Thrombin Mutant W215A/E217A Acts as a Platelet GPIb Antagonist

Michelle A. Berny, Tara C. White, Erik I. Tucker, Leslie A. Bush-Pelc, Enrico Di Cera, András Gruber, Owen J.T. McCarty

Objective—Thrombin containing the mutations Trp215Ala and Glu217Ala (WE) selectively activates protein C and has potent antithrombotic effects in primates. The aim of this study was to delineate the molecular mechanism of direct WE–platelet interaction under static and shear conditions.

Methods and Results—Purified platelets under static conditions bound and spread on immobilized wild-type but not WE thrombin. In PPACK-anticoagulated blood under shear flow conditions, platelets tethered and rolled on both wild-type and WE thrombin, and these interactions were abrogated by the presence of a glycoprotein Ib (GPIb)-blocking antibody. Platelet deposition on collagen was blocked in the presence of WE, but not wild-type thrombin or prothrombin. WE also abrogated platelet tethering and rolling on immobilized von Willebrand factor in whole blood under shear flow.

Conclusions—These observations demonstrate that the thrombin mutant WE, while not activating platelets, retains the ability to interact with platelets through GPIb, and inhibits GPIb-dependent binding to von Willebrand factor–collagen under shear. (Arterioscler Thromb Vasc Biol. 2008;28:329-334)

Key Words: platelet ■ thrombin ■ von Willebrand factor ■ GPIb

The serine protease thrombin plays a central role in both thrombosis and hemostasis. During clot formation in vivo, thrombin generation by factor Xa from prothrombin is localized to the surface of activated platelets. Additionally, thrombin also stimulates its own generation through the activation of factor XI and the cofactors V and VIII, leading to rampant thrombin generation in the presence of activated platelets. The primary procoagulant functions of thrombin are the cleavage of soluble fibrinogen to insoluble fibrin and the activation of platelets via cleavage of protease-activated receptors (PARs), resulting in platelet cytoskeletal reorganization events critical to thrombus stability. Thrombin binds to the platelet receptor GPIbα (CD42b), the major platelet receptor for von Willebrand factor (vWF) that is required for normal hemostasis.1 Thrombin has also been shown to proteolytically inactivate ADAMTS13, a reprolysin-like metalloproteinase that inactivates the large multimeric forms of vWF, which is crucial for shear-dependent platelet aggregation during hemostasis and thrombosis (see review by Jackson2).

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In the presence of thrombomodulin, however, thrombin acts as an indirect anticoagulant through the enzymatic activation of protein C. Activated protein C (APC) in turn inactivates factors Va and VIIIa, resulting in the downregulation of thrombin generation.3 Thrombin thus orchestrates a series of site-specific pro- and anticoagulant events that result in hemostatic plug formation at the site of a breach in the vasculature, while preventing intravascular expansion of the hemostatic process, thus preserving vessel patency.

A comprehensive library of Ala mutants was recently used to map the thrombin epitopes recognizing fibrinogen, protein C, and thrombomodulin to identify residues that contribute differentially to the procoagulant and anticoagulant functions of thrombin.4,5 As Trp215 and Glu217 participate in fibrinogen recognition via distinct interactions, the double mutant thrombin W215A/E217A (WE) was rationally engineered through the combination of mutations around the active site that individually reduce fibrinogen binding but not protein C activation.6 The mutant has drastically reduced (3 to 5 orders of magnitude) catalytic activity toward fibrinogen and PAR-1; however, in the presence of thrombomodulin the activity of WE toward protein C is restored to ~10% of the wild-type thrombin.7 Recent studies have shown that infusion of low doses of WE elicited safe and potent antithrombotic effects in a baboon model of platelet-dependent thrombosis.8,9 The unexpected finding from this study was that the lowest dose of WE, which produced no significant systemic anticoagulation and only a 5-fold baseline increase in circulating APC levels, was markedly more antithrombotic than infusion of exogenous APC producing detectable anticoagulation and a 9-fold baseline APC level increase. Moreover, a dispropor-
tionately high level of radiolabeled WE was detected in the thrombus. Collectively, these data suggest that the platelet-rich thrombus is capable of effectively capturing and immobilizing WE, thereby providing a possible mechanism for increased local activity via APC generation or other antithrombotic mechanisms on the thrombus surface. Thus, the goal of current study was to elucidate the molecular mechanisms mediating WE–platelet interactions under shear flow conditions that can occur on the intraluminal surface of thrombi. Our studies have demonstrated that the thrombin mutant WE, while not activating platelets, retains the ability to interact with platelets through GPIb. Furthermore, WE inhibits GPIb-dependent platelet deposition on thrombin, aggregation on collagen, and rolling on immobilized vWF under shear, suggesting that WE may act as a platelet GPIb-vWF antagonist in addition to selectively activating protein C.

**Materials and Methods**

**Reagents**

The recombinant human thrombin mutants Trp215Ala/Gln217Ala (WE), Thr172Tyr/Glu217Ala (TW), and Ser195Ala (S195A) were expressed, purified, and tested as previously described.6,10–12 Thrombin mutants were concentrated to 10 mg/mL in 50 mmol/L choline chloride, 20 mmol/L 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0. It is noteworthy that presence of diluent (1:500; corresponding to 2 µg/mL thrombin mutant) alone did not affect platelet function in any of the assays tested. vWF was a kind gift from Dr Michael Berndt (Monash University, Clayton, Australia). The anti-GPIb monoclonal antibody (mAb) 6D1 was a generous gift from Dr Barry Coller (Rockefeller University, New York), and the anti-GPⅡb mAb SZ2 was purchased from Beckman Coulter (Fullerton, Calif). Prothrombin and fibrinogen were purchased from Enzyme Research Laboratories, Inc. Chinese hamster ovary (CHO) cells stably expressing the platelet glycoprotein (GP) Ib-Ⅸ complex (CHOβⅨ cells) or only GP Ibβ and Ⅸ (CHOβⅨ cells)13 were kind gifts from Dr José López (Puget Sound Blood Center, Seattle, Wash). Other reagents were from Sigma-Aldrich Inc or previously named sources.14,15

**Preparation of Human and Murine Washed Platelets**

Human venous blood was drawn by venipuncture from healthy volunteers into sodium citrate and acid/citrate/dextrose (ACD) as previously described.14,15 The generation of mice deficient in either Rac1 or WAVE-1 was as previously described.14,16 and were kindly donated by Drs Laura Machesky and Steve Watson (University of Birmingham, UK) and Victor Tybulwicz (National Institute for Medical Research, London, UK). After centrifugation steps, purified human or murine platelets were resuspended in modified Tyrodes buffer (129 NaCl mM, 0.34 mmol/L NaH2PO4, 2.9 mmol/L KCl, 12 mM/L NaHCO3, 20 mmol/L HEPES, 5 mmol/L glucose, 1 mmol/L MgCl2; pH 7.3). Autologous human red blood cells (RBC) were isolated as previously described.14,15 Washed platelets were then reconstituted with 50% (v/v) autologous packed RBCs (reconstituted blood). All animals were maintained using housing and husbandry in accordance with local and national legal regulations, and human donors provided informed written consent, according to the Declaration of Helsinki.

**Platelet Static and Flow Adhesion Assays**

Glass coverslips were incubated with a solution of wild-type or mutant thrombin (50 µg/mL), prothrombin (50 µg/mL), collagen (100 µg/mL), fibrinogen (50 µg/mL), or vWF (50 µg/mL) overnight at 4°C as described.14,15 Surfaces were then blocked with denatured bovine serum albumin (BSA, 5 mg/mL) for 1 hour followed by washing with phosphate-buffered saline (PBS) before use in adhesion assays.

For static spreading experiments, washed platelets (2×10^7/mL) were incubated on protein-coated coverslips at 37°C for 45 minutes, followed by washing with modified Tyrodes buffer to remove unbound cells.14,15 Platelet spreading was imaged using Köhler illuminated Nomarski differential interference contrast (DIC) optics with an Axiovert 200 MPhc microscope (Carl Zeiss) and recorded using Axiovision 4.0 (Intelligent Imaging Innovations Inc). The degree of adhesion and surface area coverage was computed using Image J software as previously described.14,15

For flow adhesion assays, protein-coated coverslips were assembled onto a flow chamber (Glyotech) and mounted on the stage of an inverted microscope (Zeiss Axiovert 200 MPhc). In selected experiments, reconstituted blood or PPACK (40 µmol/L) anticoagulated whole blood was perfused through the chamber for 3 minutes at a wall shear rate of 500 to 1000 s⁻¹, and imaged using DIC microscopy in both real-time and after washing with modified Tyrodes buffer. The number of tethering and rolling platelets during the assay was determined off-line by reviewing the real-time videos as previously described.14,15

**Analysis of Data**

Statistical significance of differences between means was determined by ANOVA. If means were shown to be significantly different, multiple comparisons were performed by the Tukey test.14,15 Probability values of P<0.01 were selected to be statistically significant.

**Results**

**Molecular Mechanisms of Platelet Cytoskeletal Reorganization on Thrombin-Coated Surfaces**

To characterize the molecular basis of platelet–thrombin interactions, we gently pipetted purified platelets over surface immobilized thrombin and monitored platelet morphology using Nomarski DIC microscopy. To assess the direct ability of thrombin to support platelet binding, these experiments were performed in the presence of apyrase and indomethacin, inhibitors of the secondary mediators ADP and thromboxane A2 (TXA2), respectively. Consistent with our previous reports,17 immobilized thrombin supported human platelet adhesion and lamellipodia formation (Figure 1). In light of the fact that thrombin has been shown to induce robust activation of the small GTPase Rac1 in platelets,14 we aimed to investigate the role of Rac1 in mediating platelet spreading on immobilized thrombin. As shown in Figure 1, immobilized thrombin supported wild-type murine platelet adhesion and activation, as evidenced by generation of filopodia and full lamellipodia. Strikingly, lamellipodia formation was abolished in Rac1-deficient platelets on thrombin, although Rac1⁻/⁻ platelets retained the ability to form filopodia. In contrast, full lamellipodia formation was observed in the absence of the actin scaffolding protein WAVE-1 (Figure 1). The presence of the actin polymerization-inhibitor cytochalasin D abrogated both filopodia and lamellipodia formation for wild-type, Rac1⁻/⁻, and WAVE-1⁻/⁻ platelets on thrombin.

We next investigated the role of proteolysis in platelet adhesion to immobilized thrombin. Our results demonstrate that platelet adhesion and activation were abrogated in the presence of PPACK (40 µmol/L), which blocks the active site of thrombin (Figure 2). Similarly, platelets failed to adhere to surface-immobilized thrombin Na⁺-binding site mutants WE, TW, or TWE (Figure 2). Platelets failed to bind to the active
site mutant S195A, which has been shown to retain the Na⁺-binding properties of wild-type thrombin or to surface-immobilized prothrombin or BSA (Figure 2). It is noteworthy that the presence of WE in solution did not affect the degree of platelet adhesion to immobilized thrombin, collagen or fibrinogen under static conditions (supplemental Table I, available online at http://atvb.ahajournals.org). Moreover, both the rate and extent of thrombin-induced platelet aggregation was unaffected by the presence of WE (data not shown).

Taken together, our data show that Rac1, but not WAVE-1, is required for regulating platelet lamellipodia formation on thrombin, and that this mechanism is not activated by immobilized WE.

**Figure 1.** The role of Rac1 and WAVE-1 on spreading of platelets on immobilized thrombin. Washed human platelets (2×10⁷/mL) or murine platelets (2×10⁷/mL) from wild-type, Rac1⁻/⁻, or WAVE-1⁻/⁻ mice were placed on thrombin coated coverslips for 45 minutes at 37°C and imaged using DIC microscopy. Experiments were performed in the absence or presence of the actin-polymerization inhibitor cytochalasin D (Cyto D; 10 μmol/L). Images are representative of at least 3 experiments.

**Figure 2.** Platelet binding to wild-type or mutant thrombin. Washed human platelets (2×10⁷/mL) were placed on coverslips coated with either thrombin (Thr), PPACK-treated thrombin, WE, TW, TWE, S195A, prothrombin (PT), or BSA for 45 minutes at 37°C, and imaged using DIC microscopy. The number of adherent platelets were recorded for 3 fields of view (0.013 mm²) and expressed as mean±SEM from at least 3 experiments.

**Figure 3.** Platelet adhesion to wild-type or mutant thrombin under flow. Reconstituted human blood was perfused at a shear rate of 300 s⁻¹ for 3 minutes over thrombin-coated slides in the presence of vehicle (A), the blocking GPIb mAb SZ2 (B), exogenously added WE (C), or PPACK (D). In separate experiments, reconstituted blood was perfused over immobilized WE (E), S195A (F), prothrombin (G), or BSA (H). Images are representative of at least 3 experiments.

**Platelet Interactions With Immobilized Thrombin Under Flow**

We investigated thrombin-platelet interactions under shear stress that better characterized the microenvironment of thrombus formation in the intraluminal boundary layer of blood flow. We assessed platelet adhesion and aggregation after perfusion of reconstituted blood over immobilized thrombin at a shear rate of 300 s⁻¹. Tightly-packed platelet aggregates (33.5±1.3 aggregates/mm² × 10⁻²) consisting of 10 to 50 platelets were seen on thrombin after perfusion for 3 minutes (Figure 3A). It is noteworthy that these platelet aggregates were stable and that we did not observe a significant level of embolization. In accord with previous reports, platelet aggregation on immobilized thrombin was significantly reduced in the presence of the blocking anti-GPIb mAb SZ2 (20 μg/mL), resulting in 46.4±1.3 aggregates/mm² × 10⁻² consisting of 1 to 5 platelets (Figure 3B). Interestingly, platelet aggregation on immobilized thrombin in the presence of exogenously added WE (20 μg/mL) was reduced to a similar degree as was observed in the presence of SZ2, resulting in 45.9±1.4 aggregates/mm² × 10⁻² consisting of 1 to 5 platelets (Figure 3C). This was further evidenced by a reduction in the percentage of platelet aggregate surface coverage on thrombin in the presence of WE (58.5±4.2% versus 16.8±3.6% surface coverage on thrombin in the presence of vehicle or
or exogenously added WE (20 μg/mL) in the presence of PPACK, suggesting that a proteolytic event was required for platelet firm adhesion. The cumulative number of tethering platelets on wild-type thrombin (B) or WE (C) was recorded for a single field of view (0.013 mm²) for 30 seconds and expressed as mean±SEM from at least 3 experiments. *P<0.01 with respect to vehicle.

Figure 4. The platelet receptor GPIb binds to thrombin and WE. Human whole blood anticoagulated with PPACK was perfused over wild-type thrombin-coated or WE-coated slides at a shear rate of 300 s⁻¹ for 3 minutes. A, Representative micrographs of platelet tethering on wild-type thrombin in the presence of vehicle, the blocking GPIb mAb SZ2, or WE (20 μg/mL) are shown. B, The cumulative number of tethering platelets on wild-type thrombin (B) or WE (C) was recorded for a single field of view (0.013 mm²) for 30 seconds and expressed as mean±SEM from at least 3 experiments. *P<0.01 with respect to vehicle.

WE, respectively). Minimal platelet adhesion was observed on thrombin in the presence of PPACK (Figure 3D), whereas platelets failed to firmly adhere on WE, S195A, prothrombin, or BSA under flow (Figure 3E through 3H).

As activation of platelets is not mandatory for GPIb-mediated binding to ligands such as vWF,²⁰ we aimed to investigate whether inactive thrombin, which failed to support firm adhesion (Figure 3D), retained the ability of support GPIb-mediated events. We perfused PPACK-anticoagulated whole blood over immobilized thrombin and monitored platelet interactions under shear flow conditions in real-time using Nomarski DIC microscopy. Platelet tethering and rolling was observed on immobilized wild-type thrombin in whole blood (Figure 4A and 4B). Furthermore, platelet tethering and rolling was observed on WE thrombin, although to a lesser extent than compared with wild-type thrombin (Figure 4C). Platelets failed to arrest and adhere on wild-type or WE thrombin in the presence of PPACK, suggesting that a proteolytic event was required for platelet firm adhesion. The presence of either the function-blocking anti-GPIb mAb SZ2 or exogenously added WE (20 μg/mL) in solution abrogated platelet interactions on both immobilized wild-type and WE thrombin (Figure 4). In contrast, the presence of vehicle, wild-type thrombin, or prothrombin (20 μg/mL) in solution did not affect the extent of platelet tethering and rolling. Platelets failed to interact with immobilized prothrombin or BSA (data not shown).

To assess the ability of WE to bind to GPIbα, radiolabeled WE thrombin (¹²⁵I-WE) was incubated with CHOαβIX cells expressing GPIbα (and GPIbβ and GPIX to facilitate GPIbα expression). Our data show that GPIbα-expressing CHO cells bound 2.1-fold more ¹²⁵I-WE as compared with CHOβIX cells, which lack GPIbα (supplemental Figure I). Moreover, ¹²⁵I-WE binding to CHOαβIX cells was reduced by nearly 50% in the presence of the anti-GPIb mAb SZ2. CHOαβIX cells supported 6.4-fold more binding of ¹²⁵I-WE relative to CHO215-BSA. Additionally, CHOαβIX cells were observed to tether and roll when perfused over immobilized WE at a shear rate of 150 s⁻¹, and this rolling was inhibited by the presence of the anti-GPIb mAb SZ2 (data not shown). Overall, our data demonstrate that WE, although unable to support PAR-dependent platelet activation, retains the ability to bind GPIb, supports GPIb-dependent platelet rolling, and inhibits GPIb-dependent platelet tethering on thrombin under flow conditions that are typical of the intravascular environment.

The Thrombin Mutant WE Inhibits Platelet Adhesion to Collagen and Rolling on vWF Under Flow Conditions

It is well established that GPIb plays an essential role in mediating platelet aggregate formation on immobilized collagen under shear flow.²⁰,²¹ In accord with these reports, our data demonstrate that perfusion of PPACK-anticoagulated whole blood over collagen resulted in substantial platelet aggregate formation on collagen fibers at a shear rate of 1000 s⁻¹ (Figure 5A), whereas platelet adhesion and aggregation was severely reduced in the presence of the GPIb mAb 6D1 (Figure 5B). Strikingly, the presence of WE in solution also inhibited platelet deposition on collagen (Figure 5C) in a dose-dependent manner with an IC₅₀ of 7.98 μg/mL (supplemental Figure II). In contrast, presence of vehicle, wild-type thrombin (20 μg/mL), S195A (20 μg/mL), or prothrombin (20 μg/mL) did not reduce platelet aggregate formation on collagen (Figure 5D through 5F).

To determine the molecular mechanisms by which WE inhibits platelet aggregation on collagen under flow, we perfused PPACK-anticoagulated whole blood over immobi-
WE, in addition to serving as a protein C activator, has the ligand for thrombin. Significantly, these studies reveal that data extend previous observations that GPIb serves as a mechanism that mediate WE-platelet interactions. Specifically, the GPV in the ratio 2:2:2:1. The 282 N-terminal residues of GPV have been implicated in contributing to thrombin activation in the absence of shear, which typically occurs in wound blood. Such a mechanism of local APC activation by WE in the presence of intraluminal levels of protein C. Our findings, however, raise the possibility that the platelet surface may act as a catalyst for local protein C activation.

The platelet receptor GPIb-IX-V consists of GPIbα disulfide-linked to GPIbβ, noncovalently complexed with GPIX and GPV in the ratio 2:2:1:2. The 282 N-terminal residues of GPIbα contain binding sites for vWF, thrombin, and the coagulation factors XI and XIIa. The GPIbα leucine-rich region (LRR) 59 to 128 has been implicated in contributing to vWF binding, whereas thrombin binding is centered around an anionic region containing 3 sulfated tyrosine residues (Tyr276, Tyr278, and Tyr279). The thrombin binding site for GPIbα has been localized to thrombin exosite II. Functional assays revealed that residue Arg233 of exosite II is critical for GPIbα binding, whereas crystallography studies demonstrated that Arg233 makes contacts with several backbone oxygen atoms of the acidic region of GPIbα. Moreover, both crystal structures suggested that GPIbα might bind simultaneously to 1 thrombin molecule through exosite II and to vWF. Interestingly, both crystal structures revealed that thrombin exosite I binds GPIbα, thereby permitting 2 thrombin molecules to bind to a single GPIbα molecule, although the studies differed markedly regarding their interpretation of this binding.

It has been proposed that a conformational change in GPIbα is induced after thrombin exosite II binding, and that this conformational change permits a second thrombin molecule to bind, via exosite I, to a distinct location on GPIbα. Whether thrombin forms a dimer between 2 GPIbα molecules on the same platelet or bridges 2 GPIbα molecules on adjacent platelets has been debated (see review29) and is yet unknown.

Our studies suggest that WE specifically blocks GPIb binding to vWF, as evidenced by the ability of WE to inhibit GPIb-dependent platelet deposition on collagen and platelet rolling on vWF under shear flow. Pineda and colleagues have recently solved the x-ray crystal structure of WE, which revealed that the mutation of Trp215 and Glu217 resulted in a collapse of the 215 to 217 strand that crushes the primary specificity pocket. The collapse results from the abrogation of the stacking interaction between Phe227 and Trp215 and the polar interactions of Glu217 and Thr172 and Lys224. Other notable changes are a rotation of the carboxylate group of Asp189, breakage of the H-bond between the catalytic residues Ser195 and His57, breakage of the ion pair between Asp222 and Arg187, and significant disorder in the 186- and 220-loops that define the Na⁺-binding site. Along these lines, it is possible that the ability of WE to block GPIb-mediated binding to vWF is attributable to steric hindrance resulting from the conformational modulation of thrombin-GPIbα binding attributable to the Trp215Ala and Glu217Ala mutations. In addition, it is important to note that, in contrast to the catalytically inactive forms of thrombin (PPACK-thrombin, S195A), WE retains the ability to cleave PAR receptors, albeit at a rate that is reduced 1000-fold compared with wild-type. In light of the fact that it has been shown that thrombin binding to GPIbα facilitates PAR receptor cleavage and synergistic signaling, perhaps this dramatic increase in the residence time for the GPIb–WE–PAR receptor complex precludes the ability of vWF to bind to GPIb. This would explain why forms of thrombin which are unable to bind PAR receptors, such as S195A, fail to inhibit GPIb-vWF-mediated interactions. We do not at present know whether binding of WE to GPIb modifies the catalytic efficiency of WE toward protein C. Our findings, however, raise the possibility that the platelet surface may act as a catalyst for local protein C activation by WE in the presence of intraluminal levels of shear, eg, those that characterize the surface of nonocclusive thrombi in large vessels. Such a mechanism of local APC

**Discussion**

The studies here provide insight into the molecular mechanisms that mediate WE-platelet interactions. Specifically, the data extend previous observations that GPIb serves as a ligand for thrombin. Significantly, these studies reveal that WE, in addition to serving as a protein C activator, has the capability of functioning as a specific platelet GPIb-vWF antagonist.

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Our studies suggest that WE specifically blocks GPIb binding to vWF, as evidenced by the ability of WE to inhibit GPIb-dependent platelet deposition on collagen and platelet rolling on vWF under shear flow. Pineda and colleagues have recently solved the x-ray crystal structure of WE, which revealed that the mutation of Trp215 and Glu217 resulted in a collapse of the 215 to 217 strand that crushes the primary specificity pocket. The collapse results from the abrogation of the stacking interaction between Phe227 and Trp215 and the polar interactions of Glu217 and Thr172 and Lys224. Other notable changes are a rotation of the carboxylate group of Asp189, breakage of the H-bond between the catalytic residues Ser195 and His57, breakage of the ion pair between Asp222 and Arg187, and significant disorder in the 186- and 220-loops that define the Na⁺-binding site. Along these lines, it is possible that the ability of WE to block GPIb-mediated binding to vWF is attributable to steric hindrance resulting from the conformational modulation of thrombin-GPIbα binding attributable to the Trp215Ala and Glu217Ala mutations. In addition, it is important to note that, in contrast to the catalytically inactive forms of thrombin (PPACK-thrombin, S195A), WE retains the ability to cleave PAR receptors, albeit at a rate that is reduced 1000-fold compared with wild-type. In light of the fact that it has been shown that thrombin binding to GPIbα facilitates PAR receptor cleavage and synergistic signaling, perhaps this dramatic increase in the residence time for the GPIb–WE–PAR receptor complex precludes the ability of vWF to bind to GPIb. This would explain why forms of thrombin which are unable to bind PAR receptors, such as S195A, fail to inhibit GPIb-vWF-mediated interactions. We do not at present know whether binding of WE to GPIb modifies the catalytic efficiency of WE toward protein C. Our findings, however, raise the possibility that the platelet surface may act as a catalyst for local protein C activation by WE in the presence of intraluminal levels of shear, eg, those that characterize the surface of nonocclusive thrombi in large vessels. Such a mechanism of local APC
generation would explain the superb antithrombotic efficacy of low dose of WE observed in vivo.  

In summary, the present study dissects the molecular mechanisms of mutant thrombin WE interactions with platelets. Specifically, in contrast to the Rac1-dependent platelet lamellipodia formed on wild-type thrombin, we show that WE is unable to support platelet adhesion or cytoskeletal reorganization. We demonstrate that WE is a ligand for the platelet receptor GPIb, and that the binding of WE, but not wild-type or active-site blocked thrombin, inhibits GPIb binding to vWF. The combination of GPIb-dependent local accumulation of WE under shear and generation of APC in conjunction with inhibition of GPIb–vWF binding would provide a mechanism for the surprising hemostatic safety and antithrombotic activity of the double mutant WE thrombin.

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**Disclosures**  
None.

**References**

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Supplemental Figure I

**Figure SI. Characterization of WE binding to GPI\(\alpha\).** WE thrombin and BSA were radiolabeled with \(^{125}\text{I}\) (\(^{125}\text{I}-\text{WE}, 0.37 \mu\text{Ci} \ [10 \text{ kBq}] / \mu\text{g}; \(^{125}\text{I}-\text{BSA}, 1.15 \mu\text{Ci} \ [10 \text{ kBq}] / \mu\text{g}, >85\%\) protein bound after dialysis). 300 \(\mu\text{l}\) of \(1\times10^6\) CHO\(\alpha\beta\)IX cells/ml (which express GPI\(\alpha\)) or CHO\(\beta\)IX cells (which lack GPI\(\alpha\)) in HBSS were incubated for 30 min with 15 \(\mu\text{g/ml}\) \(^{125}\text{I}-\text{WE}\) or \(^{125}\text{I}-\text{BSA}\) in the absence or presence of the anti-GPI\(\alpha\) mAb SZ2 (20 \(\mu\text{g/ml}\)). Radiolabeled WE and BSA retention was determined and expressed as \(\mu\text{mol}\) of radiolabeled protein bound per \(1\times10^8\) cells.
Figure SII. The thrombin mutant WE inhibits platelet adhesion on immobilized collagen under flow. Human whole blood anticoagulated with PPACK was perfused over collagen-coated slides at a shear rate of 1000 s⁻¹ for 3 min in the presence of varying concentrations of WE (0.2, 2, 10, 20 μg/mL). The surface coverage of adherent platelets was recorded for 3 fields of view (0.013 mm²) and analyzed using ImageJ software. WE in solution inhibited platelet deposition on immobilized collagen in a dose-dependent manner with an IC₅₀ of 7.98 μg/mL. Values are expressed as mean ± SEM from at least three experiments.
Table S1. Effects of WE on platelet binding to immobilized ligands. Purified human platelets (2 × 10⁷/ml) were placed on BSA, thrombin, collagen, or fibrinogen-coated coverslips for 45 min at 37°C. In designated experiments, washed platelets were resuspended in buffer containing exogenously added WE (20 μg/ml) or vehicle in the presence of the ADP-scavenger apyrase and TxA₂ inhibitor indomethacin (2 U/ml Apy; 10 μM Indo) prior to exposure to the immobilized protein surface (Solution treatment). Adherent platelets are reported as mean ± SEM of 3-6 experiments.