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The glycoprotein Ib-IX-V complex contributes to tissue factor–independent thrombin generation by recombinant factor VIIa on the activated platelet surface

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Several lines of evidence suggest that recombinant factor VIIa (rFVIIa) is able to activate factor X on an activated platelet, in a tissue factor-independent manner. We hypothesized that, besides the anionic surface, a receptor on the activated platelet surface is involved in this process. Here, we showed that, in an ELISA setup, a purified extracellular fragment of GPIbα bound to immobilized rFVIIa. Surface plasmon resonance established a affinity constant (K_d) of approximately 20 nM for this interaction. In addition, CHO cells transfected with the GPIb-IX-V complex could adhere to immobilized rFVIIa, whereas wild-type CHO cells could not. Furthermore, platelets stimulated with a combination of collagen and thrombin adhered to immobilized rFVIIa under static conditions. Platelet adhesion was inhibited by treatment with O-sialoglycoprotein endopeptidase, which specifically cleaves GPIbα from the platelet surface. In addition, rFVIIa-mediated thrombin generation on the activated platelet surface was inhibited by cleaving GPIbα from its surface. In summary, 3 lines of evidence showed that rFVIIa interacts with the GPIb-IX-V complex, and this interaction enhanced tissue factor-independent thrombin generation mediated by rFVIIa on the activated platelet surface. The rFVIIa-GPIbα interaction could contribute to cessation of bleeding after administration of rFVIIa to patients with bleeding disorders. (Blood. 2008; 112:3227-3233)

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Introduction

Recombinant factor VIIa (rFVIIa) was originally developed for the treatment of hemophilia A and B, which have developed inhibitory antibodies against factor VIII and IX as a result of treatment with FVIII or FIX concentrates.[1] Nowadays, it has also been registered for use in patients with factor VII deficiency, acquired hemophilia, and inhibitor-complicated Glanzmann's thrombasthenia. Its mechanism of action is thought to involve the local enhancement of thrombin generation at the site of vessel wall damage. Enhancement of thrombin generation will result in enhanced fibrin formation as well as changes in fibrin structure that will result in a clot that is better protected against fibrinolysis.[2] Improved clot stability is also achieved through increased activation of thrombin-activatable fibrinolysis inhibitor (TAFI).[3] Finally, enhanced thrombin generation results in an acceleration of platelet activation, which will facilitate induction of hemostasis in 2 ways. First, platelet activation directly contributes to formation of the hemostatic plug. Second, activation of platelets results in an increase in thrombin generation, as platelet activation will expose procoagulant phospholipids on the platelet surface.

It has been suggested that enhancement of thrombin generation by rFVIIa is solely dependent on the presence of tissue factor (TF).[4,5] However, different independent experiments have shown that the effect of rFVIIa can proceed via TF-dependent[3,6] as well as TF-independent[7-9] pathways, and it has been postulated that both mechanisms are operative in vivo.[10]

Although the mechanisms through which rFVIIa exerts its activity have been studied extensively, there are still some unresolved questions. The now widely used standard dose of rFVIIa (90 μg/kg) results in plasma levels that by far exceed the affinity constant (K_d) for TF binding, and it has not yet been fully clarified why these high plasma concentrations of rFVIIa are required for induction of hemostasis. Furthermore, even higher doses of rFVIIa (up to 270 μg/kg) appear more efficient compared with the regular dose in treatment of inhibitor-complicated hemophilia.[11-13] In addition, relatively high doses are thought to be required for induction of hemostasis in other indications (eg, treatment of platelet-related bleeding disorders).[14-16]

It is unclear why such high doses of rFVIIa are required for the induction of hemostasis, as TF is thought to be saturated with rFVIIa at much lower doses than achieved with the regular dose of rFVIIa.[17] A possible explanation for the requirement of higher doses than initially anticipated is the inhibitory effect of zymogen factor VII on TF-FVIIa initiated coagulation.[6] An alternative explanation is that TF-independent thrombin generation accounts for the clinical efficacy of high-dose rFVIIa because the affinity of rFVIIa for the platelet surface is much lower than for TF. This latter hypothesis is supported by observations with rFVIIa variants with substantially increased TF-independent activity, but unaltered TF-dependent activity compared with wild-type rFVIIa. These rFVIIa variants were shown to be much more potent in a murine model of hemophilia compared with wild-type rFVIIa, showing that TF-independent thrombin generation accounts, at least for a substantial part, for the hemostatic efficacy of rFVIIa.[18]

Activated platelets have been shown to play an important role in the controlled enhancement of thrombin generation. rFVIIa can directly bind to negatively charged phospholipids exposed on

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activated platelets, and this supports thrombin generation independently of TF.[9,19] The activation of factor X (FX) on anionic phospholipids is a very inefficient process, and we hypothesized that, besides negatively charged phospholipids, there might be a role for a receptor on the activated platelet surface in rFVIIa-mediated thrombin generation. A possible candidate is TF, which has been reported to be present on the platelet surface after platelet activation. Several groups described the presence of TF pre-mRNA in platelets,[20,21] whereas others have reported that TF can be taken up by platelets after interaction with TF-containing microparticles.[22] Previous experiments by our group showed that rFVIIa increased platelet deposition onto collagen, and this increased platelet deposition could not be inhibited by antibodies that inhibit TF.[7] Therefore, we hypothesized that another platelet receptor is involved in the binding of rFVIIa, and we focused on the GPIb-IX-V complex because it is one of the most abundant receptors on platelets. The GPIb-IX-V complex was originally identified as the receptor for von Willebrand factor, but recent research has shown that it can also serve as a receptor for other proteins, including thrombin,[23] factor XI,[24] factor XIIa,[25] high molecular weight kininogen,[26] and (activated) protein C.[27] This led to our hypothesis that GPIbα is involved in the mechanism of action of rFVIIa.

In this study, we have investigated whether rFVIIa and GPIbα can interact with each other. Furthermore, we examined the role of GPIbα in the interaction of platelets and CHO cells transfected with the GPIb-IX-V complex to immobilized rFVIIa. Finally, we investigated whether the interaction between GPIbα and rFVIIa affects thrombin generation on the activated platelet surface.

Methods

Materials

Soluble recombinant tissue factor (residues 1-290, sTF), tissue factor pathway inhibitor, rFVIIa, recombinant factor VII, a neutralizing antibody against factor VIII, and an inhibitory antibody against TF were a generous gift from Dr M. Kjalke (Hemostasis Biology, Novo Nordisk, Malov, Denmark). The PAR1-agonist peptide serine-phenylalanine-leucine-leucine-asparagine (SFLLRN) was from Bachem (Bubendorf, Switzerland). Annexin A5 was a kind gift of Dr C. Reutelingsperger (Maastricht, The Netherlands). Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). FX was purified from fresh-frozen plasma by immunoaffinity chromatography followed by Q-Sepharose chromatography as described previously.[28] Human coagulation factors V, prothrombin, and antithrombin III (ATIII) were purchased from Haematologic Technologies (Essex Junction, VT). S2238 was purchased from Chromogenix (Milan, Italy). Pefabloc Xa and Pefachrome Xa were from Pentapharm (Basel, Switzerland). O-sialoglycoprotein endopeptidase was purchased from Cedarlane Laboratories (Hornby, ON). The Technothrombin TGA kit was obtained from Technoclone (Surrey, United Kingdom). Glycocalicin (GC) was purified as described previously.[29] Fully sulfated wild-type recombinant GPIbα, comprising residues 1 to 290, was produced and purified as described previously.[30] Essentially fatty acid free bovine serum albumin (BSA) and p-nitrophenyl phosphate substrate were obtained from Sigma-Aldrich (St Louis, MO). All other chemicals used in the experiments were of analytic grade.

Platelet preparation

Blood from healthy volunteers, who claimed not to have used aspirin or other nonsteroidal anti-inflammatory drugs for the preceding 10 days, was drawn into one-tenth volume of 3.4% sodium citrate. This was done with the approval from the Institutional Review Board from the University Medical Center Utrecht (Utrecht, The Netherlands) and in accordance with the Declaration of Helsinki. Washed platelets were prepared as described previously.[7] The blood was centrifuged at 200*g* for 15 minutes at room temperature. The platelet-rich plasma (PRP) was removed and acidified by addition of one-tenth volume of ACD (2.5% trisodium citrate, 1.5% citric acid, and 2% D-glucose). Platelets were centrifuged (500*g*, 15 minutes) and the platelet pellet was resuspended in HEPES-Tyrode buffer at pH 6.5 (10 mM of *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), 137 mM of NaCl, 2.68 mM of KCl, 0.42 mM of NaH_2PO_4, 1.7 mM of $MgSO_4$, and 5 mM of D-glucose). Prostacyclin (PGI_2, 10 ng/mL) was added to prevent platelet activation during the subsequent washing step. Platelets were centrifuged (500*g*, 15 minutes) and resuspended in a small volume of HEPES-Tyrode buffer. Platelet suspension was further diluted in HEPES-Tyrode buffer at pH 7.35 to a platelet count of 200×10^9/L (20 000/μL). GPIbα-depleted platelets were prepared by treating the platelets for 30 minutes at 37°C with 80 μg/mL O-sialoglycoprotein endopeptidase (OSE). Proteolysis of GPIb was measured by investigating binding of a GPIbα-specific antibody (AN51, Dako Denmark, Glostrup, Denmark) to platelets by fluorescent-activated cell sorter analysis. OSE treatment reduced GPIbα expression on platelets to less than 5% (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Cell culture

Wild-type Chinese Hamster Ovary (CHO) cells and CHO cells stably expressing the GPIb-IX-V (a generous gift of Dr J. A. Lopez [Seattle, WA]) were grown in a 1:1 mixture of Dulbecco modified Eagle medium and Ham's F-12 medium with 10% fetal calf serum in the presence of penicillin and streptomycin. Cells were subjected to selection by using G418 as described previously.[31]

Binding studies

rFVIIa was immobilized for 2 hours at 37°C on a Costar 96-well plate at the indicated concentrations. After blocking the wells with 2% BSA for at least 30 minutes, the wells were incubated with 2 μg/mL GC for 90 minutes at 22°C. Bound GC was detected with an in-house rabbit polyclonal antibody against GPIb, followed by a peroxidase-labeled swine-antirabbit antibody detected with TMB peroxidase substrate solution (Tebu-Bio, Heerhugowaard, The Netherlands). Results were obtained by measuring optical density at 450 nm on a SpectroMax Reader (Molecular Devices, Wokingham, United Kingdom).

For cell adhesion experiments, rFVIIa was immobilized for 2 hours at 37°C on an Immulon-2B flat-bottom microtiter plate (Dynex Technologies, Chantilly, VA) and subsequently blocked with 2% BSA for at least 30 minutes. Washed platelets (200 000/μL in HEPES-Tyrode's, pH 7.35) or CHO cells (resuspended at 10^6 cells/mL in Dulbecco modified Eagle medium:F-12, 0.5% BSA, 1 mM of $CaCl_2$, 25 μM of $ZnCl_2$) were allowed to adhere for 60 minutes at 37°C. After extensive washing with Tris-buffered saline, intrinsic phosphatase activity was measured using p-nitrophenyl phosphate (3 mg/mL dissolved in 50 mM acetic acid, 1% Triton X-100, pH 5.0), and after 30 minutes the reaction was stopped with 1 M NaOH. Optical density was measured at 405 nm.

Surface plasmon resonance analysis

Binding studies were performed using a BIAcore2000 biosensor system (BIAcore AB, Uppsala, Sweden), and surface plasmon resonance (SPR) analysis was done as previously described.[32] rFVIIa or FX was immobilized on a CM5-sensor chip, using the amine-coupling kit as prescribed by the supplier. A control channel was routinely activated and blocked in the absence of protein. Binding to coated channels was corrected for binding to noncoated channels. SPR analysis was performed in HEPES (25 mM NaCl, 125 mM HEPES, 3 mM $CaCl_2$, pH 7.4) buffer with a flow rate of 5 μL/min. Regeneration of the sensor chip surface was performed by incubating with 10 mM TDOC, (taurodeoxycholic acid) in Tris, pH 9.0, for 2 minutes at a flow rate of 5 μL/min.

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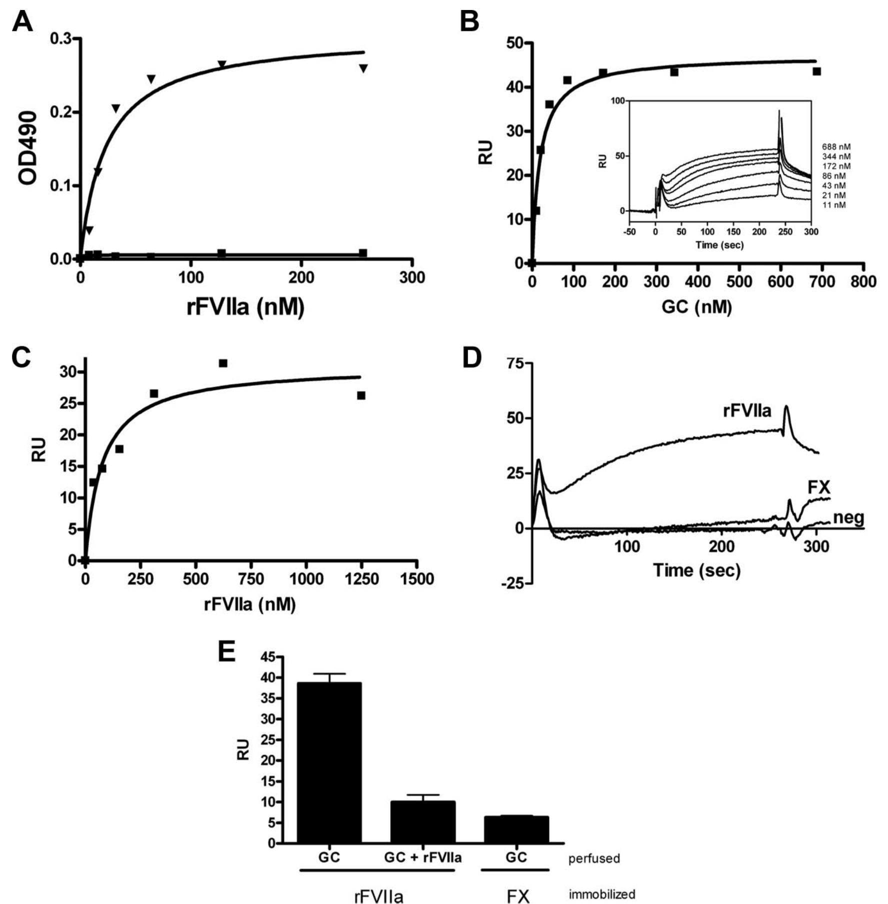

Figure 1. Glycocalicin interacts with rFVIIa. (A) rFVIIa was immobilized on a microtiter plate at concentrations indicated. Subsequently, wells were incubated with GC (5 μg/mL) or vehicle, and bound GC was detected by using a polyclonal antibody against GPIb (2 μg/mL). (B) rFVIIa (3400 RU) was immobilized on a CM5 sensor chip, and binding of GC was investigated by SPR. After adjusting for binding to a blank channel, the response of GC at equilibrium was determined and plotted against the concentration applied. Inset shows representative SPR traces of indicated concentrations of GC. (C) GC (1100 RU) was immobilized on a CM5 sensor chip, and binding of rFVIIa was investigated by SPR. (D) Representative traces of GC (67 nM) binding to a rFVIIa-coated channel, a channel coated with factor X (FX, 5400 RU) or an uncoated channel (neg). (E) Binding of GC (67 nM) to rFVIIa was investigated in the absence or presence of 2 μM rFVIIa. Representatives of at least 3 experiments are shown.

Thrombin generation

Thrombin generation experiments were performed as described previously by Monroe et al.[9] In short, isolated platelets were stimulated with a combination of convulxin (100 ng/mL) and SFLLRN (100 μM) or vehicle. Platelets were mixed with plasma concentrations of prothrombin (1.2 μM), FX (135 nM), factor V (7 μg/mL), factor VII (10 nM), antithrombin (2.5 μM), and tissue factor pathway inhibitor (TFPI, 0.1 μg/mL). Thrombin generation was initiated with indicated concentrations of rFVIIa, and at indicated time points small samples were taken and added to thrombin substrate solution (1 mM of EDTA, 50 μM Pefabloc Xa, and 0.5 mM S2238). After 25 minutes, the reaction was stopped by addition of 50% acetic acid. Optical density was measured at 405 nM. Thrombin activity was calculated by comparing the optical density to a standard range of thrombin. Thrombin activity curves were fitted using a modified Gaussian equation as described previously.[33]

For thrombin generation in plasma, isolated washed platelets were pretreated with OSE (80 μg/mL) or vehicle, before stimulation with a combination of convulxin (100 nM) and SFLLRN (100 μM). Next, platelets were reconstituted in "artificial" hemophilic plasma (pooled normal plasma, to which a neutralizing antibody against FVIII was added, final concentration, 10 Bethesda units) as described previously,[34] and thrombin generation was initiated by 25 or 100 nM rFVIIa. The formation of thrombin was monitored in time using the Technothrombin TGA kit, an assay based on a fluorescent thrombin substrate, according to the instructions of the manufacturer.

Statistical analysis

Statistical analysis was performed using the GraphPad InStat (GraphPad Software, San Diego, CA) software package. Statistical differences in binding and adhesion were analyzed by Student *t* test or standard one-way analysis of variance. Statistical differences in thrombin generation were analyzed by paired Student *t* test. Values of *P* less than .05 were considered statistically significant.

Results

Glycocalicin binds to immobilized rFVIIa

To investigate the interaction between rFVIIa and GPIbα, we tested the binding of the purified extracellular fragment of GPIbα GC to immobilized rFVIIa in an enzyme-linked immunosorbent assay (ELISA) setup. As shown in Figure 1A, GC readily bound to immobilized rFVIIa in a concentration-dependent manner. Binding of GC to rFVIIa was further investigated using SPR. rFVIIa was coated to a CM5 sensor chip via amine coupling with a maximum adsorption of 3400 RU and perfused with different concentrations of GC. Nonlinear regression analysis resulted in an affinity constant (K_d) of 19.5 (± 1.4) nM (mean ± SD, Figure 1B). Immobilized rFVIIa showed only minimal binding to FX (Figure 1D). A recombinant, truncated form of GPIbα, comprising residues 1 to 290, did not bind to immobilized rFVIIa (data not shown). In addition, when a CM5 sensor chip was coated with GC, rFVIIa bound in a dose-dependent manner with a calculated K_d of 78 nM (± 24) nM (mean ± SD, Figure 1C). The interaction of GC with immobilized rFVIIa was abrogated by addition of rFVIIa in solution, confirming the specificity of the interaction (Figure 1E).

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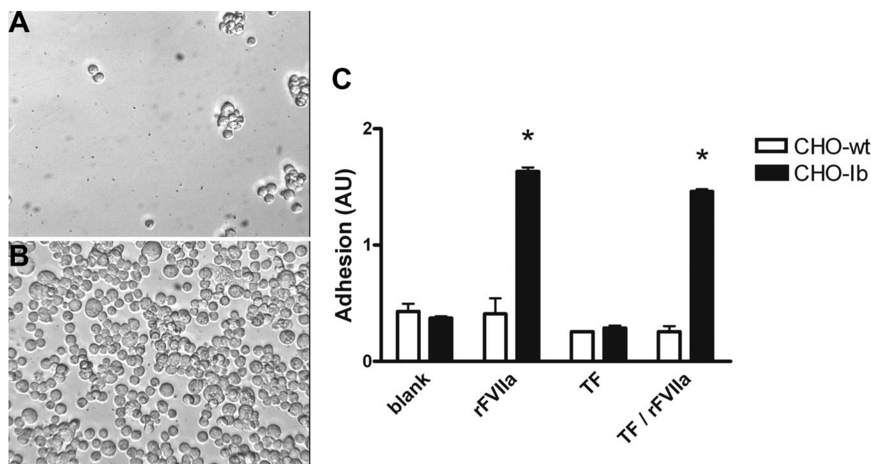

Figure 2. CHO cells transfected with the GPIb-IX-V complex bind to immobilized rFVIIa. (A,B) Mock-transfected CHO cells (CHO-wt, 10^6 cells/mL; A) and CHO cells transfected with the GPIb-IX-V complex (CHO-Ib, 10^6 cells/mL; B) were allowed to adhere under static conditions to immobilized rFVIIa (10 μg/mL) for 60 minutes at 37°C. After gentle washing, adhesion was visualized by light transmission microscopy with a Zeiss 10×/0.22 numeric aperture lens on a Leica Diavert microscope (Leica, Wetzlar, Germany). Images were acquired using a JAI-CCD camera (Copenhagen, Denmark) coupled to a matrox frame grabber (Matrox Electronic Systems, Quebec, QC) using OPTIMAS 6.2 software (Optimas, Seattle, WA); original magnification 400×. (C) CHO-wt and CHO-Ib (10^6 cells/mL) were allowed to adhere under static conditions for 60 minutes at 37°C to immobilized rFVIIa (10 μg/mL) or recombinant soluble tissue factor (TF; 10 μg/mL). Subsequently, TF-immobilized wells were blocked with 2% BSA, before incubation with rFVIIa or vehicle ("TF/rFVIIa" and "TF," respectively). Adhesion was quantified by measuring the intrinsic phosphatase activity, and optical density is depicted as arbitrary units of adhesion (AU). Images and graph are representative of at least 3 independent experiments performed in triplicate. Error bars represent SD. $^*P < .01$ compared with blank.

CHO cells expressing the GPIb/IX/V-complex interact with immobilized rFVIIa

To further investigate the interaction between rFVIIa and GPIbα, we allowed mock-transfected CHO cells (CHO-wt) as well as CHO cells expressing the GPIb-IX-V complex (CHO-Ib) to adhere to immobilized rFVIIa under static conditions. Expression of GPIb was confirmed by fluorescent-activated cell sorter analysis (Figure S2). As shown in Figure 2, CHO-Ib cells interacted with immobilized rFVIIa, whereas CHO-wt did not, suggesting a specific interaction between rFVIIa and the GPIb-IX-V complex. However, pretreatment of the CHO-Ib cells with OSE, which removed all GPIbα from the cells (which was confirmed by flow cytometry), did not affect adhesion to rFVIIa.

TF did not interfere in the interaction between the GPIb-IX-V complex expressed on CHO cells, and immobilized rFVIIa, as adhesion of CHO-Ib cells to immobilized rFVIIa was not affected by an inhibitory antibody against TF or by preincubation of immobilized rFVIIa with sTF (data not shown). Furthermore, CHO-Ib cells readily adhered to rFVIIa in complex with immobilized sTF (Figure 2C). Again, this shows that the binding of rFVIIa to TF does not interfere with the interaction between rFVIIa and the GPIb-IX-V complex.

Proteolysis of GPIbα reduces adhesion of activated platelets to immobilized rFVIIa

To investigate whether the interaction between rFVIIa and GPIbα is sufficiently strong to bind platelets, washed platelets were allowed to adhere to immobilized rFVIIa under static conditions. Nonstimulated platelets did not bind to immobilized rFVIIa to a substantial extent. However, on stimulation with collagen (4 μg/mL) or a combination of collagen (4 μg/mL) and thrombin (1 U/mL) for 15 minutes, platelet adhesion was substantially increased compared with nonstimulated platelets (Figure 3A). Adhesion was dependent on negatively charged phospholipids, as the addition of annexin A5 decreased adhesion of activated platelets to rFVIIa (Figure 3B). Subsequently, we pretreated platelets with OSE, which cleaves the extracellular portion of GPIbα. When platelets were pretreated with OSE (80 μg/mL, 30 minutes, 37°C), adhesion to immobilized rFVIIa was inhibited by approximately 40% (Figure 3B), suggesting an important role for GPIbα in the interaction of platelets with rFVIIa. Platelet adhesion in the presence of an inhibitory antibody against TF was only slightly decreased, suggesting only a minor role for platelet-exposed TF (Figure 3B).

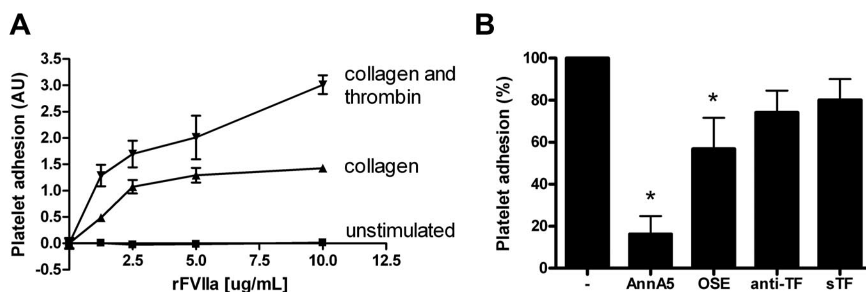

Figure 3. Platelet adhesion to immobilized rFVIIa is dependent on negatively charged phospholipids and GPIbα. (A) Washed platelets were stimulated with collagen (4 μg/mL) or a combination of collagen (4 μg/mL) and thrombin (1 U/mL). Subsequently, platelets were allowed to adhere under static conditions for 60 minutes at 37°C to immobilized rFVIIa at the concentrations indicated. (B) Washed platelets were pretreated with annexin A5 (30 μg/mL, AnnA5), OSE (80 μg/mL), an inhibitory antibody against TF (0.5 mg/mL, anti-TF), sTF (10 μg/mL), or vehicle (−) before stimulation with a combination of collagen (4 μg/mL) and thrombin (1 U/mL) and subsequently allowed to adhere to rFVIIa. Adhesion of stimulated platelets to rFVIIa (5 μg/mL) is indicated as 100% platelet adhesion. Graph shows mean platelet adhesion of at least 2 independent experiments performed in triplicate. $^*P < .05$ compared with control.

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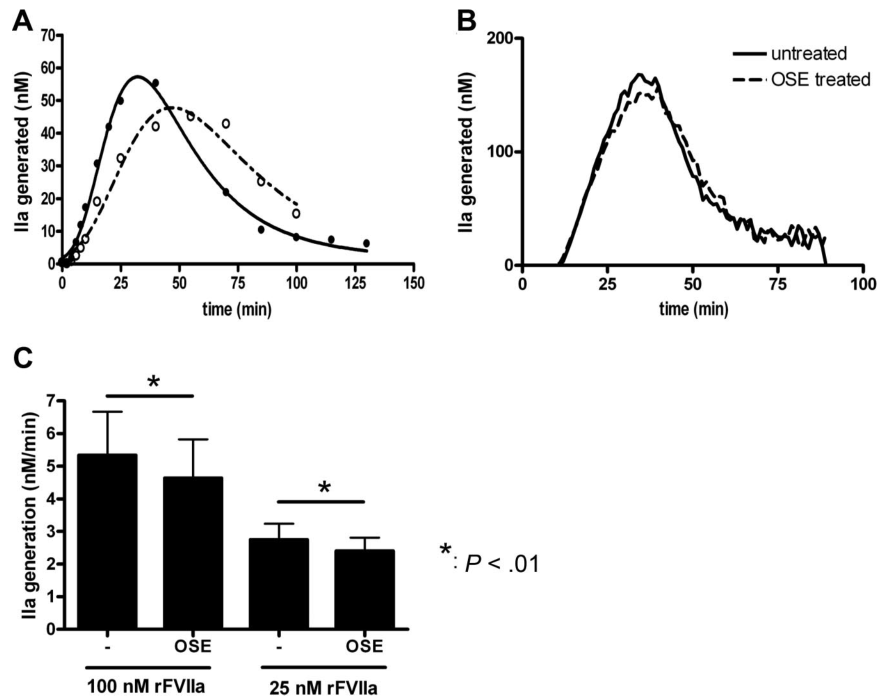

Figure 4. GPIbα accelerates rFVIIa-mediated thrombin generation. Platelets were pretreated with OSE (160 μg/mL, ----, ○) or vehicle (—, ●) for 30 minutes at 37°C and subsequently stimulated with a combination of convulxin (100 ng/mL) and SFLLRN (100 μM). (A) Next, thrombin generation was followed in time after addition of 25 nM VIIa in the presence of plasma concentrations of coagulation factors VII, X, II, V, and Ca^{2+}, and inhibitors TFPI and antithrombin III. Samples were taken at indicated time points, and the amount of thrombin generated was calculated. (B) Platelets were reconstituted in "artificial" hemophilic plasma (final concentration, 200 000/μL), and coagulation was started by the addition of 25 or 100 nM rFVIIa. The formation of thrombin was followed in time using a commercially available thrombin generation assay. (C) Quantification of the initial slope of plasma-based thrombin generation curves as shown in panel B. Shown are mean values from thrombin generation curves generated from 8 different donors in which thrombin generation curves were generated in "artificial" hemophilic plasma with 25 or 100 nM of rFVIIa in the absence or presence of OSE pretreatment. Error bars represent SD.

Proteolyis of GPIbα reduces thrombin formation on activated platelets by rFVIIa

To study whether GPIbα plays a role in the generation of thrombin on the activated platelet surface, we investigated thrombin generation under hemophilic conditions using a modification of the cell-based model as described by Monroe et al.[9] In the setup used in our experiments, coagulation was started by high-dose rFVIIa in the absence of a TF-bearing cell, in combination with platelet activation by SFLLRN/convulxin. The initial slope of the curve was taken as a measure for the rate of thrombin generation. As shown in Figure 4, pretreatment of the platelets with OSE showed small but significant inhibition of thrombin generation in the presence of 100 nM rFVIIa, whereas it showed more pronounced inhibition in the presence of 25 nM rFVIIa (100 nM: 2.99 ± 0.79 vs 3.47 ± 0.64, $P < .01$; 25 nM: 0.99 ± 0.36 vs 1.39 ± 0.56, $P < .05$ [mean ± SD, n = 5], respectively). However, this inhibition was only observed in 5 of 10 donors tested. The donors that did not respond to OSE treatment showed comparable peak thrombin generation compared with control. The cause of this donor variability remains to be elucidated.

Furthermore, in the absence of rFVIIa, hardly any thrombin generation was observed during the investigated time period (data not shown). The area under the curve, a measure for total thrombin generation, was similar comparing treated versus nontreated platelets, indicating that GPIbα does not influence the total amount of FX converted to FXa but only affects the rate of conversion of FX into factor Xa.

To study the effect of GPIb in thrombin generation in a more physiologic model, we used a commercially available thrombin generation assay, in which we reconstituted platelets in "artificial" hemophilic plasma. Again, the initial slope of the curve was taken as a measure for the rate of thrombin generation. As shown in Figure 4B, thrombin generation was significantly inhibited on treatment with OSE compared with controls (100 nM rFVIIa: 4.64 ± 1.19 vs 5.34 ± 1.33, $P < .01$; 25 nM rFVIIa: 2.40 ± 0.41 vs 2.75 ± 0.48, $P < .05$ [mean ± SD, n = 8], respectively).

Discussion

In this study, 3 lines of evidence show that rFVIIa is able to interact with the GPIb-IX-V complex. First, we showed that rFVIIa and purified GC interact with each other, both in an ELISA setup as well as in SPR analysis. Furthermore, CHO cells transfected with the GPIb-IX-V complex were able to adhere to immobilized rFVIIa, whereas wild-type cells were not. Finally, we demonstrated that, on activation, platelets were able to adhere to immobilized rFVIIa in a GPIbα-dependent manner. Furthermore, the formation of thrombin on the activated platelet surface was reduced on proteolysis of GPIbα. Taken together, these observations suggest that the interaction of rFVIIa with GPIbα plays an important role in TF-independent enhancement of thrombin generation by rFVIIa on the activated platelet surface. Surprisingly, proteolysis of GPIbα did affect platelet adhesion but not adhesion of transfected CHO cells to immobilized rFVIIa. At least in the CHO cell system, it thus appears that other components of the GPIb-IX-V complex are involved in the interaction with rFVIIa. At present, tools to investigate the interaction of rFVIIa with other components of the complex are not available, and the role of the other subunits in the interaction remains to be established. However, because rFVIIa-mediated platelet adhesion and thrombin generation on the activated platelet are affected by specific proteolysis of GPIbα, we are convinced that GPIbα is the main physiologic receptor for rFVIIa within the GPIb-IX-V complex.

Activated platelets are thought to play an important role in enhancing thrombin generation when the initial exposed TF of the vessel wall is covered with platelets and is not accessible anymore for plasma FVIIa. First, it has been shown that TF is incorporated in the growing thrombus by recruiting TF-containing microvesicles in a mechanism dependent on P-selectin and PSGL-1.[22,35] Second, activation of platelets results in exposure of negatively charged phospholipids, which is important in the propagation of coagulation. We propose a third mechanism by which platelets can

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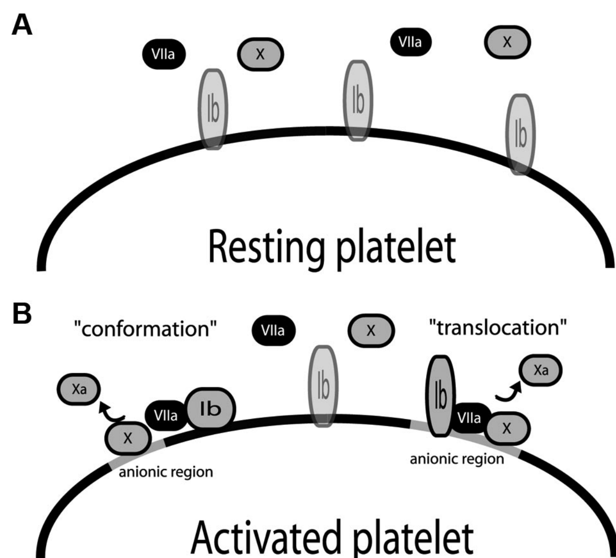

Figure 5. Hypothetical model for the role of GPIbα in the mechanism of action of rFVIIa. (A) On the resting platelet, the interaction between rFVIIa and the GPIb-IX-V complex (depicted as "Ib") does not occur, as the resting platelet does not express negatively charged phospholipids, and presumably because the GPIb-IX-V complex is not in a rFVIIa-binding membrane environment. (B) After activation of the platelet, rFVIIa is able to interact with the GPIb-IX-V complex. This interaction is critically dependent on the negatively charged surface but may also involve translocation of the GPIb-IX-V complex to an anionic or lipid raft region of the platelet surface. Alternatively, the interaction might require a conformational change with the GPIb-IX-V complex. The interaction of rFVIIa with GPIb results in acceleration of Xa generation.

propagate secondary hemostasis: GPIbα expressed on activated platelets acts in localizing rFVIIa on the platelets surface, thereby enhancing its enzymatic efficacy.

rFVIIa interacts with purified GC, but a recombinant soluble form of GPIbα, consisting of residues 1 to 290, does not interact with rFVIIa, whereas recombinant soluble GPIbα does bind both von Willebrand factor and thrombin, 2 other ligands for GPIbα. This suggests that the glycosylated stack of the GPIbα molecule plays a role in the interaction with rFVIIa or that GC has a different conformation compared with recombinant soluble GPIbα. Furthermore, rFVIIa does not bind to resting platelets, whereas GPIbα is abundantly present on the surface of resting platelets. In addition, CHO-cells expressing the GPIb-IX-V complex readily adhere to immobilized rFVIIa without the need for prior activation. An interesting explanation for these contradictory results could be that GPIbα needs to be located in the proper lipid microenvironment. Alternatively, platelet activation could result in a conformational change within the GPIb-IX-V complex, which changes the complex into an rFVIIa-binding conformation. Apparently, the GPIb-IX-V complex expressed on the CHO cells already has the sufficient characteristics necessary for binding of rFVIIa. In platelets, after activation, the lipid microenvironment of GPIbα could change, and the specific combination of lipids and GPIbα could be responsible for the binding of rFVIIa. In this way, activation of the platelets could serve 2 roles: (1) exposure of negatively charged phospholipids at the platelet surface, which is a prerequisite for rFVIIa binding to platelets; and (2) translocation of GPIbα to these negatively charged regions, which provides the perfect surface for the propagation of coagulation or an activation-induced conformational change in the complex (Figure 5). In this way, rFVIIa can interact with the negatively charged surface via its Gla domain and (perhaps simultaneously) with GPIbα via another part of the molecule, in which the interaction with GPIbα accelerates propagation of coagulation. However, other explanations for the discrepancy observed between the 2 cell types cannot be ruled out; therefore, future experiments will have to prove this model system.

Recent reports have suggested the presence of TF on activated platelets. However, it is improbable that in our model TF is involved in the interaction of rFVIIa with platelets. As shown by Monroe et al,[9] inhibitory antibodies against TF showed no reduction in thrombin generation on the activated platelet surface. In addition, in our model a delay in thrombin generation was observed after cleavage of GPIbα, irrespective of whether TF is involved or not. Furthermore, we have shown that the binding of CHO-Ib cells to immobilized rFVIIa is not influenced by the presence of TF. Panes et al[21] reported the colocalization of GPIbα and TF on the activated platelet surface, which leaves the possibility that both GPIbα and TF interact with rFVIIa at the same time, possibly serving different roles in the mechanism of action of rFVIIa.

Our results clearly show that rFVIIa can interact with GPIbα. The most important cellular receptor for rFVIIa is TF, but besides TF, several receptors have been reported to interact with rFVIIa. The TF/rFVIIa complex is able to bind and signal via a protease-activated receptor, presumably PAR-2 (reviewed by Riewald and Ruf[36]). Furthermore, recent reports show that the endothelial protein C receptor is able to bind rFVIIa at the endothelial cell surface.[37-40] However, the effect of binding of rFVIIa to these receptors on procoagulant activity of rFVIIa is still a matter of debate, as the groups show contradictory results on activation of protein C and generation of FXa. It remains to be elucidated if and how all these receptors interplay in the mechanism of action of rFVIIa.

Recently, Tranholm et al reported a variant of rFVIIa with improved TF-independent, but similar TF-dependent thrombin generation capacity compared with wild-type rFVIIa.[18] An interesting application of the results presented in this manuscript would be to construct a new variant that has an improved interaction with GPIbα, which can ultimately lead to an enhanced efficacy of rFVIIa. This could improve current therapy because lower doses of rFVIIa or less frequent administration of rFVIIa would be necessary, which would obviously benefit the patient.

In conclusion, our findings suggest an important role for GPIbα in the mechanism of action of rFVIIa; therefore, the development of rFVIIa variants with an improved binding to GPIbα could ultimately lead to improved therapy for hemophilia patients.

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Authorship

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References

1. Hedner U. Recombinant activated factor VII as a universal haemostatic agent. Blood Coagul Fibrinolysis. 1998;9(suppl 1):S147-S152.
2. He S, Blomback M, Jacobsson EG, Hedner U. The role of recombinant factor VIIa (FVIIa) in fibrin structure in the absence of FVIII/FIX. J Thromb Haemost. 2003;1:1215-1219.
3. Lisman T, Mosnier LO, Lambert T, et al. Inhibition of fibrinolysis by recombinant factor VIIa in plasma from patients with severe hemophilia A. Blood. 2002;99:175-179.
4. Butenas S, Brummel KE, Branda RF, Paradis SG, Mann KG. Mechanism of factor VIIa-dependent coagulation in hemophilia blood. Blood. 2002;99: 923-930.
5. Butenas S, Brummel KE, Bouchard BA, Mann KG. How factor VIIa works in hemophilia. J Thromb Haemost. 2003;1:1158-1160.
6. van't Veer C, Mann KG. The regulation of the factor VII-dependent coagulation pathway: rationale for the effectiveness of recombinant factor VIIa in refractory bleeding disorders. Semin Thromb Hemost. 2000;26:367-372.
7. Lisman T, Moschatsis S, Adelmeijer J, Nieuwenhuis HK, de Groot PG. Recombinant factor VIIa enhances deposition of platelets with congenital or acquired alpha IIb beta 3 deficiency to endothelial cell matrix and collagen under conditions of flow via tissue factor-independent thrombin generation. Blood. 2003;101:1864-1870.
8. Hoffman M, Monroe DM, Roberts HR. Human monocytes support factor X activation by factor VIIa, independent of tissue factor: implications for the therapeutic mechanism of high-dose factor VIIa in hemophilia. Blood. 1994;83:38-42.
9. Monroe DM, Hoffman M, Oliver JA, Roberts HR. Platelet activity of high-dose factor VIIa is independent of tissue factor. Br J Haematol. 1997;99: 542-547.
10. Lisman T, de Groot PG. Mechanism of action of recombinant factor VIIa. J Thromb Haemost. 2003;1:1138-1139.
11. Cooper HA, Jones CP, Campion E, Roberts HR, Hedner U. Rationale for the use of high dose rF-VIIa in a high-titre inhibitor patient with haemophilia B during major orthopaedic procedures. Haemophilia. 2001;7:517-522.
12. Kenet G, Lubetsky A, Luboshitz J, Martinowitz U. A new approach to treatment of bleeding episodes in young hemophilia patients: a single bolus megadose of recombinant activated factor VII (NovoSeven). J Thromb Haemost. 2003;1:450-455.
13. Santagostino E, Mancuso ME, Rocino A, et al. A prospective randomized trial of high and standard dosages of recombinant factor VIIa for treatment of hemarthroses in hemophiliacs with inhibitors. J Thromb Haemost. 2006;4:367-371.
14. Monroe DM, Hoffman M, Oliver JA, Roberts HR. A possible mechanism of action of activated factor VII independent of tissue factor. Blood Coagul Fibrinolysis. 1998;9(suppl 1):S15-S20.
15. Kjalke M, Ezban M, Monroe DM, et al. High-dose factor VIIa increases initial thrombin generation and mediates faster platelet activation in thrombocytopenia-like conditions in a cell-based model system. Br J Haematol. 2001;114:114-120.
16. Roberts HR, Monroe DM, White GC. The use of recombinant factor VIIa in the treatment of bleeding disorders. Blood. 2004;104:3858-3864.
17. Hedner U, Erhardtsen E. Potential role for rFVIIa in transfusion medicine. Transfusion. 2002;42: 114-124.
18. Tranholm M, Kristensen K, Kristensen AT, et al. Improved hemostasis with superactive analogs of factor VIIa in a mouse model of hemophilia A. Blood. 2003;102:3615-3620.
19. Kjalke M, Kjellev S, Rojkjaer R. Preferential localization of recombinant factor VIIa to platelets activated with a combination of thrombin and a GPVI receptor agonist. J Thromb Haemost. 2007;5: 774-780.
20. Schwertz H, Tolley ND, Foulks JM, et al. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. J Exp Med. 2006;203:2433-2440.
21. Panes O, Matus V, Saez CG, et al. Human platelets synthesize and express functional tissue factor. Blood. 2007;109:5242-5250.
22. Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood. 2005;106:1604-1611.
23. Harmon JT, Jamieson GA. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin: a soluble radioreceptor assay for the interaction of thrombin with platelets. J Biol Chem. 1986;261:13224-13229.
24. Baglia FA, Badellino KO, Li CQ, Lopez JA, Walsh PN. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. J Biol Chem. 2002;277:1662-1668.
25. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. J Biol Chem. 2000;275:22756-22763.
26. Joseph K, Nakazawa Y, Bahou WF, Ghebrehiwet B, Kaplan AP. Platelet glycoprotein Ib: a zinc-dependent binding protein for the heavy chain of high-molecular-weight kininogen. Mol Med. 1999; 5:555-563.
27. White TC, Berny MA, Fernández JA, Griffin JH, Gruber A, McCarty OJ. Glycoprotein Ib medaties platelet recruitment, activation and aggregation on immobilized protein C and activated protein C under shear. J Thromb Haemost. 2007;5(suppl 2):OM-030.
28. Hackeng TM, van't Veer C, Meijers JC, Bouma BN. Human protein S inhibits prothrombinase complex activity on endothelial cells and platelets via direct interactions with factors Va and Xa. J Biol Chem. 1994;269:21051-21058.
29. Weeterings C, Adelmeijer J, Myles T, de Groot PG, Lisman T. Glycoprotein Ibalpha-mediated platelet adhesion and aggregation to immobilized thrombin under conditions of flow. Arterioscler Thromb Vasc Biol. 2006;26:670-675.
30. Huizinga EG, Tsuji S, Romijn RA, et al. Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. Science. 2002;297: 1176-1179.
31. Lopez JA, Leung B, Reynolds CC, Li CQ, Fox JE. Efficient plasma membrane expression of a functional platelet glycoprotein Ib-IX complex requires the presence of its three subunits. J Biol Chem. 1992;267:12851-12859.
32. Lenting PJ, Neels JG, van den Berg BM, et al. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. J Biol Chem. 1999;274:23734-23739.
33. Allen GA, Wolberg AS, Oliver JA, et al. Impact of procoagulant concentration on rate, peak and total thrombin generation in a model system. J Thromb Haemost. 2004;2:402-413.
34. Lisman T, de Groot PG, Lambert T, Rojkjaer R, Persson E. Enhanced in vitro procoagulant and antifibrinolytic potential of superactive variants of recombinant factor VIIa in severe hemophilia A. J Thromb Haemost. 2003;1:2175-2178.
35. Giesen PL, Rauch U, Bohrmann B, et al. Blood-borne tissue factor: another view of thrombosis. Proc Natl Acad Sci U S A. 1999;96:2311-2315.
36. Riewald M, Ruf W. Orchestration of coagulation protease signaling by tissue factor. Trends Cardiovasc Med. 2002;12:149-154.
37. Preston RJ, Ajzner E, Razzari C, et al. Multifunctional specificity of the protein C/activated protein C Gla domain. J Biol Chem. 2006;281:28850-28857.
38. Ghosh S, Pendurthi UR, Steinoe A, Esmon CT, Rao LV. Endothelial cell protein C receptor acts as a cellular receptor for factor VIIa on endothelium. J Biol Chem. 2007;282:11849-11857.
39. Lopez-Sagaseta J, Montes R, Puy C, et al. Binding of factor VIIa to the endothelial cell protein C receptor reduces its coagulant activity. J Thromb Haemost. 2007;5:1817-1824.
40. Steinoe A, Sorensen BB, Stennicke HR. Recombinant activated human factor VII (FVIIA) binds to the endothelial protein C receptor (EPCR) on human umbilical vein endothelial cells (HUVEC). J Thromb Haemost. 2007;5(suppl 2):P-T-015.

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