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Thrombin interaction with a recombinant N-terminal extracellular domain of the thrombin receptor in an acellular system

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The cDNA of the human endothelial cell thrombin receptor has been cloned and a chimeric fusion protein consisting of glutathione-S-transferase (GST) and the portion 25–97 corresponding to the N-terminal first extracellular domain of the thrombin receptor (TRE) has been expressed in *Escherichia coli*. Introduction of a factor Xa cleavage site in the fusion protein allowed purification of TRE after removal from the GST carrier protein. Purified GST–TRE or TRE have been tested in solution for their ability to interact with thrombin. α-Thrombin cleaved the fusion protein at position Arg-41–Ser-42 of TRE in a time- and concentration-dependent manner and GST–TRE competed with the tripeptidic substrate S-2238 for hydrolysis by thrombin ($K_i = 0.5\ \mu$M). γ-Thrombin that lacks the anion-binding exosite was 100-fold less potent than α-thrombin at cleaving GST–TRE. TRE competed with polymerizing fibrin monomers for binding to thrombin ($K_i = 7.5\ \mu$M). The cleavage of GST–TRE by α-thrombin was inhibited by several α-thrombin exosite ligands such as the C-terminal peptide of hirudin, thrombomodulin and fibrin(ogen) fragment E. In contrast, platelet glycocalicin did not inhibit GST–TRE cleavage. In conclusion, the use of purified soluble GST–TRE allowed us to derive an affinity constant for thrombin interaction with the N-terminal domain of the receptor and to confirm the location of the cleavage site at Arg-41–Ser-42 of the receptor. The importance of the thrombin anion-binding exosite for thrombin receptor recognition is highlighted by the low reactivity of γ-thrombin for GST–TRE and by competition experiments, which in addition indicate that binding sites for fibrin(ogen), thrombomodulin and GST–TRE are overlapping. In contrast, binding of thrombin to GST–TRE and glycocalicin are not mutually exclusive, indicating that glycocalicin and TRE interact with discrete subsites within the large groove that constitutes the anion-binding exosite.

INTRODUCTION

Thrombin is a serine protease which plays an essential role in blood clotting [1] and also acts as a potent agonist for platelet activation, inducing a wide range of biochemical and morphological changes [2]. On the platelet surface, several thrombin-binding proteins and substrates have been identified, including glycoprotein Ib (GPIb) [3,4], glycoprotein V [5,6], and protease nexin I [7], but little was known until 1991 about the structure of the effective platelet thrombin receptor (TR). Molecular cloning of the TR from human megakaryocytic cell lines [8], hamster lung fibroblasts [9] and rat smooth-muscle cells [10] constituted an important step in understanding the mechanism of thrombin-induced platelet activation. The TR belongs to the seven-transmembrane-domain G-protein-coupled receptor family, and is characterized by an original mechanism of action. The receptor binds thrombin and is activated after a unique proteolytic cleavage which exposes a new N-terminal sequence supposed to act as a tethered ligand capable of self-activating the receptor [11,12]. The putative thrombin cleavage site (Arg-41–Ser-42) is, as in protein C, characterized by an acidic residue (Asp-39) in position P3 which is known to be unfavourable for the interaction with the thrombin catalytic site [13]. The cleavage site is followed by a sequence with some similarities with the C-terminal tail of hirudin and is proposed to bind to the thrombin anion-binding exosite [14].

In order to define more precisely the interaction of thrombin with its receptor, we have constructed a recombinant fusion protein composed of a carrier protein, glutathione-S-transferase (GST), coupled to the N-terminal extracellular domain of TR (TRE). We have studied in a purified system the interaction of thrombin with the soluble fusion protein and the effect of several proteins known to interact with thrombin such as: (i) the C-terminal segment 54–65 of hirudin which binds to the thrombin anion-binding exosite, (ii) thrombomodulin, an integral membrane glycoprotein found on the surface of endothelial cells that binds to the thrombin exosite and modifies thrombin specificity [15], (iii) fibrin(ogen) fragment E, a soluble fragment corresponding to the N-terminal part of fibrin(ogen), devoid of fibrinopeptides and containing the counterpart of the thrombin exosite [16], and (iv) glycocalicin, a soluble proteolytic fragment derived from GPIbα, which binds thrombin and competes with fibrin(ogen) and thrombomodulin for occupancy of the exosite [17].

MATERIALS AND METHODS

Protein purification

Human α-thrombin and γ-thrombin were purified as previously described [18]. The thrombin preparations used throughout this study were more than 97% α-thrombin, as judged by SDS/PAGE, and specific activity was 3000 ± 200 units/mg of protein.

Abbreviations used: GST, glutathione-S-transferase; TR, thrombin receptor; TRE, thrombin receptor first extracellular domain; GPIb, glycoprotein Ib; PPACK, D-phenylalanylprolylarginine chloromethyl ketone; S-2238, H-D-phenylalanylpipecolylarginine-*p*-nitroanilide; IC_{50}, concentration required to obtain 50% inhibition.

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The γ-thrombin preparation contained negligible amounts of α-thrombin, as judged by the inability of hirudin to inhibit platelet aggregation induced by γ-thrombin. α-Thrombin was ^{125}I-labelled using the solid-phase reactant Iodogen (Pierce, Rockford, IL, U.S.A.) and Na^{125}I (Amersham, Les Ulis, France) [19]. Glycocalicin was kindly provided by Dr. K. J. Clemetson and was purified as described previously [20]; no contaminants were apparent as judged by SDS/PAGE. Fibrin monomers were prepared as described previously [17]. Rabbit lung thrombomodulin [21], fibrin(ogen) fragment E [16] and factor Xa [22] were isolated according to published procedures.

Cloning of the endothelial cell TR cDNA

Total RNA, corresponding to three confluent 60-mm-diam. dishes of human saphenous vein endothelial cell monolayers, was prepared according to a described procedure [23]. A sample (100 ng) of total RNA was reverse transcribed with 20 units of murine Moloney virus reverse transcriptase (Gibco-BRL, Cergy-Pontoise, France) for 30 min at 37 °C using 3 pmol of non-coding primer 5′-CGGTCGACCTTTTTAACCTCCCAGCAG-TC-3′ corresponding to nucleotides 1530–1510 of the published Dami cell TR cDNA sequence [8]. The full-length cDNA was obtained after PCR amplification and assembly of a 5′-fragment corresponding to nucleotides 208–765 amplified using sense primer 5′-GCGAATTCGCGCAGAGCCCGGGACAATGG-3′ and antisense primer 5′-ATGCTGCAGTGACGACGAA-GCGACACAATT-3′, and a 3′-fragment corresponding to nucleotides 761–1530 amplified using sense primer 5′-TCAC-TGCATTTTACTGTAACATGTACG-3′ and antisense primer 5′-CGGTCGACCTTTTTAACCTCCCAGCAGTC-3′. Following PCR amplification the 5′- and 3′-fragments were digested with *Eco*RI and *Pst*I, and with *Pst*I and *Sal*I respectively and were ligated together in the *Eco*RI–*Sal*I sites of the pBluescript vector (Stratagene, San Diego, CA, U.S.A.). The resulting full-length 1322 nt cDNA insert was then verified by sequencing.

Production of TRE in *E. coli* and antibody generation

A *Sma*I–*Alu*I 220 nt fragment corresponding to nucleotides 297–517 of TR was isolated from the full-length receptor in pBluescript and subcloned in the *Sma*I site of pBluescript. After cleavage of the pBluescript construct with *Bam*HI and *Eco*RI, the fragment was finally ligated in the *Bam*HI–*Eco*RI sites of the PGEX-3X bacterial expression vector (Pharmacia, St. Quentin en Yvelines, France). This resulted in an in-frame insertion resulting in a fusion protein consisting of GST and the N-terminal extracellular portion of TR (TRE) corresponding to amino acids 25–98. The plasmid was transformed in the *E. coli* DH5α strain. The recombinant protein was produced from a 450 ml liquid culture after induction with 0.1 mM isopropylthiogalactoside (IPTG) for 2.5 h at 37 °C. After centrifugation at 3300 ***g*** for 20 min at 4 °C, the bacterial pellet was resuspended in 50 ml of ice-cold PBS buffer containing 0.1 mg/ml antipain, 0.2 mg/ml aprotinin, 1 mg/ml benzamidine, 0.1 mg/ml chymostatin (Boerhinger, Mannheim, Germany), 0.05 mg/ml leupeptin (Fluka, St. Quentin en Yvelines, France), and 5 μg/ml amidinophenylmethanesulphonyl fluoride (aPMSF) (Calbiochem, La Jolla, CA, U.S.A.). After mild sonication, 1 % (v/v) Triton X-100 was added and incubated for 30 min at 4 °C. The bacterial lysate was then centrifuged at 3300 ***g*** for 20 min at 4 °C and the supernatant was incubated with 5 ml of 50 % (v/v) glutathione–Sepharose beads (Pharmacia) for 5 min at room temperature. Beads were washed twice with PBS and the fusion protein was eluted by two washes with 10 mM reduced glutathione in 50 mM Tris/HCl, pH 8, and analysed by SDS/PAGE. From eight separate experiments starting with 60 to 500 ml of culture, the yield for GST–TRE was 8.6 ± 5.2 mg/l of culture (mean ± S.E.M.). For removal of the GST tail, the fusion protein coupled to the glutathione–Sepharose beads was cleaved in the presence of 1 μg/ml of purified factor Xa and 2 mM Ca^{2+} for 30 min at 37 °C. The eluted TRE polypeptide was then further purified by gel-filtration chromatography on a Superdex 75HR 10/30 column (Pharmacia) equilibrated with PBS followed by concentration over a 1000 Da cutoff Filtron membrane (Filtron France, Coignières, France). The final yield after factor Xa cleavage and gel-filtration chromatography was 2.1 ± 0.7 mg/l of culture (mean ± S.E.M., $n = 4$). The protein concentrations were estimated by measuring the A_{280} using absorption coefficients of 1.4275 $M^{-1} \cdot cm^{-1}$ for GST–TRE and 1.609 $M^{-1} \cdot cm^{-1}$ for TRE. These were calculated based on the known amino acid composition of the two polypeptides using the PC-GENE software (Intelligenetics, Mountain View, CA, U.S.A.). An identical procedure was followed to purify the TRE fragment after cleavage at the internal thrombin cleavage site at position 41/42 of the receptor after incubation of the beads with 60 units/ml of purified human α-thrombin. N-terminal sequencing was performed on 2 μg of purified thrombin-cleaved TRE using the Edman degradation procedure on an Applied Biosystems 477A peptide sequencer (Applied Biosystems SARL, Roissy, France). For antibody generation, 100 μg of GST–TRE fusion protein was injected into a rabbit in complete Freund's adjuvant, and seven subsequent boosters were given at days 15, 30, 50, 110, 140, 200 and 250 in incomplete Freund's adjuvant. At day 280 the animal was bled for serum isolation. Anti-GST–TRE IgGs were purified according to a published procedure [24] after precipitation of non-IgG proteins with caprylic acid followed by precipitation with ammonium sulphate. F(ab′)$_2$ fragments from 2 mg of IgGs were prepared after overnight digestion with 50 μg of pepsin in 0.14 M sodium acetate buffer, pH 4.5, and purified by gel filtration on a Superdex 200HR 10/30 column (Pharmacia) equilibrated with PBS. The anti-GST–TRE antibodies were tested by an e.l.i.s.a. against GST–TRE-, TRE- and GST-purified proteins, and were shown to cross-react with all three proteins with respective 1/25000, 1/3200 and 1/15000 titres.

Effect of GST–TRE on thrombin amidolytic activity

The hydrolysis of S-2238 (H-D-phenylalanylpipecolylarginine-*p*-nitroanilide, Chromogenix, Mölndal, Sweden) by 0.4 nmol/l thrombin was measured at 37 °C in 10 mmol/l Hepes, 10 mmol/l Tris, 100 mmol/l NaCl, pH 7.8, 0.1 % (w/v) poly(ethylene glycol) (PEG 8000) in the presence or absence of the recombinant GST–TRE. The rate of *p*-nitroanilide liberation was followed at 405 nm on a LABsystems iEMS microwell-plate reader (Helsinki, Finland).

Effect of GST–TRE on thrombin binding to fibrin

Thrombin interaction with fibrin was performed as previously described by measuring at equilibrium the association of ^{125}I-thrombin to repolymerizing purified fibrin monomers [25]. Briefly, reaggregation of fibrin monomers from acetic acid solutions was initiated by diluting an aliquot of solubilized fibrin into 50 mmol/l Tris, 50 mmol/l NaCl, pH 7.5, 1 % (w/v) BSA in the presence of 5 nmol/l iodinated thrombin. After 2 h at 37 °C samples were centrifuged and clot-bound thrombin quantified by γ-counting [17].

Thrombin-induced hydrolysis of GST–TRE

Recombinant GST–TRE was incubated at 37 °C with 5 nmol/l α-thrombin in a 20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4, buffer containing 2 mg/ml BSA (fraction V, Sigma Chemical Corp, St. Louis, MO, U.S.A.). At selected times, the reaction was stopped by addition of 2 % (w/v) SDS, 10 mM *N*-ethylmaleimide and boiling for 5 min. The hydrolysis reaction was also performed with γ-thrombin or in the presence of different thrombin ligands such as hirudin (Serbio, Asnières, France), PPACK (D-Phe-Pro-Arg-chloromethyl ketone), the C-terminal peptide 54–65 of hirudin (Bachem Feinchemikalien AG, Bubendorf, Switzerland), thrombomodulin, fibrin(ogen) fragment E, or glycocalicin. Samples were run on 12 % (w/v) polyacrylamide slab gels (Mini-Protean II, Bio-Rad, Ivry, France) and stained with Coomassie Blue. Alternatively, when low concentrations of GST–TRE were used, the fusion protein and its cleavage products were detected by Western-blot analysis using the polyclonal anti-GST–TRE antibody and peroxidase-coupled goat anti-(rabbit IgG) antibodies revealed by enhanced chemiluminescence (Amersham International Plc., U.K.). The extent of GST–TRE hydrolysis was determined after densitometric scanning of the bands corresponding to GST–TRE and its proteolytic fragments (Shimatzu CS-930 densitometer, Japan).

RESULTS

Cloning of the endothelial cell TR cDNA

A full-length 1322 nt cDNA coding for endothelial cell TR was obtained after PCR amplification of endothelial cell RNA and assembly of two fragments covering nucleotides 208–1530 of the published platelet sequence [8]. The endothelial cell TR sequence was found to be identical with the platelet sequence with the exception of a change of CG to GC at positions 935–936 that changes Val-238 into Leu, and a conservative G to C change at position 1289. A similar Val to Leu change at position 238 of the TR has been previously reported for endothelial cells [26].

Production of a recombinant GST–TRE fusion protein and polyclonal antibody

A 220 nt fragment encoding amino acids 25–98 of endothelial cell TR was subcloned into the PGEX-3X plasmid. A recombinant protein was produced after transformation into *E. coli*. The protein migrated as a major 33 kDa band on SDS/PAGE in 12 % gels corresponding to 24 kDa for the GST carrier (228 residues) and 9 kDa for the N-terminal extracellular domain 25–97 of TR. The factor Xa-catalysed removal of the GST portion of the fusion protein was assessed by the reduction of the molecular mass of the protein on SDS/PAGE. The protein could also be cleaved by thrombin, generating a shorter TRE fragment that was purified by gel filtration and was subjected to N-terminal sequence analysis. The sequencing readout corresponded to the sequence 42–54 of the cloned receptor, which provided evidence for the identity of the recombinant protein and for the cleavage by thrombin at position 41–42. An anti-GST–TRE polyclonal antibody was produced that recognized, in an e.l.i.s.a. and by immunoblotting, the purified GST–TRE and TRE proteins, and also recognized two synthetic peptides corresponding to amino acids TR 43–56 and TR 53–64.

Interaction of GST–TRE with the thrombin catalytic site

The thrombin-induced hydrolysis of GST–TRE was studied after analysis of the cleavage products on SDS/12 % PAGE. The intensity of the band corresponding to the fusion protein (33 kDa) decreased after incubation with thrombin and was accompanied by a parallel increase of a 26.5 kDa band corresponding to GST coupled to the N-terminal 20 residues of TRE after cleavage of the Arg-41–Ser-42 bond by thrombin (Figure 1). Disappearance of the GST–TRE band and appearance of the 26.5 kDa band were used to follow hydrolysis, as the C-terminal TRE 6.5 kDa peptide could not be clearly visualized on Coomassie Blue-stained gels. Hydrolysis was time- and concentration-dependent with 100 % hydrolysis of GST–TRE obtained after 3 min with 5 nM thrombin. Hydrolysis was completely prevented by pre-

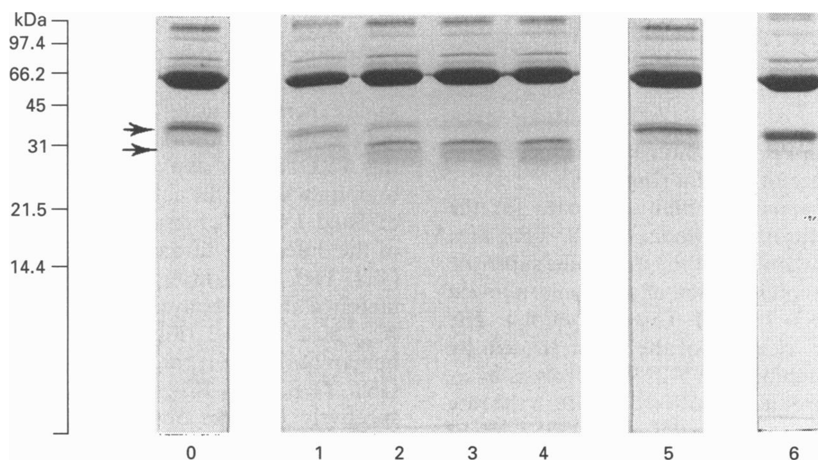

Figure 1 Cleavage of GST–TRE by α-thrombin

GST–TRE (2.7 μmol/l) was incubated with 5 nmol/l α-thrombin in 20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4, 2 mg/ml BSA, at 37 °C for 0, 10, 30, 50 and 60 s (lanes 0, 1, 2, 3 and 4 respectively). Digestion products were analysed on a non-reduced SDS/12% PAGE. Molecular-mass standards are indicated on the left. The upper arrow indicates the 33 kDa band corresponding to the GST–TRE 25–97 fusion protein and the lower arrow indicates the 26.5 kDa band corresponding to the GST–TRE 25–41 fusion protein. GST–TRE hydrolysis was inhibited when performed in the presence of 5 nmol/l PPACK-treated thrombin (lane 5), or in the presence of 2.5 units/ml of hirudin (lane 6).

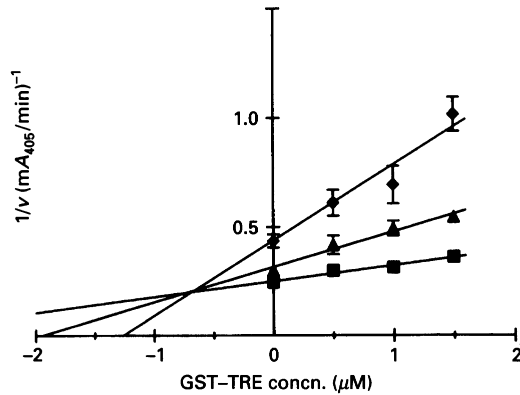

Figure 2 The fusion protein GST–TRE inhibits thrombin amidolytic activity

α-Thrombin (0.4 nmol/l) was incubated with the thrombin chromogenic substrate S-2238 [◆, 5 μmol/l; ▲, 10 μmol/l; or ■, 20 μmol/l in 10 mmol/l Tris, 10 mmol/l Hepes, 100 mmol/l NaCl, pH 7.8, 0.1% (w/v) PEG 8000] in the presence of increasing amounts of GST–TRE. The initial rate of hydrolysis was measured and its reciprocal plotted versus GST–TRE concentration. Results are the mean ± S.E.M. of three experiments.

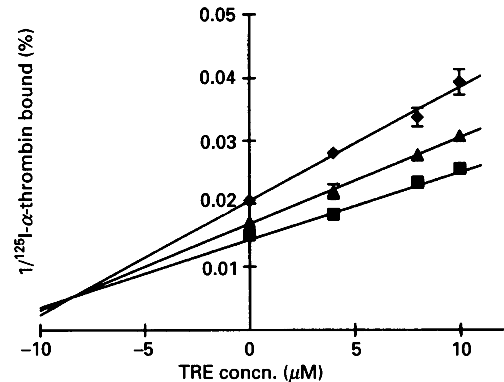

Figure 4 TRE inhibits thrombin binding to fibrin

^{125}I-α-thrombin (5 nmol/l) was incubated with fibrin monomers (◆, 0.4 μmol/l; ▲, 0.6 μmol/l; or ■, 0.8 μmol/l) which were allowed to polymerize in 50 mmol/l Tris, 50 mmol/l NaCl, pH 7.4, 10 mg/ml BSA, in the presence of increasing amounts of TRE for 2 h at 37 °C. After centrifugation of the samples, fibrin-bound thrombin was quantified by γ-counting of the clots. A Dixon representation of the results (means ± S.E.M. of three experiments) is presented.

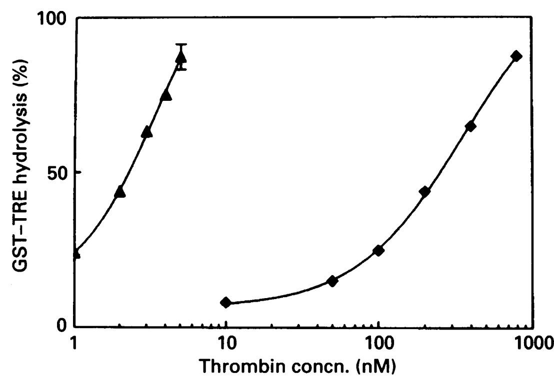

Figure 3 γ-Thrombin has a reduced ability to hydrolyse GST–TRE

GST–TRE (2.7 μmol/l in 20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4, 0.2% BSA) was incubated with increasing amounts of α- (▲) or γ-thrombin (◆) for 1 min at 37 °C. After SDS/PAGE, hydrolysis of GST–TRE was quantified by densitometric scanning of the Coomassie Blue-stained gels. Results are presented as the percentage of GST–TRE hydrolysis versus the concentration of thrombin.

treatment of α-thrombin with PPACK and when the incubation was performed in the presence of hirudin (Figure 1).

In order to measure an apparent affinity constant for the binding of GST–TRE to α-thrombin, we used GST–TRE as a competitive substrate in the hydrolysis of the tripeptidic substrate S-2238 (Figure 2). A Dixon representation of the results allowed us to calculate a K_i for GST–TRE of 0.5 ± 0.04 μmol/l. The purified TRE obtained after cleavage of the fusion protein by factor Xa was also a potent inhibitor of S-2238 hydrolysis by α-thrombin, 0.1 μmol/l TRE producing a 50% decrease in the rate of S-2238 hydrolysis. In contrast, the carrier protein GST, up to 2 μmol/l, did not modify the rate of S-2238 hydrolysis by thrombin.

Interaction of GST–TRE with the thrombin anion-binding exosite

As the N-terminal extracellular domain of TRE contains a negatively charged sequence similar to that of the C-terminal tail of hirudin [14] it has been proposed to interact with the anion-binding exosite of thrombin [27]. Disruption of the anion-binding exosite is observed upon trypsin-catalysed conversion of α- into γ-thrombin. γ-Thrombin hydrolysed GST–TRE but with a reduced efficiency when compared with α-thrombin, 50% hydrolysis being observed after 1 min of incubation with 250 nmol/l γ-thrombin as compared with 2 nmol/l for α-thrombin (Figure 3). In addition, GST–TRE, even used at 1.5 μmol/l, did not significantly inhibit the hydrolysis of S-2238 by γ-thrombin (results not shown), indicating that the GST–TRE cleavage site bound weakly to the catalytic site of γ-thrombin.

Several proteins including fibrin(ogen), thrombomodulin and GPIb are known to bind to structures of α-thrombin located within the anion-binding exosite [25,28,29]. In order to examine the overlap between the structures of α-thrombin engaged in the interactions with TRE and these three proteins, we have performed competition experiments.

The binding of ^{125}I-α-thrombin to repolymerizing fibrin was inhibited by purified TRE (Figure 4). As TRE digestion by thrombin presumably occurs during the course of the reaction and thus could modify the equilibrium, we have performed the same experiment using PPACK-active-site-inhibited ^{125}I-thrombin (results not shown). Similar results were obtained, indicating that TRE behaved as a competitive inhibitor of thrombin binding to fibrin whether the active site was blocked or accessible with K_i of 9 and 7.4 μmol/l respectively. We have then studied the effect of the different α-thrombin exosite ligands on the hydrolysis of GST–TRE by α-thrombin. The C-terminal peptide of hirudin inhibited the proteolysis of GST–TRE by α-thrombin with an IC_{50} of 27 μmol/l (Figure 5a). Rabbit thrombomodulin and human fibrin(ogen) fragment E both inhibited the hydrolysis of GST–TRE, with IC_{50} values of 10 nmol/l and 30 μmol/l respectively (Figures 5b and 5c). In contrast with fibin(ogen) fragment E, thrombomodulin and hirudin 54–65, glycocalicin (up to 5 μmol/l) did not inhibit the hydrolysis of GST–TRE by α-thrombin. In order to decrease the GST–TRE concentration in the proteolysis assay to 70 nmol/l, we used a sensitive immunoblot for the detection of GST–TRE and its proteolytic fragment. Even in these conditions glycocalicin (5 μmol/l) failed to modify α-thrombin-induced hydrolysis of GST–TRE (results not shown). Using the immunoblot assay we were able to confirm the

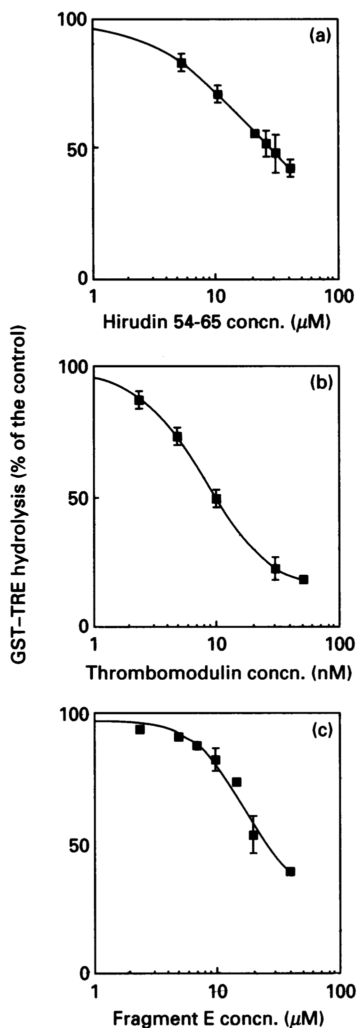

Figure 5 Inhibition of thrombin-induced GST–TRE cleavage by hirudin, thrombomodulin and fibrinogen fragment E

GST–TRE (2 μmol/l in 20 mmol/l Tris, 150 mmol/l NaCl, pH 7.4, 2 mg/ml BSA) and α-thrombin (2 nmol/l) were incubated for 1 min at 37 °C in the presence of increasing amounts of hirudin 54–65 (**a**), thrombomodulin (**b**) and fibrin(ogen) fragment E (**c**). The samples were solubilized with 2% (w/v) SDS and electrophoresed on 12% (w/v) acrylamide gels. Hydrolysis was quantified as in Figure 3. The amount of GST–TRE hydrolysis is presented as the percentage of the GST–TRE hydrolysed versus the concentration of hirudin 54–65 (**a**), thrombomodulin (**b**), or fibrin(ogen) fragment E (**c**). Results are the mean ± S.E.M. of three experiments.

inhibition of GST–TRE cleavage by hirudin fragment 54–65 (results not shown).

DISCUSSION

To date, the mechanism of activation of TR by thrombin and the mode of interaction of thrombin with its receptor have been essentially studied on cells expressing the natural or recombinant forms of TR. The efficacy of receptor cleavage estimated from the intensity of cellular responses to thrombin in cells expressing recombinant receptor is dependent on the level of receptor expression and intracellular signalling efficiency, and does not allow direct measurement of thrombin affinity for TR [30]. In the present study we have devised a system allowing the study of thrombin–TR interaction in a purified system using soluble purified proteins. We describe the production and characterization of a recombinant fusion protein of 33 kDa which is composed of the first N-terminal extracellular domain corresponding to residues 25–97 of the TR (9 kDa) coupled to a GST carrier protein (24 kDa). Cleavage by factor Xa of the purified GST–TRE produced the liberation of TR 25–97 from the GST protein. A similar chimeric protein consisting of GST and portion 1–160 of the TR has been described previously [26] and used to produce a polyclonal rabbit antibody directed against the putative thrombin cleavage site on the TR. This antibody inhibited thrombin-induced platelet activation, but not platelet activation induced by the agonist peptide [31].

The present construction appears to be a valid tool for studying *in vitro* the interaction of thrombin with its receptor since GST–TRE is cleavable by α-thrombin. The apparent K_i of 0.5 μM on the hydrolysis of a tripeptidic substrate reflects the affinity of α-thrombin for the soluble GST–TRE. Such an approach has been previously used by Lundblad et al. [32], with fibrinogen acting as a competitive inhibitor of S-2238 hydrolysis by α-thrombin with a K_i of 8.3 μM. This value approximates the K_m for fibrinogen Aα-chain cleavage [33], indicating the validity of the approach. The apparent affinity of thrombin for GST–TRE so determined is higher than the affinity reported by Vu et al. [27] who found a K_m of 10 μM for TR 28–60 hydrolysis by thrombin, but it is close to the IC_{50} value of 0.4 μM reported for the non-cleavable peptide TR 38–64 on thrombin-induced platelet activation [34]. In the system we used, the affinity of α-thrombin for GST–TRE appears to be at least 10-fold greater than its affinity for fibrinogen. The apparent affinity of thrombin for GST–TRE (~ 0.5 μM) is at least two orders of magnitude lower than the concentration of the platelet receptor (0.75 nM) estimated for a suspension of 3×10^8 platelets/ml, assuming 1500 copies of TR per platelet [35].

The involvement of the anion-binding exosite in thrombin binding to TR has been indicated by studies using whole cells expressing recombinant TR [8,11,36]. The C-terminal segment of hirudin was found to inhibit cellular responses to thrombin [14,29,36]. Furthermore, cells expressing a mutant TR deleted for the hirudin-like domain need 100-fold more thrombin to be activated when compared with cells expressing wild-type TR [27]. The present study provides a series of arguments that confirms the direct interaction of TR with thrombin through the anion-binding exosite. Our experimental conditions allowed the direct comparison of thrombin interaction with TRE to thrombin interaction with several anion-binding exosite ligands. First, we have observed that hirudin 54–65 inhibited thrombin-induced TRE cleavage. Secondly, the facts that TRE competed with fibrin for binding to α-thrombin and that fibrin(ogen) fragment E inhibited thrombin-induced hydrolysis of GST–TRE are in favour of an overlap between fibrinogen- and TRE-binding sites on thrombin. These results are in good agreement with our previous observation of an inhibition by fibrin(ogen) fragment E of α-thrombin-induced platelet activation [16]. Thirdly, α-thrombin-induced TRE cleavage was inhibited by thrombomodulin, in agreement with the reported inhibition of α-thrombin-induced platelet activation by thrombomodulin [37]. Fourthly, we have shown that γ-thrombin, which lacks the anion-binding exosite I, can still cleave GST–TRE, but with a 100-fold decreased potency when compared with α-thrombin. This property is comparable with the low capacity of γ-thrombin to induce platelet activation [38]. It thus appears that TR cleavage and the resulting platelet activation can take place even in the absence of binding to the receptor hirudin-like domain, but with a greatly reduced efficiency.

GPIb has been shown earlier to be a platelet thrombin-binding

protein. Thrombin binding to GPIb does not seem to be directly involved in signal transduction [39] but rather to increase the sensitivity and the rate of platelet responses to thrombin. For example, platelets with absent or defective GPIb such as platelets from Bernard–Soulier patients [40], platelets treated with monoclonal antibodies specific for GPIb [41,42], or platelets lacking GPIb after protease treatment [43–46] are less responsive to thrombin than control platelets. Glycocalicin, a water-soluble fragment of the GPIb α-chain retains the ability to bind thrombin [4] with an affinity similar to that reported for intact GPIb on platelets [47]. The thrombin-binding site has been located on the N-terminal 45 kDa tryptic fragment of glycocalicin [46]. Glycocalicin contains a stretch of negatively charged residues between Asp-269 and Asp-287 and behaves as a thrombin anion-binding exosite ligand since it competes with the hirudin 54–65 peptide, fibrin(ogen) and thrombomodulin for binding to thrombin [17]. Using purified proteins, we could not detect any competition between GST–TRE and glycocalicin for interaction with thrombin. This could be explained by a better affinity of α-thrombin for GST–TRE compared with glycocalicin. Some arguments argue against this possibility. First, when used as a competitor of α-thrombin binding to fibrin, glycocalicin has a K_i of 0.1 μM [17] compared with a K_i of 7.5 μM for TRE. Secondly, equilibrium binding studies on platelets also indicate that GPIb binds thrombin with high affinity, with a K_d estimated at 0.3 nM [43]. In conclusion, the absence of competition between glycocalicin and TRE for the binding to thrombin observed in this study indicates that thrombin bound to glycocalicin is still able to cleave GST–TRE and suggests that glycocalicin and TRE interact with discrete subsites within the thrombin exosite. In regard to the geometry of the large groove that constitutes the anion-binding exosite I [48], it appears possible for two different ligands to be docked at the same time in this area. Since thrombin binds similarly to glycocalicin and to GPIb [47] we can postulate that the observations made with glycocalicin are valid for GPIb. The existence of independent binding sites for TR and GPIb on thrombin could explain why, on platelets, GPIb is not a competitor for TR-dependent platelet responses. In contrast, thrombin interaction with GPIb is known to accelerate TR-coupled platelet activation by increasing the rate of platelet responses, as well as platelet sensitivity to α-thrombin [39,40,42]. In our acellular system, glycocalicin does not increase the cleavage of TR by thrombin. It thus appears that the promoting effect of GPIb on thrombin-induced platelet activation requires membrane-bound proteins and procedes by a mechanism that has still to be determined.

In conclusion, this study has provided direct evidence for the important role of the thrombin anion-binding exosite for the interaction with TR and its efficient cleavage. In addition, we have shown that TR and GPIb, two proteins containing similar negatively charged domains, could interact with discrete binding sites on thrombin. The model used in this study could be useful for the further characterization of the interaction of thrombin with its receptor, and also to study potential pharmacological inhibitors of thrombin-induced platelet activation.

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