Thrombin-induced platelet activation via PAR4: pivotal role for exosite II

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Summary
Thrombin-induced platelet activation via PAR1 and PAR4 is an important event in haemostasis. Although the underlying mechanisms responsible for ensuring efficient PAR1 activation by thrombin have been extensively studied, the potential involvement of recognition sites outside the active site of the protease in thrombin-induced PAR4 activation is largely unknown. In this study, we developed a new assay to assess the importance of exosite I and II for PAR4 activation with α- and γ-thrombin. Surprisingly, we found that exosite II is critical for activation of PAR4. We also show that this dependency on exosite II likely represents a new mechanism, as it is unaffected by blockage of the previously known interaction between thrombin and glycoprotein Ibα.

Introduction
The serine protease thrombin potently activates platelets by proteolytic cleavage of two protease-activated receptors, PAR1 and PAR4. Although the evolutionary benefits of this seemingly redundant dual receptor configuration are unknown, emerging clinical and experimental evidence support the notion that the two receptors have distinct and complementary roles in platelet biology. For example, PAR1 is more sensitive than PAR4 to low concentrations of thrombin (1) and is more effective in rapidly mobilising platelet haemostatic functions, such as the release of bioactive cargo stored in granules (2). While platelets respond with a transient “spike” in the intracellular calcium concentration upon stimulation of PAR1, PAR4 stimulation gives rise to a much more prolonged calcium mobilisation pattern (3).

The catalytic activity and specificity of thrombin is highly dependent on two intramolecular recognition sites located distant from the active site. These domains, designated fibrinogen recognition site and heparin binding site, or exosite I and II, facilitate proteolysis by interacting with anionic surfaces on various substrates, and are the target of several physiologically important thrombomodulatory agents such as serpins. It has previously been shown that cleavage of PAR1 is facilitated by two interactions involving exosite I and II: i) exosite II-mediated binding of thrombin to glycoprotein (Gp)Ibα (4) and ii) exosite I-mediated binding of thrombin to the hirudin-like domain of PAR1 (5). PAR4, unlike PAR1, does not contain a hirudin-like binding motif for interaction with exosite I on thrombin, but it has been proposed that it makes use of dual proline residues and an anionic cluster to effect direct binding to the active site and to slow down dissociation of the protease (6). Experimental evidence suggests that PAR1 and PAR4 form heterodimers on the platelet surface in human platelets (7). It has been proposed that this spatial organisation facilitates PAR4 cleavage by a mechanism analogous to that in mice, wherein a heterodimeric configuration promotes PAR4 cleavage by providing a binding site for exosite I on PAR3 (8, 9). However, to our knowledge, no studies have examined the potential involvement of exosite II in thrombin-induced PAR4 activation.

In this study, we developed an assay that allowed us to quantify the contribution of PAR4 to thrombin-induced platelet activation. Using the DNA aptamers HD1 and HD22, which specifically inhibit exosite I and II, respectively, we investigated the effects of blocking these binding sites on the activation of PAR4 with α- and γ-thrombin. These results were confirmed with complementary techniques such as western blotting and correlations of cytosolic calcium mobilisation patterns. We also used different techniques to explore the role of GpIbα in this context. Surprisingly, blockage of exosite II on thrombin with HD22 or heparin strongly inhibited PAR4 activation. As blockage or proteolytic cleavage of GpIbα did not affect platelet activation via PAR4, the observed dependency of thrombin upon exosite II for effective PAR4 activation cannot be attributed to the previously demonstrated interaction between thrombin and GpIbα.
Methods

Materials
The FITC-conjugated monoclonal antibody (mAb) PAC-1 was from BD Biosciences (San Jose, CA, USA). The mAb SZ2 shown to block the von Willebrand factor-binding domain on GpIba was from Immunotech (Marseille, France). mAbs towards GpIba (clone AN51), glycoprotein IIa (Clone Y2/51) and control IgG1 were from Dako (Glostrup, Denmark). The mAb 5F4 was from Abnova (Taipei, Taiwan). Secondary antibodies for western blots were from Cell Signalling Technology (Boston, MA, USA). PPACK and the peptides SFLLRN (PAR1-AP) and AYPGKF (PAR4-AP), which are specific agonists of the thrombin receptor subtypes PAR1 and PAR4 respectively, were from Bachem (Waldam Rhein, Germany). The DNA-aptamers HD1 and HD22 were from Biotmers.net (Ulm, Germany). The fibrin polymerisation inhibitor Pefablock FG (GPRP) was from Pentapharm (Basel, Switzerland). Heparin was from Leo Pharma (Ballerup, Denmark), Bovine and human α- and γ-thrombin, chemicals for the HEPES buffer (composed of 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM HEPES and 10 mM glucose, pH 7.4), as well as all other reagents used, were obtained from Sigma-Aldrich (St. Louis, MO, USA). For thrombin, activity units (IU/ml) were converted to molar concentrations using previous guidelines (10, 11). Nk protease was purified from the venom of Naja kaouthia as described by Wijeyewickrema et al. (12), and was a generous gift from Prof. Robert Andrews (Monash University, Melbourne, Australia).

Platelet preparation
In accordance with an informed consent procedure approved by the Ethics Committee at Linköping University Hospital, whole blood from healthy volunteers was collected in sampling tubes with 129 mM sodium citrate. The supernatant (PRP) was collected after centrifugation at 140 × g for 12 minutes (min) for subsequent experiments. For platelet isolation, a citrate dextrose solution (ACD) was mixed with venous heparinised blood (1/5; v/v), as well as all other reagents used, were obtained from Sigma-Aldrich (St. Louis, MO, USA). For thrombin, activity units (IU/ml) were converted to molar concentrations using previous guidelines (10, 11). Nk protease was purified from the venom of Naja kaouthia as described by Wijeyewickrema et al. (12), and was a generous gift from Prof. Robert Andrews (Monash University, Melbourne, Australia).

Flow cytometry
Flow cytometry was performed on a Coulter Epics XL MCL flow cytometer with Expo 32 ADC software (Beckman Coulter, Miami, FL, USA). For the titration experiments with α- and γ-thrombin, PRP (n=4) or isolated platelets in a final dilution of 1:10 were added to HEPES buffer together with 2 mM of the fibrin polymerisation blocker GPRP and FITC-conjugated PAC-1 antibody (1:2.5). Where indicated, 20 μg/ml SZ2 or 20 μg/ml control IgG was added to the platelet suspensions. For the thrombin titration experiments, 0.02-70 nM α-thrombin or 1-81 nM γ-thrombin, either alone or pre-incubated for 10 min with either 5 nM PPACK, 1 μM HD1 and/or 1 μM HD22, was added in the presence or absence of 100 μM PAR1-AP (SFLLRN). In other experiments, only 100 μM PAR1-AP and/or 500 μM PAR4-AP (AYPGKF) was added in the presence or absence of HD1 or HD22 in the concentrations stated above. Where indicated, experiments were performed after depletion of GpIba with Nk protease. The platelet suspensions were incubated for 16 min, after which the activation was quenched by diluting the suspensions 1:20 with HEPES.

Western blotting
Washed platelets in KRG buffer at a concentration of 4.5 × 10^9/ml were incubated for 20 min with agonists, and then mixed with Tris-HCl 50 mM pH 7.5 with 2% Tween and protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Samples were frozen and thawed twice, with successive vortexing, and protein extract was collected after pelleting debris by centrifugation at 10,000 × g for 1 min. Electrophoresis of proteins in NuPage precast gels (Invitrogen, Thermo Scientific) was run at constant voltage of 125 V in MES buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3) (Invitrogen). Electrotransfer to PVDF membranes (BioRad, Hercules, CA, USA) was made at 125 mA per membrane. Membranes were blocked with TBS-T with 5% skimmed milk during 1 h at gentle shaking, followed by incubation with primary antibody (5F4 at a dilution of 1/1,000 or 2/1,000) overnight at 4°C under constant shaking. After three washes the secondary antibody was incubated at a dilution of 1/3,000 or 1/4,000 for 1 h at RT. The detection was performed using a commercial ECL reagent (Merck Millipore, Billerica, MA, USA) and exposure was made in a dark chamber (FUJI LAS-1000, Fujifilm, Tokyo, Japan) with Image Reader software V2.6.

GpIba-depletion with the snake venom Nk protease
Washed platelets were incubated with Nk protease (0.49 mg/ml) at 37°C for 30 min and then immediately used for further experiments. Cleavage efficiency was assessed with flow cytometry, using the RPE-labelled mAb AN51 (dilution 1/10; v/v) as a marker for GpIba, the FITC-labelled mAb Y2/51 towards GpIIa as a receptor control and relevant isotype controls.

Aggregometry
Aliquots (0.5 ml) of platelet suspensions (2.5 × 10^8 platelets/ml) were preincubated at 37°C for 5 min, either alone or in the presence of 20 μg/ml SZ2, 20 μg/ml control IgG or 1 μM HD22. Platelet aggregation was induced by adding 27 nM γ-thrombin. Changes in light transmission were recorded using a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log, Haverton, PA, USA).
Measurements of cytosolic Ca$^{2+}$

Platelets were loaded with fura-2 by incubating PRP with 3 μM fura-2/AM (Molecular Probes, Life Technologies, Carlsbad, CA, USA) for 45 min at RT, after which platelets were isolated as described above. Platelets were pre-incubated and stimulated as indicated at 37°C and fluorescence was recorded using a Hitachi F-7000 spectrophotometer at 510 nm with alternating excitation at 340 nm and 380 nm. Cytosolic calcium [Ca$^{2+}$] was expressed as a fluorescence ratio (340/380 nm).

Statistical analysis

Data are expressed as mean ± SD. The inhibitory effect of HD1 on thrombin was assessed with the use of two-tailed Student t-test and considered significant when p<0.05. The EC50 values were calculated by fitting data to a four parameter logistic curve using Sigmaplot® software (Systat Software Inc., San Jose, CA, USA).

Figure 1: Activation of PAR4 by α-thrombin; critical dependence on exosite II. A) Platelet-rich plasma mixed with FITC-conjugated PAC-1 antibody was incubated with 100 μM PAR1-AP (first column), 500 μM PAR4-AP (second column) and 100 μM PAR1-AP plus 500 μM PAR4-AP (third column) for 16 min, after which the mean fluorescence intensity (MFI) was measured with flow cytometry. B) Platelets were incubated for 16 min with increasing concentrations of thrombin, either alone or preincubated with 1 μM HD1, 1 μM HD22 or 1 μM HD1 plus 1 μM HD22. C) The experiment in (B) was repeated in the presence of 100 μM PAR1-AP to study the contribution of PAR4. D) PAR4-mediated platelet activation was calculated from the results in (B-C) by subtracting the signal obtained with 100 μM PAR1-AP from the signal with thrombin in the presence of 100 μM PAR1-AP. The normalised PAR4-mediated activation calculated by this method is shown in (E). Results in (A-D) are expressed as a percentage of the maximal MFI obtained with the control sample for each donor (thrombin in the concentration range 0.007–70 nmol/l). In (E), results are expressed as a percentage of the maximal calculated PAR4 activation obtained with the control sample. Data represent mean ± SD, n≥3.
Results

PAR4 activation by α-thrombin can be assessed with a new flow cytometric assay

Flow cytometric titration experiments revealed that maximal PAC-1 binding as a response to stimulation with one of the specific PAR1- and PAR4-activating hexapeptides, SFLLRN (PAR1-AP) and AYPGKF (PAR4-AP) was achieved with 100 μM PAR1-AP and 500 μM PAR4-AP (Suppl. Figure 1 A-B, available online at www.thrombosis-online.com). The signal acquired with a combination of 100 μM PAR1-AP and 500 μM PAR4-AP amounted to more than 90% of the maximal signal obtained with α-thrombin (Figure 1A) in PRP. Intriguingly and essential for the development of this assay, maximal PAC-1 binding as a response to the previously defined saturating concentrations of activating peptides was approximately 60% higher for PAR4-AP alone than for PAR1-AP alone, and 76% higher upon maximal stimulation of both receptors than with PAR1-AP alone (Figure 1A). Surplus activity above the threshold of maximal PAR1 stimulation is therefore derived from PAR4. Furthermore, the dose-response curve for PAC-1 binding upon activation with PAR4-AP was shown to be virtually identical in the presence or absence of PAR1-AP (Suppl. Figure 2B, available online at www.thrombosis-online.com), allowing for a straightforward interpretation of results where thrombin-induced PAR4 activation was assessed in the presence of PAR1-AP. We then performed thrombin titration experiments with and without simultaneous addition of 100 μM PAR1-AP (Figure 1B-C), which enabled us to calculate a dose-response curve for the activation of PAR4 with α-thrombin (Figure 1D-E).

Since it has been suggested that thrombin might have the capacity to activate platelets independently of PAR1 and PAR4 (13), we then conducted titration experiments with PPACK-thrombin, which is proteolytically inactive but retains its capacity to bind and interact with platelet receptors. As these experiments showed no platelet activation in the concentration range used (0.017-69.6 nM, Suppl. Figure 1C, available online at www.thrombosis-online.com), this possibility could be ruled out. Also, as shown in Suppl. Figure 2B (available online at www.thrombosis-online.com), neither the addition of HD1, HD22, the monoclonal antibody SZ2 nor negative control IgG had any impact on PAC-1 binding upon activation with PAR1-AP and/or PAR4-AP, excluding the possibil-
ity of unspecific interactions affecting PAC-1 binding downstream of receptor activation for these reagents.

**Blocking exosite II strongly inhibits PAR4 activation by α- and γ-thrombin**

In order to determine whether proteolytic cleavage of PAR4 is facilitated by anchoring of thrombin via the high-affinity binding sites exosite I or II, we performed titrations with α-thrombin in the presence of the DNA aptamers HD1 and HD22, which selectively block anion-binding exosite I and II, respectively (14, 15). Interestingly, virtually no activation of either PAR1 or PAR4 was observed in the presence of a combination of 1 μM HD1 and 1 μM HD22 (▶ Figure 1B). Blockage of exosite I with HD1 had a statistically significant inhibitory effect (p<0.05, paired samples t-test) on PAR4 activation in the concentration range 1.1–4.4 nM, while selective blockage of exosite II with HD22 resulted in a complete blockage of PAR4-mediated platelet activation in the entire throm-

![Figure 3: Kinetic profiles of thrombin-triggered calcium transients reveal differential inhibition of PAR receptors with HD1 and HD22. A-C) Changes in intracellular Ca\textsuperscript{2+} upon thrombin stimulation (7 nM) alone or in the presence of 1 μM HD22 or HD1 were recorded. D-E) Ca\textsuperscript{2+} profiles obtained with 100 μM PAR1-AP alone or 500 μM PAR4-AP alone are provided for comparison. F-H) A combination matrix of the PAR1 and PAR4 calcium response profiles with variation in amplitude (0–100 %) were fitted to the calcium signal induced by thrombin with or without HD1 or HD22, where best fit can be seen in red as the minimum sum of squared residuals. Data represent the average of three independent experiments conducted on platelets from different donors.](image-url)
bin concentration range. As shown in Figure 4, the addition of HD22 had a similar impact on PAR4-activation by γ-thrombin, a degradation product of α-thrombin which lacks exosite I and has been shown to selectively activate PAR4 (13, 16), which was consistent in the concentration range used (1–81 nM).

The results shown in Figure 1D-E reveal that exposure to α-thrombin activates PAR4 at surprisingly low thrombin concentrations, with an EC50 estimated to 0.7 nM. To validate this finding, we performed western blot analysis to assess cleavage of PAR4 on protein lysates from washed platelets treated with α-thrombin. Immunoblotting with the mAb 5F4 which specifically recognises PAR4, revealed the appearance of an extra band with a molecular weight corresponding to that of the cleaved PAR4 receptor as platelets were exposed to 0.7 or 14 nM thrombin, but not when exposed to PAR4-AP (Figure 2A).

To confirm the observed exosite II-dependency of thrombin-mediated PAR4 activation with alternative methods, measurements of changes in cytosolic calcium levels upon thrombin stimulation (7 nM) in the presence or absence of HD1 and HD22 were conducted (Figure 3). The temporal calcium profiles obtained were compared with those obtained with only PAR1 and PAR4 stimulation with PAR1-AP and PAR4-AP, respectively. This was accomplished with computational fitting, finding the residual minimum in a matrix containing combinations of 0–100% PAR1 response and 0–100% PAR4 response. The results shown in Figure 3 clearly demonstrate that the addition of HD22 shifts the concentration profile to closely mimic that of PAR1 stimulation, whereas the presence of HD1 gives rise to a temporal concentration profile characteristic of PAR4 stimulation. Additionally, western blotting with the mAb 5F4 against PAR4 in the presence of HD1 or HD22, confirmed the finding that PAR4 cleavage is attenuated by HD22 (Figure 2B). Immunoblotting for PAR4 with 5F4 and subsequent densitometry revealed that the attenuation of PAR4 band density obtained when exposing platelets to 7 nM thrombin was partially restored when adding HD22 (Figure 2C-D).

To verify that the inhibitory effects of HD22 on PAR4 activation were solely mediated by exosite II inhibition, and not caused by potential unknown unspecific interactions between HD22 and thrombin, control experiments using the oligosaccharide heparin, which is known to specifically bind to thrombin’s exosite II (17) were performed, showing a dose-dependent inhibition of PAR4-mediated platelet activation by γ-thrombin with heparin concentrations similar to those used in the clinical setting (Figure 4A). As these experiments were conducted with washed platelets, samples did not contain antithrombin, and the effects could therefore not stem from the well-known anticoagulant properties of the heparin-antithrombin complex.

GpIba does not support thrombin-induced PAR4 activation

The role of GpIba in PAR4 activation by α- and γ-thrombin was explored with thrombin titration experiments in the presence of the monoclonal antibody SZ2 as well as after depletion of GpIba with the snake venom Nk protease (Figure 5), a protease that has been shown to cleave off GpIba at a site very close to that of mocularhaegin (12). In Figure 5B, the graph shows the PAR4 response upon stimulation with α-thrombin, calculated using the same assay as in Figure 1D-E. As treatment with Nk protease requires removal of plasma components, these experiments were conducted with isolated platelets. Treatment of platelets with Nk protease and staining with a RPE-labelled antibody towards GpIba resulted in a decrease in MFI from 21.6 ± 6.5 to 1.7 ± 0.6, as compared with the MFI after staining for GpIba which was 34.1 ± 4.2 and 34.2 ± 5.3, respectively, before and after treatment (n=11). As a reference, MFI for a RPE-labelled isotype control antibody was 1.2 ± 0.2 before and 1.0 ± 0.1 after incubation with the protease,

![Figure 4: HD22 and heparin, but not SZ2, inhibit platelet aggregation induced by γ-thrombin. A) Washed platelets incubated with PAC-1 antibody were exposed to 27 nM γ-thrombin alone or together with 1 nM HD22, or the indicated concentrations of heparin for 16 min, after which the mean fluorescence intensity (MFI) was measured with flow cytometry. B-C) Aggregation after exposure to 27 nM γ-thrombin, measured as light transmission, was recorded for untreated PRP, and PRP pre-incubated with 1 µM HD22, 20 µg/ml control mAb or 20 µg/ml SZ2. Data represent mean ± SD (n = 3–5) in (A) and (C), whereas (B) shows one representative original trace.](image-url)
implying a cleavage efficiency of 96.6%. In accordance with results from previous studies (4), depletion of GpIbα and addition of the monoclonal antibody SZ2 had an inhibitory effect on PAR1 activation by α-thrombin (data not shown). However, PAR4 activation by α- or γ-thrombin was not significantly affected (Figure 5A–B). Also, as shown in Figure 4C, pre-incubation with HD22 completely inhibited platelet aggregation induced by 27 nM γ-thrombin, while incubation with 20 μg/ml SZ2 had no inhibitory effect.

Discussion

The present study demonstrates that blockage of exosite II has dramatic effects on the activation of PAR4 by α- and γ-thrombin. Although unexpected, indirect support for this finding can be inferred from previous studies. For example, it has been shown that the addition of YD-3, a selective inhibitor of PAR4, does not significantly increase the inhibitory effect of HD22 on thrombin-induced platelet activation, while producing a synergistic inhibition in combination with the exosite I inhibitor HD1 (18). Interestingly, our results implicate that targeting of PAR4 could be achieved indirectly by pharmacological interventions involving exosite II inhibitors. Our finding also sheds new light on the thrombomodulating function of GpIbα and exosite II on thrombin or by depletion of GpIba from the platelet surface. These findings strongly indicate that exosite II-mediated thrombin-PAR4 interactions via binding to exosite II. We herein show that the inhibitory effect of HD22 or heparin could not be reproduced by the addition of the monoclonal antibody SZ2 which inhibits the interaction between GpIba and exosite II on thrombin or by depletion of GpIba from the platelet surface. These findings strongly indicate that exosite II-mediated binding of thrombin to GpIba does not support thrombin-induced PAR4 activation. As it was previously demonstrated that blockage of exosite II does not affect the ability of thrombin to hydrolyse a PAR4(44–66) peptide (20), that binding of polyphosphates to exosite II does not alter the structure of thrombin (21) and that HD22 used in the concentrations employed in this study only has marginal effects on the ability of thrombin to cleave various substrates (14), the inhibitory effect observed herein with HD22 is not likely to stem from allosteric changes affecting the catalytic properties of thrombin.

Presently, the structural information available regarding the interaction between thrombin and PAR4 are confined to a crystal

![Figure 5: GpIba does not support PAR4 activation with α- or γ-thrombin. Washed platelets incubated with PAC-1 antibody were exposed to different concentrations of (A) α-thrombin or (B) γ-thrombin. Titrations were performed in the presence or absence of PAR1–AP (100 μM). The PAR4 response shown in (B) was then calculated as described in Figure 1. Titrations were performed without further treatment (control), in the presence of 20 μg/ml SZ2, after treatment with Nk protease for depletion of GpIba, or after incubation with 1 μM of HD22. Results are expressed as a percentage of the maximal mean fluorescence intensity obtained in each thrombin titration series. Each experiment was repeated at least three times with blood from different donors. Data represent mean ± SD.](564)
What is known about this topic?
• Exosites I and II are important for regulating the catalytic activity and specificity of thrombin.
• Previous studies have shown that thrombin-induced platelet activation via PAR1 is facilitated by platelet-thrombin interactions involving both exosite I and II. Less is known about whether these important regulatory domains support the activation of PAR4.

What does this paper add?
• Thrombin-induced PAR4 activation is critically dependent on exosite II.
• This dependency does not seem to be related to the well-known interaction between thrombin’s exosite II and glycoprotein Ib.

structure of a small N-terminal extracellular fragment of PAR4 bound to murine thrombin in complex with PAR3 (27). While this study eloquently demonstrates how the N-terminal region of PAR4 is spatially arranged in order to enable interaction between thrombin’s exosite I and PAR3, it cannot provide guidance regarding potential interactions between exosite II and other extracellular regions of PAR4. Interestingly, introduction of the mutation W215A, located close to exosite II on thrombin, has been reported to reduce PAR4 cleavage 280-fold (28), supporting the notion that regions outside the immediate vicinity of the active site might be important for the interaction between thrombin and PAR4. The potential existence of an additional cofactor responsible for facilitating thrombin-induced cleavage of PAR4 via exosite II also warrants further investigation. Certainly, there are possible candidates which have been shown to interact with thrombin on the platelet surface, most prominently GpV (29).

In conclusion, our results indicate that previously unknown interactions involving exosite II on thrombin are essential for thrombin-mediated PAR4 activation. Apart from shedding new light on the thrombomodulatory effects of naturally occurring regulators of haemostasis such as serpins, this finding may also provide new targets for pharmaceutical intervention aimed at the inhibition of thrombin-mediated platelet activation.

Conflicts of interest
None declared.

References