Interaction of the 268–282 region of glycoprotein Ibα with the heparin-binding site of thrombin inhibits the enzyme activation of factor VIII

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Activation of factor VIII (FVIII) by thrombin plays a fundamental role in the amplification of the coagulation cascade and takes place through specific proteolytic cleavages at Arg^{372}, Arg^{740} and Arg^{1689}. Full FVIII activation requires cleavage at Arg^{372}, a process involving the α-thrombin exosite-II; referred to as heparin-binding site (HBS). The present study was aimed at investigating the effect of glycoprotein Ibα (GpIbα; 1–282 fragment) binding to thrombin HBS on FVIII activation. Similar experiments were also performed using a synthetic peptide modelled on the 268–282 sequence of GpIbα, and sulphated successfully at all tyrosine residues present along its sequence, at positions 276, 278 and 279. Both GpIbα 1–282 and the sulphated GpIb 268–282 peptides induced a progressive decrease (up to 70 %) in activated FVIII generation, assessed by coagulation and FXa-generation assays. Furthermore, SDS/PAGE and Western-blot experiments showed that the specific appearance of the 44 kDa A2 domain on cleavage of the FVIII Arg^{372}–Ser^{373} peptide bond was delayed significantly in the presence of either GpIbα 1–282 or GpIb 268–282 peptide. Moreover, the effect of the latter on thrombin-mediated hydrolysis of a peptide having the sequence 341–376 of FVIII was investigated using reverse-phase HPLC. The k_{cat}/K_m values of the FVIII 341–376 peptide hydrolysis by thrombin decreased linearly as a function of the GpIbα 268–282 peptide concentration, according to a competitive inhibition effect. Taken together, these experiments suggest that the sulphated 268–282 region of GpIbα binds to thrombin HBS, and is responsible for the inhibition of the Arg^{372}–Ser^{373} bond cleavage and activation of FVIII.

Key words: factor VIII, haemostasis, platelet glycoprotein Ib, thrombin.

INTRODUCTION

The mature factor VIII (FVIII) is composed of 2332 amino acids and contains three different domains: an A domain which is repeated three times, a central B domain and a twice-repeated C domain. FVIII activation by thrombin is of paramount importance in the amplification of the coagulation cascade and takes place through specific proteolytic cleavages in both the heavy and the light chains, namely at Arg^{372}, Arg^{740} and Arg^{1689} [1,2]. From the latter cleavage, von Willebrand factor (vWF), which under physiological conditions is complexed with FVIII [1,2], dissociates from it allowing the phospholipid-binding region in the C domains to be exposed, which is mandatory for the cofactor activity of activated FVIII (FVIIIa) [1]. These processes take place primarily on the surface of activated platelets, which accumulate at the site of a vascular damage *in vivo* [3]. The cleavage in the light chain of FVIII and the release of vWF on activation result in a considerable higher affinity of FVIIIa for activated platelets as compared with zymogen FVIII [4].

The surface of platelets also contains many copies of glycoprotein Ib (GpIb), which forms a non-covalent complex with GpV and GpIX [5]. GpIb belongs to the family of the 'leucine-rich repeat' proteins, which are often involved in cell signalling [6]. GpIb is composed of two chains, α and β, held together by a disulphide bridge [7], whereby the former is involved in binding to both vWF and α-thrombin [5,8,9]. Sulphation of three tyrosine residues (Tyr^{276}, Tyr^{278} and Tyr^{279}) at the anionic C-terminal end of fragment 1–282 is a key element for the binding of GpIbα to both vWF and α-thrombin. In this respect, the truncated 1–275 fragment (lacking the sulphated tyrosine residues) binds vWF with a 10-fold reduced affinity [10], whereas desulphation of tyrosine residues inhibited binding to thrombin [11]. Very recently, crystal structures of GpIbα N-terminal domain in the free form [12] and in complex with vWF A1 domain [13] have been reported. The overall fold of GpIbα is retained on complex formation. In particular, the concave face of GpIbα interacts with the globular A1 domain of vWF, whereas the C-terminal anionic region (encompassing residues 268–282) is disordered, as in the free, unbound GpIbα [13]. GpIbα–vWF interaction mediates platelet adhesion to the subendothelium of injured vessels [13–15], whereas GpIbα–thrombin interaction (a) positively affects the thrombin-induced platelet activation mediated by protease-activated receptor 1 cleavage by thrombin [16]; and (b) allosterically reduces the efficiency of fibrinogen cleavage by thrombin [17]. Recent studies [17–19] demonstrated that GpIbα binds via its C-terminal anionic segment 272–282 to the α-thrombin exosite-II referred to as heparin-binding site (HBS), located in the C-terminal region of the enzyme and involved in the interaction with glycosaminoglycans [20–22] and prothrombin F1 + 2 fragment [23]. In addition, HBS (together with exosite-I) appears to be involved in the interaction of thrombin with FV and FVIII [24–26].

Co-localization of thrombin–FVIII interaction and thrombin–GpIb interaction on the surface of activated platelets prompted us to investigate the functional consequences of GpIbα binding on the thrombin-mediated proteolytic activation of recombinant human FVIII. The possible relevance of these findings in the design of novel anticoagulant molecules is also discussed.

Abbreviations used: a.m.u., atomic mass units; Fmoc, fluoren-9-ylmethoxycarbonyl; FRS, fibrinogen-recognition site; FVIII, factor VIII; FVIIIa, activated FVIII; GpIb, glycoprotein Ib; HBS, heparin-binding site; mAb, monoclonal antibody; PEG, poly(ethylene glycol); RP, reverse phase; TFA, trifluoroacetic acid; vWF, von Willebrand factor.

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EXPERIMENTAL

Materials

Human full-length recombinant FVIII was purchased from BAXTER S.p.A. (Milano, Italy). Human α-thrombin, purified from purified human prothrombin, was purchased from Haematologic Technologies (Essex Junction, VT, U.S.A.). Briefly, prothrombin activation was obtained using *Ecarin carinatus* venom (10 μg/ml) for 60 min at 37 °C. α-Thrombin was subsequently purified by a sulphopropyl cation-exchange HPLC column (MA7S; Bio-Rad Laboratories, Hercules, CA, U.S.A.) as described previously [27]. Concentration of the purified material was measured spectrophotometrically at 280 nm, using a molar absorption coefficient equal to 65 879 $M^{-1} \cdot cm^{-1}$. Active-site titration, performed with *p*-nitrophenyl-guanidinobenzoate as described previously [27], showed that the preparation was 95 ± 10 % active. The FVIII peptide with the 341–376 sequence, NH_2-E-E-A-E-D-Y-D-D-D-L-T-D-S-E-M-D-V-V-R-F-D-D-D-N-S- P-S-F-I-Q-I-R-S-V-A-K-CO_2H, modified by a phosphate group at Tyr^{346}, was synthesized by PRIMM s.r.l. (Milano, Italy). The peptide was > 95 % pure, as revealed by reverse-phase (RP) HPLC. High-resolution molecular-mass determination yielded a value of 4246.5 a.m.u. (atomic mass units), in agreement with the theoretical value (4247.3 a.m.u.). The peptide was stored in dimethylformamide at a concentration of 10 mg/ml at − 80 °C until use, when it was diluted 100–200-fold in the appropriate aqueous buffer solutions for kinetic experiments. The soluble form of GpIbα 1–282 was purified from outdated platelet concentrates, as described previously [18]. Fluoren-9-ylmethoxycarbonyl (Fmoc)-Tyr(SO_3H)-OH sodium salt was purchased from Bachem (Torrance, CA, U.S.A.). Purified human FX, activated FX (FXa) and activated FIX (FIXa) were purchased from Calbiochem (San Diego, CA, U.S.A.). The anti-FVIII monoclonal antibodies (mAbs), OBT0037A and FVIIIC, from Accurate Chemical and Scientific (San Diego, CA, U.S.A.) were purchased from Unimed Scientifica (Rome, Italy). The OBT0037A mAb is specific for the heavy-chain A2 domain, allowing detection of the product of cleavage at Arg^{372}, whereas the FVIIIC mAb is specific for the 80 kDa light chain of FVIII [26]. The single-stranded DNA aptamer, referred to as HD22, having the sequence AGTCCGTGGTAGGGCAGGTT-GGGGTGACT-3′ and binding specifically to thrombin HBS [18], was a gift from Dr Jeffrey I. Weitz (Hamilton Civic Hospitals Research Centre and McMaster University, Ontario, Canada). Reagents and solvents for peptide synthesis were from Fluka (Rolconkoma, NY, U.S.A.).

Synthesis and characterization of the fully sulphated GpIbα 268–282 peptide

Sulphated GpIbα 268–282 peptide [NH_2-G-D-E-G-D-T-D-L-Y(SO_3H)-D-Y(SO_3H)-Y(SO_3H)-P-E-E-CO_2H] was synthesized by the solid-phase Fmoc method [28] on a *p*-alkoxybenzyl ester polystyrene resin [29] [47 mg, 1 % (w/w) divinylbenzene cross-linked] derivatized with Fmoc-Glu(*t*-But) (1.07 mmol/g), using a model 431 automated peptide synthesizer purchased from Applied Biosystems (Norwalk, CT, U.S.A.). Coupling reaction was performed with the 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole activation procedure [30], using a 4-fold molar excess of protected amino acids. *t*-Butyl-protecting group was used for aspartate, glutamate and threonine amino acid side chains. After the assembly of the peptide chain 268–282 was completed, the side-chain-protected peptidyl resin was treated for 15 min at 4 °C with a mixture (2 ml) of trifluoroacetic acid (TFA)/water (95:5, v/v), and the crude peptide was recovered by the addition of ice-cold diethyl ether precipitation. The peptide mixture was fractionated using an analytical Vydac (The Separations Group, Hesperia, CA, U.S.A.) C-18 column (4.6 mm × 250 mm; 5 μm particle size; 300 Å porosity, 1 Å = 10^{-10} m) eluted with a linear acetonitrile–0.05 % TFA gradient from 5 to 30 % in 30 min at a flow rate of 0.8 ml/min and recording the absorbance of the effluent at 226 nm. The peptide material eluted in correspondence with the chromatographic peaks was collected in 1.5 ml polypropylene tubes, freeze-dried in a SpeedVac concentrator (Savant, Holbrook, NY, U.S.A.). The chemical identity of the purified material was established by high-resolution MS in negative-ion mode on a Mariner electrospray ionization-time-of-flight instrument from Perseptive Biosystems (Foster City, CA, U.S.A.), which gave mass values in agreement with the expected amino acid composition within 5–10 p.p.m. mass accuracy. For preparative purposes, aliquots (0.5–1 mg) of the crude peptide in 5 M guanidinium chloride were injected on to a semi-preparative Vydac C-18 column (1 cm × 25 cm; 10 μm particle size) eluted with a linear acetonitrile–0.05 % TFA gradient from 10 to 25 % in 20 min, at a flow rate of 1.5 ml/min and recording the absorbance of the effluent at 226 nm. The material eluted in correspondence with the major chromatographic peak (approx. 1 ml), corresponding to the fully sulphated GpIbα 268–282 peptide, was added to an equal volume of ice-cold 0.1 M ammonium acetate buffer (pH 5.0), frozen at − 80 °C, freeze-dried in a SpeedVaac concentrator.

The concentration of the fully sulphated GpIbα 268–282 peptide was determined by UV absorption at 262 nm on either a double-beam PerkinElmer λ-2 (Norwalk, CT, U.S.A.) or a Varian Cary 2200 spectrophotometer (Assoc. Inc., Sunnyvale, CA, U.S.A.), using a molar absorption coefficient of 960 $M^{-1} \cdot cm^{-1}$. This value was obtained using a molar absorption coefficient of 320 $M^{-1} \cdot cm^{-1}$ at 262 nm for Tyr(SO_3H) in 0.1 M NaOH [31].

Activation of FVIII by thrombin

Coagulation assay was performed to detect FVIII activation by thrombin as described previously with minor modifications [32,33]. Briefly, 100 nM recombinant FVIII (Baxter) diluted in Tris-buffered saline [50 mM Tris, 0.15 M NaCl, 0.1 % poly-(ethylene glycol) (PEG) and 2 % (w/v) BSA, pH 7.50] was incubated with 15 nM human α-thrombin at 37 °C. After specific time intervals, samples (10 μl) were taken from the mixture and the activation was stopped by 1 mM PMSF, followed by a 1000-fold dilution with Tris-buffered saline at 4 °C. Each sample was tested for the FVIII activity in a one-stage clotting assay, using an automatic coagulometer (MLA, Electra 1800C; Instrumentation Laboratory, Milano, Italy), synthetic phospholipids with $CaCl_2$ and FVIII-deficient plasma (Hemoliance, Instrumentation Laboratory). The presence of diluted thrombin and PMSF in the diluted samples did not affect the FVIII activity in the coagulation assay. Results were expressed as the percentage of coagulant FVIII activity compared with certified normal plasma (Reference Assay Plasma from Hemoliance, used at 1 unit/ml). For experiments on the effects of GpIbα (1–282) and GpIbα (268–282) peptides on FVIII activation by thrombin, these ligands were incubated at various concentrations with thrombin for 15 min at 37 °C before the addition of FVIII.

Assay for generation of FXa

The rate of conversion of purified FX into FXa was monitored to analyse the activation of FVIII by thrombin. In a two-stage

assay, 50 μl of 100 nM rFVIII was incubated with 0.2 nM human α-thrombin in 10 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$ and 0.1 % PEG 6000 (pH 7.50) at 37 °C (buffer A). The pH of the buffer solution was measured at 25 °C, using a measured $\Delta pH/\Delta T$ value of −0.013. At various times, the reaction was stopped by adding 10 μM D-phenyl-prolyl-arginyl-chloromethane. The reaction products were diluted 250-fold in 200 μl of buffer A, containing synthetic phospholipids (Synthasil; Hemoliance); again diluted 1.25-fold in buffer A and in 2 nM FIXa. Subsequently, 100 nM FX was added to the solution. The reaction was stopped after 60 s, by adding 25 μl of 0.25 M EDTA (pH 7.50). The amount of FXa generated was determined by adding 0.5 mM benzyloxycarbonyl-D-Arg-Gly-Arg-*p*-nitroanilide (Chromogenix; Instrumentation Laboratory) and measuring the rate of substrate hydrolysis at 405 nm, using a microplate spectrophotometric reader from Grifols (Triturus™, Barcelona, Spain). The rate of *p*-nitroaniline production was converted into FXa concentration, by using a reference curve constructed with purified FXa at known concentrations.

SDS/PAGE and Western-blot analysis of FVIII activation products

FVIII (50 nM) and 1 nM thrombin in buffer A were incubated from 1 to 90 min and were subsequently subjected to SDS-loading buffer and boiled for 5 min. They were subjected to SDS/PAGE [4–15 % (w/v) gel; Bio-Rad Laboratories] and then were stained with Silver stain plus kit (Bio-Rad Laboratories). Gels were Western-blotted and probed with the mAb OBT0037, which binds to a high-chain A2 epitope. In separate experiments, the mAb FVIIIC was used to detect the cleavage of the 80 kDa light chain. Detection was achieved using goat anti-mouse IgG–horseradish peroxidase. The secondary antibody signal was detected using the ECL® system (Amersham Biosciences, Cologno Monzese, Italy).

Hydrolysis of FVIII 341–376 peptide by thrombin

Hydrolysis of the FVIII 341–376 peptide by thrombin was followed by RP-HPLC. In one set of experiments, hydrolysis of FVIII 341–376 by thrombin was studied as a function of the substrate concentration (6–400 μM), in the absence and presence (40 μM) of GpIbα 268–282 peptide. These experiments allowed the calculation of separate and accurate values of k_{cat} and K_m parameters, using the Michaelis–Menten equation. In different experiments, performed under pseudo-first-order conditions, a solution containing 5 μM FVIII peptide, that is $\ll K_m$ values (see below under Results section), was incubated with 50 nM α-thrombin in 10 mM Hepes, 0.15 M NaCl, 0.1 % PEG 6000 (pH 7.5) at 25 °C. Under the same experimental conditions, the effect of the synthetic GpIbα 268–282 peptide and HD22 aptamer on the hydrolysis of the FVIII 341–376 peptide was studied by using the former in a 20–80 μM and the latter over a 100–500 nM concentration range respectively. At specific time intervals (from 1 to 90 min), the reaction was stopped with 0.3 M $HClO_4$ and the cleaved peptide was measured by RP-HPLC, using a 250 mm × 4.6 mm RP-304 column (Bio-Rad Laboratories). The chromatographic run was performed by applying the following conditions: from 5 to 65 % acetonitrile in 0.1 % (v/v) TFA in 20 min. The peaks were detected at 226 nm. Under the pseudo-first-order conditions of the study, the concentration of FVIII peptide P_t cleaved at time t, was fitted to the following equation:

$$P_t = P_\infty[1 - \exp(-k_{obs}t)] \quad (1)$$

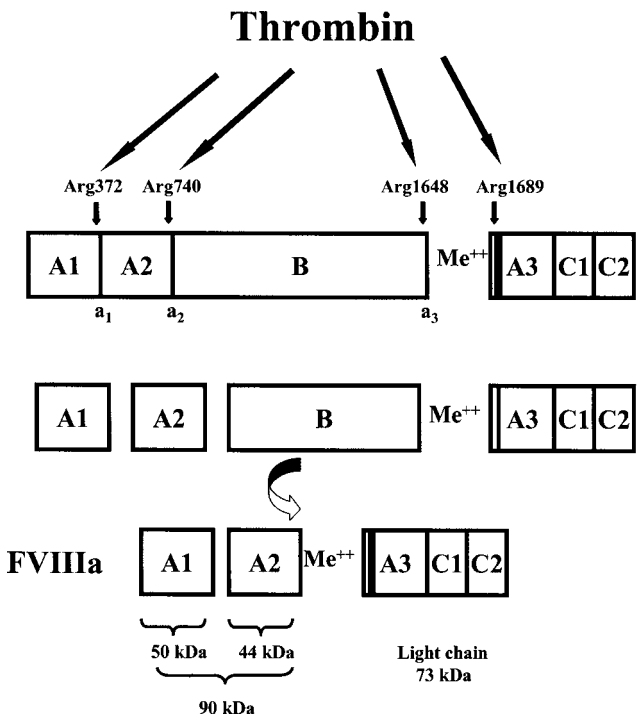

Figure 1 Schematic representation of various FVIII forms produced by thrombin activity

The short a1, a2 and a3 sequences are acidic regions, which play an important role in the specificity of FVIII interaction with thrombin and vWF. The metal ion (Me^{2+}), probably copper, which links the heavy and light chains, is also reported schematically.

where P_∞ is the FVIII 341–376 peptide concentration at $t = \infty$ and k_{obs} is the pseudo-first-order rate of its hydrolysis, equal to $e_0 k_{cat}/K_m$ (e_0 is the thrombin concentration).

RESULTS

Chemical synthesis of the fully sulphated GpIbα 268–282 peptide

In the present study, we have used the fully sulphated synthetic GpIbα 268–282 peptide, together with the natural GpIbα 1–282 fragment, to investigate the effects on thrombin–FVIII interaction. However, solid-phase chemical synthesis of Tyr(SO_3H)-containing peptides is still a great challenge [34,35], mainly due to the intrinsic lability of Tyr(SO_3H) in acidic conditions [80–95 % (v/v) aqueous TFA], to which the synthetic peptide is exposed during cleavage from the resin and side-chain deprotection, leading to the corresponding desulphated peptide species. Thus cleavage reaction of the GpIbα 268–282 synthetic peptide from the solid support was performed in 95 % aqueous TFA for 15 min only, at 4 °C. MS analysis of the material eluted from the RP-HPLC column reveals that the fully sulphated peptide (2016.12 a.m.u.) is eluted after shorter time periods, whereas the three disulphated (1936.20 a.m.u.) and monosulphated (1856.18 a.m.u.) species are eluted later (Figures 1 and 2). To minimize tyrosine desulphation, also occurring during the freeze-drying step, the material eluted with the major chromatographic peak, corresponding to the fully sulphated GpIbα 268–282 peptide, was added to an equal volume of ice-cold 0.1 M ammonium acetate buffer (pH 5.0), frozen at −80 °C and freeze-dried. This procedure allowed us to obtain highly homogeneous (>98 %) samples of fully sulphated 268–282 peptide. Before and after each binding experiment reported in the

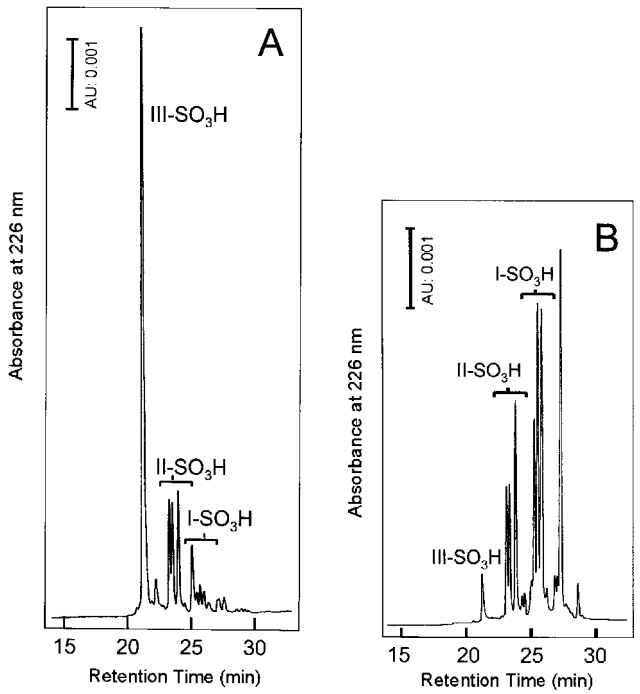

Figure 2 RP-HPLC analysis of the crude synthetic peptide GpIbα 268–282 after resin cleavage and side-chain deprotection

For comparison, the reaction was conducted in 95% aqueous TFA at 4 °C for 15 min (**A**), and at room temperature (22–24 °C) for 60 min. Note how the different temperatures affect the desulphation reaction of the fully sulphated peptide species (**B**). In both cases, the peptide mixture was precipitated with ice-cold diethyl ether. An aliquot (50–100 μg) of the dried precipitate was dissolved in 0.1 M ammonium acetate, quickly loaded on to a Vydac C-18 analytical column (4.6 mm × 150 mm, 5 μm), and eluted as described in the Experimental section. I-SO_3H, II-SO_3H and III-SO_3H correspond to GpIbα 268–282 with one, two and three sulphated tyrosine residues respectively. The unlabelled peak in (**B**) refers to the desulphated species. The fully sulphated peptide derivative elutes at shorter retention times, as expected from its more pronounced hydrophilic character. AU, absorbance unit.

present study, the extent of tyrosine desulphation was determined by RP-HPLC analysis and MS, and always found to be $<8\%$.

Thrombin-mediated activation of full-length FVIII: effect of GpIbα on coagulation assays

Purified GpIbα, over a 0.25–5 μM concentration range, inhibited the activation by thrombin of full-length FVIII progressively in plasma, detected by one-stage coagulation assay (Figure 3A). Inactivation of FVIIIa, due to subunit dissociation, is responsible for the bell-like shape of the curve, in agreement with previous studies [32,33]. In the absence of GpIbα 1–282, the initial rate of FVIIIa production is approx. 10 times greater than that in the presence of 5 μM GpIb. Likewise, the peak height of FVIII activation was approx. 7 times lower in the presence of 5 μM GpIbα, compared with control. It may be noted that a high concentration (0.5 μM) of the HD22 aptamer resulted in severe inhibition of FVIII activation as well (Figure 3A). An inhibition pattern, similar to that of GpIbα, was also obtained by using the GpIbα 268–282 peptide, used over a 10–80 μM concentration range (Figure 3B).

Notably, in a global coagulation assay sensitive to the activation of FVIII, such as the activated partial thromboplastin time, addition to normal control plasma of either increasing GpIbα 1–282 concentrations from 0.5 to 5 μM or GpIb 268–282 peptide from 10 to 80 μM caused in all cases a progressive increase in the

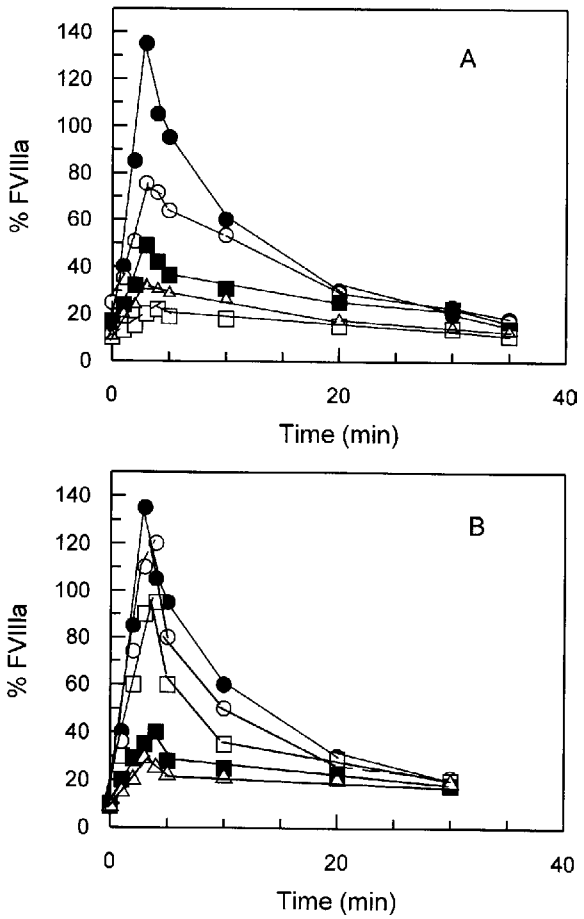

Figure 3 Time course of FVIII activation by thrombin

(**A**) Activation assessed by coagulation assays in the presence of sulphated GpIbα 1–282 fragment. ●, Control; ○, 0.25 μM GpIbα; ■, 2 μM GpIbα; and □, 5 μM GpIbα. The data set pertaining to the effect of HD22 aptamer (△), used at 0.5 μM, is also reported for comparison. (**B**) Activation in the presence of GpIbα 268–282 peptide used at 0 (●), 10 (○), 20 (□), 40 (■) and 80 μM (△) concentrations.

clotting time from 30 ± 3 to 80 ± 8 s and from 30 ± 3 to 70 ± 9 s respectively.

FXa generation, SDS/PAGE and Western-blot analysis of FVIII activation products

The results obtained with recombinant FVIII and purified FIX/phospholipids/Ca^{2+} confirmed the findings obtained in the coagulation assays. In Figure 4, the time course of FXa generation by the thrombin–FVIIIa is reported. Also this kind of experiment showed that increasing concentrations of GpIbα up to 5 μM induced a progressive and considerable decrease (up to 70%) in FVIIIa generation, responsible for the reduced activation of FX by the FIXa–FVIIIa complex. Similar results were also obtained with both HD22 (results not shown) and the GpIb 268–282 peptide. At a concentration of 80 μM, GpIb 268–282 peptide caused approx. 4-fold reduction of both the initial rate of FX activation and the peak of FX generation (Figure 4).

These findings prompted us to investigate, using SDS/PAGE and Western-blot experiments, the effect of GpIbα 1–282 on the rate of production of FVIII fragments by the activity of thrombin. The OBT0037 mAb, which is specific for the heavy-chain A2 domain of FVIII allowed us to monitor the cleavage of the

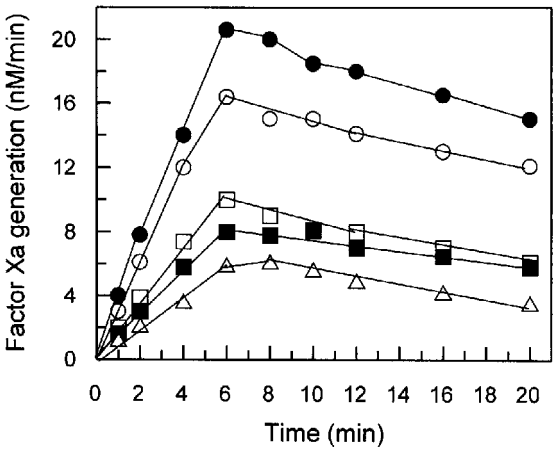

Figure 4 Kinetics of FVIII activation by thrombin assessed by FXa-generation assay, using purified enzymes in the presence of either GpIbα 1–282 domain or sulphated GpIbα 268–282 peptide

●, Control; ○, 0.25 μM GpIbα; □, 2 μM GpIbα; and ■, 5 μM GpIbα. The data set pertaining to the effect of 80 μM GpIbα 268–282 peptide (△) is also reported for comparison.

heavy-chain heterodimers by the specific appearance of the 44 kDa A2 domain on cleavage of the Arg^{372}–Ser^{373} peptide bond [26]. In the presence of 5 μM GpIbα 1–282, 80 μM fully sulphated GpIb 268–282 and 400 nM HD22, the appearance of the 44 kDa A2 domain was delayed significantly, as it began to appear at approx. 2 min in the absence, and at approx. 8–16 min in the presence of the various effectors (Figures 5A–5D). This result is in agreement with the lower rate of FVIII activation, observed in the coagulation as well as in the FXa-generation assay, as described above. In contrast, HD22 (400 nM), GpIb 1–282 (5 μM) and the sulphated GpIb 268–282 peptide (80 μM) did not affect the cleavage of 80 kDa FVIII by thrombin, as shown in Figures 5(E) and 5(F). Altogether, these findings suggested strongly that GpIbα 1–282 binding to the HBS of thrombin competitively inhibited only thrombin-mediated cleavage of the FVIII Arg^{372}–Ser^{373} peptide bond, without altering the light-chain cleavage at Arg^{1689}.

Effect of GpIbα and HD22 aptamer on thrombin-mediated hydrolysis of FVIII 341–376 peptide

The effect of this GpIbα peptide on thrombin hydrolysis of a peptide with the sequence 341–376 of FVIII was investigated using RP-HPLC. The FVIII 341–376 peptide contains the Arg^{372}-Ser^{373} peptide bond, whose cleavage has been indicated to involve thrombin HBS very recently [26]. RP-HPLC allowed us to discriminate the cleaved fragment 341–372 from the native FVIII 341–376 peptide, the latter eluting 2 min later than the cleaved species (results not shown). Thrombin cleaved the FVIII peptide with an apparent k_{cat}/K_m value of $(3 \pm 0.3) \times 10^4$ $M^{-1} \cdot s^{-1}$, calculated under pseudo-first-order conditions, as shown in Figure 6. This value decreased linearly as a function of both the GpIbα 268–282 peptide and HD22 concentrations, both of which bind to thrombin HBS. In fact, when the K_m/k_{cat} values were analysed as a function of these ligands' concentration, a linear dependence was obtained (Figures 7 and 8). This finding is compatible with a competitive inhibition, whereby binding of GpIbα species to thrombin impedes FVIII binding and vice versa. Steady-state experiments were in agreement with these findings. In fact, in the absence of GpIbα 268–282, the k_{cat} and the K_m values were equal to 3.24 ± 0.3 s^{-1} and 112 ± 22 μM respectively (Figure 9). Thus the measured k_{cat}/K_m ratio was equal to 2.89×10^4 $M^{-1} \cdot s^{-1}$, in good agreement with the approximate value obtained under pseudo-first-order conditions. Moreover, in the presence of 40 μM GpIbα 268–282 peptide, the k_{cat} and the K_m values were equal to 3.06 ± 1 s^{-1} and 295 ± 130 μM respectively (Figure 9). These results are compatible with a competitive effect exerted by the GpIbα 268–282 peptide, since only the K_m value was enhanced by the GpIb peptide. Thus the observed K_m/k_{cat} values, calculated under pseudo-first-order conditions, were fitted to the following equation:

$$(K_m/k_{cat})_{obs} = (K_m/k_{cat})^{\circ}[1 + (P/K_i)] \quad (2)$$

where $(K_m/k_{cat})^{\circ}$ is obtained in the absence of the GpIb peptide, K_i is the equilibrium dissociation constant of the GpIbα 268–282 peptide binding to thrombin and P is the GpIb peptide concentration.

The K_i value for GpIb peptide was found to be equal to 8.8 μM, whereas that of HD22 was equal to 25 nM, in good agreement with previous findings [18]. It is noteworthy that the non-sulphated GpIbα 268–282 peptide has a K_i value of 54.1 ± 14 μM, i.e. an affinity for thrombin approx. 6-fold lower when compared with the fully sulphated molecule, as shown in Figure 7. In contrast, a peptide with the same composition of the desulphated GpIbα 268–282, but with the scrambled sequence NH_2-E-D-G-T-D-G-Y-L-D-Y-P-E-Y-E-D-CO_2H did not significantly influence the FVIII 341–376 peptide hydrolysis.

These experiments showed indeed that both the 268–282 region of GpIbα and HD22 DNA aptamer, which were demonstrated previously to be involved in binding to thrombin HBS [18], are also responsible for the inhibition of cleavage of the Arg^{372}–Ser^{373} scissile bond and activation of FVIII. Furthermore, these results showed that the complete sulphation of the three tyrosine residues contained in the 268–282 region of GpIbα enhances the affinity for thrombin by approx. 4.18 kJ/mol (1 kcal/mol).

DISCUSSION

Proteolytic activation of FVIII by thrombin plays a crucial role in the blood coagulation process. In the absence of vWF, the cleavage at Arg^{372} is the necessary and sufficient condition for a complete FVIIIa cofactor activity, whereas in the presence of vWF, hydrolysis of the peptide bond at Arg^{1689} is also needed for FVIII activation [1]. The crucial importance of cleavages at positions 372 and 1689 for the activation of FVIII is also illustrated by the finding of severe haemophilia A patients with missense mutations at either of these residues [36,37].

Extensive alanine-scanning mutagenesis studies have shown recently that both the fibrinogen-recognition site (FRS) and, to a minor extent, the HBS are involved in FVIII recognition and activation [24,26]. In particular, FRS is relevant for cleavage at Arg^{1689}, whereas Arg^{101} in HBS is important for cleavage of the FVIII Arg^{372}–Ser^{373} peptide bond [26]. In the same study, it has also been shown that alanine mutations at the Na^+-binding site and at the level of Trp^{60} → Asp loop (defining the S2 specificity site of thrombin) dramatically reduce FVIII activation by thrombin [26]. Although these experiments showed that both FRS and HBS are necessary for FVIII activation, the authors were not able to indicate unequivocally whether these exosites are involved directly in this inhibition and/or exert their effects by inter-domain allosteric phenomena [26]. With this in mind, it has been reported that a negative allosteric linkage exists between the FRS and HBS on α-thrombin [38], although very recently no inter-exosite coupling has been documented [39]. The functional experiments reported in the present study with GpIbα 1–282 and the sulphated 268–282 fragments are in agreement

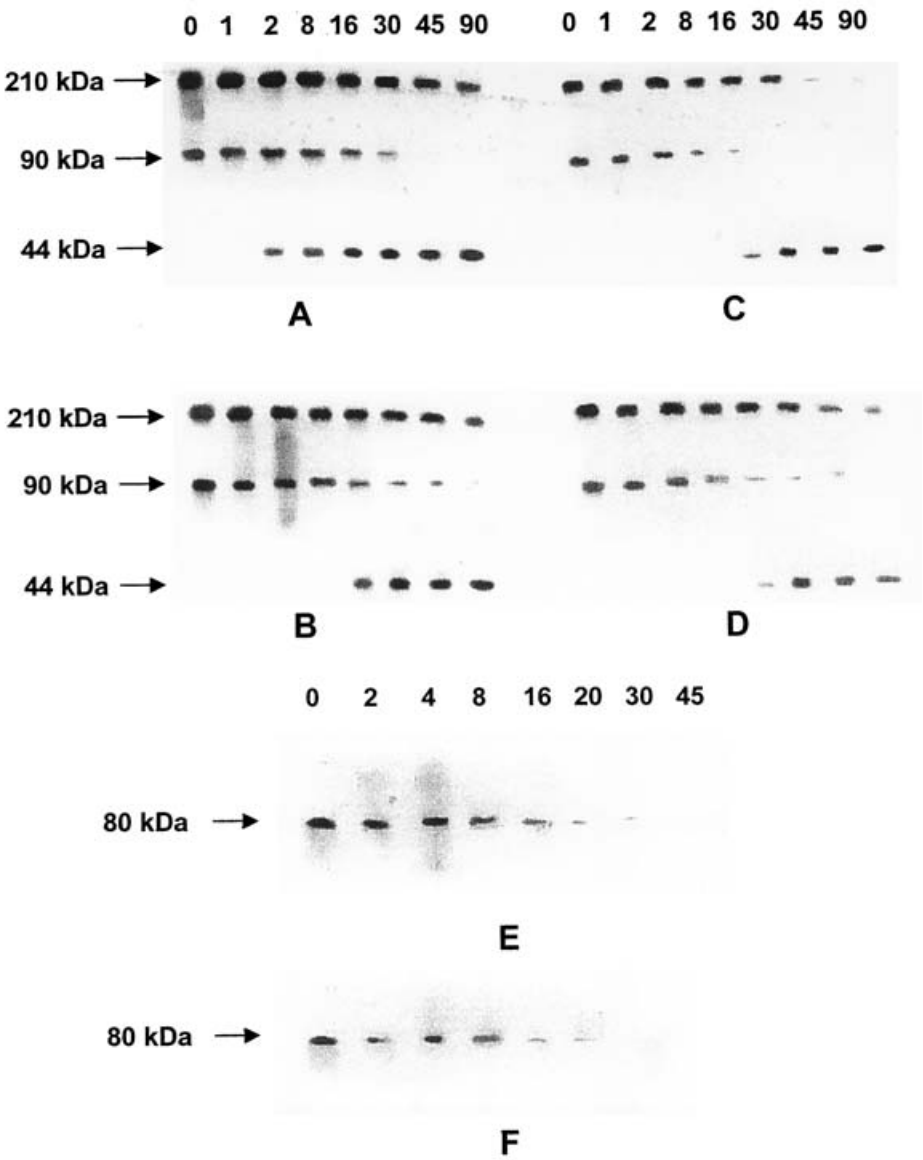

Figure 5 Cleavage products of FVIII activation by thrombin assessed by SDS/PAGE and Western blotting

Results were obtained in the absence (**A**) and in the presence of (**B**) 5 μM GpIbα 1–282 domain, (**C**) 80 μM fully sulphated GpIbα 268–282 peptide and (**D**) 400 nM HD22 aptamer. Cleaved domains of FVIII, obtained after the indicated time periods (expressed in min at the top of each gel lane), were subjected to SDS/PAGE and Western blotted by using the OBT0037 anti-FVIII mAb, as described in the Experimental section. Also shown are the cleavage products of FVIII light-chain hydrolysis at Arg^{1689} by thrombin in the absence (**E**) and in the presence (**F**) of 80 μM sulphated GpIbα 268–282 peptide.

qualitatively with the site-directed mutagenesis studies [26]. The integrated approach used in the present study has shown that cleavage at Arg^{372} involves HBS of thrombin directly. In fact, the DNA aptamer HD22, which is known to bind to HBS without causing allosteric transitions in the thrombin molecule [18,40], inhibited FVIII 341–376 peptide hydrolysis competitively, as shown in Figure 8. A similar result was also obtained with the fully sulphated GpIbα 268–282 peptide. Altogether, these findings indicate that two different HBS ligands can inhibit similarly the thrombin-dependent FVIII activation. It is noteworthy that previous studies proved that non-fractionated heparin could also inhibit thrombin-mediated cleavage at Arg^{372} [24], probably by the same competitive mechanism.

In contrast, the FVIII region located N-terminally with respect to the cleavage site at Arg^{372} could be predicted to interact with HBS, as it bears a stretch of 14 anionic residues, such as $Glu^{341-342}$, Glu^{344}, Asp^{345}, sulphated-Tyr^{346}, $Asp^{347-349}$, Asp^{352}, Glu^{354}, Asp^{356}, $Asp^{361-363}$, within a region of 36 amino acids. This chemical feature renders this segment suitable in particular to make ionic interactions with the most basic domain of thrombin, i.e. HBS [20]. GpIbα 1–282 interacts with a vast area of HBS encompassing many basic residues, such as Arg^{93}, Arg^{97}, Arg^{101}, Arg^{233}, Lys^{236} and Lys^{240} [17,19]. HBS is formed by a patch of 13 positively charged amino acids, covering an approximate area of 200 $Å^2$ on thrombin surface and extending just beyond the S2–S3 recognition sites. In particular, arginine residues 173, 175 and 97 of HBS comprised two loops surrounding the catalytic site, within 10–15 Å distance [20].

The present findings show that the same area or part of it is also involved in the interaction with the FVIII domain centred on

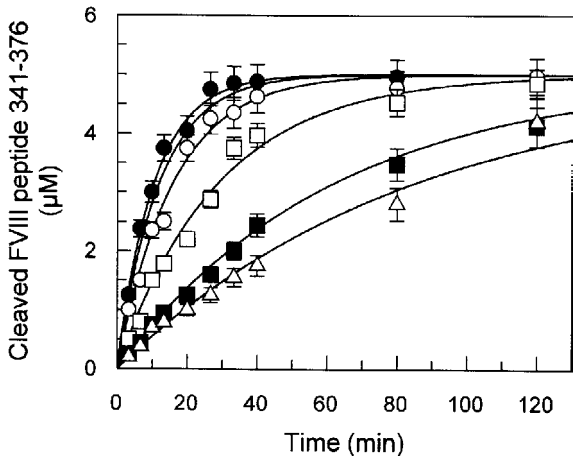

Figure 6 Kinetics of thrombin-mediated hydrolysis of FVIII 341–376 peptide in the presence of different concentrations of sulphated GpIbα 268–282 peptide

Kinetics of hydrolysis of 5 μM FVIII 341–376 peptide by 50 nM thrombin as a function of different concentrations of sulphated GpIbα 268–282 peptide: ●, no peptide; ○, 20 μM; □, 40 μM; and ■, 80 μM GpIb peptide. The data set pertaining to the effect of HD22 aptamer (△), used at 0.5 μM, is also reported for comparison. The continuous lines were drawn according to the best-fit k_{obs} values as follows: $1.58 \pm 0.08 \times 10^{-3}$ s^{-1}, $1.04 \pm 0.06 \times 10^{-3}$ s^{-1}, $0.58 \pm 0.07 \times 10^{-3}$ s^{-1} and $0.27 \pm 0.03 \times 10^{-3}$ s^{-1} at 0, 20, 40 and 80 μM GpIbα 268–282 peptide concentrations respectively. The best-fit k_{obs} value calculated at 0.5 μM HD22 was equal to $0.2 \pm 0.03 \times 10^{-3}$ s^{-1}.

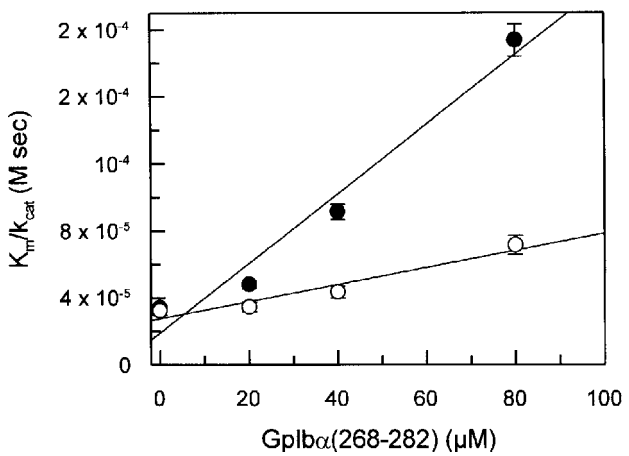

Figure 7 Competitive inhibition plot pertaining to the effects of fully sulphated (●) and non-sulphated (○) GpIbα 268–282 peptides on thrombin-induced FVIII 341–376 peptide hydrolysis

The inverse of the observed k_{cat}/K_m values was analysed as a function of the GpIb peptide concentration according to eqn (2). The derived values of K_i were equal to 8.79 ± 0.9 and 54.1 ± 14 μM for the sulphated and the non-sulphated peptides respectively. The vertical bars represent the S.E.M. from two different experiments.

Arg[372], which thus impedes competitively the thrombin interaction with GpIbα. The present findings are thus in agreement with the hypothesis that the FVIII 341–376 peptide, as well as the corresponding segment of the FVIII molecule, would bridge-bind to the catalytic site by its C-terminal segment, whereas the acidic N-terminal domain would interact with thrombin HBS. This binding mode would resemble partially the one followed by haemadin, a protein inhibitor (57 amino acids) of thrombin isolated from the leech *Haemadipsa sylvestris*, which

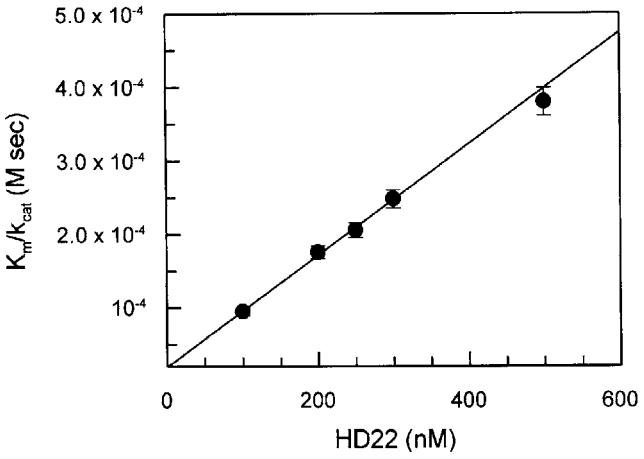

Figure 8 Competitive inhibition plot pertaining to the effect of HD22 aptamer on thrombin-induced FVIII activation

The inverse of the observed k_{cat}/K_m values was analysed as a function of the GpIb peptide concentration according to eqn (2). The derived value of K_i was equal to 25 ± 3 nM. The vertical bars represent the S.E.M. from two different experiments.

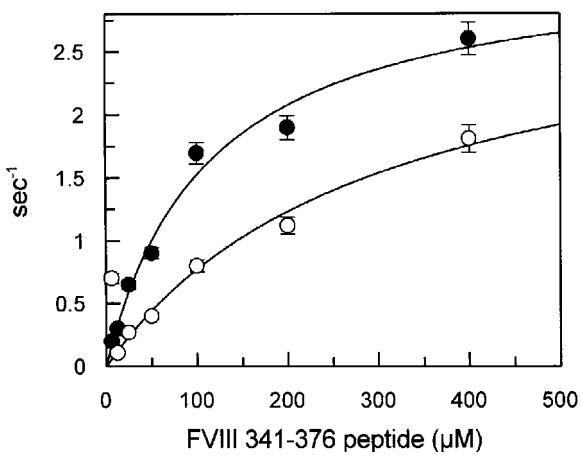

Figure 9 Steady-state hydrolysis of FVIII 341–376 peptide by thrombin in the absence (●) and presence (○) of 40 μM fully sulphated GpIbα 268–282 peptide

Experimental data were fitted to the Michaelis–Menten equation and the continuous lines were drawn using the best-fit k_{cat} and K_m values equal to 3.24 ± 0.3 s^{-1} and 112 ± 22 μM respectively in the control, and 3.06 ± 1 s^{-1} and 295 ± 130 μM respectively in the presence of 40 μM GpIb peptide. The vertical bars represent the S.E.M. from two different experimental points.

has a three-dimensional structure very similar to that of hirudin [41]. However, it differentiates from hirudin, since its negatively charged C-terminal tail does not interact with FRS, but it binds to HBS [41]. Although this hypothesis needs to be confirmed structurally by X-ray or NMR studies, it is in agreement with the functional results obtained in the present study.

Results from the present study may have therapeutic implications. It is a well-known fact that GpIb participates *in vivo* through interaction with vWF in platelet spreading and aggregation, especially at high shear rates in arterial circulation [42]. Moreover, once activated, platelets provide a procoagulant surface on which thrombin is generated, stabilizing the growing thrombus [43]. Activation of FVIII is a fundamental reaction to amplify the coagulation cascade. In those situations in which

deep vascular damage occurs, there is exposure of tissue factor resulting in thrombin generation, which in turn facilitates platelet interaction. Therapy with anti-thrombin drugs has been shown to be effective in preventing platelet deposition in experimental thrombosis models [44,45]. On the other hand, the controversy about the efficacy and relative roles of anti-platelet and anti-thrombin strategies in arterial anti-thrombotic therapy is still ongoing [46,47]. Inhibition of the vWF–GPIb interaction is being perceived as a promising therapeutic strategy aimed at preventing platelet interactions with damaged vessels [48,49]. Recent studies also showed that exposure of platelets to surface-bound thrombin promotes platelet adhesion and aggregation and that this effect is not blocked by anti-platelet strategies directed at GPIIb-IIIa, whereas it is inhibited strongly by antibodies blocking thrombin–GpIb interaction [50].

In view of this, analogues of fragment 268–282 of GpIbα, also containing non-hydrolysable Tyr(SO_3H) derivatives (e.g. 4-sulphonomethyl-L-phenylalanine), would allow different steps of blood coagulation (i.e. GpIb interaction with and FVIII activation by thrombin) to be controlled at the same time, opening the way to a novel strategy in anti-thrombotic therapy, especially for arterial thrombosis. Further studies are needed to verify whether GpIbα-like compounds could inhibit both the thrombin–GpIb interaction and the thrombin-induced FVIII activation *in vivo*.

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REFERENCES

1 Hill-Eubanks, D. C., Parker, C. G. and Lollar, P. (1989) Differential proteolytic activation of factor VIII–von Willebrand factor complex by thrombin. Proc. Natl. Acad. Sci. U.S.A. **86**, 6508–6512
2 Curtis, J. E., Helgerson, S. L., Parker, E. T. and Lollar, P. (1994) Isolation and characterization of thrombin-activated human factor VIII. J. Biol. Chem. **269**, 6246–6251
3 Monroe, D. M., Hoffman, M. and Roberts, H. R. (2002) Platelets and thrombin generation. Arterioscler. Thromb. Vasc. Biol. **22**, 1381–1389
4 Saenko, E. L., Scandella, D., Yakhyaev, A. V. and Greco, N. J. (1998) Activation of factor VIII by thrombin increases its affinity for binding to synthetic phospholipid membranes and activated platelets. J. Biol. Chem. **273**, 27918–27926
5 Ware, J. (1998) Molecular analysis of the platelet glycoprotein Ib-IX-V receptor. Thromb. Haemostasis **79**, 466–478
6 Buchanan, S. S. T. C. and Gay, N. J. (1996) Structural and functional diversity in the leucine-rich repeat family of proteins. Prog. Biophys. Mol. Biol. **65**, 1–44
7 Lopez, J. A. and Dong, J. F. (1997) Structure and function of the glycoprotein Ib-IX-V complex. Curr. Opin. Hematol. **4**, 323–329
8 Matsushita, T., Meyer, D. and Sadler, J. E. (2000) Localization of von Willebrand factor-binding sites for platelet glycoprotein Ib and botrocetin by charged-to-alanine scanning mutagenesis. J. Biol. Chem. **275**, 11044–11049
9 Soslau, G., Class, R., Morgan, D. A., Foster, C., Lord, S. T., Marchese, P. and Ruggeri, Z. M. (2001) Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. J. Biol. Chem. **276**, 21173–21183
10 Ward, C. M., Andrews, R. K., Smith, A. I. and Berndt, M. C. (1996) Identification of the sulfated tyrosine/anionic sequence Tyr^{276}-Glu^{282} of glycoprotein Ibα as a binding site for von Willebrand factor and α-thrombin. Biochemistry **35**, 4929–4938
11 Marchese, P., Murata, M., Mazzucato, M., Pradella, P., De Marco, L., Ware, J. and Ruggeri, Z. M. (1995) Identification of three tyrosine residues of glycoprotein Ibα with distinct roles in von Willebrand factor and α-thrombin binding. J. Biol. Chem. **270**, 9571–9578
12 Uff, S., Clemetson, J. M., Harrison, T., Clemetson, K. J. and Emsley, J. (2002) Crystal structure of the platelet glycoprotein Ibα N-terminal domain reveals an unmasking mechanism for receptor activation. J. Biol. Chem. **277**, 35657–35663
13 Huizinga, E. G., Tsuji, S., Romijn, R. A., Schiphorst, M. E., de Groot, P. G., Sixma, J. J. and Gros, P. (2002) Structures of glycoprotein Ibα and its complex with von Willebrand factor A1 domain. Science **297**, 1176–1179
14 Vasudevan, S., Roberts, J. R., McClintock, R. A., Dent, J. A., Celikel, R., Ware, J., Varughese, K. I. and Ruggeri, Z. M. (2000) Modeling and functional analysis of the interaction between von Willebrand factor A1 domain and glycoprotein Ibα. J. Biol. Chem. **275**, 12763–12768
15 Sadler, J. E. (2002) Biomedicine. Contact – how platelets touch von Willebrand factor. Science **297**, 1128–1129
16 De Candia, E., Hall, S. W., Rutella, S., Landolfi, R. and De Cristofaro, R. (2001) Binding of thrombin to the glycoprotein Ib accelerates the hydrolysis of PAR-1 on intact platelets. J. Biol. Chem. **276**, 4692–4698
17 Li, C. Q., Vindigni, A., Sadler, J. E. and Wardell, M. R. (2001) Platelet glycoprotein Ibα binds to thrombin anion-binding exosite II inducing allosteric changes in the activity of thrombin. J. Biol. Chem. **276**, 6161–6168
18 De Cristofaro, R., De Candia, E., Rutella, S. and Weitz, J. I. (2000) The Asp^{272}-Glu^{282} region of platelet Gplbα interacts with the heparin binding site of thrombin and protects the enzyme from the heparin-catalyzed inhibition by antithrombin III. J. Biol. Chem. **275**, 3887–3895
19 De Cristofaro, R., De Candia, E., Rutella, S., Landolfi, R. and Hall, S. W. (2001) Structural and functional mapping of the thrombin domain involved in binding to the platelet Gplb. Biochemistry **40**, 13268–13273
20 Bode, W., Turk, D. and Karshikov, A. (1992) The refined 1.9 Å crystal structure of D-Phe-Pro-Arg-chloromethylketone inhibited human α-thrombin. Structure analysis, overall structure, electrostatic properties, detailed active site geometry, structure–function relationships. Protein Sci. **1**, 426–471
21 Gan, Z. R., Li, Y., Chen, Z., Lewis, S. D. and Shafer, J. A. (1994) Identification of basic amino acid residues in thrombin essential for heparin-catalyzed inactivation by antithrombin III. J. Biol. Chem. **269**, 1301–1305
22 Sheehan, J. P. and Sadler, J. E. (1994) Molecular mapping of the heparin-binding exosite of thrombin. Proc. Natl. Acad. Sci. U.S.A. **91**, 5518–5522
23 Arni, R. K., Padmanabhan, K., Padmanabhan, K. P., Wu, T. P. and Tulinsky, A. (1993) Structures of the noncovalent complexes of human and bovine prothrombin factor 2 with human PPACK-thrombin. Biochemistry **32**, 4727–4737
24 Esmon, C. T. and Lollar, P. (1996) Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and VIII. J. Biol. Chem. **271**, 13882–13887
25 Myles, T., Yun, T. H., Hall, S. W. and Leung, L. L. (2001) An extensive interaction interface between thrombin and factor V is required for factor V activation. J. Biol. Chem. **276**, 25143–25149
26 Myles, T., Yun, T. H. and Leung, L. L. (2002) Structural requirements for the activation of human factor VIII by thrombin. Blood **100**, 2820–2826
27 De Cristofaro, R., Rocca, B., Bizzi, B. and Landolfi, R. (1993) The linkage between binding of the C-terminal domain of hirudin and amidase activity in human α-thrombin. Biochem. J. **289**, 475–480
28 Atherton, E. and Sheppard, R. C. (1987) Peptide synthesis. In The Peptides, vol. 9 (Udenfriend, S. and Meienhofer, J., eds.), pp. 1–39, Academic Press, New York
29 Wang, S. S. (1973) *p*-Alkoxybenzyl alcohol resin and *p*-alkoxybenzyloxycarbonylhydrazide resin for solid phase synthesis of protected peptide fragments. J. Am. Chem. Soc. **95**, 1328–1333
30 Knorr, R., Trzeciak, A., Bannwarth, W. and Gillessen, D. (1989) New coupling reagents in peptide chemistry. Tetrahedron Lett. **30**, 1927–1930
31 Moroder, L., Wilschowitz, L., Jaeger, E., Knof, S., Thamm, P. and Wünsch, E. (1979) Synthese von tyrosin-*O*-sulfat-haltigen peptiden. Hoppe Seyler's Z. Physiol. Chem. **360**, 787–790
32 Michnick, D. A., Pittman, D. D., Wise, R. J. and Kaufman, R. J. (1994) Identification of individual tyrosine sulfation sites within factor VIII required for optimal activity and efficient thrombin cleavage. J. Biol. Chem. **269**, 20095–20102
33 Fulcher, C. A., Roberts, J. R. and Zimmerman, T. S. (1983) Thrombin proteolysis of purified factor VIII procoagulant protein: correlation of activation with generation of a specific polypeptide. Blood **61**, 807–811
34 Yagami, T., Shiwa, S., Futaki, S. and Kitagawa, K. (1993) Evaluation of the final deprotection system for the solid-phase synthesis of Tyr(SO_3H)-containing peptides with Fmoc-strategy and its application to the synthesis of (CCK)-12. Chem. Pharm. Bull. **41**, 376–380
35 Kitagawa, K., Aida, C., Fujiwara, H., Yagami, T., Futaki, S., Kogire, M., Ida, J. and Inoue, K. (2001) Facile solid-phase synthesis of sulfated tyrosine-containing peptides: synthesis of human big gastrin-II and (CCK)-39. J. Org. Chem. **66**, 1–10
36 Gitschier, J., Kogan, S., Levinson, B. and Tuddenham, E. G. (1988) Mutations of factor VIII cleavage sites in hemophilia A. Blood **72**, 1022–1028
37 Arai, M., Inaba, H., Higuchi, M., Antonarakis, S. E., Kazazian, Jr, H. H., Fujimaki, M. and Hoyer, L. W. (1989) Direct characterization of factor VIII in plasma: detection of a mutation altering a thrombin cleavage site (arginine-372 → histidine). Proc. Natl. Acad. Sci. U.S.A. **86**, 4277–4281

38 Fredenburgh, J. C., Stafford, A. R. and Weitz, J. I. (1997) Evidence for allosteric linkage between exosites 1 and 2 of thrombin. J. Biol. Chem. **272**, 25493–25499
39 Verhamme, I. M., Olson, S. T., Tollefsen, D. M. and Bock, P. E. (2002) Binding of exosite ligands to human thrombin. Re-evaluation of allosteric linkage between thrombin exosites I and II. J. Biol. Chem. **277**, 6788–6798
40 Tasset, D. M., Kubik, M. F. and Steiner, W. (1997) Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. J. Mol. Biol. **272**, 688–698
41 Richardson, J. L., Kroger, B., Hoeffken, W., Sadler, J. E., Pereira, P., Huber, R., Bode, W. and Fuentes-Prior, P. (2000) Crystal structure of the human α-thrombin–haemadin complex: an exosite II-binding inhibitor. EMBO J. **19**, 5650–5660
42 Savage, B., Almus-Jacobs, F. and Ruggeri, Z. M. (1998) Specific synergy of multiple substrate–receptor interactions in platelet thrombus formation under flow. Cell (Cambridge, Mass.) **94**, 657–666
43 Gast, A., Tschopp, T. B. and Baumgartner, H. R. (1994) Thrombin plays a key role in late platelet thrombus growth and/or stability. Effect of a specific thrombin inhibitor on thrombogenesis induced by aortic subendothelium exposed to flowing rabbit blood. Arterioscler. Thromb. **14**, 1466–1474
44 Ohyama, T., Hori, T., Moriike, M., Asano, T., Hayashi, H., Iwade, K. and Hosoda, S. (1998) Anti-thrombotic effects of CX-397, a recombinant hirudin analog, in a canine model of coronary artery thrombosis. Thromb. Haemost. **79**, 423–430
45 Heras, M., Chesebro, J. H., Webster, M. W., Mruk, J. S., Grill, D. E., Penny, W. J., Bowie, E. J., Badimon, L. and Fuster, V. (1990) Hirudin, heparin, and placebo during deep arterial injury in the pig. The *in vivo* role of thrombin in platelet-mediated thrombosis. Circulation **82**, 1476–1484
46 Patrono, C., Coller, B., Dalen, J. E., FitzGerald, G. A., Fuster, V., Gent, M., Hirsh, J. and Roth, G. (2001) Platelet-active drugs: the relationships among dose, effectiveness, and side effects. Chest **119** (1 Suppl.), 39S–63S
47 Weitz, J. I. and Hirsh, J. (2001) New anticoagulant drugs. Chest **119** (Suppl. 1), 95S–107S
48 McGhie, A. I., McNatt, J., Ezov, N., Cui, K., Mower, L. K., Hagay, Y., Buja, L. M., Garfinkel, L. I., Gorecki, M. and Willerson, J. T. (1994) Abolition of cyclic flow variations in stenosed, endothelium-injured coronary arteries in nonhuman primates with a peptide fragment (VCL) derived from human plasma von Willebrand factor–glycoprotein Ib binding domain. Circulation **90**, 2976–2981
49 Mruk, J. S., Webster, M. W. I., Heras, M., Reid, J. M., Grill, D. E. and Chesebro, J. H. (2000) Flavone-8-acetic acid (Flavonoid) profoundly reduces platelet-dependent thrombosis and vasoconstriction after deep arterial injury *in vivo*. Circulation **101**, 324–328
50 Diaz-Ricart, M., Estebanell, E., Lozano, M., Aznar-Salatti, J., White, J. G., Ordinas, A. and Escolar, G. (2000) Thrombin facilitates primary platelet adhesion on to vascular surfaces in the absence of plasma adhesive proteins: studies under flow conditions. Haematologica **85**, 280–288

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