Coagulation factor VIIa: prohemostatic drug and biomarker for thrombosis

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The N-terminal region of glycoprotein Ibα modulates factor IXa-mediated factor Xa-generation on the activated platelet surface

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Abstract

Background: The vitamin K-dependent coagulation factor IX(a) binds to the surface of activated platelets. Previous studies have indicated that this interaction is not critically dependent on the Gla domain of FIX(a), which suggests that proteins present on the surface of platelets are involved. A possible candidate is the GPIb-IX-V complex, which is the second most abundant receptor complex on the surface of platelets in which GPIbα is the most important ligand binding moiety. Objective: To determine whether the GPIb-IX-V complex assists in the binding of FIX(a) to the surface of activated platelets. Methods and Results: Activated, but not resting platelets, adhered to immobilized factor IX (FIX) and activated factor IX (FIXa) under static conditions. CHO-cells expressing the GPIb-IX-V complex, but not wild-type CHO cells, adhered to immobilized FIX(a). Proteolysis of the N-terminal region of GPIbα by O-sialoglycoprotein endopeptidase (OSE) from the surface of activated platelets did not reduce platelet binding to FIX(a). However, FIXa-mediated factor Xa-generation on the activated platelet surface was slightly, although not significantly, accelerated by proteolysis of the N-terminal region of GPIbα. Conclusion: FIX(a) interacts with the GPIb-IX-V complex expressed on CHO cells. Although proteolysis of the N-terminal region of GPIbα did not reduce platelet binding to FIX(a), it did appear to accelerate FIXa-mediated factor Xa-generation on the surface of activated platelets. Further studies are required to examine the binding site of FIX(a) on the GPIb-IX-V complex. Furthermore, our results may indicate that FVIII and/or FX interact with the GPIb-IX-V complex as well.

Introduction

The surface of activated platelets plays a vital role in supporting coagulation reactions. At first it was thought that the negatively charged phospholipids at the surface of activated platelets were sufficient for the binding of vitamin K-dependent coagulation factors. Over the years, however, studies provided evidence for a role of platelet receptors in the interaction of coagulation factors with the platelet membrane. A first clue for a role of factors other than negatively charged phospholipids in localizing coagulation factors to the platelet membrane came from studies showing distinct differences between coagulation factor binding to platelets as compared to binding to synthetic phospholipid vesicles [1-3]. For example, the binding affinity of coagulation factor IX(a) to synthetic phospholipid vesicles is >100 times worse compared to the binding to platelets, and critically dependent on the presence of the gamma-carboxyglutamic acid-rich (Gla) domain within FIX(a) [1]. The binding of FIX(a) to platelets is not solely dependent on the presence of its Gla domain, as des-Gla FIXa binds with a similar affinity to platelets than native FIXa [2]. These observations strongly suggests that binding proteins present on the surface of platelets are involved. A possible candidate is the glycoprotein (GP) Ib-IX-V complex, which is the second most abundant receptor on platelets. The GPIb-IX-V complex consist of the molecules GPIbα, GPIbβ, GPIX and GPV (ratio 2:2:2:1 respectively), in which the GPIbα subunit is the most important ligand binding molecule. The GPIb-IX-V receptor is involved in both primary and secondary hemostasis, which includes the binding of the GPIb-IX-V receptor to collagen-bound von Willebrand factor and the binding of several coagulation factors respectively. Known ligands of the GPIb-IX-V complex are FII(a) [4], FVII(a) [5], FXI(a) [6], FXII(a) [7], high molecular weight kininogen (HMWK) [8], and activated protein C (aPC) [9]. Previous work from our group showed that proteolysis of GPIbα from the surface of activated platelets resulted in a slightly reduced tissue factor-independent thrombin generation by rFVIIa [5]. Another study showed that the FXII-GPIbα interaction inhibits thrombin-induced platelet aggregation by inhibiting the binding of thrombin to platelets [7]. Thus, besides assisting in localizing coagulation factors to the platelet membrane, GPIbα appears to modulate coagulation factor function. This unique feature of the GPIb-IX-V complex makes the complex an interesting potential therapeutic target for anti-thrombotic drug development. The present study is part of an ongoing study in our laboratory in which we systematically studying the interaction of coagulation factors with GPIbα and potential functional consequences of these interactions.

Material and methods

Platelet isolation

Blood was drawn from healthy volunteers who denied ingestion of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) for the preceding 10 days into 3.2% (v/v) sodium citrate (9:1) vacuum tubes (Greiner Bio-One B.V., Alphen aan den Rijn, the Netherlands). Blood samples were stored at room temperature for a maximum of 1 h after blood collection. Blood samples were centrifuged at 200 g for 15 min at room temperature to obtain platelet rich plasma (PRP). To prevent platelet activation and aggregation during the platelet isolation, 10 ng/ml prostacyclin (PGI-2) was added. Subsequently, the PRP was centrifuged at 500 g for 15 min at room temperature, and the platelet pellet was resuspended in Hepes-Tyrode buffer (HT buffer; 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 145 mM NaCl, 5 mM KCl, 0.6 mM $NaH_2PO_4 \cdot H_2O$, 1.0 mM $MgSO_4 \cdot 7\ H_2O$, 5 mM D-glucose), pH 6). PGI-2 was added, and the platelets were centrifuged at 500 g for 15 min at room temperature. The obtained platelet pellet was resuspended in Hepes-Tyrode buffer (pH 7.35).

Cell culture

Wild type Chinese Hamster Ovary (CHO-wt) cells and cells expressing the GPIb-IX-V complex (CHO-Ib cells, a generous gift of Dr. J.A. Lopez) were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium (Lonza, Basel, Switzerland). The medium was supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., MA, USA). CHO-Ib cells were subjected to selection by addition of 400 μg/ml geneticin (G418) (Thermo Fisher Scientific Inc., MA, USA) to the medium. Cells were cultured at 37°C in 5% CO_2/95% air.

Binding studies

FIX (Enzyme Research Laboratories, Swansea, United Kingdom), FIXa (Enzyme Research Laboratories, Swansea, United Kingdom), rFVIIa (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark) were immobilized at a concentration of 1.25 μg/ml (platelet adhesion) or 2.5 μg/ml (CHO-cell adhesion) on a 96-well Immulon-2B flatbottom microtiter plate (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 2 h at 37°C. Subsequently, the plate was washed three times with Tris-buffered Saline (TBS; 5 mM Tris, 150 mM NaCl, pH 7.5), and blocked with 2% (w/v) BSA in TBS for a minimum of 30 min at 37°C. The plate was emptied, washed with TBS, and $20x10^6$ platelets/well in HT buffer (pH 7.35) or $100x10^6$ CHO- cells/well in a 1:1 mixture of DMEM and Ham's F12 supplemented with

0.5% (w/v) BSA and 3 mM $CaCl_2$ were added for one hour at 37°C. Platelets were pre-treated with 50 μg/ml O-sialoglycoprotein endopeptidase (OSE, Cedarlane, Burlington, Ontario, Canada) or vehicle for 30 min at 37°C. Platelets were activated by addition of 15 μM Thrombin Receptor Agonist Peptide (TRAP, Bachem, Bubendorf, Switzerland) and 200 ng/ml convulxin (GPVI receptor agonist, CVX, Enzo Life Sciences, Antwerpen, Belgium). After the incubation, the platelets or cells were removed and the plate was washed with TBS. After the final washing step, the plate was emptied and 100 μl 3mg/ml p-nitrophenyl phosphate substrate (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added in 50 mM acetic acid with 1% (v/v) triton X-100 (pH 5) for 15 min protected from light. The substrate conversion was stopped by adding 50 μl of NaOH and the optical density was measured at 405 nm using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

FACS

Platelets were isolated from whole blood as described earlier. The isolated platelets were diluted to 200.000 platelets/μl and incubated with 0 (vehicle), 6.25, 12.5, 25, 50, 80, and 100 μg/ml OSE (Cedarlane, Burlington, Ontario, Canada) for 30 minutes at 37°C. Proteolysis of GPIbα was measured by binding of a GPIbα-specific antibody (monoclonal mouse anti-human CD42b, platelet glycoprotein Ib/RPE clone AN51, Dako, Glostrup, Denmark). Platelet activation was monitored by measuring the P-selectin expression on the platelet surface using a mouse anti-human CD62P-PE (BD biosciences, Breda, the Netherlands). Appropriate negative controls were taken along in every experiment; mouse IgG2a-PE (CD42b isotype control, Dako, Glostrup, Denmark) and mouse IgG1-PE (P-selectin isotype control, BD biosciences, Breda, the Netherlands). Antibodies were incubated for 20 min at 37°C. Samples were fixed with 0.2% (v/v) formaldehyde in 0.9% NaCl solution. FACS measurements were performed using a BD FACS Calibur (BD Biosciences, San Jose, CA, USA).

Intrinsic tenase activity

Platelets were isolated as described before, and diluted to a final concentration of 200.000/μl in HT buffer. For the proteolysis of GPIb, 50 μg/ml OSE (Cedarlane, Burlington, Ontario, Canada) was added to the platelets and incubated for 30 min at 37°C. Next, the platelets pre-treated with OSE or vehicle were activated by addition of 15 μM TRAP and 200 ng/ml CVX, and incubated for 15 min at 37°C. Subsequently, 30 μl of activated platelets pre-treated with OSE or vehicle were added to 2.5 μg/ml FIXa (Enzyme Research Laboratories, Swansea, United Kingdom), 1 U/ml FVIII (ADVATE, Baxter, Newbury,

United Kingdom), and to a concentration range of 0-250 nM of FX (Enzyme Research Laboratories, Swansea, United Kingdom) in HEPES buffer (25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% v/v BSA, pH 7.4). After 2 min of incubation at 37°C, 20 μl of sample was added to 100 μl cold 12 mM (w/v) EDTA in TBS and kept on ice. These coagulation reactions were performed in plastic disposables (Greiner Bio-One B.V., Alphen aan den Rijn, the Netherlands). Subsequently, 100 μl of this mix was added to 20 μl S-2765 (Chromogenix, Instrumentation Laboratory, Bedford, MA, USA) in a 96-well Immolin-2B flatbottom microtiter plate (Thermo Fisher Scientific Inc., Waltham, MA, USA). Immediately, the substrate conversion was followed at 405 nm using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Color intensity was translated to FXa levels formed using a calibration curve of known concentrations of FXa (Enzyme Research Laboratories, Swansea, United Kingdom).

Statistical analysis

Statistical differences in the platelet and cell adhesion experiments were analyzed by the unpaired Student's t-test and the paired Student's t-test, respectively. To make a statistical comparison between the FXa formed on the activated platelet in the presence or absence of the GPIb-IX-V receptor, the areas under the curve (AUC) were calculated and analyzed using the paired Student's t-test. P values ≤0.05 were considered statistically significant. Statistical Analysis was performed using the GraphPad Prism 5.1 Software Inc. package (La Jolla, CA, USA).

Results

Binding of activated platelets to immobilized factor IX and IXa

To determine the interaction of factor IX and IXa with platelets, resting and activated platelets were allowed to adhere under static conditions to immobilized factor IX and IXa. The results are shown in Figure 1. Activated platelets adhered readily to immobilized FIX and FIXa, whereas the interaction between resting platelets and FIX(a) was similar to background platelet adhesion to immobilized BSA.

Binding of CHO cells expressing the GPIb-IX-V complex to immobilized factor IX and IXa

CHO-wt and CHO-Ib cells were allowed to adhere under static conditions to immobilized factor IX and IXa. The results are shown in Figure 2. CHO cells expressing the GPIb-IX-V complex (CHO-Ib cells) adhered readily to immobilized FIX and FIXa,

whereas the interaction between wild type CHO cells (CHO-wt) and FIX(a) was similar to background cell adhesion to immobilized BSA.

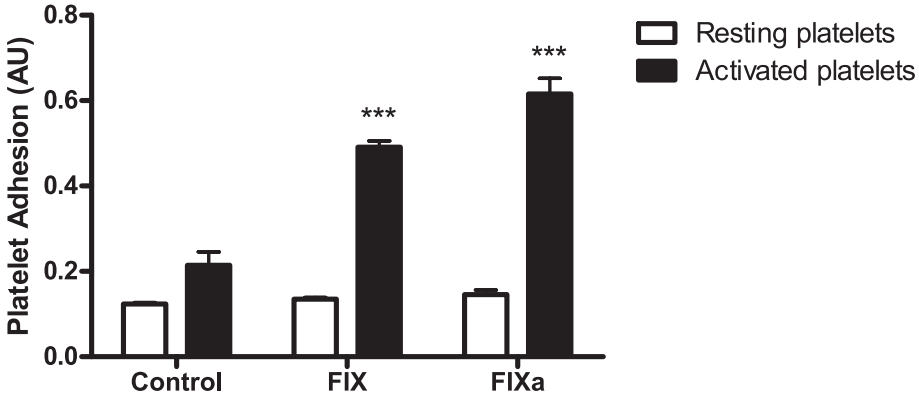

Figure 1 Platelets were isolated from whole blood and allowed to adhere under static conditions to control (vehicle), immobilized factor IX and IXa. Activated platelets were stimulated with a combination of TRAP and convulxin. Platelet adhesion was quantified by measuring the intrinsic phosphatase activity at an optical density of 405 nm, indicated as arbitrary units (AU). Shown are means of 5 independent experiments each performed in triplicate, and error bars indicate standard deviation. ***p≤0.0001, compared to control.

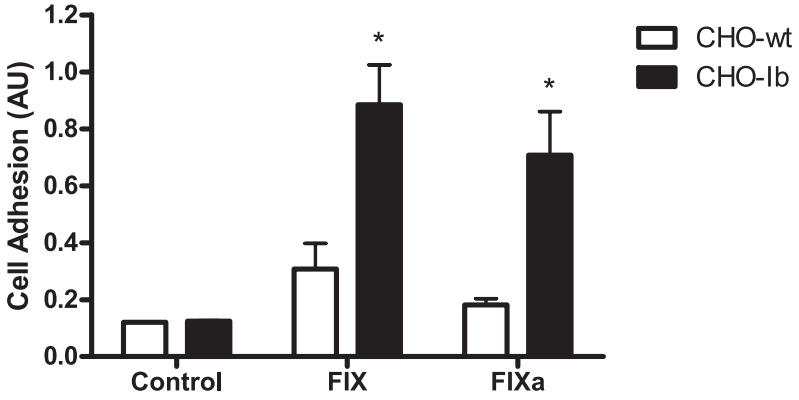

Figure 2 CHO-wt and CHO-Ib cells were allowed to adhere under static conditions to control (vehicle), immobilized factor IX and IXa. Cell adhesion was quantified by measuring the intrinsic phosphatase activity at an optical density of 405 nm, indicated as arbitrary units (AU). Shown are means of 4 independent experiments each performed in quadruplicate, and error bars indicate standard error of the mean. *p≤0.05, compared to control.

Proteolysis of GPIb from the platelet surface by OSE

Activated platelets, as well as CHO-Ib cells, bind to immobilized FIX and FIXa. In order to assess whether GPIbα is involved in the binding of platelets to immobilized FIX(a), we first determined the concentration of OSE required for full removal of the N-terminal part of GPIbα and assessed the extent of platelet activation by OSE. The results are shown in Figure 3. An OSE concentration of 50 μg/ml or more resulted in full removal of GPIb from the surface of platelets. The P-selectin expression did not appreciably change by OSE treatment.

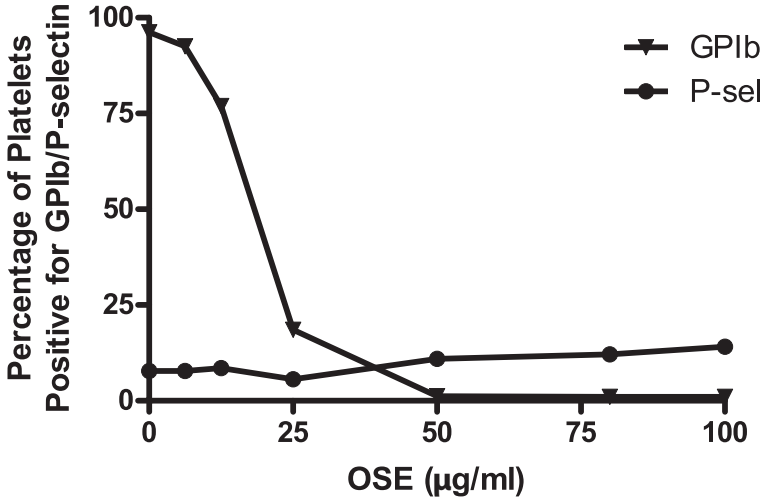

Figure 3 Platelets were isolated from whole blood and incubated with different concentrations of OSE for 30 min at 37°C. GPIb and P-selectin (P-sel) expression were assessed by flow cytometry using specific antibodies. Graph shows results of a representative single experiment.

Binding of activated platelets to immobilized factor FIX(a) is not mediated by the N-terminal part of GPIbα

The involvement of the N-terminal region of GPIbα in the binding of FIX(a) to activated platelets was assessed by allowing platelets treated with or without OSE, to adhere under static conditions to immobilized factor IX and IXa. The results are shown in Figure 4. OSE treatment did not affect adhesion of activated platelets to immobilized FIX and FIXa.

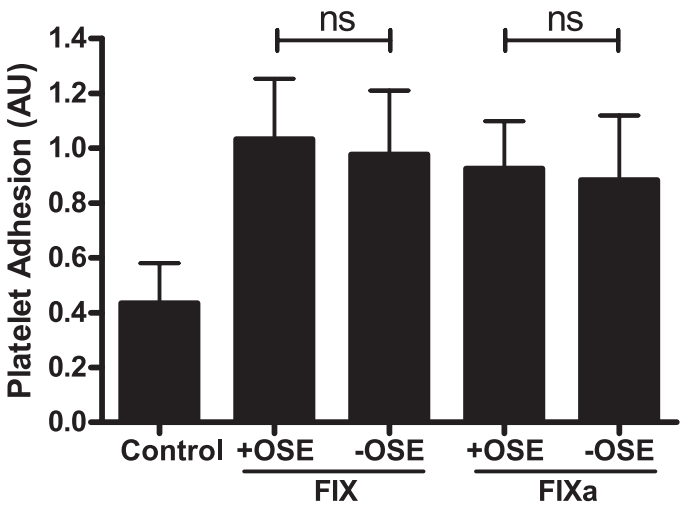

Figure 4 Activated platelets were incubated with 50 μg/ml OSE or vehicle and allowed to adhere to control (vehicle), immobilized factor IX and IXa. Platelet adhesion was quantified by measuring the intrinsic phosphatase activity at an optical density of 405 nm, indicated as arbitrary units (AU). Graph shows mean adhesion of 3 experiments each performed in triplicate. Error bars indicate standard error of the mean. ns indicates not significant.

Proteolysis of the N-terminal region of GPIbα from the activated platelet surface by OSE results in an accelerated FIXa-mediated FXa-generation

Although FIX(a) binds to the GPIb-IX-V complex on CHO cells, we could not demonstrate that FIX(a) binds to the N-terminal region of GPIbα on platelets, which is the major ligand binding site of the GPIb-IX-V complex. Although we did not demonstrate this region to be involved in FIX(a) binding, we examined whether this region is involved in FIXa-mediated FXa-generation. The results are shown in Figure 5. Activated platelets pre-treated with OSE showed an accelerated FIXa-mediated FXa-generation compared to activated platelets without OSE treatment (area under the curve (AUC) of 1589 ± 336 [mean ± SEM] vs 1365 ± 232 respectively). However, the difference in FXa formation at the surface of activated platelets did not reach statistical significance ($p = 0.28$).

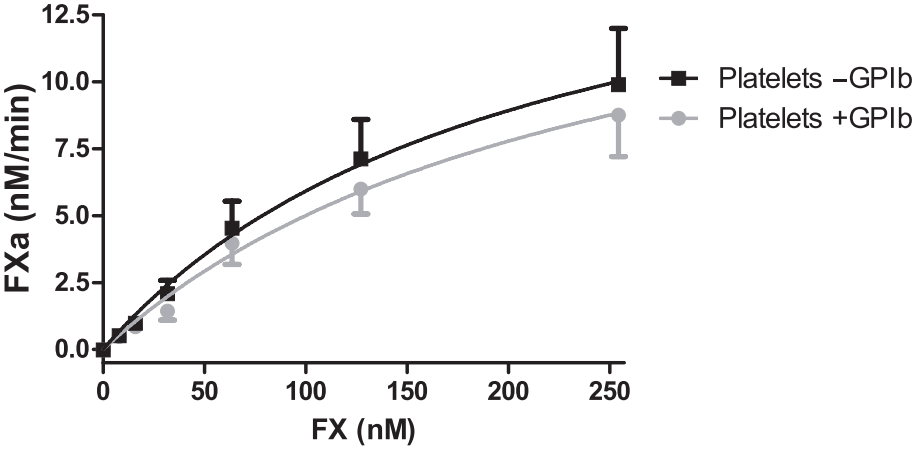

Figure 5 Proteolysis of GPIb accelerates FIXa-mediated FXa-generation on the activated platelet surface. Platelets pre-treated with 50 μg/ml OSE (platelets -GPIb) or vehicle (platelets +GPIb) were activated, and coagulation factors IXa (2.5 μg/ml), FVIII (1 U/ml) and FX (0-250 nM) were added. FXa generation was followed by means of substrate conversion. Color intensity was translated to FXa levels using a calibration curve of known concentrations of FXa. Shown are means of six independent experiments, and error bars indicate standard error of the mean.

Discussion

Previous studies strongly suggests that platelet membrane components other than phosphatidylserine are involved in the binding of FIX(a) to the surface of activated platelets. The identification of a putative FIX(a) receptor has been a topic of interest for many years, but has not yet been identified. In the present study, we investigated the hypothesis that the GPIb-IX-V complex is the binding site for FIX(a) on the platelet membrane. A number of coagulation proteins have already been shown to interact with this platelet receptor complex, in particular with the GPIbα moiety [4-9]. Our results show that activated platelets, as well as CHO cells expressing the GPIb-IX-V complex, bind to immobilized factor IX and IXa. Nevertheless, we could not definitively show that the GPIb-IX-V complex is the FIX(a) receptor on platelets. Experiments in which the N-terminal region of GPIbα, to which most GPIb-IX-V complex ligands bind, was removed from the platelet surface did not reduce FIX(a) binding to platelets. We also investigated the binding of FIX(a) to glycocalicin (GC), the extracellular fragment of GPIbα, using a BIAcore2000 biosensor system. Unfortunately, these experiments resulted in inconclusive results (data not shown).

We could not provide evidence that the GPIb-IX-V receptor is involved in the localization of FIX(a) to the surface of platelets. However, as it may be that FIX(a) binds to parts of the GPIb-IX-V complex not cleaved by OSE, we did proceed in assessing a potential effect of GPIbα on FIXa-mediated propagation of coagulation. We showed a slight, but consistent, enhancement of FIXa-mediated factor Xa-generation on OSE-treated platelets suggesting that binding of FIX(a) to GPIbα reduces procoagulant activity of FIXa. Alternatively, the enhancement of intrinsic tenase activity by OSE treatment could indicate that factor X and/or FVIII interacts with GPIbα.

The discrepant results between FIX(a) binding to GPIb-IX-V on CHO cells and lack of effect of OSE treatment of platelets on FIX(a) binding may indicate that either the FIXa binding site on GPIb-IX-V is outside the OSE-cleaved region, or that the N-terminal region of GPIbα is not the only binding protein for FIX(a) on platelets. Other coagulation proteins including FXI [6,10], activated protein C [11] and beta 2-glycoprotein I (β_2GPI) [12,13] have multiple binding partners on the platelet membrane. These multiple interactions may be required to bring coagulation factors in close proximity of each other. For example, the binding of GPIbα with coagulation factor XI [6]. In addition, GPIbα localizes FXI to lipid rafts present on the platelet surface [14], to which GPIbα is redistributed upon platelet activation [15]. The binding of FXI to activated platelets is further assisted by the platelet apolipoprotein E receptor 2 (ApoER2) [10].

In conclusion, FIX(a) binds to the GPIb-IX-V complex, and this interaction appears to modify intrinsic tenase activity. Whether other binding partners for FIX(a) on the platelet surface exist, whether FVIII and/or FX also interact with the GPIb-IX-V complex, and how the GPIb-IX-V complex affects the complete coagulation cascade requires further study.

References

1. Jones ME, Griffith MJ, Monroe DM, Roberts HR, Lentz BR. Comparison of lipid binding and kinetic properties of normal, variant, and gamma-carboxyglutamic acid modified human factor IX and factor IXa. Biochemistry 1985; 24: 8064-9.
2. Rawala-Sheikh R, Ahmad SS, Monroe DM, Roberts HR, Walsh PN. Role of gamma-carboxyglutamic acid residues in the binding of factor IXa to platelets and in factor-X activation. Blood 1992; 79: 398-405.
3. Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. Arterioscler Thromb Vasc Biol 2002; 22: 1381-9.
4. De Marco L, Mazzucato M, Masotti A, Ruggeri ZM. Localization and characterization of an alpha-thrombin-binding site on platelet glycoprotein Ib alpha. J Biol Chem 1994; 269: 6478-84.
5. Weeterings C, de Groot PG, Adelmeijer J, Lisman T. The glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. Blood 2008; 112: 3227-33.
6. 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: 1662-8.
7. 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: 22756-63.
8. 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: 555-63.
9. White TC, Berny MA, Tucker EI, Urbanus RT, de Groot PG, Fernandez JA, Griffin JH, Gruber A, McCarty OJ. Protein C supports platelet binding and activation under flow: Role of glycoprotein Ib and apolipoprotein E receptor 2. J Thromb Haemost 2008; 6: 995-1002.
10. White-Adams TC, Berny MA, Tucker EI, Gertz JM, Gailani D, Urbanus RT, de Groot PG, Gruber A, McCarty OJ. Identification of coagulation factor XI as a ligand for platelet apolipoprotein E receptor 2 (ApoER2). Arterioscler Thromb Vasc Biol 2009; 29: 1602-7.
11. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C: Biased for translation. Blood 2015; 125: 2898-907.
12. Pennings MT, van Lummel M, Derksen RH, Urbanus RT, Romijn RA, Lenting PJ, de Groot PG. Interaction of beta2-glycoprotein I with members of the low density lipoprotein receptor family. J Thromb Haemost 2006; 4: 1680-90.
13. Pennings MT, Derksen RH, van Lummel M, Adelmeijer J, VanHoorelbeke K, Urbanus RT, Lisman T, de Groot PG. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: Glycoprotein Ibalpha and apolipoprotein E receptor 2'. J Thromb Haemost 2007; 5: 369-77.
14. Baglia FA, Shrimpton CN, Lopez JA, Walsh PN. The glycoprotein Ib-IX-V complex mediates localization of factor XI to lipid rafts on the platelet membrane. J Biol Chem 2003; 278: 21744-50.

15. Shrimpton CN, Borthakur G, Larrucea S, Cruz MA, Dong JF, Lopez JA. Localization of the adhesion receptor glycoprotein Ib-IX-V complex to lipid rafts is required for platelet adhesion and activation. J Exp Med 2002; 196: 1057-66.