Cleavage of the thrombin receptor: identification of potential activators and inactivators

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The kinetic parameters were determined for the hydrolysis of a peptide based on the activation site of the thrombin receptor (residues 38–60) by thrombin and 12 other proteases. The k_{cat} and K_m values for the cleavage of this peptide (TR^{38-60}) by thrombin were 107 s^{-1} and 1.3 μM; the k_{cat}/K_m of TR^{38-60} is among the highest observed for thrombin. A model is presented that reconciles the parameters for cleavage of the peptide with the concentration dependence of cellular responses to thrombin. Cleavage of TR^{38-60} was not specific for thrombin. The pancreatic proteases trypsin and chymotrypsin hydrolysed TR^{38-60} efficiently ($k_{cat}/K_m > 10^6$ $M^{-1}\cdot s^{-1}$). Whereas trypsin cleaved TR^{38-60} at the thrombin activation site (Arg^{41}-Ser^{42}), chymotrypsin hydrolysed the peptide after Phe^{43}. This chymotryptic cleavage would result in inactivation of the receptor. The efficient cleavage of TR^{38-60} by chymotrypsin ($k_{cat}/K_m \approx 10^6$ $M^{-1}\cdot s^{-1}$) was predominantly due to a low K_m value (2.8 μM). The proteases factor Xa, plasmin, plasma kallikrein, activated protein C and granzyme A also hydrolysed TR^{38-60} at the Arg^{41}-Ser^{42} bond, but exhibited k_{cat}/K_m values that were at least 10^3-fold lower than that observed with thrombin. Both tissue and urokinase plasminogen activators as well as granzyme B and neutrophil elastase were unable to cleave TR^{38-60} at appreciable rates. However, neutrophil cathepsin G hydrolysed the receptor peptide after Phe^{55}. Like the chymotryptic cleavage, this cleavage would lead to inactivation of the receptor, but the cathepsin G reaction was markedly less efficient; the k_{cat}/K_m value was almost four orders of magnitude lower than that for thrombin. In addition to the above cleavage sites, a secondary site for thrombin and other arginine-specific proteases was identified at Arg^{46}, but the cleavage at this site only occurred at very low rates and is unlikely to be significant *in vivo*.

INTRODUCTION

Thrombin is a trypsin-like serine protease whose activity is central to haemostasis. It has procoagulant and anticoagulant activities and exhibits remarkable specificity towards its substrates and inhibitors. Thrombin is generated after activation of the coagulation cascade and, once produced, it cleaves fibrinogen to yield fibrin monomers that polymerize to form a haemostatic plug. Thrombin further stimulates its own production by activating factors V and VIII [1]. When complexed with the endothelial cell protein thrombomodulin, thrombin activates protein C, which turns off the coagulation cascade by degrading factors Va and VIIIa [2].

In addition to its interaction with soluble components of the coagulation cascade, thrombin also induces a broad range of cellular responses, many of which are mediated by a G-protein-coupled receptor [3,4]. Thrombin cleaves the N-terminal extracellular domain of this receptor at a site that is similar to the thrombin cleavage site in protein C. The new N-terminus created by thrombin cleavage binds to the body of the receptor and triggers activation. The thrombin receptor contains two recognition sites for thrombin. In addition to the activation site, an acidic sequence is found on the C-terminal side of this site and this region of the receptor binds to the anion-binding exosite of thrombin [5,6] .

The wide expression of this thrombin receptor and the range of responses it evokes upon activation suggest that its activity might help to mediate the inflammatory and proliferative responses to injury [7]. A number of the cells involved in these responses express the thrombin receptor (e.g. platelets, lymphocytes, fibroblasts and endothelial cells). In addition, numerous proteases are present during different phases of the response to injury. Enzymes of the coagulation cascade are present during blood clot formation. Fibrinolytic proteases are generated to promote dissolution of the clot, and infiltrating neutrophils and lymphocytes also release proteases. These proteases have the potential to activate or inactivate the thrombin receptor. Cleavage of the receptor at the site used by thrombin, Arg^{41}-Ser^{42}, leads to receptor activation. In contrast, cleavage of the receptor at a position on the C-terminal side of this site inactivates the receptor. Studies with synthetic peptides have shown that an interaction with the α-amino group of Ser^{42} is absolutely essential for receptor activation [8–10].

To assess whether activation and/or inactivation of the thrombin receptor by other proteases could occur at physiologically relevant rates, we have determined the kinetic parameters for cleavage of a peptide based on the thrombin activation site by 13 different serine proteases involved in blood clotting, fibrinolysis, inflammation and immunity. Receptor cleavage was not limited to thrombin, and the results indicate that activation or inactivation of the thrombin receptor by other proteases could occur in situations of disease or injury.

MATERIALS AND METHODS

Materials

Human α-thrombin was prepared as described by Stone and Hofsteenge [11] and was fully active as determined by active site

Abbreviations used: APC, activated protein C; tPA, tissue plasminogen activator; TR^{38-60}, a synthetic peptide corresponding to residues 38–60 of the thrombin receptor; uPA, urokinase-type plasminogen activator.

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titration with 4-methylumbelliferyl *p*-guanidinobenzoate [12]. Bovine factor Xa was prepared as described by Le Bonniec et al. [13]. Human plasmin, bovine pancreatic trypsin (treated with tosylphenylalanylchloromethane), bovine type II chymotrypsin, human melanoma two-chain tissue plasminogen activator (tPA), human plasma kallikrein and 4-methylumbelliferyl *p*-guanidinobenzoate were from Sigma (Poole, Dorset, U.K.). Human activated protein C (APC) and granzymes A and B were prepared as previously described [14,15]. Human urine low-molecular-mass urokinase (uPA) was from Calbiochem (Nottingham, Notts., U.K.). Human neutrophil cathepsin G and human leucocyte elastase were from Athens Inc. (Athens, GA, U.S.A.). The synthetic peptide corresponding to residues 38–60 of the thrombin receptor (TR^{38-60}; LDPRSFLLRNDNDKYEPFWEDEE) was a gift from Biogen (Cambridge, MA, U.S.A.). Amino acid analysis was used to confirm its composition and to determine its concentration. BSA was fraction V from Boehringer Mannheim (Lewes, E. Sussex, U.K.). The serpins α_1-antitrypsin and α_1-antichymotrypsin were produced as described by Hopkins et al. [16] and Rubin et al. [17]. Hirudin was produced as previously described [18] and D-Phe-Pro-Arg-CH_2Cl was a gift from Dr. E. Shaw (Friedrich-Miescher-Institut, Basel, Switzerland). Other chemicals used were of the highest grade available commercially.

Reaction conditions

All experiments were performed at 37 °C in 0.05 M Tris/HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.1 % poly(ethylene glycol), 6 kDa. BSA (0.25 %) was added to the reaction mixture when incubations were performed with APC, urokinase, kallikrein and cathepsin G; 0.1 % (w/v) Triton X-100 was added to the assays with cathepsin G and neutrophil elastase.

Determination of protease concentrations

The concentration of thrombin was determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate [12]. The active concentrations of factor Xa, trypsin, APC, plasma kallikrein, tPA, uPA and plasmin were determined by titration with D-Phe-Pro-Arg-CH_2Cl [19], which was standardized against thrombin. Chymotrypsin and neutrophil elastase were titrated against α_1-antitrypsin, whereas α_1-antichymotrypsin was used to determine the active concentration of cathepsin G.

Determination of the kinetic parameters for cleavage of TR^{38-60}

TR^{38-60} was incubated with proteases for different periods and the reaction was stopped either by the addition of hirudin (thrombin) or by the addition of HCl to lower the pH of the reaction buffer to 3 (other proteases). The reaction products were then separated by reverse-phase HPLC on a 0.5 ml C4 column. The gradient used was 0–35 % (v/v) acetonitrile in 0.1 % (v/v) trifluoroacetic acid over 30 min. The flow rate was 0.5 ml/min. Cleavage products were collected and subjected to N-terminal sequencing and/or mass spectroscopy analysis to identify the cleavage sites. N-terminal sequencing and matrix-assisted laser desorption ionization mass spectroscopy were performed by the Protein Chemistry Facility, Department of Biochemistry, University of Cambridge. Cleavage by all proteases resulted in the generation of a small (less than six residues) and a large (more than 14 residues) fragment. Under the conditions of the HPLC analysis, the small fragment did not bind to the C4 column and cleavage rates were calculated on the basis of the area of the peak of the large fragment. When the reaction was stopped by lowering the pH, slow cleavage of the Asp^{39}-Pro^{40} bond in TR^{38-60} occurred [20]. This resulted in the generation of an additional peak on HPLC analysis. This peak was separated sufficiently from the larger fragment generated by proteolytic cleavage so as not to interfere with quantification of the latter. In initial experiments, fixed concentrations of TR^{38-60} and the protease were used and samples were removed at various time points. From the results obtained in these experiments, an incubation time and protease concentration were chosen so that less than 10 % substrate hydrolysis occurred during the course of the reaction. For the determination of kinetic parameters, assays were performed in duplicate with seven concentrations of TR^{38-60}; the range chosen included at least one concentration above and below the K_m. Calibration curves were constructed by using fully digested samples of TR^{38-60}. A linear relationship between the amount of cleavage product and peak area was observed for the concentration ranges studied. Initial velocities at different substrate concentrations were calculated from peak areas with the use of these standard curves. The values were then fitted to the Michaelis–Menten equation by using nonlinear regression to yield estimates for k_{cat} and K_m.

RESULTS

Kinetic parameters for cleavage of TR^{38-60} by proteases with a preference for basic residues

TR^{38-60} was a very efficient substrate for thrombin. Under initial-rate conditions (less than 10 % cleavage), hydrolysis occurred only at the Arg^{41}-Ser^{42} bond; the site of cleavage was identified by the N-terminal sequence of the peptide generated and by its amino acid composition. The K_m and k_{cat} values for cleavage of TR^{38-60} at this site were 1.3 ± 0.1 μM and 107 ± 2 s^{-1} (Table 1). The k_{cat}/K_m for TR^{38-60} was equivalent to those observed for the best synthetic substrates of thrombin [21] and approx. 8-fold higher than that observed for fibrinogen [22]. Prolonged incubation of TR^{38-60} with high concentrations of thrombin (more than 10 nM) resulted in rapid cleavage of the Arg^{41}-Ser^{42} bond

Table 1 Kinetic parameters for the cleavage of TR^{38-60}

The initial rate of cleavage of TR^{38-60} was determined in duplicate for seven different concentrations by HPLC analysis as described in the Materials and methods section. These values were fitted to the Michaelis–Menten equation by nonlinear regression to yield the estimates of k_{cat} and K_m shown, along with the standard errors obtained from the regression analysis. Unless otherwise stated, the kinetic parameters are for hydrolysis of the Arg^{41}-Ser^{42} bond.

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Thrombin	1.3 ± 0.1	107 ± 2	$(8.0 \pm 0.7) \times 10^7$
Trypsin	18 ± 1	103 ± 5	$(5.7 \pm 0.4) \times 10^6$
Plasmin	13 ± 1	0.60 ± 0.02	$(4.6 \pm 0.4) \times 10^4$
Granzyme A	7.9 ± 2.2	0.18 ± 0.02	$(2.3 \pm 0.7) \times 10^4$
APC	8.9 ± 1.5	0.026 ± 0.002	$(2.9 \pm 0.5) \times 10^3$
Kallikrein	84 ± 22	0.20 ± 0.04	$(2.4 \pm 0.4) \times 10^3$
Factor Xa	–	–	$4 \times 10^{2*}$
tPA	–	–	$< 10^*$
uPA	–	–	$< 10^*$
Chymotrypsin†	2.8 ± 0.2	3.1 ± 0.1	$(1.1 \pm 0.1) \times 10^6$
Cathepsin G‡	44 ± 5	0.62 ± 0.06	$(1.4 \pm 0.2) \times 10^4$
Elastase	–	–	$< 100^*$
Granzyme B	–	–	$< 10^*$

* These values were calculated using the relationship given in eqn. (1) to estimate the specificity constant, k_{cat}/K_m (or its upper limit).

† These parameters are for the hydrolysis of the Phe^{43}-Leu^{44} bond.

‡ These parameters are for the hydrolysis of the Phe^{55}-Trp^{56} bond.

and subsequent cleavage after Arg^{46}; the peptide generated by the second cleavage had the N-terminal sequence Asn-Pro-Asn-Asp-Lys; the mass of the peptide was consistent with the sequence ^{47}NPNDKYEPFWEDEE^{60} (1812.7 Da observed, 1811.8 Da predicted). This second cleavage, however, proceeded relatively slowly. After incubation of 4 μM TR^{38-60} with 400 nM thrombin for 100 min, only approx. 10 % of the peptide generated by the first cleavage (residues 42–60, TR^{42-60}) had been further hydrolysed at the second position to yield residues 47–60 (TR^{47-60}). This secondary cleavage reaction also occurred when TR^{38-60} was incubated with other trypsin-like proteases (kallikrein, plasmin, trypsin and APC). However, the extent of this reaction was negligible compared with the proteolytic cleavage at the Arg^{41}-Ser^{42} under the conditions used. As with thrombin, incubations for long periods with high enzyme concentrations (more than 10 nM) were necessary to observe significant cleavage at the Arg^{46}-Asn^{47} site. The observed cleavage at Arg^{46}-Asn^{47} after prolonged incubations is consistent with the appearance of peptides generated by this cleavage in the mother liquor from crystallographic studies of thrombin–receptor peptide complexes [6].

TR^{38-60} was also efficiently cleaved by trypsin. Trypsin cleaved TR^{38-60} predominantly at the Arg^{41}-Ser^{42} bond; the peptide generated had the N-terminal sequence Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn and its mass agreed with that predicted for a single cleavage at Arg^{41}-Ser^{42} (2429.6 Da observed, 2428.6 Da predicted). The K_m and k_{cat} values for the trypsin cleavage of TR^{38-60} were 18 ± 1 μM and 103 ± 5 s^{-1} (Table 1). Thus trypsin exhibited the same k_{cat} as thrombin but the K_m was 14-fold higher.

TR^{38-60} was also cleaved relatively well by plasmin and granzyme A. Both proteases hydrolysed TR^{38-60} predominantly at the Arg^{41}-Ser^{42} bond as determined by N-terminal sequencing and mass spectroscopy of the generated peptide; the N-terminal sequence was Ser-Phe-Leu-Leu-Arg- and the observed molecular mass differed by only 0.1 % from the predicted one. The K_m and k_{cat} values for plasmin were 13 ± 1 μM and 0.60 ± 0.02 s^{-1} (Table 1); thus the K_m was 10-fold higher than that observed with thrombin, and the k_{cat} value was 180-fold lower. The major difference between granzyme A and thrombin was also due to a much lower k_{cat} with granzyme A. The K_m and k_{cat} values for TR^{38-60} with granzyme A were 8 ± 2 μM and 0.18 ± 0.02 s^{-1} (Table 1); although the K_m with granzyme A was only 6-fold higher, the k_{cat} was approx. 600-fold lower.

Plasma kallikrein and APC were approx. 10^4-fold less efficient than thrombin in cleaving TR^{38-60} (Table 1). Both proteases cleaved TR^{38-60} at the Arg^{41}-Ser^{42} activation site as determined by N-terminal sequencing and mass spectroscopy of the generated peptide; the observed molecular masses differed from the expected values by less than 0.1 %. Plasma kallikrein and APC exhibited similar values for the specificity constant (k_{cat}/K_m). For APC the low value of the specificity constant was due to a very low k_{cat}, whereas plasma kallikrein had a high K_m (Table 1).

TR^{38-60} was not cleaved to an appreciable extent by factor Xa, tPA or uPA. Incubation of TR^{38-60} (4 μM) with bovine factor Xa (10 nM) for 1 h resulted in only 1 % hydrolysis of the Arg^{41}-Ser^{42} bond. However, overnight incubation (18 h) of 4 μM TR^{38-60} with 100 nM factor Xa resulted in 94 % cleavage of this bond. An estimate of the rate constant for the cleavage reaction could be obtained from these results. If the K_m for TR^{38-80} is significantly greater than 4 μM (as it was for all proteases except thrombin), then the cleavage reaction will follow first-order kinetics and an estimate of the specificity constant for the reaction can be obtained from the following relationship:

$$k_{cat}/K_m = \{\ln(1 - [P]/[P]_\infty)\}/[E]_0 t \quad (1)$$

where $[P]/[P]_\infty$ is the fraction of TR^{38-60} hydrolysed at time t and $[E]_0$ is the concentration of protease. By using this relationship, estimates of 300 and 400 $M^{-1} \cdot s^{-1}$ were obtained for the specificity constant with factor Xa from the fraction of TR^{38-60} cleaved at 1 and 18 h respectively (Table 1). Overnight (18 h) incubation of 4 μM TR^{38-60} with 100 nM uPA or tPA did not result in proteolysis. Assuming that 5 % hydrolysis of TR^{38-60} would have been detected, the relationship given in eqn. (1) was used to calculate an upper limit of 10 $M^{-1} \cdot s^{-1}$ for the specificity constants of tPA and uPA.

Kinetic parameters for cleavage of TR^{38-60} by proteases with a preference for hydrophobic or acidic residues

The extracellular domain of the thrombin receptor contains a number of potential cleavage sites for proteases with a preference for hydrophobic or acidic residues. Cleavage at a number of these sites would lead to inactivation of the receptor. To investigate this possibility, the kinetics of cleavage of TR^{38-60} by chymotrypsin, cathepsin G, neutrophil elastase and granzyme B were determined. Neutrophil elastase and cathepsin G are released in high concentrations on activation of neutrophils [23]. Overnight (16 h) incubation of 4 μM TR^{38-60} with 6 nM human neutrophil elastase did not lead to proteolysis of the peptide. Assuming that 5 % hydrolysis of TR^{38-60} would have been detected, the relationship given in eqn. (1) can be used to estimate an upper limit of 100 $M^{-1} \cdot s^{-1}$ for the specificity constant for cleavage of TR^{38-60} by neutrophil elastase (Table 1). Incubation of TR^{38-60} with 100 nM cathepsin G for 30 min resulted in the generation of a number of small peptides. However, shorter incubation times with less enzyme (e.g. 10 min with 10 nM cathepsin G) resulted in a single cleavage. Mass spectroscopy identified the cleavage site as Phe^{55}-Trp^{56}; the molecular mass of the generated peptide was 2221.8 Da, which corresponded to the predicted mass of 2221.5 Da for ^{38}LDPRSFLLRNPNDKYE-PF^{55}. The K_m and k_{cat} values for the cleavage of TR^{38-60} at this site by cathepsin G were 44 ± 5 μM and 0.62 ± 0.06 s^{-1} (Table 1). The K_m was approx. 30 times higher than that found for the TR^{38-60}–thrombin interaction, and the k_{cat} was 170-fold lower. Compared with thrombin, the specificity constant was reduced 5700-fold.

Incubation of TR^{38-60} with chymotrypsin resulted in cleavage of the receptor peptide at Phe^{43}; the N-terminal sequence of the generated peptide was Leu-Leu-Arg-Asn-Pro-Asn-Asp- and the observed molecular mass of 2194.4 Da corresponded to that predicted for the peptide ^{44}LLRNPNDKYEPFWEDEE^{60} (2194.3 Da). This cleavage reaction was specific and no proteolysis occurred at other aromatic residues even after prolonged incubations. The k_{cat} and K_m values for the interaction of TR^{38-60} with chymotrypsin were 3.1 ± 0.1 s^{-1} and 2.8 ± 0.2 μM (Table 1). The observed K_m for chymotrypsin was comparable to that of thrombin, but the k_{cat} was 35-fold lower.

Despite the specificity of granzyme B for aspartic acid residues, mass spectroscopic analysis revealed that cleavage of TR^{38-60} with the preparation of granzyme B occurred at Arg^{41}; the observed molecular mass (2429.6) of the peptide generated corresponded well to that predicted for the peptide corresponding to residues 42–60 (2428.6). The initial rate for cleavage at this site by the granzyme B preparation was approx. 1/30 as fast as that observed with granzyme A. Because SDS/PAGE analysis of the granzyme B preparation indicated the presence of a minor contamination of granzyme A, it was concluded that the observed cleavage was due to granzyme A. Under the conditions used (4 μM TR^{38-60} with 100 nM granzyme B for 16 h), no cleavage occurred at the three aspartic residues present in TR^{38-60}. Thus it

was concluded that the specificity constant for cleavage of the peptide by granzyme B was less than 10 $M^{-1} \cdot s^{-1}$ (Table 1).

DISCUSSION

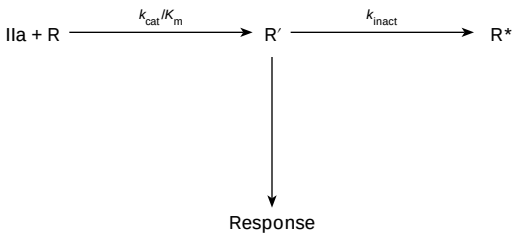
Cleavage of the thrombin receptor by thrombin

The kinetic parameters for the cleavage of TR^{38-60} by thrombin indicate that this peptide is one of the best substrates known for thrombin. The efficient cleavage of this peptide by thrombin is due to interactions both at the active site and at the anion-binding exosite. The peptide contains an optimal P_2–P_2' sequence (Pro-Arg-Ser-Phe) and a C-terminal sequence (^{52}YEPFWED-EE^{60}) that binds to the anion-binding exosite of thrombin [6,24]. This exosite interaction makes a large contribution to the efficiency of activation of the receptor on cells [5]. For example, disruption of the anion-binding exosite in γ-thrombin leads to a 10^3-fold increase in the EC_{50} for the activation of the receptor on neuroblastoma cells [25]. The exosite interactions with the receptor peptide are apparently able to overcome the presence of the unfavourable aspartic residue in the P_3 position of the sequence [26]. The structural basis for the efficient interactions of TR^{38-60} with the active site and exosite have been provided by the studies of Mathews et al. [6], who have solved crystal structures for several complexes of thrombin with receptor peptides. The receptor peptides were expected to bind to thrombin in a manner analogous to hirulog [27], with ^{38}Leu-Asp-Pro-Arg^{41} occupying the active site and ^{52}Tyr-Glu-Pro-Phe-Trp-Glu^{57}-binding to the exosite. The crystallographic studies of the receptor peptide–thrombin complexes demonstrated that ^{38}Leu-Asp-Pro-Arg^{41} was able to dock in thrombin's active site in a manner analogous to D-Phe-Pro-Arg in the hirulog- and D-Phe-Pro-Arg-CH_2-thrombin structures [27,28], and that ^{52}Tyr-Glu-Pro-Phe^{55} occupies the same position in the anion-binding exosite of thrombin as ^{55}Asp-Phe-Glu-Glu-Ile^{59} in hirudin [29–31]. However, the expected bidentate docking was not achieved in a single crystal. An alternative intermolecular bridging mode of peptide–thrombin binding was revealed and seemed to be the result of a dominant S-like conformation of the receptor peptide in solution. In uncleaved receptor peptides, ^{52}Tyr-Glu-Pro-Phe^{55} bound to the exosite of one thrombin molecule and ^{38}Leu-Asp-Pro-Arg^{41} docked in an unusual non-productive mode with the active centre of a neighbouring molecule [6]. Our studies indicate that these non-productive interactions are probably crystallization artifacts. The highly efficient cleavage of TR^{38-60} would not be observed if the non-productive interactions occurred to a significant extent in solution.

Although it is difficult to calculate the concentration of the thrombin receptor on cell surfaces, it seems reasonable to assume that under most conditions the concentration of thrombin ([IIa]) will be greater than that of the receptor ([TR]); i.e., [IIa] $\gg$ [TR]. Under these conditions, the rate of cleavage of the receptor (v) will be described by:

$$v = k_{cat}[IIa][TR]/([IIa] + K_m) \quad (2)$$

This equation predicts that the rate of activation of the thrombin receptor will display a hyperbolic dependence on the concentration of thrombin. Such a concentration dependence is usually observed for the activation of the receptor on cells, but the EC_{50} of thrombin is usually much lower than the K_m observed for TR^{38-60}. The EC_{50} of thrombin for cellular responses is usually in the nanomolar range, whereas the K_m for TR^{38-60} was 1.3 μM. Although it is possible that the K_m for the cleavage of the receptor on cells is substantially lower than that observed for the cleavage of TR^{38-60} in solution, it seems more likely that the EC_{50} does not measure the affinity of thrombin for its receptor on cells.



Scheme 1

The K_m for TR^{38-60} is already among the lowest observed for thrombin substrates, and TR^{38-60} uses the two binding regions of thrombin (active site and anion-binding exosite) that are known to make substantial contributions to the binding of substrates. It is difficult to envisage additional interactions that would lead to a decrease in the K_m of three orders of magnitude. Moreover, the specificity constant for TR^{38-60} is close to diffusion-controlled; i.e. any increase in this value is unlikely. Thus any decrease in the K_m for the thrombin receptor must be accompanied by a corresponding decrease in the k_{cat}. The fact that the EC_{50} values observed for cellular effects are lower than the K_m for TR^{38-60} can be explained on the basis of the Scheme 1, where R and R′ represent the uncleaved and cleaved receptor respectively. R′ is the activated form of the receptor that couples to intracellular second-messenger systems to yield the observed signal. This signalling response is halted by the conversion of the active form of the receptor to an inactive form (R*) through phosphorylation or another mechanism. The concentration of active receptor and hence the magnitude of the response depends on the rate of formation of R′ and the rate of its inactivation. If the concentration of thrombin is less than the K_m for the cleavage reaction, the rate of formation of R′ equals (k_{cat}/K_m)[IIa][R]. For a particular concentration of thrombin, the concentration of R′ increases while the rate of formation of R′ is greater than its rate of inactivation and decreases when the rate of inactivation becomes greater. The peak response occurs when the rate of formation of R′ equals the rate of its inactivation; i.e. when (k_{cat}/K_m)[IIa][R] equals k_{inact}[R′]. If it is assumed that the concentration of R* is negligible compared with those of R and R′ when the peak response occurs, the following equation can be derived for the magnitude of the peak response:

$$\text{Resp.} = \text{Resp.}_{max}[IIa]/([IIa] + K_m k_{inact}/k_{act}) \quad (3)$$

where Resp. and Resp._{max} represent respectively the observed response and the maximum response observed at an infinite concentration of thrombin. Eqn. (3) also predicts a hyperbolic dependence of the response on the concentration of thrombin. However, the observed EC_{50} will not equal the K_m for the cleavage reaction, but will equal $K_m k_{inact}/k_{cat}$. If the rate constant for the inactivation reaction is lower than k_{cat}, the observed EC_{50} will be lower than the K_m. Thus this mechanism is consistent with the data if k_{cat} is approx. 10^3-fold higher than k_{inact}. In conclusion, the mechanism presented in Scheme 1 is qualitatively consistent with the observed concentration dependence of cellular responses to receptor activation and can reconcile the differences between the K_m for the cleavage reaction and the EC_{50} for the cellular response. This mechanism also predicts that EC_{50} values might vary between cell types depending on the magnitude of k_{inact} for a particular cell and thus would explain some of differences observed in EC_{50} values for cellular responses to thrombin.

The observation of cleavage at the Arg^{46}-Asn^{47} bond in digests performed with high concentrations of thrombin (and other

proteases) and in crystallographic studies [6] indicates that thrombin cleaves the receptor peptide not only at the reported activation site but also at a secondary site. Cleavage of the thrombin receptor at its activation site results in the ^{42}SFLLR^{46} sequence acting as a tethered ligand for the receptor. Desensitization of the receptor occurs by a number of mechanisms including phosphorylation and internalization [4]. It seems theoretically possible that cleavage of the receptor at Arg^{46}-Asn^{47} could contribute to desensitization. This cleavage would cut the tether of the ligand and allow it to diffuse away from its binding pocket. The significance of this cleavage *in vivo*, however, is likely to be limited. This cleavage only occurred to a limited extent even after prolonged incubation with high concentrations of thrombin. Moreover, access to the Arg^{46}-Asn^{47} bond in the activated receptor is likely to be restricted because the side chain of Arg^{46} seems to interact with the tethered-ligand-binding site [8–10].

Cleavage of the thrombin receptor by other plasma proteases

In comparison with thrombin, a number of other proteases that normally occur in blood plasma cleaved TR$^{38–60}$ relatively slowly; the specificity constants were all at least three orders of magnitude smaller. Factor Xa is responsible for the production of thrombin from prothrombin and its structural features suggested that it might be a potential activator of the receptor. In contrast with thrombin, the active site cleft of factor Xa is rather open, being more like a groove than a canyon [32,33]. It features a hydrophobic S_4 pocket that can accommodate bulky residues, and a hydrophilic S_3 site surrounded by positively changed residues. Despite the presence of an arginine residue at P_1, an aspartic residue at P_3 and a leucine residue at P_4, the thrombin receptor peptide was not cleaved efficiently by factor Xa. However, factor Xa possesses an acidic region at a location similar to the anion-binding exosite of thrombin. This might represent the factor Va-binding site for the formation of the prothrombinase complex [33]. In the TR$^{38–60}$ interaction with factor Xa, this acidic patch in the factor Xa molecule might lead to electrostatic repulsion with the ^{52}YEPFWEDEE^{60} sequence of the thrombin receptor peptide, thus hindering efficient binding and cleavage.

Activation of platelets by plasmin is observed only at high concentrations (more than 0.2 μM) of this protease, whereas lower plasmin concentrations cause a dose- and time-dependent inhibition of platelet aggregation in response to thrombin [34,35]. The proteolytic activity of plasmin is essential in this process [36]. The kinetic parameters obtained for the plasmin cleavage of TR$^{38–60}$ suggest that the dual effects of plasmin on platelets may be due to cleavage of the thrombin receptor. Because of the low specificity constant for plasmin (1700-fold lower than that for thrombin), relatively high concentrations are required for efficient receptor activation. At low concentrations, activation of a limited number of receptors occurs. However, a response to receptor activation (in this case, platelet aggregation) requires activation of a minimum number of receptors. Thus low concentrations of plasmin would not induce aggregation. At the same time, cleavage of the receptors by plasmin could lead to homologous desensitization, such that the platelets become insensitive to activation by thrombin. A similar effect of low concentrations of thrombin has been observed [37,38]. Thus only high concentrations of plasmin would cleave the receptor efficiently, leading to its activation. Such concentrations could be generated locally at the site of clot formation or as a result of the use of plasminogen activators during thrombolytic therapy. This therapy is used to treat coronary thrombosis and is accomplished by infusion of streptokinase, uPA or tPA to activate plasminogen. Plasmin cleaves fibrin to dissolve the thrombus. However, plasmin generated in excess of the capacity of α_2-antiplasmin, the natural inhibitor of the protease, can cause unwanted platelet aggregation after thrombolysis, with resultant reocclusion [39].

The effects of recombinant tPA on platelets are similar to those of plasmin [40]. The results presented here suggest that neither tPA nor uPA is able to cleavage the thrombin receptor. Therefore the platelet effects of thrombolytic agents *in vivo* are likely to result from the plasmin generated by these agents. In contrast with uPA and tPA, plasma kallikrein and APC were able to cleave TR$^{38–60}$ at appreciable rates. However, the low values of the specificity constant for these proteases (approx. 30000-fold lower than that for thrombin) indicate that activation of the thrombin receptor by these proteases is unlikely to occur *in vivo*. This is particularly true of APC because the plasma concentration of protein C is approx. 1/20 of that of prothrombin [41].

Cleavage of the thrombin receptor by pancreatic serine proteases

Trypsin mimics the effects of thrombin on platelets [42–44]. Moreover the time course, magnitude and pertussis-toxin sensitivity of the trypsin response are indistinguishable from those of thrombin in the megakaryocytic cell line HEL [45]. Prior treatment of these cells with trypsin or thrombin precludes a further response to either protease. Trypsin also induces Ca^{2+} mobilization in Jurkat T-cells, and analysis of the dose–response curves of Ca^{2+} mobilization by thrombin and trypsin indicate that trypsin is only 3 to 5 times less potent than thrombin [46]. The results of our kinetic studies suggest that trypsin would be capable of evoking cellular responses by cleavage of the thrombin receptor at the Arg^{41}-Ser^{42} activation site. The specificity constant for trypsin was only 14-fold lower than that of thrombin. However, not all cellular responses to trypsin are due to activation of the thrombin receptor. Trypsin is also capable of activating proteinase activated receptor 2, a recently identified homologue of the thrombin receptor [47].

Chymotrypsin treatment of platelets strongly inhibits thrombin-induced aggregation and release of serotonin [48]. In contrast, there is little difference between the responses of control and chymotrypsin-treated platelets to the receptor agonist peptide SFLLRN [49]. Because chymotrypsin also cleaves glycoprotein Ib on platelets [50], Kinlough-Rathbone et al. [49] proposed two hypotheses to explain the effects of this protease on platelets. Cleavage of glycoprotein Ib by chymotrypsin could remove an interaction that is important for activation of the receptor by thrombin. However, because SFLLRN directly binds to the receptor to effect activation, removal of glycoprotein Ib would not influence SFLLRN-dependent activation. As an alternative, Kinlough-Rathbone et al. [49] proposed that chymotrypsin could cleave the receptor at a site on the C-terminal side of the activation site. This would result in the loss of an essential portion of the tethered ligand, but would not affect the site for the tethered-ligand binding. Our results are consistent with the second hypothesis. Chymotrypsin cleaved TR$^{38–60}$ after Phe^{43}. This cleavage would result in the removal of Ser^{42} and Phe^{43} from the tethered ligand. Interactions with the α-amino group of Ser^{42} and the side chain of Phe^{43} in the tethered ligand are essential for receptor activation [8–10]. The cleavage by chymotrypsin was also relatively specific: no cleavage occurred at residues Tyr^{52}, Phe^{55} or Trp^{56} of the thrombin receptor peptide. However, further proteolysis at other aromatic residues located closer to the first transmembrane domain (Tyr^{69}, Phe^{87} and Tyr^{95}) cannot be excluded. Interestingly, TR$^{38–60}$ was a very good substrate for chymotrypsin with a specificity constant of 1.1×10^6 $M^{-1} \cdot s^{-1}$. This efficient cleavage was mainly due to a low K_m; the affinity

of chymotrypsin for TR^{38-60} was equivalent to that thrombin (Table 1).

Cleavage of the thrombin receptor by proteases from neutrophils and lymphocytes

The proteases elastase and cathepsin G are found in the dense granules of neutrophils and are secreted after activation of neutrophils. Neutrophil elastase cleaves substrates at peptide bonds where the P_1 residue has a small alkyl side chain [51], whereas cathepsin G prefers an aromatic residue in this position [52]. The powerful proteolytic activities of both elastase and cathepsin G are essential for the migration of neutrophils through connective tissue and for the destruction of bacterial invaders. The effects of cathepsin G on a number of cells have been well characterized. Selak [53] has recently proposed that cathepsin G can activate human platelets through the cleavage of its own receptor. However, treatment of endothelial cells with cathepsin G at concentrations achieved *in vivo* inhibits thrombin-induced prostacyclin release and increases in intracellular $[Ca^{2+}]$ [23], suggesting that cathepsin G cleaves and inactivates the thrombin receptor. Results obtained in the present study and those of Molino et al. [54] support the hypothesis that cathepsin G cleaves and inactivates the thrombin receptor. Recently, Molino et al. [54] reported the cleavage of a thrombin receptor peptide by cathepsin G. Analysis of the peptide proteolytic fragments identified three cleavage sites for cathepsin G: Arg^{41}-Ser^{42}, Phe^{43}-Leu^{44} and Phe^{55}-Trp^{56}. Our studies indicate that cleavage at Phe^{55}-Trp^{56} occurs before cleavage at other sites. Despite its chymotrypsin-like activity, cathepsin G cleaved TR^{38-60} at a site different from that of chymotrypsin (Phe^{55}-Trp^{56}, compared with Phe^{43}-Leu^{44} for chymotrypsin). The cleavage by cathepsin G in the sequence Pro-Phe/Trp is consistent with the preference of cathepsin G for large aromatic residues at the P_1 position and proline at P_2 [52]. Because the predominant cathepsin G cleavage site is located on the C-terminal side of the activation site, it will lead to receptor activation and thus would account for the observed effects of cathepsin G on thrombin-induced activation of platelets and endothelial cells. Moreover, the observed specificity constant for TR^{38-60} ($10^4\ M^{-1}\cdot s^{-1}$), coupled with the observation that high concentrations of cathepsin G can be released locally from neutrophils [23], indicates that this potential inactivation of the receptor might be relevant in certain pathological conditions.

The response of several cell lines to neutrophil elastase has also been studied in detail [23,46,53,55]. Although platelets are not activated by elastase, it potentiates cathepsin G-induced platelet activation and inhibits thrombin-induced responses in platelets, endothelial and Jurkat T-cells [23,46,53,55]. If elastase cleaves and inactivates the thrombin receptor on these cells, the site of cleavage must be between residue 61 and the first transmembrane domain; elastase did not cleave between residues 38 and 60.

A number of highly homologous serine proteases termed 'granzymes' (for granule-associated enzymes) have been shown to be expressed by cytotoxic T-lymphocytes and natural killer cells. The cleavage of TR^{38-60} by two granzymes was examined. Granzyme B is found only in the granules of activated cytolytic T cells, natural killer cells and lymphokine-activated killer cells and is thought to play a role in lymphocyte-induced apoptosis of target cells [56]. Although granzyme B exhibits a specificity for aspartic residues, it did not cleave after any of the three aspartic residues in TR^{38-60}. In contrast, Granzyme A cleaved TR^{38-60} at the Arg^{41}-Ser^{42} activation site. Granzyme A exhibits a specificity for arginine and is unusual among serine proteases in that it exists as a disulphide-linked homodimer of molecular mass 60 kDa [57]. In a neuronal cell line and astrocytes, granzyme A causes profound morphological changes that are apparently mediated through activation of the thrombin receptor [58]. The EC_{50} for these effects of granzyme A was, however, 1000-fold higher than that of thrombin in the same assays [25,58]. This low potency of granzyme A is consistent with the observed difference in the specificity constants of both proteases with TR^{38-60}; the value for thrombin was 3000-fold higher than that for granzyme A (Table 1). Although the specificity constant for granzyme A is relatively low ($2\times10^4\ M^{-1}\cdot s^{-1}$), high local concentrations of granzyme A might occur. The concentration of granzyme A in granules has been estimated at 0.1 mM [59]. Thus immediately after degranulation of cytotoxic T-lymphocytes, a high concentration of granzyme A occurs in the microenvironment surrounding the cell. In this context it is interesting to note that cytotoxic T-lymphocytes and natural killer cells, which contain granzyme A, also express the thrombin receptor [60], suggesting a possible autocrine activation of the thrombin receptor in these cells.

Incubation of platelets with up to 230 nM granzyme A does not lead to platelet aggregation but abolishes aggregation in response to a subsequent stimulation with 1 nM thrombin [58]. There are at least three possible explanations for these observations: (1) granzyme A could abolish the response of platelets to thrombin by inactivating the thrombin (granzyme A was still present durin thrombin treatment of platelets); (2) activation of the receptor by suboptimal concentrations of granzyme A could lead to its down-regulation, as has been described for stimulation with suboptimal levels of thrombin [37,38]; or (3) granzyme A could cleave and inactivate a protein such as glycoprotein Ib that is necessary for optimal thrombin-induced platelet aggregation. Incubation of 4 μM thrombin with 0.1 μM granzyme A for up to 15 min did not lead to the degradation of thrombin as judged by SDS/PAGE (results not shown) and thus the first explanation can be rejected. As pointed out above, the specificity constants for granzyme A and thrombin differed by over 10^3-fold, such that 230 nM granzyme A would cleave TR^{38-60} at the same rate as approx. 0.1 nM thrombin. This level of thrombin is somewhat less than is usually required for optimal platelet aggregation, and desensitization of the receptor by cleavage with subliminal levels of granzyme A remains a possible explanation for the observed lack of thrombin response after granzyme A treatment. The third possibility, of granzyme A cleavage and inactivation of other platelet proteins necessary for thrombin-induced aggregation, remains to be tested.

Conclusions

Our results suggest that although the thrombin receptor is cleaved very efficiently by thrombin, other proteases should be capable of activating or inactivating the receptor. The pancreatic proteases trypsin and chymotrypsin cleave the thrombin receptor peptide TR^{38-60} efficiently at sites that would lead to receptor activation and inactivation respectively. Although other proteases cleaved TR^{38-60} more slowly, the results indicated that activation of the receptor by plasmin and granzyme A as well as its inactivation by cathepsin G might occur at physiologically relevant rates.

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