A thrombin receptor function for platelet glycoprotein Ib–IX unmasked by cleavage of glycoprotein V

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Glycoprotein (GP) V is a major substrate cleaved by the protease thrombin during thrombin-induced platelet activation. Previous analysis of platelets from GP V-null mice suggested a role for GP V as a negative modulator of platelet activation by thrombin. We now report the mechanism by which thrombin activates GP V in platelets. We show that proteolytically inactive forms of thrombin induce robust stimulatory responses in GP V null mouse platelets, via the platelet GP Ib–IX–V complex. Because proteolytically inactive thrombin can activate wild-type mouse and human platelets after treatment with thrombin to cleave GP V, this mechanism is involved in thrombin-induced platelet aggregation. Platelet activation through GP Ib–IX depends on ADP secretion, and specific inhibitors demonstrate that the recently cloned P2Y12 ADP receptor (G<sub>i</sub>-coupled ADP receptor) is involved in this pathway, and that the P2Y<sub>1</sub> receptor (G<sub>i</sub>-coupled ADP receptor) may play a less significant role. Thrombosis was generated in GP V null mice only in response to catalytically inactive thrombin, whereas thrombosis occurred in both genotypes (wild type and GP V null) in response to active thrombin. These data support a thrombin receptor function for the platelet membrane GP Ib–IX–V complex, and describe a novel thrombin signaling mechanism involving an initiating proteolytic event followed by stimulation of the GP Ib–IX via thrombin acting as a ligand, resulting in platelet activation.

Methods

Generation of CHO-Expressed Thrombins. Plasma-derived thrombin and diisopropylphospho-(DIP)-thrombin were purchased from Haematologic Technologies (Burlington, VT). Activity of plasma DIP-thrombin was between 0% and 0.03% by chromogenic assay with tosyl-Gly-Pro-Arg-4-nitroanilide (Chromozyme TH; Roche Molecular Biochemicals) as the substrate. DIP-thrombin was treated with repeated doses of diisopropyl fluorophosphate (DFP), until no chromogenic activity could be detected. Fluorimetric measurements of hirudin binding to either plasma-derived DIP-thrombin or proteolytically active thrombin were similar. CHO-expressed prothrombins [wild type (wt), S205A, R89/R93/E94, and R98A] were expressed and purified as described (15). Activation of thrombin was carried out by using the prothrombinase complex [for wt and S205A (ref. 16)], or by using <i>Echis carinatus</i> venom as described (ref. 15; for R89/R93/E94 and R98A). CHO-expressed wt thrombin was 70% less active compared with plasma-derived thrombin in fibrinogen clotting assays with 10 μM purified fibrinogen (Enzyme Research Laboratories, South Bend, IN). Higher concentrations of the CHO-expressed proteins were required to elicit a response in the GP V null platelets (1–2 μM) than in the plasma-derived thrombin (100–400 nM). DFP-treatment of CHO-derived proteins was carried out as described (17). Loss of proteolytic activity was determined by chromogenic assay with Chromozyme TH and S2238, a p-nitroanilide thrombin substrate (DiaPharma, West Chester, OH).

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Abbreviations: GP, glycoprotein; GP V f1, cleaved GP V; vWF, von Willebrand factor; PAR, protease-activated receptor; CHO, Chinese hamster ovary; DIP-, diisopropylphospho-; DFP, diisopropylfluorophosphate; WP, washed platelets; wt, wild type; PGI<sub>2</sub>, prostacyclin; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>.

See commentary on page 1330.

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Affinity Purification of Ab 3584. Rabbit polyclonal Ab 3584 was kindly provided by B. Steiner and S. Meyer (Hoffman-LaRoche, Basel). We first purified the human extracellular domain of GP Ibα (Glycocalcin) as described (18). Ab 3584 was then affinity purified on a glycocalcin column. The affinity-purified Ab 3584 IgG recognized human glycocalcin as assessed by Western blotting and mouse platelets by FACS analysis.

Aggregation. Washed platelets (WP) were isolated as described previously (13). Platelets were resuspended in Tyrode–Hepes buffer and rested for 15 min before use in aggregation. Wt platelets were incubated with repeated doses (four) of 10 pM thrombin in calcium-free Tyrode-Hepes buffer with gentle mixing. Platelets were rested for at least 2 min before the addition of DIP-thrombin. Aggregation was measured as the change in transmittance obtained after the addition of agonist by using a Chronolog lumi-aggregometer. For experiments using inhibitors, aggregation was initiated after a brief incubation with inhibitors. The αIIbβ3 inhibitors used in this study have been described (19), and were shown to inhibit mouse αIIbβ3 (D. A. Law, personal communication). Whereas full aggregation varied in different platelet preparations (50–70% transmittance), in all cases DIP-thrombin-induced aggregation was equivalent to the full aggregation obtained with 10 nM thrombin.

Human platelets were isolated from freshly drawn blood anticoagulated in acid/citrate/dextrose (ACD)/prostacyclin (PGI2) containing apyrase (50 units/ml, 1 μl/10 ml of blood), and washed as described (20). Platelets were resuspended in Tyrode-Hepes buffer containing 1 mM each CaCl2 and MgCl2 at 2–2.5 × 106/ml. GP V on WP was cleaved by pretreatment with thrombin (10 pM) added four times sequentially with gentle mixing. WP were rested for 2 min and then incubated with PAR 1 antagonist [40 μM (ref. 21)] or DMSO control, and aggregation was initiated by the addition of DIP-thrombin (0.4 μM). This concentration of DIP-thrombin resulted in full aggregation.

Detection of Cleaved GP V (GP V f1) in Supernatants of wt Platelets Treated with Thrombin. wt WP were isolated from 20 mice, and incubated with or without 50–100 pM thrombin for 30 min without stirring at 37°C. Platelets were centrifuged at 100,000 × g to remove microparticles, and the supernatant was lyophilized, reconstituted in 1×3M 2-mercaptoethanol/0.15 mM NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS (reducing RIPA buffer) and boiled for 5 min. Reduced samples were buffer-exchanged by dialysis into nonreducing RIPA buffer and immunoprecipitations were carried out with rabbit Ab 808 [previously shown to recognize an epitope not available in native GP V (13)] or control rabbit IgG. Samples were electrophoresed by reducing SDS/PAGE, and Western analysis was done with Ab 808.

Thrombosis Model in Mice. This model is a modification of that described by Leon et al. (22). Briefly, mice were anesthetized and the jugular vein was exposed surgically. Retro-orbital blood sample was obtained. The platelet counts in the supernatant were aggregated with DIP-thrombin (100 nM). The data are the average of duplicate experiments.

Results

Effect of Proteolytically Inactive Thrombin on GP V-Deficient Platelets. To evaluate whether proteolytically inactive thrombin potentiates the activity of active thrombin, we conducted aggregation experiments with GP V−/− platelets and DIP-thrombin. Surprisingly, DIP-thrombin induced platelet aggregation in GP V−/− platelets without the addition of any active thrombin, whereas wt platelets showed no response (Fig. L4). Aggregation of GP V−/− platelets required approximately 10-fold more DIP-thrombin (100 nM) than untreated thrombin (10 nM), confirming the Kd values determined in recent studies (23). Platelets from wt mice pretreated with suboptimal doses (40–50 pM) of active thrombin (Fig. 1B) aggregated in response to inactive thrombin, but were nonresponsive without pretreatment. Western analysis showed that thrombin...
pretreatment hydrolyzed GP V from the platelet surface, as determined by the release of GP V αI, the thrombin hydrolytic fragment of GP V (Fig. 1B Inset). In other experiments, we also found that GP V null platelets aggregated in response to recombinant thrombin carrying a mutation [S205A (ref. 15)] that inactivates its proteolytic capacity (Fig. 1C). CHO-expressed wt thrombin inactivated by DFP also caused aggregation in GP V null platelets. Aggregation of mutant and wt platelets in response to all forms of thrombin was sensitive to inhibition by an antagonist of the αIIbβ3 integrin (19), indicating that the aggregation reactions involved the characteristic agonist-induced pathway (data not shown).

Role of GP Ib–IX in Platelet Aggregation Induced by Proteolytically Inactive Thrombin. Ab 3584 recognizes GP Ib–IX on human and mouse platelets (13). Because GP Ibα is a candidate receptor for thrombin, we tested whether the antibody could inhibit platelet aggregation in response to proteolytically inactive thrombin. Ab 3584 (Fig. 2A) and affinity-purified Ab 3584 (which recognizes only the GP Ibα subunit; Fig. 2C; see Methods) effectively blocked aggregation of GP V-deficient platelets caused by DIP-thrombin or S205A-thrombin response (Fig. 2D), but had only a slight effect on aggregation induced by native untreated thrombin (Fig. 2B). Ab 3584 also inhibited DIP-thrombin-mediated aggregation of wt mouse platelets that had been pretreated with suboptimal doses of thrombin (data not shown). Further, Ab LJ-1B10, which inhibits thrombin binding to GP Ibα (24), inhibits the aggregation in human platelets rendered GP V deficient by thrombin pretreatment (data not shown). These data implicate thrombin binding to GP Ibα in the aggregation of platelets induced by proteolytically inactive thrombin.

Role of the Heparin Binding Exosite in DIP-Thrombin-Induced Aggregation. The exosite II of thrombin binds heparin and may also be involved in the interaction with GP Ibα (23). We therefore examined whether aggregation induced by DIP-thrombin could be inhibited by heparin. Within the standard therapeutic dose range [0.3 unit/ml; standard range 0.2–0.7 unit/ml (ref. 25)] heparin significantly inhibited DIP-thrombin-induced platelet aggregation and completely reversed the response of both GP V null (Fig. 3A) and thrombin-pretreated wt platelets (Fig. 3C) within 5 min. In contrast, this concentration of heparin provided only marginal inhibition of aggregation caused by native thrombin applied to either GP V null (Fig. 3B) or wt platelets (Fig. 3D). Thus, the data suggest that binding between exosite II of thrombin and GP Ibα on platelets is essential for platelet activation in response to proteolytically inactive thrombin. Because heparin blocks this interaction at concentrations used therapeutically, this mechanism may also be involved in the antithrombotic activity of heparin.

Signaling Pathways Mediated by GP Ib–Thrombin Interaction. GP Ib–IX can signal in response to the binding of vWF (5, 26) and induce platelet activation, probably through 14–3–3 (27, 28), a signaling molecule constitutively associated with the cytoplasmic tail of GP Ibα that is phosphorylated at Ser-609 (29). Other studies have shown that agents such as PGI2 and prostaglandin E2 (PGE2) negatively regulate GP Ib–IX signaling. The mechanism of inhibition by the adenylyl cyclase activators seems to be through the activation of the cAMP-dependent kinase and phosphorylation of the cytoplasmic domain of GP Ibα (30, 31). To determine whether the thrombin signaling function of GP Ib–IX–V is similarly regulated, we evaluated the role of the cAMP-dependent pathway in DIP-thrombin-induced signaling. Fig. 3E shows that PGI2 completely inhibited DIP-thrombin-induced shape change and aggregation of GP V (PGI2, another activator of adenylyl cyclase, also inhibited thrombin-induced aggregation in GP V (30)). PGI2, inhibited DIP-thrombin-induced aggregation in both mouse (Fig. 3G) and human platelets pretreated with suboptimal doses of active thrombin. In contrast, thrombin-induced aggregation was not affected significantly by PGI2 even at high concentrations (4.45 μM, Fig. 3F and H). In fact, PGI2-treated platelets that failed to respond to DIP-thrombin were fully responsive to 10 nM α-thrombin (data not shown). Thus, the thrombin-signaling function of GP Ib–IX–V is ablated in the presence of adenylyl cyclase activators. This finding also suggests that incubation of isolated platelets with suboptimal concentrations of heparin could reduce the sensitivity of platelet aggregation induced by DIP-thrombin.
platelets with PGI2 or PGE1 could suppress this signaling pathway. In previous studies reported by Kahn et al. (12, 32), only PAR signaling was observed in the platelet response to thrombin. The inclusion of PGE1 during platelet isolation and desensitization studies described therein may have inhibited signaling through GP Ib–IX–V.

Previous studies have implicated secreted ADP in platelet aggregation by low doses of thrombin. ADP activates platelets via three distinct pathways (20): a Gq-coupled pathway mediated by the P2Y1 receptor, a Gi-coupled pathway mediated by the recently cloned P2Y12 receptor (33), and a calcium channel (P2X). We conducted platelet aggregation studies with GP V−/− platelets both in the absence and in the presence of specific antagonists to the ADP receptors P2Y12 and P2Y1. Interestingly, we found that 83 nM 2-methylthioadenosine monophosphate, a specific P2Y12 antagonist, completely inhibited platelet aggregation mediated by DIP-thrombin (Fig. 4A). However, the P2Y1 antagonist adenosine 3′,5′-bis-phosphate (2 μM) had little effect on the DIP-thrombin-induced aggregation (Fig. 4B). These data imply that the secreted ADP preferentially activates the P2Y12 receptor. Adenosine 3′,5′ diphosphate did show a partial (50%) effect at 4 μM (not shown), but it seems that the Gi-coupled ADP receptor is the major contributor to the signaling pathway. Both compounds could independently inhibit ADP-mediated aggregation in mouse platelets (data not shown). These results support a role for secreted ADP in platelet activation via the thrombin-GP Ibo interaction, and specifically a role for P2Y12 in human platelets. RWJ 56110, a PAR 1 antagonist, could be mediated by inhibition of the ADP response.

Kahn et al. (11, 12) have shown that PAR 1 activation occurs at low thrombin concentrations, whereas PAR 4 activation requires high concentrations of thrombin. Because human platelets and wt mouse platelets were pretreated with low doses of thrombin to cleave GP V and render the platelets responsive to DIP-thrombin, we evaluated the potential contribution of PAR 1 in the response to DIP-thrombin in human platelets. RWJ 56110, a PAR 1 antagonist, could be mediated by inhibition of the ADP response.

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Physiological Consequences of Thrombin–GP Ib Interaction. To evaluate the physiological significance of the ligand binding function of thrombin, we examined the effect of a systemic infusion of DIP-thrombin into mice. Infusion of a platelet agonist induces a decrease in platelet counts because of (ongoing) thrombosis (22). As can be seen in Fig. 5, there was significant platelet loss after the infusion of 10 nM thrombin in both GP V null and wt mice (Fig. 5C), with no statistical difference observed between the two groups. Injection of 1 nM thrombin had a marginal effect on platelet counts in either GP V null mice or wt mice (Fig. 5B). In contrast, GP V null mice showed a significant platelet loss when injected with 100 nM DIP-thrombin compared with wt mice (Fig. 5A), in which platelet loss was minimal. As additional controls, we used mutant CHO-expressed thrombins (15) in which the exosite II had been mutated (R89/R93/E94 and R98A). These exosite II mutants were inactivated by using DFP, and then injected into either GP V null or wt mice. As can be seen in Fig. 5E and F, there was no major loss of platelets in either group. In contrast, 460 nM CHO-expressed thrombin inactivated with DFP (Fig. 5D) caused a significant platelet loss in GP V null mice, with little effect in wt mice. These results show that binding of inactive forms of thrombin to GP V-deficient platelets can occur in vivo, and that the binding results in platelet activation and thrombosis. Our data further substantiate the view that the binding site for GP Ib is exosite II of thrombin. These results support a role for GP Ibα in thrombin-induced signaling in platelets that results in aggregation and thrombosis.

Discussion

The platelet GP Ib–IX–V complex plays a critical role in thrombus formation under conditions of high shear (34, 35). A role for GP Ib–IX–V in the inside-out activation of αIIb/β3 after vWF binding was shown by using a heterologous expression system (36). Recently, a role for GP Ib in platelet procoagulant activity has been proposed on the basis of observations that inhibition of thrombin binding to GP Ib inhibited annexin V binding to the complex rendered the platelets more responsive to thrombin, and GP V −/− mice were found to have a shorter bleeding time (13). We now show that absence of GP V results in the ability of the platelets to signal (upon thrombin binding) to GP Ibα.

It is well established that thrombin-induced platelet activation occurs via the PAR family of thrombin receptors (11). Studies also indicate that the PAR 1 cleavage occurs at thrombin concentrations, whereas PAR 4 cleavage occurs at high thrombin concentrations, and inhibition of both PAR1 and PAR4 in human platelets eliminated thrombin-induced aggregation (12). Further, thrombin-mediated aggregation seemed equivalent in GP V −/− and wt mice generated by Kahn et al. (32). However, our studies now suggest that the GP Ib–IX complex also contributes to thrombin-induced aggregation. To reconcile this apparent difference, we examined the effect of treatment conditions used by Kahn et al. on DIP-thrombin-induced aggrega-

Fig. 5.  Thrombosis in wt and GP V null mice. The mice were injected with 100 nM DIP-thrombin (A), 1 nM thrombin (B), 10 nM thrombin (C), 0.46 μM CHO-expressed wt DIP-thrombin (D), 0.75 μM CHO-expressed DIP-R89/R93/E94 thrombin (exosite II mutant, E) or 0.75 μM CHO-expressed DIP-R98A-thrombin (exosite II mutant, F). The number of animals used and the statistical significance is shown in each graph.
We have found that agents such as PGE\textsubscript{1} that activate adenyl cyclase completely inhibited the aggregation induced by proteolytically inactive thrombin, but not by native thrombin. Thus the conditions used in these studies (including PGE\textsubscript{1} and EDTA) would inhibit platelet aggregation via GP Ib–IX, but not via the PARs. The data suggest that repression of adenyl cyclase is critical for platelet activation through the GP Ib–IX complex. Further evidence for the importance of GP Ib–IX in thrombin-induced platelet activation is demonstrated when DIP-thrombin-induced platelet activation is carried out in the presence of a PAR 1 antagonist. Our data show that inhibition of PAR 1 only has a slight effect on the signaling response by DIP-thrombin, and this effect may be because of the inhibition of the signaling response to low doses of active thrombin used in the pretreatment. These data also suggest that inhibition of PAR 1 alone may not prevent platelet activation by thrombin, because thrombin can also signal via the GP Ib–IX complex.

Our data support a model (Fig. 6) for thrombin-induced platelet activation that involves not only the established pathway mediated by the PARs, but also another pathway in which the presence of GP V in the GP Ib–IX–V complex inhibits the ability of thrombin to function as a receptor ligand. After the loss of GP V upon cleavage by thrombin, thrombin binding to GP Ib\alpha results in activation of αIIbβ3 and consequently in aggregation. As shown here, the GP Ibα-bound thrombin need not be catalytically functional for this response to occur. Binding of thrombin to GP Ib\alpha results in ADP secretion, which contributes to platelet activation through the P2Y\textsubscript{12} receptor (33). The data show not only a previously unknown functional role for thrombin, but also a mechanism by which this pathway can mediate thrombosis independent of proteolytic activity. The binding of thrombin to GP Ib\alpha occurs via the heparin binding exosite and prevents the inactivation of thrombin by antithrombin III (23). Our data indicate that this GP Ibα-bound thrombin can itself initiate additional signaling responses in platelets. The two signaling pathways may have evolved to mediate a more robust aggregation response particularly under conditions of arterial flow. Effective antithrombotic therapies targeting thrombin-induced platelet activation would thus require the inhibition of both pathways. Further, the data suggest a central role for ADP and the P2Y\textsubscript{12} receptor in thrombin-induced signaling responses mediated by GP Ib\alpha. The findings therefore reveal another arena for therapeutic intervention in cardiovascular disease.

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Fig. 6. Thrombin-induced platelet activation. Two pathways exist on the platelet for activation by thrombin. The pathway described in this article involves the initial cleavage of GP V from the GP Ib–IX–V complex at low thrombin concentrations, resulting in a hyperresponsive platelet. Occupancy of the binding site on GP Ibα by thrombin results in a signaling response that leads to αIIbβ3 activation. Additionally, thrombin can cleave the PARs on platelets, and this cleavage will then also stimulate platelet aggregation.