Structural and functional characterization of tissue factor pathway inhibitor following degradation by matrix metalloproteinase-8

Anna C. CUNNINGHAM*, Karen A. HASTY†‡, Jan J. ENGHILD§ and Alan E. MAST*†[1]

*Department of Pathology, The University of Tennessee, Memphis, TN 38163, U.S.A., †Research and Pathology Services, Department of Veterans Affairs, Memphis, TN 38104, U.S.A., ‡Department of Anatomy and Neurobiology, The University of Tennessee, Memphis, TN 38163, U.S.A., and §Department of Molecular and Structural Biology, University of Arhus, Arhus, Denmark

Vascular injury results in the activation of coagulation and the release of proteolytic enzymes from neutrophils and connective-tissue cells. High concentrations of these inflammatory proteinases may destroy blood coagulation proteins, contributing to coagulation and bleeding disorders associated with severe inflammation. Matrix metalloproteinase-8 (MMP-8) is released from neutrophils at sites of inflammation and vascular disease. We have investigated the effect of MMP-8 degradation on the anticoagulant function of tissue factor pathway inhibitor (TFPI) as a potential pathological mechanism contributing to coagulation disorders. MMP-8 cleaves TFPI following Ser^{174} within the connecting region between the second and third Kunitz domains ($k_{cat}/K_m \sim 75\ M^{-1} \cdot s^{-1}$) as well as following Lys^{20} within the NH_2-terminal region. MMP-8 cleavage of TFPI decreases the anticoagulant activity of TFPI in factor Xa initiated clotting assays as well as the ability of TFPI to inhibit factor Xa in amidolytic assays. Yet, MMP-8 cleavage does not alter the ability of TFPI to inhibit trypsin. Since the inhibition of both factor Xa and trypsin is mediated by binding to the second Kunitz domain, these results suggest that regions of TFPI other than the second Kunitz domain may directly interact with factor Xa. ^{125}I-factor Xa ligand blots of TFPI fragments generated following MMP-8 degradation were used for probing binding interactions between factor Xa and regions of TFPI, other than the second Kunitz domain. In experiments performed under reducing conditions that disrupt the Kunitz domain structure, ^{125}I-factor Xa binds to the C-terminal fragment of MMP-8-degraded TFPI. This fragment contains portions of TFPI distal to Ser^{174}, which include the third Kunitz domain and the basic C-terminal region. An altered form of TFPI lacking the third Kunitz domain, but containing the C-terminal region, was used to demonstrate that the C-terminal region directly interacts with factor Xa.

Key words: blood coagulation, neutrophil collagenase, proteolysis.

INTRODUCTION

Tissue factor is an approx. 50 kDa integral membrane protein located on subendothelial fibroblasts and smooth-muscle cells [1,2]. Following vascular injury, tissue factor binds plasma factor VII/VIIa to form a catalytic complex that rapidly converts both factors IX and X from inactive zymogen forms into active serine proteinases, thereby initiating the coagulation cascade. Since tissue factor is the primary protein that initiates blood coagulation *in vivo*, control of its activity is an important determinant in the extent of blood clot formation following vascular injury. The primary inhibitor of the factor VIIa/tissue factor catalytic complex is tissue factor pathway inhibitor (TFPI), a trivalent Kunitz-type serine proteinase inhibitor located on the surface of endothelial cells. The physiological importance of TFPI is most clearly demonstrated in mice lacking TFPI activity, which die *in utero* from disseminated intravascular coagulation [3]. The activity of TFPI is thought to be mediated by simultaneous active-site-directed inhibition of factor VIIa/tissue factor and factor Xa, by the first and second Kunitz domains respectively [4]. Simultaneous inhibition of the two proteinases most likely occurs before the dissociation of the newly activated factor X from the factor VIIa/tissue factor catalytic complex [5]. Although the third Kunitz domain and C-terminal region of TFPI do not directly inhibit proteinase activity, they are required for the effective inhibition by TFPI in both factor Xa and factor VIIa/tissue factor initiated plasma clotting assays [6–9]. Therefore TFPI is susceptible to loss of its anticoagulant activity by limited proteolytic degradation within the regions connecting the Kunitz domains or within the C-terminal region.

Neutrophils and connective-tissue cells release a variety of proteinases within inflammatory loci. These enzymes function to degrade the extracellular matrix, allowing tissue remodelling and wound healing following the injury. This proteolytic activity is tightly regulated by both intra- and extra-vascular proteinase inhibitors. However, in severe inflammatory conditions, the concentrations of proteolytic enzymes within the wound may overwhelm the capacity of the inhibitors present, resulting in an environment in which TFPI may be susceptible to degradation. Matrix metalloproteinase-8 (MMP-8), also called neutrophil collagenase, is a zinc-dependent proteinase stored in the specific granules of neutrophils and released at sites of acute inflammation [10,11]. In addition to being the predominant collagenase present in healing wounds, MMP-8 is also produced by endothelial cells and is present within atherosclerotic lesions [12–14]. Due to its prominent role in inflammatory and vascular diseases, we have investigated the effects of MMP-8 degradation on the anticoagulant function of TFPI as a potential mechanism, which may contribute to the coagulation disorders associated with severe inflammation and atherosclerotic vascular disease.

EXPERIMENTAL

Reagents

The chromogenic factor Xa substrate (methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine *p*-nitroanilide acetate) was pur-

Abbreviations used: HBSA, human BSA; MMP, matrix metalloproteinase; TFPI, tissue factor pathway inhibitor.

[1] To whom correspondence should be addressed, at Research Service-151, VA Hospital, 1030 Jefferson Ave., Memphis, TN 38104, U.S.A. (e-mail alan.mast3@med.va.gov).

chased from American Diagnostica (Greenwich, CT, U.S.A.). The chromogenic trypsin substrate (D-Pro-Phe-Arg-*p*-nitroanilide dihydrochloride) was purchased from Sigma. The fluorogenic peptide substrate for MMP-8 [Dnp-Pro-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(*N*-Me-Abz)-NH_2] was purchased from Bachem (Torrance, CA, U.S.A.). Rabbit brain cephalin was a gift from Marc Goldford (Sigma). Fresh frozen plasma was obtained from the Memphis Veterans Hospital Blood Bank.

Proteins

Recombinant full-length Ala-TFPI produced in *Escherichia coli* was a gift from Chiron Corporation (Emoryville, CA, U.S.A.) and Searle Corporation (Skokie, IL, U.S.A.). TFPI-160, an altered form of TFPI truncated after Gly^{160}, was produced in *E. coli* and purified as described previously [15]. A rabbit polyclonal TFPI C-terminal antibody, recognizing the last 12 amino acids in TFPI, and K1K2C, an altered form of TFPI containing the first two Kunitz domains and the basic C-terminal region, were gifts from Dr George Broze, Jr (Washington University, St. Louis, MO, U.S.A.). The K1K2C form of TFPI contains a Met-Ala-Asp-Ser sequence connected to Glu^{15} at the N-terminus. The protein is truncated at Gly^{150} following the second Kunitz domain, and the basic C-terminal region (amino acids Phe^{243} to Met^{276}) is attached. MMP-8 was purified as described previously [16]. NC-855, an altered form of MMP-8 truncated after nt 855 (amino acid 262) that lacks the C-terminal collagen recognition loop, was synthesized and purified as described previously [17]. Factor Xa was purchased from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Trypsin was purchased from Sigma.

Determination of protein concentrations

Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's instructions. Enzyme samples were compared with a standard curve based on the absorbance (A_{595}) of known concentrations of BSA to approximate protein concentrations.

Equipment

A Storm™ PhosphorImager from Molecular Dynamics (Sunnyvale, CA, U.S.A.) was used to analyse gels of proteolytic fragments of TFPI stained with SYPRO Orange (Bio-Rad) using ImageQuant 5.1 software (Molecular Dynamics). A Spectra MAX Plus spectrophotometer from Molecular Devices Corporation (Sunnyvale, CA, U.S.A.) was used to monitor amidolytic assays. Data were collected and analysed by the Soft Max Pro™ 2.1 software package. An ST-4 clot detection system from Diagnostica Stago (Parsippany, NJ, U.S.A.) was used to monitor coagulation assays.

SDS/PAGE

Proteins were analysed using continuous 5–15 % linear gradient gels in the 2-amino-2-methylpropane-1,3-diol/glycine/HCl buffer system. Before electrophoresis, some samples were mixed with the sample buffer containing 1 % SDS, boiled for 3 min, and were either non-reduced or reduced with 50 mM dithiothreitol as indicated. Proteins were visualized either by Coomassie Blue staining or by SYPRO Orange staining and quantified using the Storm™ PhosphorImager software.

Western-blot analysis

After SDS/PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.) and incubated for 1 h in 3 % (w/v) non-fat milk reconstituted in PBS to block non-specific protein binding. Blots were immunostained with a polyclonal TFPI C-terminal primary antibody, which recognizes the last 12 amino acids of the TFPI protein. Proteins were visualized using ECL® Western-blotting detection reagents (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.).

Protein iodination

Factor Xa was iodinated using Iodobeads® according to instructions provided by the manufacturer (Pierce Chemical Co., Rockford, IL, U.S.A.).

^{125}I-factor Xa ligand blots

After SDS/PAGE under non-reducing or reducing conditions, proteins were transferred to nitrocellulose and incubated with 1–5 μg/ml ^{125}I-factor Xa in 3 % non-fat dry milk for 16 h at 4 °C. The blots were rinsed extensively with 3 % non-fat dry milk over 6–8 h to remove non-specifically bound factor Xa. The nitrocellulose was allowed to dry and autoradiography was performed.

MMP-8 cleavage of TFPI

TFPI (1.8 μmol/l) was incubated with MMP-8 or NC-855 (2.04 μmol/l) in 0.2 mol/l Tris buffer (pH 7.4), containing 0.015 mol/l $CaCl_2$ and 0.2 % sodium azide, 2.5 mol/l glucose and 0.01 mol/l 4-aminophenylmercuric acetate at room temperature for specified times. Cleavage reactions were stopped by the addition of EDTA (10 mM) and placing samples on ice. Samples were analysed by SDS/PAGE.

Amino acid sequencing

TFPI cleaved with MMP-8 for 3 h was subjected to SDS/PAGE to separate the TFPI fragments. After transfering to a polyvinylidene difluoride membrane (Bio-Rad) and staining with Coomassie Blue, automated Edman degradation of the protein fragments was performed to obtain N-terminal amino acid sequences.

Collagenase activity assay

A fluorometric assay was used to monitor the effect of TFPI on NC-855 catalytic activity using the fluorogenic peptide substrate Dnp-Pro-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(*N*-Me-Abz)-NH_2 [18]. The assay buffer comprised 50 mmol/l Tris, 200 mmol/l NaCl, 5 mmol/l $CaCl_2$, 1 μmol/l ZnCl and 0.05 % Brij 35 (pH 7.6). In black polystyrene 96-well microtitre plates (Costar, Corning, NY, U.S.A.), latent NC-855 was activated upon incubation with 1 mmol/l 4-aminophenylmercuric acetate in 100 μl of assay buffer for 30 min at 37 °C. TFPI was added to active NC-855 and incubated for 1 h. This was followed by the addition of freshly prepared 100 μmol/l fluorogenic substrate. Fluorescence was monitored with an excitation wavelength of 365 nm and an emission wavelength of 450 nm for 1 h at 23 °C.

Estimation of k_{cat}/K_m for the cleavage of TFPI by MMP-8

Densitometric scanning of the SDS/PAGE gel containing the proteolytic fragments of TFPI was performed to obtain a crude estimate of the k_{cat}/K_m using the equation $k_{cat}/K_m = \ln 2/([E] \cdot t/2)$ [19], where [E] is the enzyme concentration and $t/2$ is the time for half of the native TFPI to be cleaved into the

23 and 13 kDa fragments. With increasing time, multiple simultaneous cleavages of TFPI occur. Since each cleavage may have independent effects on TFPI inhibitory activity, a more accurate determination of k_{cat}/K_m values was not attempted.

TFPI inhibition of factor Xa

TFPI samples (5 nmol/l) were mixed with factor Xa substrate (0.5 mmol/l) in 50 mmol/l Hepes (pH 7.4), 100 mmol/l NaCl, 5 mmol/l $CaCl_2$, 0.1% human BSA (HBSA) and 5 mmol/l EDTA at room temperature. The reaction was started by the addition of human factor Xa (0.2 nmol/l), and ΔA_{405} was measured for 1 h.

TFPI inhibition of trypsin

Trypsin substrate (166.7 μmol/l) was added to TFPI samples (final concentration, 5 nmol/l) in HBSA plus 5 mmol/l EDTA at room temperature. The reaction was initiated by the addition of trypsin (0.5 nmol/l), and the ΔA_{405} was monitored for 1 h.

Estimation of K_i (initial)

To estimate the affinity of the initial encounter complex between factor Xa and TFPI or cleaved TFPI, progress curves measuring factor Xa inhibition (ΔA_{405}/min) within the first minute of the reaction were analysed [20]. In a microtitre plate, various concentrations of factor Xa substrate (50, 100, 200 and 500 μmol/l) were made in HBSA. TFPI or cleaved TFPI at a final concentration of 5 nmol/l was then added. The reaction was initiated by the addition of factor Xa (0.5 nmol/l), and A_{405} was continuously recorded. An Eadie–Hofstee plot of the velocity (ΔA_{405}/min) divided by the concentration of factor Xa substrate versus the velocity yielded $K_{m(apparent)}$. K_i (initial) values were then determined by the equation $K_m[I]/(K_{m(apparent)} - K_m)$, where [I] is the initial concentration of the inhibitor present in the reaction. K_m was determined independently to be 85.6 μM for factor Xa and the factor Xa substrate.

Determination of K_i (final)

The K_i (final) was determined by analysing progress curves [21]. Various final concentrations of TFPI or MMP-8-cleaved TFPI (0.5, 1, 2 and 4 nmol/l) were added to the factor Xa substrate (500 nmol/l). The reaction was initiated by the addition of factor Xa (0.4 nmol/l) and was monitored until the ΔA_{405}/min was linear. $K_{i(apparent)}$ is defined as the reciprocal of the slope of the line generated by a plot of $V_o/(V_i - 1)$ versus concentration of the inhibitor, where V_o is the velocity of the reaction in the absence of the inhibitor and V_i is the velocity of the reaction in the presence of the inhibitor. These values were determined by calculating the slopes of the progress curves at equilibrium. K_i (final) is calculated by correcting for the concentration of the substrate using the equation $K_{i(apparent)}/[1 + ([S]/K_m)]$, where [S] is the final concentration of the substrate used in the reaction. K_m for factor Xa and substrate was determined independently as described above.

Factor Xa initiated plasma coagulation assays

In a cuvette, 50 μl of rabbit brain cephalin, prepared as described by the manufacturer (Sigma) and diluted 10 times into HBSA, 50 μl $CaCl_2$ (5 mmol/l) and 50 μl of factor Xa (0.2 nmol/l) were incubated together at 37 °C. A 50/50 mixture (100 μl) of TFPI sample (at various concentrations) and normal human pooled plasma were incubated together for 5 min at room temperature. The clotting reaction is initiated after the addition of the TFPI/plasma mixture to the cuvette. The degree of apparent factor Xa inhibition is determined by comparing the clotting time with a standard curve generated by performing the assay at different concentrations of factor Xa without the inhibitor. The apparent factor Xa inhibitory activity for cleaved TFPI samples was then normalized to full-length TFPI and displayed as the percentage of full-length TFPI inhibitory activity.

RESULTS

Cleavage of TFPI by MMP-8

Progressive limited proteolysis of TFPI occurs during incubation with MMP-8 at 23 °C. SDS/PAGE analysis of the TFPI cleavage products reveals an initial cleavage producing 23 and 13 kDa fragments (Figure 1). Subsequent cleavages follow, which reduce the size of both native TFPI and the 23 kDa fragment by 2 kDa, whereas the 13 kDa fragment is not altered (Figure 1). The N-terminal amino acid sequence of the 23 kDa band is that of native Ala-TFPI (ADSEEDEE), whereas that of the 13 kDa band is TKVPSLFE, indicating that the first cleavage occurs between Ser^{174} and Thr^{175} within the region connecting the second and third Kunitz domains (Figure 2). N-terminal amino acid sequence analysis of the 21 kDa fragment is LMHSFC, indicating that MMP-8 also cleaves between Lys^{20} and Leu^{21}, within the N-terminal region of TFPI. Since the C-terminal region of TFPI has been proven to be susceptible to proteolysis in tissue culture and by other MMPs [8,22], Western-blot analysis using an antibody directed against the final 12 amino acids (254–276) of the C-terminus was performed. This antibody reacted strongly with native TFPI, Lys^{20}-cleaved TFPI (34 kDa) and the 13 kDa fragment, but not with the 23 kDa or the 21 kDa fragments (Figure 3). Importantly, MMP-8 does not appear to degrade the C-terminal region of TFPI; thus, the 13 kDa fragment is intact from Thr^{175} to the C-terminus end of native TFPI. When TFPI is allowed to incubate with MMP-8 for 24 h, additional low-molecular-mass bands are generated, indicating that MMP-8 slowly further degrades TFPI (Figure 1).

To determine whether the C-terminal region of MMP-8, which is necessary for its activity against native collagen, plays a role in the recognition of the TFPI substrate, the time-course expermiments were repeated with a truncated form of MMP-8,

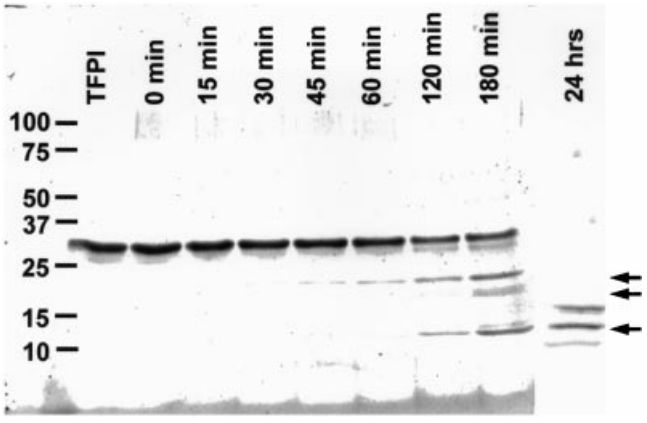

Figure 1 TFPI cleavage by MMP-8

TFPI was incubated with MMP-8 at 23 °C for the indicated times. Following SDS/PAGE, proteins were quantified with SYPRO Orange using the Storm PhosphorImager. Arrows indicate the 23, 21 and 13 kDa peptides subjected to amino acid sequence analysis.

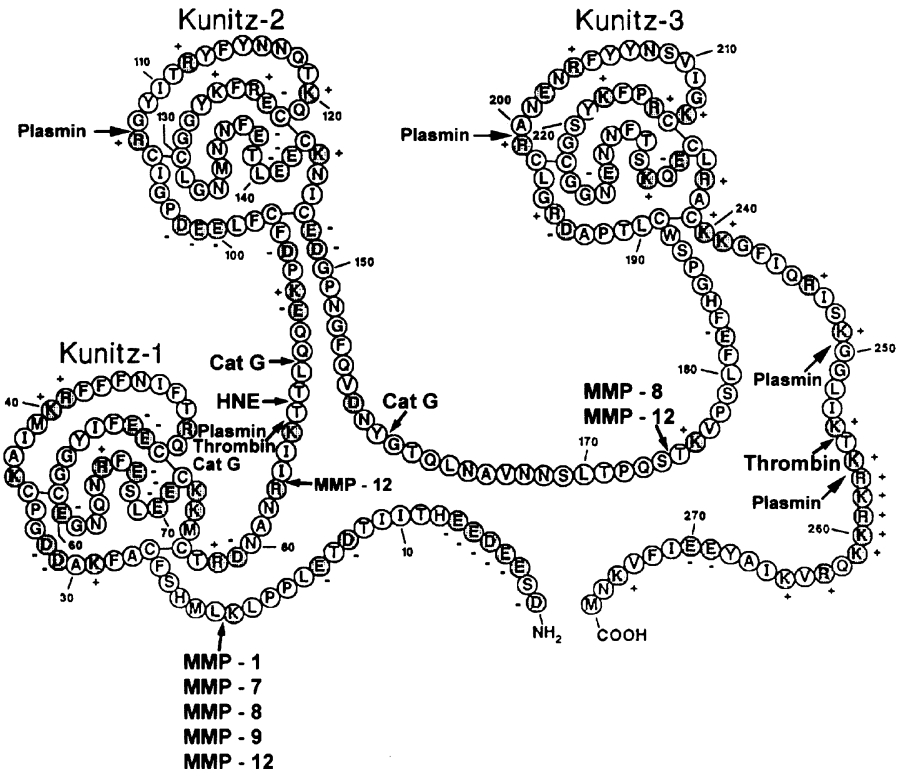

Figure 2 Sites within TFPI susceptible to proteolytic degradation

Cleavage sites were determined through NH_2-terminal amino acid sequencing of peptides purified by SDS/PAGE. Sites of cleavage by MMP-8 and other proteinases, including MMP-1, -7, -9, -12 [22], human neutrophil elastase (HNE) [20], thrombin [34], plasmin [35] and cathepsin G (Cat G) [36] are denoted by arrows on the diagram of the primary amino acid sequence and Kunitz domain structure.

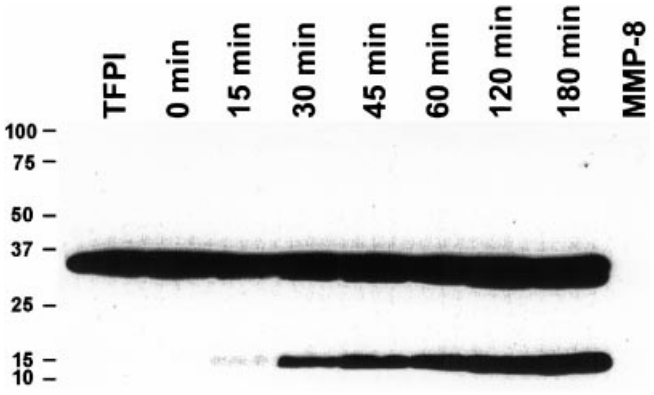

Figure 3 Effect of MMP-8 on the C-terminal region of TFPI

A time course of MMP-8 cleavage of TFPI was subjected to Western-blot analysis using an antibody recognizing the final 12 amino acids of the C-terminus of TFPI. Binding of the C-terminal antibody to the 13 kDa band reveals that the TFPI C-terminus remains intact after cleavage at Ser^{174}.

NC-855, which lacks this collagen-binding region [17]. In these experiments, NC-855 degraded TFPI in a manner identical with that of full-length MMP-8, indicating that the collagen recognition region is not required for cleavage of TFPI by MMP-8 (results not shown). Since both forms of MMP-8 cleave TFPI identically, they were used interchangeably in experiments. The Figure legends indicate the form of MMP-8 used in the results presented.

To determine the apparent rate of MMP-8 proteolytic cleavage at Ser^{174} of TFPI, k_{cat}/K_m was estimated by densitometric scanning of SDS/PAGE experiments, similar to that shown in Figure 1. These gels demonstrated that one-half of the TFPI is cleaved following Ser^{174} in 75.6 min in the presence of 2.04 μmol/l NC-855. When calculated as described in the Experimental section, k_{cat}/K_m for cleavage following Ser^{174} of TFPI by NC-855 is estimated to be approx. 75 $M^{-1} \cdot s^{-1}$. It is quite probable that this value represents a low estimate of the true rate of cleavage at this site, because MMP-8 also simultaneously cleaves TFPI following Lys^{20}.

Effect of MMP-8 proteolysis on TFPI inhibitory activity

Progress curves measuring the cleavage of an amidolytic substrate by factor Xa in the presence of TFPI degraded by MMP-8 for various times demonstrate that MMP-8 degradation causes a progressive loss of factor Xa inhibitory activity over 24 h (Figure 4A). To quantify the loss of activity, inhibition constants were determined for native TFPI and TFPI cleaved for 24 h with MMP-8. TFPI is a slow tight-binding inhibitor of factor Xa. As such, it forms an immediate encounter complex with factor Xa, which then slowly isomerizes to a final tightened complex [23]. The affinity of the initial encounter complex between factor Xa and MMP-8-cleaved TFPI is reduced for K_i(initial) increasing from 8 to 91 nM. The affinity of the final isomerized complex is also reduced for K_i(final) increasing from 68 to 950 pM. To

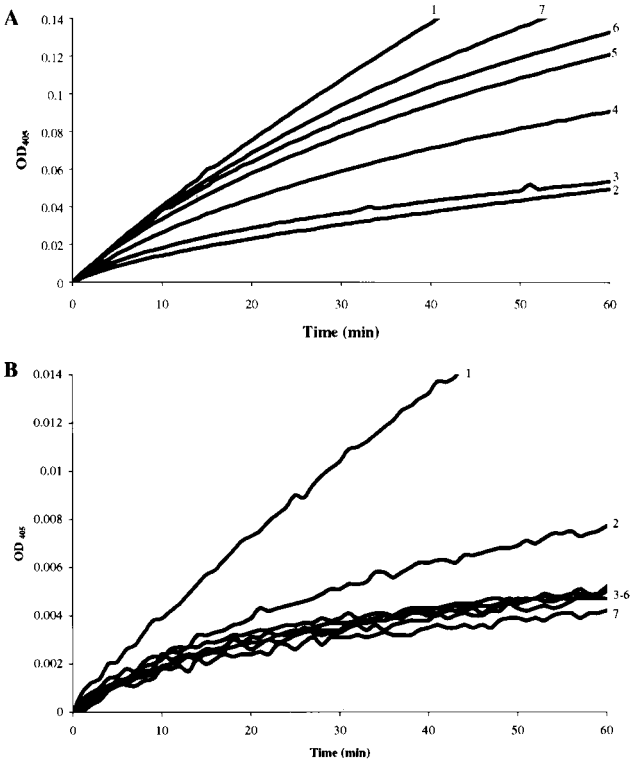

Figure 4 Inhibition of factor Xa or trypsin by TFPI following MMP-8 cleavage

(**A**) Cleavage of 500 μmol/l factor Xa substrate is monitored continuously in reactions initiated with 0.5 nmol/l factor Xa, analysing the effects of 5 nmol/l TFPI or TFPI cleaved by 2.04 μmol/l NC-855 at the indicated time points. Samples are as follows: 1, no inhibitor; 2, TFPI; 3, 1 h cleavage; 4, 2 h cleavage; 5, 3 h cleavage; 6, 6 h cleavage; 7, 24 h cleavage. (**B**) Cleavage of 166.7 μmol/l trypsin substrate is monitored continuously in reactions initiated with 0.5 nmol/l trypsin, analysing the effects of 5 nmol/l TFPI or TFPI cleaved by 2.04 μmol/l NC-855 at the indicated time points. Samples are as follows: 1, no inhibitor; 2, TFPI; 3, 1 h cleavage; 4, 2 h cleavage; 5, 3 h cleavage; 6, 6 h cleavage; 7, 24 h cleavage.

Table 1 Inhibition of factor Xa initiated clotting assay following cleavage of TFPI by MMP-8

Inhibitor (5 nmol/l)	TFPI inhibitory activity (%)
Full-length TFPI	100
TFPI cleaved 1 h	47.2 $\pm$ 8.1
TFPI cleaved 2 h	50.4 $\pm$ 2.15
TFPI cleaved 3 h	19.2 $\pm$ 1.3
TFPI cleaved 6 h	28.2 $\pm$ 3.8
TFPI cleaved 24 h	15.5 $\pm$ 0.3

assess potential physiological effects of this loss of activity, the anticoagulant activity of the same TFPI samples was examined using factor Xa-initiated plasma clotting assays. In the clot-based assays, the apparent inhibition of factor Xa by MMP-8-cleaved TFPI appears to occur in two steps, with about half of the activity lost at 1 h and the remainder lost at 3 h (Table 1). Experimental variability probably accounts for the increase in TFPI activity observed at 6 h, as the values presented in Table 1 represent the average and standard deviation of a sample from a single time point repeated four times. This increase was not consistently reproducible in other time-course experiments performed. In contrast with factor Xa, when these same samples were used to measure the ability of TFPI to inhibit trypsin, the inhibitory activity slightly increases as TFPI is progressively degraded by MMP-8. Full trypsin inhibitory activity remains even after 24 h of degradation, which abrogates essentially all of the anti-factor Xa activity (Figure 4B). Since trypsin is inhibited primarily by the second Kunitz domain, these results indicate that this domain remains intact and is capable of proteinase inhibition after TFPI has been fully degraded by MMP-8.

TFPI does not inhibit MMP-8 catalytic activity

To determine if TFPI inhibits MMP-8 activity in addition to being degraded, increasing concentrations of TFPI were added to MMP-8 and the fluorogenic peptide substrate Dnp-Pro-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH_2. MMP-8 cleavage of the fluorogenic substrate was not altered by concentrations of TFPI up to 900 nmol/l (results not shown), indicating that TFPI is not an inhibitor of MMP-8 catalytic activity.

Binding interactions between factor Xa and regions of TFPI other than the second Kunitz domain

The inhibition of trypsin, but not factor Xa, by MMP-8-cleaved TFPI demonstrates that effective inhibition of factor Xa by full-length TFPI does not occur through the independent action of the second Kunitz domain. Other regions of TFPI are required and may act by maintaining the second Kunitz domain in a conformation that optimally binds factor Xa or by providing secondary binding sites for factor Xa. TFPI fragments produced by MMP-8 were probed using ^{125}I-factor Xa ligand blots under reducing and non-reducing conditions to determine whether regions other than the second Kunitz domain directly bind factor Xa. Under non-reducing conditions, ^{125}I-factor Xa binds avidly to TFPI fragments that contain the second Kunitz domain (Figure 5A). In addition, ^{125}I-factor Xa binds to a high-molecular-mass (approx. 200 kDa) heterogeneous band, which is not present under non-reducing conditions. This probably represents non-specific aggregates of TFPI and MMP-8. When the samples are reduced, ^{125}I-factor Xa no longer binds to TFPI fragments containing the second Kunitz domain, demonstrating that the disulphide-defined structure of the reactive site loop is required for binding to factor Xa. However, binding of ^{125}I-factor Xa to the 13 kDa C-terminal TFPI fragment, which only contains the third Kunitz domain and C-terminal region, becomes apparent following prolonged exposure (approx. 2 weeks) of the ligand blot (Figure 5A). Since this binding occurs under reducing conditions, the binding is not dependent on the presence of an intact reactive site loop in the third Kunitz domain. To determine whether ^{125}I-factor Xa binds to the third Kunitz domain or the C-terminal region of the 13 kDa TFPI fragment, ligand blots were performed using TFPI-160, a truncated form of TFPI containing the first two Kunitz domains, and K1K2C, an altered form of TFPI containing only the first two Kunitz domains and the basic C-terminal region (Figure 5B). Since both altered forms of TFPI contain the second Kunitz domain, ^{125}I-factor Xa binds tightly under non-reducing conditions. However, under reducing conditions, binding to K1K2C, but not TFPI-160, is observed, signifying that the C-terminal region of TFPI directly interacts with factor Xa.

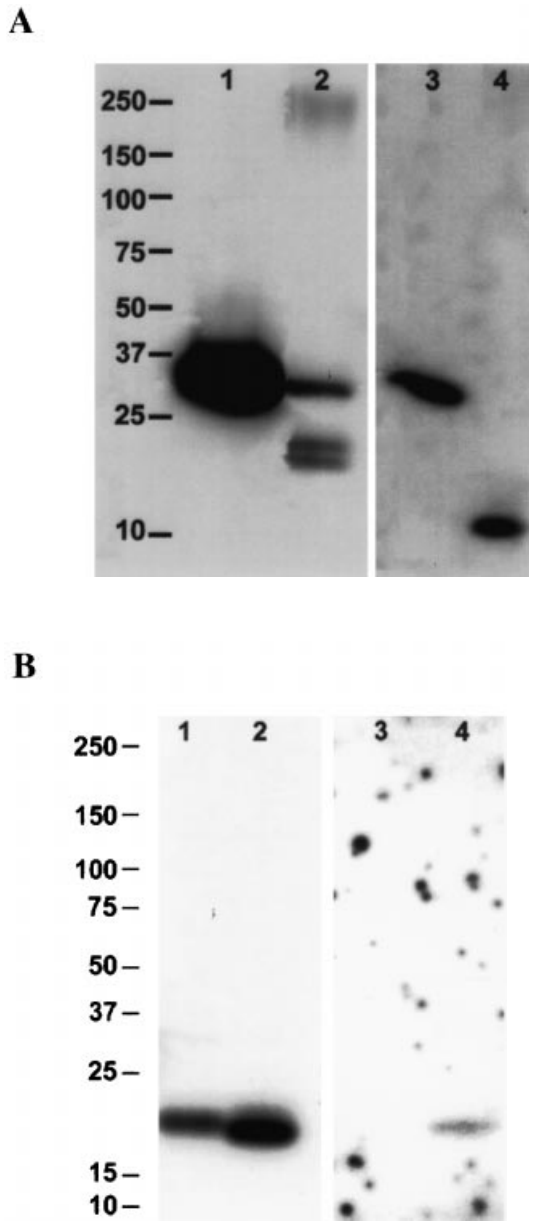

Figure 5 ^{125}I-factor Xa binds to the C-terminal 13 kDa fragment of TFPI in a manner not dependent on an intact Kunitz domain structure

(**A**) Non-reducing conditions: ^{125}I-factor Xa binds to full-length TFPI (lane 1) and to TFPI fragments, containing the second Kunitz domain in the MMP-8-cleaved TFPI sample (lane 2). Reducing conditions: prolonged exposure of the autoradiogram reveals binding of ^{125}I-factor Xa to full-length TFPI (lane 3) and to the 13 kDa C-terminal fragment of TFPI that contains only the third Kunitz domain and C-terminal region (lane 4). (**B**) Non-reducing conditions: ^{125}I-factor Xa binds to both TFPI-160 (lane 1) and K1K2C (lane 2). Reducing conditions: ^{125}I-factor Xa does not bind to TFPI-160 (lane 3), but does bind to K1K2C (lane 4).

DISCUSSION

The prototypical Kunitz-type proteinase inhibitor is bovine pancreatic trypsin inhibitor (aprotinin), a 58-amino-acid protein containing three disulphide bonds, which structurally stabilize the reactive-site loop region of the inhibitor for binding within the active site of a target proteinase. TFPI consists of three Kunitz-type serine proteinase inhibitory domains connected in tandem by respective stretches of 20 and 41 amino acids [24]. The connecting regions of TFPI, as well as the acidic N-terminal and basic C-terminal regions, are susceptible to limited proteolytic degradation by several proteinases (Figure 2). Our results show that MMP-8 proteolysis separates the second and third Kunitz domains and greatly reduces the anticoagulant activity of TFPI, even though the reactive-site loops of the individual Kunitz domains remain intact. Therefore this mechanism for the catalytic inactivation of TFPI is distinct from that of the serpin-type serine proteinase inhibitors, which are susceptible to inactivation through proteolytic cleavage directly within the reactive-site loop region by proteinases that they do not inhibit [25].

MMP-8 is a member of a family of closely related enzymes that degrade the extracellullar matrix. The family members contain similar structural domains, yet they have distinct specificity for different matrix components [17,26,27]. MMP-8 has a C-terminal disulphide-defined loop that is required for collagenolytic activity, but not for catalytic activity against more general substrates, such as casein [17]. MMP-8 preferentially cleaves TFPI following Ser^{174} in the connecting region between the second and third Kunitz domains as well as following Lys^{20} within the N-terminal region of TFPI, a site that is susceptible to cleavage by several other MMPs [22]. NC-855, a truncated form of MMP-8 lacking the C-terminal loop, cleaves TFPI in a pattern identical with the full-length enzyme. Thus the cleavage sites in TFPI are recognized directly by the catalytic region of MMP-8 without the extended recognition-site interactions that are necessary for collagenolytic activity. The finding that TFPI is a general catalytic substrate for MMP-8 is consistent with it having a pattern of proteolytic degradation very similar to that of MMP-12 (macrophage elastase), which also cleaves TFPI following Ser^{174} and Lys^{20}. However, MMP-12 also readily cleaves following Arg^{83} within the connecting region between the first and second Kunitz domains, a cleavage site that was not identified for MMP-8 [22]. It has been reported that TFPI-2, a trivalent Kunitz-type inhibitor similar to TFPI, is an inhibitor of several metalloproteinases (MMP-1, -2, -9 and -13) [14]. Therefore we considered the possibility that in addition to being degraded by MMP-8, TFPI also directly inhibits MMP-8 activity. In experiments using a fluorogenic peptide substrate for MMP-8, TFPI did not inhibit the rate of substrate cleavage, even at a concentration of 900 nM, indicating that TFPI is not an effective inhibitor of MMP-8 activity.

The cleavage of TFPI by MMP-8 rapidly decreases its anticoagulant activity in factor Xa initiated clotting assays and decreases its ability to inhibit factor Xa in amidolytic assays. The loss of anticoagulant activity occurs more quickly than the loss of direct factor Xa inhibitory activity (cf. Figure 4A and Table 1), suggesting that the 13 kDa cleavage fragment, which includes the third Kunitz domain and the basic C-terminal region, may be inhibiting the anticoagulant activity of the remaining fully intact TFPI. The C-terminal region does not directly inhibit factor Xa catalytic activity, but studies using truncated forms of TFPI have demonstrated that it is required for optimal inhibition of factor Xa by the second Kunitz domain [6,8]. Therefore the 13 kDa fragment may prevent TFPI anticoagulant activity by blocking interactions between the C-terminal region of full-length TFPI and factor Xa.

MMP-8 cleavage slightly increases the ability of TFPI to inhibit trypsin in amidolytic assays. The strikingly different effects of MMP-8 cleavage on the ability of TFPI to inhibit factor Xa and trypsin clearly demonstrate that extended macromolecular interactions involving the third Kunitz domain or C-terminal region, both components of the 13 kDa fragment, and non-active-site regions of factor Xa occur during the rapid inhibition of factor Xa by TFPI. However, these interactions are

not necessary for inhibition of trypsin. Loss of factor Xa, but not trypsin, inhibitory activity also has been observed when TFPI is cleaved by human neutrophil elastase following Thr^{87} between the first and second Kunitz domains [20]. Thus regions, both proximal and distal to the second Kunitz domain of TFPI, are required for the effective inhibition of factor Xa, either through direct binding interactions with factor Xa and/or by maintaining the second Kunitz domain in a conformation necessary for binding to factor Xa.

^{125}I-factor Xa ligand blots of MMP-8-cleaved TFPI were used to probe potential direct binding interactions between factor Xa and regions of TFPI other than the second Kunitz domain. These experiments demonstrate direct binding between the 13 kDa fragment of MMP-8-cleaved TFPI and factor Xa. Since the binding occurs under reducing conditions, these data indicate that factor Xa binds either to the C-terminal region or to the third Kunitz domain in a manner that is not dependent on the presence of an intact, disulphide bonded, reactive site loop within the third Kunitz domain. To define further which region of TFPI is binding to factor Xa, ligand blots were performed using two altered forms of TFPI, TFPI-160 and K1K2C. Both of these forms of TFPI lack the third Kunitz domain, but K1K2C has the C-terminal region attached to the end of the second Kunitz domain, whereas TFPI-160 does not. In these experiments, factor Xa bound to K1K2C, but not to TFPI-160, under reducing conditions, indicating that factor Xa directly binds to the C-terminal region of TFPI. We have previously demonstrated that an interaction between the Gla domain of factor Xa and the C-terminal region of TFPI is necessary for rapid inhibition of factor Xa by TFPI in amidolytic assays [6]. Therefore it is probable that the binding interaction observed here occurs through association of the basic C-terminal residues of TFPI with the acidic residues within the Gla domain of factor Xa. Binding to the 23 kDa fragment of MMP-8-cleaved TFPI, which contains the first and second Kunitz domains, was not observed under reducing conditions, suggesting that the first Kunitz domain may function in the inhibition of factor Xa by maintaining the second Kunitz domain in an optimal inhibitory conformation rather than directly binding to a factor Xa exosite.

Inactivation of TFPI by proteolysis within the connecting regions between the Kunitz domains is a mechanism capable of generating local procoagulant environments. The probability of physiologically significant amounts of TFPI being inactivated by MMP-8 is dependent upon the concentrations of MMP-8 and its controlling inhibitors at a local site and the rate at which TFPI is cleaved by MMP-8. The k_{cat}/K_m for the cleavage of TFPI following Ser^{174} is estimated to be approx. $75\ M^{-1} \cdot s^{-1}$. Whereas this rate is slower than the rate at which MMP-8 cleaves human type I collagen ($k_{cat}/K_m \sim 2538\ M^{-1} \cdot s^{-1}$), it is comparable with the rate for human type III collagen cleavage ($k_{cat}/K_m \sim 130\ M^{-1} \cdot s^{-1}$) [16]. Although these rates for cleavage of proteinase inhibitor substrates by the MMPs are relatively slow, it has been shown that cleavage and inactivation of α_1-proteinase inhibitor, a major inhibitor of neutrophil elastase by MMP-9, is a critical pathological event in the neutrophil elastase-mediated development of skin blisters in a mouse model of the autoimmune disease bullous pemphigoid [28]. In addition, the results presented here indicate that partial degradation of TFPI generates fragments of TFPI that can inhibit the anticoagulant activity of the remaining intact TFPI. Thus the cleavage of TFPI by MMP-8 within specific locations may contribute either to normal physiology or to the development of disease. MMP-8 is a predominant collagenase in healing wounds with peak concentrations occurring at day 4 after injury in a human wound model [12]. It is reasonable to hypothesize that cleavage of TFPI by MMP-8 may result in unregulated tissue factor activity within a wound and thereby contribute to the coagulation disorders associated with severe inflammation. In addition, tissue factor [29], TFPI [30] and MMP-8 [31] have been immunolocalized within atherosclerotic plaques, and it has been hypothesized that MMPs degrade the fibrous structure of the plaque, making it more prone to rupture [32,33]. Cleavage of TFPI by MMP-8, as well as by other MMPs, such as MMP-1, -7, -9 and -12 [22], within the plaque would decrease its ability to abrogate plaque tissue factor activity, resulting in a highly thrombogenic surface being exposed to flowing blood following plaque rupture and increased risk for the formation of an occlusive vascular thrombosis. The studies described here were performed using recombinant TFPI in an *in vitro* system. It is difficult to predict accurately *in vivo* sites where proteolytic degradation of endogenous TFPI may play a pathological role due to a lack of knowledge of the concentrations of different proteinases and their controlling inhibitors. However, the susceptibility of the regions connecting the Kunitz domains to limited proteolytic degradation with the resultant loss of anticoagulant activity suggests that it does occur and may contribute to the development of coagulation disorders.

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