) in a 96-well plate (Immulum 2 Dynex; Stago, Paris, France) maintained at 37°C. The accumulation of fluorescence from cleaved thrombin substrate: Z-GGR-AMC (41 μmol/L) in the presence of CaCl₂ (1.7 mmol/L) was measured continuously at excitation and emission wavelengths of 390 and 460 nm, respectively (Fluoroskan Ascent; ThermoLab Systems, Helsinki, Finland). The area under the curve, also called the endogenous thrombin potential (ETP), corresponds to the total amount of thrombin formed in the sample. Peak corresponds to the maximal concentration of thrombin generated.

Measurement of the thrombus volume

Rectangular glass microcapillaries (VitroCom, Mountain Lakes, NJ) were coated with type I collagen (2.5 mg/mL) overnight at 4°C and blocked with PBS-1% HSA for 30 min at room temperature. Hirudinated (100 U/mL) mouse whole blood labeled with DIOC₆ (1 μmol/L) was perfused through the collagen-coated channels at 37°C and various shear rates using a programmable syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA). To determine the thrombus volume, the fluorescence emitted in the range 490-595 nm after excitation with a 488 nm argon-ion laser was measured using a confocal Leica SP5 inverted microscope with a resonant scanner and a 40x oil objective. Series of optical sections in xyz were recorded from the base to the peak of the thrombi at designated time points. The images were then stacked and analyzed with ImageJ software (National Institute of Health,
Bethesda, MD) to determine the volume of the thrombi. 3D reconstructed images were obtained using Image surfer (Center for Computer-Integrated Systems for Microscopy and Manipulation, Chapel Hill, NC).

**Mice**

C57BL/6 WT mice were obtained from Charles River (L’Arbresle, France) and all mice were maintained in the animal facilities of the Etablissement Français du Sang-Alsace. We used 8-week-old male mice. All procedures for animal experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals as defined by European laws.

**Tail bleeding time**

The tail bleeding time was measured as previously reported. The time required for the arrest of bleeding and the volume of blood lost were determined over a 30 min period.

**Arterial thrombosis models**

Mice were anesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and injected with a fluorescent dye (DIOC₆, 5 µL of a 100 µM solution/g of body weight) to label platelets and allow visualization of a thrombus.Mesenteric arterioles were injured with a laser and the resultant thrombus growth was monitored as previously described. Alternatively, thrombosis was induced mechanically by compression of the abdominal aorta with forceps for 60 s. Thrombus formation was then monitored in real time with a fluorescence microscope coupled to a charge-coupled device camera (Roper Scientific, Evry, France) and the images were analyzed with the Metamorph software (Molecular Devices, Roper Scientific). The mice were maintained and the experiments were performed in the animal facilities of the EFS-Alsace.
Statistics

All values are reported as the mean ± standard error of the mean (SEM) unless otherwise indicated. The data of Figures 1, 2, 3, 4, S1 and S2 were analysed with a two-tailed paired t-test. The data of Figures 5 and 6 were analysed with a Grubbs’ test followed with a two-tailed unpaired t-test. Differences were considered to be significant for p<0.05. All statistical tests were carried out using Prism software (GraphPad, La Jolla, CA).
REFERENCES


Supplemental Figure I: Xia.B2, does not decrease TF-induced thrombin generation. 

A-B, CAT experiments were performed in mouse citrated PRP. The reaction was triggered by addition of TF (0.5 pmol/L) in the presence of Ca$^{2+}$ and the fluorogenic substrate Z-GGR-AMC. Thrombin activity was determined from the accumulation of the fluorescent product and calculated relative to a thrombin calibrator. A, The bar graphs correspond to the endogenous thrombin potential (ETP) values, representing the total amount of thrombin generated. B, The bar graphs represent the peak values which correspond to the maximal thrombin concentration attained. The results are presented as the mean ± SEM in three independent experiments for each condition.
Supplemental figure II: RAM.1 F(ab)'2 inhibits GPIb-mediated filopodia extension after platelet adhesion to immobilized VWF. A-B, Washed mouse platelets (3 x 10^7/mL) incubated with Integrilin (40 μg/mL) were treated with control or RAM.1 F(ab)'2 fragments (10 μg/mL) and allowed to adhere for 15 min to a VWF matrix in the presence of botrocetin (1 μg/mL). Adherent platelets were fixed and examined by scanning electron microscopy. The total number of adherent platelets (A) and the percentage of platelets extending 0-1, 2-4 and >5 filopodia (ns: P>0.05) (B) were analysed in five random fields (n=3). Results represent the mean ± SEM in three independent experiments (*P<0.05).