

# Factor Xa Cleavage of Tissue Factor Pathway Inhibitor Is Associated with Loss of Anticoagulant Activity\*

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## Summary

Tissue factor : factor VIIa induced activation of blood coagulation is inhibited by the complex between factor Xa and tissue factor pathway inhibitor (factor Xa : TFPI). We recently reported that phospholipid-bound factor Xa reduces the high binding affinity of factor Xa : TFPI for negatively charged phospholipids by a partial degradation of TFPI (17). The present study was undertaken to elucidate the factor Xa cleavage sites in TFPI and to delineate the consequences of this proteolysis with respect to the inhibitory activity of factor Xa : TFPI. We found that phospholipid-bound factor Xa cleaves in TFPI the peptide bonds between Lys<sup>86</sup>-Thr<sup>87</sup> and Arg<sup>199</sup>-Ala<sup>200</sup>. Interestingly, Arg<sup>199</sup> is the P1 residue of the third Kunitz-type protease inhibitor domain. The fast cleavage of the Arg<sup>199</sup>-Ala<sup>200</sup> bond results in a 50-70% reduction of the anticoagulant activity of factor Xa : TFPI, as determined with a dilute tissue factor assay, but is not associated with a diminished inhibitory activity of factor Xa : TFPI towards TF : factor VIIa catalyzed activation of factor X. On the other hand, the slower cleavage of the Lys<sup>86</sup>-Thr<sup>87</sup> peptide bond was associated with both a diminished anticoagulant and anti-TF : factor VIIa activity. Dissociation of factor Xa from the cleaved TFPI was not observed. These data provide evidence for a dual role of factor Xa since it is the essential cofactor in the TFPI-controlled regulation of TF-dependent coagulation as well as a catalyst of the inactivation of TFPI.

## Introduction

Tissue factor pathway inhibitor (TFPI) is the main regulator of tissue factor induced blood coagulation (1, 2). TFPI inhibits the generation of factor Xa and factor IXa by the tissue factor (TF) : factor VIIa complex in a unique manner (3). First, TFPI binds to factor Xa and then in a second step, a quaternary complex between factor Xa : TFPI and TF : factor VIIa is formed, thereby neutralizing the proteolytic activity of the TF : factor VIIa complex.

TFPI is a plasma protein that circulates predominantly in complex with lipoproteins at a concentration of 2-3 nmol/l (4). The mature molecule consists of 276 amino acid residues, including 18 cysteines that are all involved in disulfide bonds (5). Post-translational modifica-

tions such as glycosylation and phosphorylation seem to be of minor importance for its inhibitory functions, since recombinant TFPI produced in *Escherichia coli* is indistinguishable from recombinant TFPI produced by mammalian cell lines (6). TFPI is a member of the Kunitz family of serine protease inhibitors consisting of three tandemly repeated Kunitz-type inhibition domains (each containing 6 Cys residues) flanked by an acidic N-terminus and a basic C-terminal tail (5). Using site directed mutagenesis (7), it was demonstrated that the Kunitz-1 domain is essential for the interaction with factor VIIa and that the Kunitz-2 domain interacts with the active site of factor Xa. No inhibitory function could be attributed to the Kunitz-3 domain (8). However, this domain was shown to be involved in the interaction of TFPI with heparin (9) and lipoproteins (10).

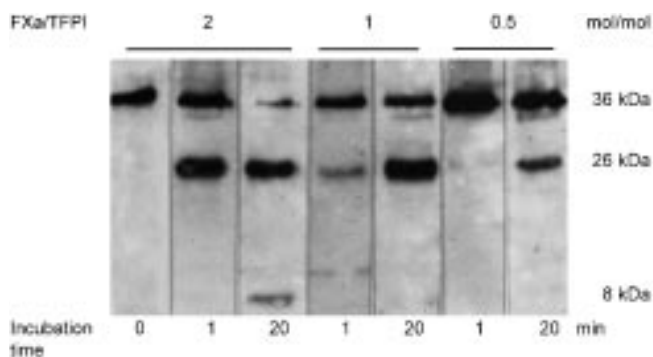
Various data suggest that the basic C-terminus is essential to the anticoagulant activity of TFPI. TFPI without this positively charged C-terminus (TFPI<sub>1-250</sub>) has been reported to have only 1% of the anticoagulant effect of full length TFPI when assayed in a dilute thromboplastin prothrombin time assay (11). Also, studies on the interaction between factor Xa and TFPI showed that the C-terminus of TFPI is required for optimal inhibition of factor Xa (6, 12). Previous studies from our laboratory demonstrated that the rate of association of factor Xa with TFPI was 10-fold higher than with TFPI lacking the C-terminus (13). Furthermore, we showed that negatively charged lipid membranes accelerated the rate of association of factor Xa with TFPI but not the association with the C-terminal truncated variants TFPI<sub>1-250</sub> and TFPI<sub>1-161</sub> (14). This enhanced rate of formation of the factor Xa : TFPI complex results in an accelerated rate of inhibition of the TF : factor VIIa catalyzed activation of factor X. It should however be mentioned that another study, in which factor Xa was used in excess over TFPI, showed no effect of lipids on the rate of factor Xa : TFPI association (15).

Valentin and Schousboe (16) showed that TFPI binds to anionic phospholipids presumably via electrostatic interactions. We observed minor binding for TFPI but found that the affinity of factor Xa for a planar phospholipid bilayer composed of 25 mol% dioleoylphosphatidylserine and 75 mol% dioleoylphosphatidylcholine was greatly enhanced when complexed with TFPI (17), but not with C-terminus truncated TFPI. In the same study we showed that in the presence of an excess factor Xa, factor Xa : TFPI complex shows a transient high affinity binding to negatively charged phospholipid membranes. This loss in binding affinity appeared to be associated with factor Xa mediated cleavage of TFPI in complex with factor Xa.

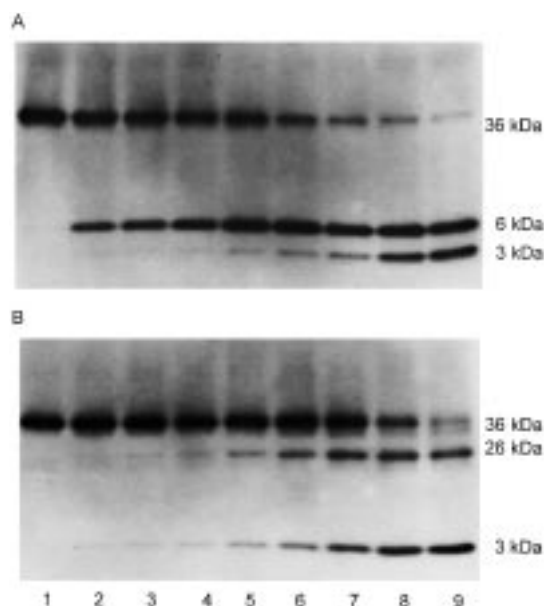
Proteolytic cleavage of TFPI has been reported earlier for human leukocyte elastase (HLE) (18, 19) and cathepsin G (19). The cleavage of TFPI by factor Xa is, however, of particular interest, because factor Xa is the only protease that under physiological conditions interacts with TFPI to form the inhibitor of its own activating complex (TF : factor VIIa). We, therefore, investigated the functional consequences of

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**Fig. 1** Cleavage of TFPI as a function of free factor Xa concentration. TFPI (6 nmol/l) was incubated with 12, 6 or 3 nmol/l factor Xa in the presence of PSPC vesicles (10 μmol/l). At timed intervals (1 and 20 min) aliquots were removed from the incubation mixtures and the reduced samples were subjected to SDS-PAGE (12%) and Western blotting using a goat anti-human TFPI<sub>1-161</sub> antibody



**Fig. 2** Progress of factor Xa-catalyzed degradation of TFPI visualized with an antibody against TFPI<sub>254-276</sub>. TFPI (50 nmol/l) was incubated with Factor Xa (75 nmol/l) for 10 min at 37° C. Timed samples were taken after phospholipids were added to a final concentration of 5 μmol/l and subjected to gradient SDS-PAGE (4-20%) and Western blotting using an antibody against the C-terminus of TFPI. Samples were taken at 0, 0.5, 1, 2, 5, 10, 20, 40, and 60 min (lanes 1- 9). A, reduced samples and B, unreduced samples

factor Xa mediated proteolysis of TFPI with respect to the inhibition of blood coagulation in plasma, inhibition of TF : factor VIIa activation of factor X and inhibition of factor Xa activity. Furthermore, we identified the two cleavage sites in TFPI associated with its inactivation by using immunoblot analysis and N-terminal sequence analysis of the cleavage products.

**Materials and Methods**

**Materials.** Bovine serum albumin (BSA, essentially fatty acid free) was obtained from Sigma (St. Louis, MO). The chromogenic substrate for factor Xa, S2765, was purchased from Chromogenix (Mölnådal, Sweden). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) were obtained from Avanti Polar Lipids (Alabaster, AL). The plasma used was citrated (13 mmol/l) pooled human platelet free plasma. Electrophoresis reagents were from Bio-Rad (Richmond, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagents and Hyperfilm™-ECL were provided by Amersham Life Science (Buckinghamshire, England). Nitrocellulose transfer membrane was obtained from Protran (Dassel, Germany). Immobilon-P membranes were purchased from Millipore (Bedford, MA). All other reagents used were of analytical grade.

**Proteins.** Human factor Xa was prepared by activation of purified factor X (20) with the factor X activating protein from Russell’s Viper venom (Sigma, St. Louis, MO) and isolated as was described for bovine factor Xa (21). The molar concentration was determined by active site titration with p-nitrophenyl p-guanidinobenzoate hydrochloride (pNPGb) (22). Recombinant human tissue factor pathway inhibitor (TFPI), produced in *Escherichia coli* (23), was kindly supplied by Searle/Chiron (Chesterfield, MO). This recombinant TFPI preparation, not being glycosylated, is very similar to the native highly glycosylated protein expressed in mammalian cells with respect to anticoagulant activity (6, 23). Truncated TFPI preparations were kindly supplied by Dr. Ole Nordfang (Novo Nordisk, Bagsvaerd, Denmark). The C-terminal truncated variant of TFPI lacking only the C-terminal basic region (TFPI1-250) was obtained from transfected baby-hamster kidney cells as described previously (24) and was isolated by cation-exchange chromatography (11). This preparation of truncated TFPI consists of a mixture of molecules terminated at amino acid Ile-247, Ser-248, Gly-250 or Thr-255 (11). The C-terminal truncated variant of TFPI lacking the third Kunitz-type domain and the C-terminal tail (TFPI<sub>1-161</sub>) was expressed in *Saccharomyces cerevisiae* and purified as described previously (25). The molar concentrations of the TFPI preparations were determined by titration with known amounts of factor Xa (13). Recombinant human tissue factor thromboplastin (Dade Innovin) was purchased from Baxter Diagnostics (Deerfield, IL). The lyophilized powder was dissolved according to the manufacturer’s description. Aliquots were stored at -80° C. Recombinant human tissue factor was a kind gift of Dr. Yale Nemerson (Mount Sinai School of Medicine, New York, NY) and relipidated as described (14). Recombinant factor VIIa was a kind gift of Dr. Ulla Hedner (Novo Nordisk,

Cycle	Phenylthiohydantoin amino acid (pmol)													
	26 kDa fragment			8 kDa fragment			20 kDa fragment			30 kDa fragment				
1	(Met)	2.8		Thr	3.2		Thr	5.8	Ala	7.8	(Met)	2.5	Ala	13.3
2	Asp	4.3		Thr	3.0		Thr	5.9	Asn	6.8	Asp	6.2	Asn	9.4
3	Ser	2.4		Leu	4.2		Leu	9.0	Glu	5.3	Ser	4.2	Glu	7.0
4	Glu	2.3		Gln	3.5		Gln	6.0	Asn	5.6	Glu	4.0	Asn	7.2
5	Glu	4.0		Gln	4.9		Gln	6.8	Arg	5.3	Glu	5.5	Arg	6.7
6	Asp	2.5		Glu	4.1		Glu	6.4	Phe	5.0	Asp	4.1	Phe	8.6
7	Glu	3.0		Lys	2.7		Lys	6.2	Tyr	5.0	Glu	4.2	Tyr	6.6
8	Glu	3.8		Pro	3.1		Pro	5.8	Tyr	6.5	Glu	4.7	Tyr	7.0
9	His	0.6		Asp	2.9		Asp	5.9	Asn	7.1	His	1.6	Asn	7.8

**Table 1** N-terminal sequence data of TFPI degradation fragments

Proteolysis of TFPI and the N-terminal sequence procedure are performed as described in Materials and Methods. The 26 kDa and 8 kDa fragments (obtained after 60 and 40 min of incubation respectively) were isolated from SDS-PAGE under reducing conditions and the 20 kDa and 30 kDa fragments (respectively obtained after 120 and 2 min) from SDS-PAGE under non-reducing conditions. Further details are described in the text.

Bagsvaerd, Denmark). Swine anti-goat IgG, labeled with horse radish peroxidase, was purchased from Caltag laboratories (San Francisco, CA). Peroxidase labeled goat anti-rabbit IgG was from Jackson Immuno Research Laboratories Inc. (West Grove, PA). Polyclonal goat anti-human rTFPI<sub>1-161</sub> antibodies (26, 27) were a kind gift of Dr. Ole Nordfang (Novo Nordisk, Bagsvaerd, Denmark). Rabbit polyclonal antibodies directed against a synthetic peptide corresponding to amino acids 254-276 of TFPI C-terminus were prepared at Monsanto Company (Chesterfield, MO).

**Phospholipids.** Small unilamellar phospholipid vesicles composed of 25 mol% DOPS and 75 mol% DOPC (PSPC) and 100 mol% DOPC vesicles (PC) were prepared as described previously (28).

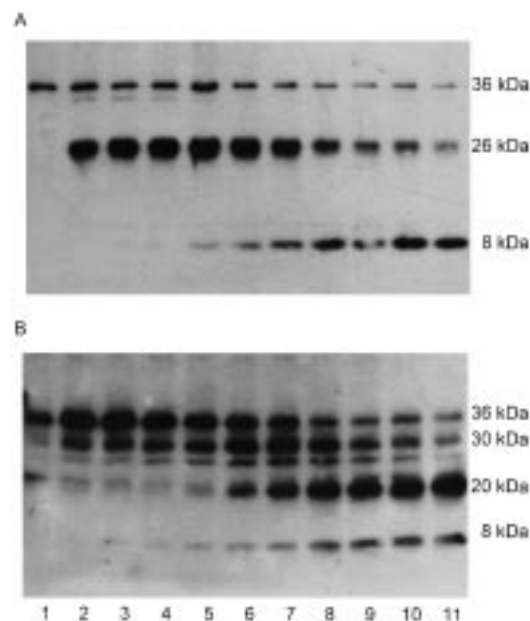
**Incubation mixtures of TFPI and factor Xa.** Preformed factor Xa : TFPI complexes were prepared by incubating factor Xa and TFPI at indicated concentrations for 10 min in a Tris-buffer containing 50 mmol/l Tris-HCl, pH 7.9, 175 mmol/l NaCl with 5 mmol/l CaCl<sub>2</sub>, and 0.5 mg/ml BSA. This incubation time was sufficiently long to obtain an equilibrium in complex formation. In order to start factor Xa-mediated proteolysis of TFPI, phospholipid vesicles (of different composition) were added at indicated concentrations. All reactions were carried out at 37° C. At timed intervals, aliquots were removed from the incubation mixture and diluted 1.5-fold in Tris-buffer containing 20 mmol/l EDTA to stop further proteolysis. Samples were frozen (-80° C) or kept on ice until usage for SDS-PAGE/Immunoblotting, a factor Xa assay, a dilute tissue factor clotting assay and TF : factor VIIa activity measurements.

**Gel-electrophoresis and immunoblot analysis.** The proteolytic fragments of TFPI were identified by electrophoresis on either 12% or gradient (4-20%) SDS-polyacrylamide separating gels, according to Laemmli (29), and subsequent immunoblot analysis with two different antibodies. The samples for electrophoresis were incubated with 8 mmol/l Tris-HCl, pH 6.7 containing 0.7% SDS, 10% glycerol and 0.25% bromophenol blue, under non-reducing conditions or after reduction with 1.25% β-mercaptoethanol, at 37° C for 30 min. As molecular weight standards, prestained low range (SDS-PAGE standard, Bio-Rad Laboratories, Hercules, CA) or broad range (See Blue™ standard, Novex, San Diego, CA) molecular weight markers were used.

After electrophoresis on a 12% SDS-PAGE the TFPI fragments were electrophoretically transferred to nitrocellulose transfer membrane (0.2 μm pore size) by semi-dry blotting at 150 mA for 2 h, using 40 mmol/l glycine, 50 mmol/l Tris (pH 8.5), 0.04% (w/v) SDS, and 20% (v/v) methanol as blotting buffer. Thereafter the blots were blocked with 5% non-fat dry milk in PBS buffer (10 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 150 mmol/l NaCl, pH 7.22). Subsequently the nitrocellulose membrane was incubated overnight with a polyclonal antibody directed against human TFPI<sub>1-161</sub>, in PBS/Tween buffer (0.3% v/v Tween-20) containing 1 mg/ml BSA. A secondary, swine anti-goat antibody conjugated with peroxidase, was diluted 1:4000 in PBS/Tween buffer and used for antigen detection on the nitrocellulose, by the chemiluminescence system of Amersham (Buckinghamshire, England), according to the manufacturer's description.

In order to specifically identify the low molecular weight C-terminal TFPI fragments as well as the C-terminus containing larger fragments, samples were also subjected to gradient SDS-PAGE (4-20% gradient gel) under reducing and non-reducing conditions. After electroblotting, the proteolytic fragments were visualized with a rabbit polyclonal antibody directed against the TFPI C-terminus (residues 254-276). A secondary goat anti-rabbit antibody conjugated with peroxidase was diluted 1:2000 in PBS/Tween/BSA buffer (0.1% Tween-20, 0.5% BSA) and used for antigen detection on the nitrocellulose with the chemiluminescence system of Amersham.

**Amino terminal sequence analysis.** Reduced and non-reduced samples of incubation mixtures of TFPI (3 μmol/l), factor Xa (6 μmol/l), 200 μmol/l PSPC and 5 mmol/l CaCl<sub>2</sub> in Tris-buffer were subjected to a 12% SDS-PAGE and electroblotted onto Immobilon-P membrane (0.45 μm pore size) by semi dry blotting as described above, using 50 mmol/l Tris (pH 8.4), 50 mmol/l boric acid and 20% (v/v) methanol as blotting buffer. The amount of (cleaved) TFPI applied on gel was 72 pmol. The blots were stained with Coomassie blue (0.01% w/v Coomassie Brilliant Blue® in 35% v/v methanol and 5% v/v acetic acid). The protein bands of interest were excised and used for direct N-terminal amino acid sequencing by automated Edman degradation, performed by Sequencing Centre Utrecht (SeCU), Utrecht, The Netherlands, using

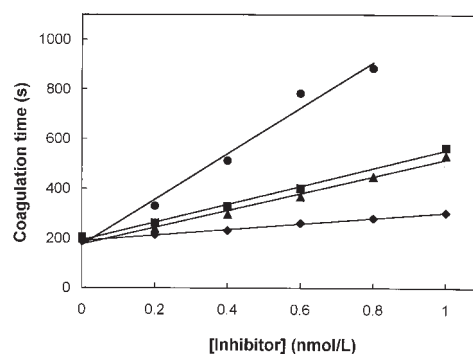


**Fig. 3** Progress of factor Xa-catalyzed degradation of TFPI visualized with an antibody against TFPI<sub>1-161</sub>. TFPI (50 nmol/l) was incubated with factor Xa (75 nmol/l) in the presence of phospholipids (5 μmol/l) at 37° C. Samples were taken at 0, 0.5, 1, 2, 5, 10, 20, 40, 60, 80 and 100 min (lanes 1-11) and subjected to SDS-PAGE (12%) and Western blotting using an antibody against TFPI<sub>1-161</sub>. A, reduced samples and B, unreduced samples

an Applied Biosystem 476A pulsed liquid sequencer (Applied Biosystems, Warrington, UK). Analysis of the sequencing data was performed with the 610A data analysis system from Applied Biosystems.

**Factor Xa assay.** The residual factor Xa activity after incubation with TFPI, whether or not in the presence of phospholipids, was assayed by adding 5 μl of the samples to polystyrene cuvettes containing 445 μl of 50 μmol/l Tris-HCl, pH 7.9, 175 mmol/l NaCl, 20 mmol/l EDTA and 0.5 mg/ml BSA, and 50 μl of 3 mmol/l chromogenic substrate S2765. The conversion of the chromogenic substrate was followed at 37° C on a dual wavelength spectrophotometer at 405 nm (reference wavelength 500 nm), and the factor Xa concentrations were calculated from standard curves of known amounts of the enzyme (22).

**Dilute tissue factor clotting assay.** Tris-EDTA buffer diluted samples (10 μl) of incubation mixtures containing TFPI, factor Xa and phospholipid were added to 190 μl plasma and incubated for 4 min at 37° C. Then 50 μl of this sample-plasma mixture was added to 100 μl thromboplastin solution (Innovin, 1/500 diluted in Tris-buffer pH 7.9, containing 30 mmol/l Ca<sup>2+</sup>) and



**Fig. 4** Effect of TFPI and preformed factor Xa : TFPI complexes on dilute tissue factor clotting time. The preformed complexes, factor Xa : TFPI (●), factor Xa : TFPI<sub>1-161</sub> (■) and factor Xa : TFPI<sub>1-250</sub> (▲), or free TFPI (◆) were added to the assay system at the indicated concentrations. Further details are described under Materials and Methods

**Table 2** Prolongation of the clotting time upon addition of TFPI or factor Xa : TFPI complexes to the dilute tissue factor assay

Inhibitor	Prolongation of the clotting time * (seconds/nmol/L)
Factor Xa:TFPI <sub>1-161</sub>	360
Factor Xa:TFPI <sub>1-250</sub>	340
Factor Xa:TFPI	1000
TFPI <sub>1-161</sub>	7
TFPI <sub>1-250</sub>	16
TFPI	113

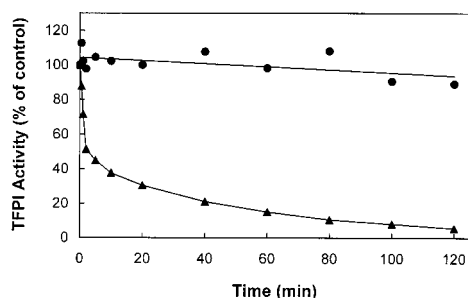
\* Calculated from slopes as shown in Fig. 4.

clotting times were measured on an ACL 300R coagulation analyzer from Instrumentation Laboratories (Lexington, MA) (11). The clotting time was related to an anticoagulant activity (expressed as % of control) using a reference curve constructed with known amounts of preformed factor Xa : TFPI complex.

**Inhibition of tissue factor : factor VIIa activity by TFPI.** Tris-EDTA buffer diluted samples of incubation mixtures containing TFPI, factor Xa and phospholipid were added to a mixture containing relipidated tissue factor (0.1 nmol/l), factor X (100 nmol/l), CaCl<sub>2</sub> (5 mmol/l) and PSPC (10 μmol/l) in Tris-buffer, resulting in a final concentration of 2.5 nmol/l (proteolytically cleaved) TFPI : factor Xa. This mixture was then incubated for 3 min at 37° C and factor X activation was started by adding factor VIIa (1 nmol/l). The amount of factor Xa formed was determined by averaging the factor Xa concentrations in the samples taken between 12 and 20 min after the addition of factor VIIa. This maximum amount of factor Xa formed was related to TFPI activity using a reference curve constructed under the same conditions from known amounts of preformed factor Xa : TFPI complex.

## Results

**Factor Xa catalyzed proteolysis of TFPI.** Previous work has indicated that TFPI is cleaved in the presence of a molar excess of factor Xa and a negatively charged phospholipid surface (17). Western blot analysis of incubation mixtures of TFPI with varying amounts of factor Xa and in the presence of PSPC vesicles instead of a planar phospholipid bilayer, confirm these earlier observations. The left panel of Fig. 1 shows that incubation of 12 nmol/l factor Xa with 6 nmol/l TFPI in the presence of 10 μmol/l PSPC in Tris-buffer containing 5 mmol/l CaCl<sub>2</sub> gives within 1 min rise to the appearance of a goat-anti human TFPI<sub>1-161</sub> positive band with a molecular weight of 26 kDa.



**Fig. 5** Effect of factor Xa cleavage on the anticoagulant activity of TFPI. Samples of the incubation mixtures of Fig. 3 were used to measure the anticoagulant activity of preformed factor Xa : TFPI complexes incubated with 5 μmol/l PSPC (▲) or 5 μmol/l PC (●) with the dilute tissue factor clotting assay

Additionally, after 20 min of incubation, a faint band of approximately 8 kDa becomes visible, whereas TFPI has almost disappeared. When the concentration of factor Xa was lowered to become equal to the molar concentration of TFPI (6 nmol/l, middle panel), proteolysis proceeded much slower as judged from the change in intensity of the 26 kDa band during the incubation. Furthermore, the 8 kDa fragment is absent and only a slight decrease is observed in the intensity of the band corresponding with TFPI. Under these conditions, when equimolar amounts of factor Xa and TFPI were incubated, the measured free factor Xa concentration was 0.5 nmol/l in accordance with the earlier reported dissociation constant of the factor Xa : TFPI complex in the presence of phospholipids (14). When the molar concentration of factor Xa (3 nmol/l) is half that of TFPI (Fig. 1, right panel), the appearance of cleaved TFPI, observed within an incubation time of 20 min, was strongly reduced. Omitting the PSPC vesicles or replacement with pure PC vesicles when TFPI was incubated with a two-fold molar excess of factor Xa largely reduced the rate of proteolysis. In both cases only a very faint band corresponding to the 26 kDa fragment appeared after a 60 min incubation period (data not shown). These experiments thus confirm that: 1) proteolysis of TFPI in complex with factor Xa depends on the concentration of free factor Xa and 2) proteolysis is drastically accelerated by the presence of a phospholipid membrane that contains phosphatidylserine.

**Identification of the factor Xa cleavage sites in TFPI.** To identify the cleavage pattern of TFPI caused by the factor Xa mediated proteolysis, we performed Western blot analysis and N-terminal sequencing of the degradation fragments. Fig. 2A shows the Western blot after gradient SDS-PAGE (4-20%) of reduced samples taken at timed intervals from an incubation mixture that contained factor Xa (75 nmol/l), TFPI (50 nmol/l), PSPC (5 μmol/l) and 5 mmol/l CaCl<sub>2</sub> in Tris-buffer. The degradation fragments were visualized with an antibody directed specifically against the C-terminus of TFPI (i.e. residues 254-276). It is shown that the band corresponding with TFPI (36 kDa) disappears concomitantly with the appearance of a ~6 kDa fragment. Upon prolonged incubation an additional ~3 kDa fragment arises. Because the antibody against TFPI<sub>254-276</sub> solely detects degradation peptides that contain the C-terminus, it is evident that the ~3 kDa and ~6 kDa fragments result from cleavages in the C-terminal region of TFPI. We note that the late appearing ~3 kDa fragment is seen on blots of reduced and unreduced samples (Figs. 2A and B). This peptide probably results from cleavage of TFPI or C-terminal degradation peptides, at sites Lys<sup>249</sup>-Gly<sup>250</sup> and Arg<sup>246</sup>-Ile<sup>247</sup> (Li et al., unpublished data). The ~6 kDa fragment on the other hand, is only seen in the blot of reduced samples (Fig. 2A) and not in the blot of the unreduced samples (Fig. 2B). This suggests that a cleavage within the Kunitz-3 domain produces a two-chain form of TFPI, where disulfide bridges link the ~6 kDa fragment to the N-terminal cleavage fragment. The non-reduced blot (Fig. 2B) shows in addition to the ~3 kDa peptide also ~26 kDa fragment upon prolonged incubation. It is feasible that this fragment originates from another factor Xa cleavage site near the N-terminus of TFPI. From these results we conclude that factor Xa cleaves at least 3 peptide bonds in TFPI, namely at the N-terminus (~26 kDa fragment), at the C-terminus (~3 kDa fragment) and within the Kunitz-3 domain resulting in a two-chain TFPI due to the disulfide bridges.

To detect also TFPI degradation fragments that do not contain residues 254-276, we used an antibody against TFPI<sub>1-161</sub> to visualize the factor Xa cleavage pattern of TFPI. Fig. 3A shows the Western blot after SDS-PAGE (12%) of the reduced samples taken at timed intervals from an incubation mixture as described for Fig. 2. Apparently, TFPI is rapidly cleaved into a ~26 kDa fragment. An ~8 kDa peptide appears

only after considerably delay. A comparison with Fig. 2A suggests that this ~26 kDa fragment is the result of a cleavage in the Kunitz-3 domain. This was confirmed by the N-terminal amino acid sequence analysis of the ~26 kDa fragment. We found that, according to the known primary structure of human TFPI (5, 7), the sequence (M)DSEEDDEEH (see also Table 1) corresponds to residues 1-8 of TFPI. The methionine residue preceding the TFPI sequence, originates from the recombinant expression of TFPI. The ~6 kDa C-terminal fragment appearing in Fig. 2A remains invisible in Fig. 3A because it is not recognized by the antibody directed against TFPI<sub>1-161</sub>.

Fig. 3B shows the immunoblot of the unreduced samples, taken from the same incubation mixture as described for Fig. 3A and visualized with an antibody against TFPI<sub>1-161</sub>. A major difference with the cleavage pattern seen in Fig. 2B (the blot of unreduced samples that used an antibody against TFPI<sub>254-276</sub>) is the fast appearance of a protein band of ~30 kDa. The N-terminal amino acid sequencing of this cleavage product yielded two sequences: (M)DSEEDDEEH and ANENRFYYN, which correspond to residues 1-8 and 200-208, respectively (Table 1). The peptide bond that is cleaved, Arg<sup>199</sup>-Ala<sup>200</sup>, is located within the Kunitz-3 domain. Because the two peptide chains are held together by disulfide bonds, it is quite remarkable to see that under non-reducing conditions, the cleavage product has an apparent molecular weight (~30 kDa) that is significantly lower than observed for uncleaved TFPI. Reduction of the molecular weight because of an additional cleavage in the C-terminal region is unlikely because none of the blots of non-reduced samples showed the appearance of a small fragment with the same time course of appearance as the ~30 kDa protein band. On the contrary, the simultaneous appearance of a ~6 kDa C-terminal peptide recognized by anti-TFPI<sub>254-276</sub> in reduced samples (Fig. 2A), and that of a ~26 kDa N-terminal peptide recognized by anti-TFPI<sub>1-161</sub> in reduced samples (Fig. 3A) strongly supports the notion that the protein band that corresponds with ~30 kDa peptide is a two-chain TFPI with an intact C-terminus. The late appearance of the anti TFPI<sub>1-161</sub> positive ~8 kDa (Fig. 3A/B) and ~20 kDa (Fig. 3B) fragments simultaneously with the appearance of a ~26 kDa fragment recognized by the antibodies against TFPI<sub>254-267</sub> (Fig. 2B) indicates a further cleavage of the two-chain TFPI by factor Xa. The ~8 kDa fragment in Fig. 3A/B is not visible in Fig. 2A/B but appears simultaneously with the ~20 kDa fragment in Fig. 3B indicating that the ~8 kDa fragment represents an N-terminal cleavage peptide. Thus besides a fast cleavage at Arg<sup>199</sup>-Ala<sup>200</sup>, resulting in two-chain TFPI, a slower cleavage somewhere between Kunitz-1 and Kunitz-2 domain occurs. The N-terminal amino acid sequencing of the ~8 kDa fragment seen in the immunoblot was impeded by the fact that the protein blot of reduced samples, after electrophoresis on a 16% SDS-PAA-gel, showed a triplet in the low molecular weight region. We could identify a peptide of ~8 kDa with TTLQEQKPD as the N-terminal sequence (Table 1). The ~20 kDa fragment (Fig. 3B) presumably arising from the same cleavage, in contrast, could easily be isolated and yielded two sequences: TTLQEQKPD and ANENRFYYN. The first sequence corresponds to residues 87-95 and the latter one corresponds to residues 200-208 (Table 1). The ~26 kDa fragment in Fig. 2B has probably the same origin as the ~20 kDa band in Fig. 3B. That is, they both contain the Kunitz-2 domain and the Arg<sup>199</sup>-Ala<sup>200</sup> cleaved Kunitz-3 domain. The ~20 kDa band however, possibly also lacks its C-terminus because in the same time also the ~3 kDa fragment appears and a faint intermediate is observed in Fig. 3B between the ~30 kDa and ~20 kDa band. This could explain why this ~20 kDa fragment is not visible in Fig. 2B since this blot is obtained with the antibody raised against the C-terminus. In summary, factor Xa cleaves TFPI in the peptide bond between

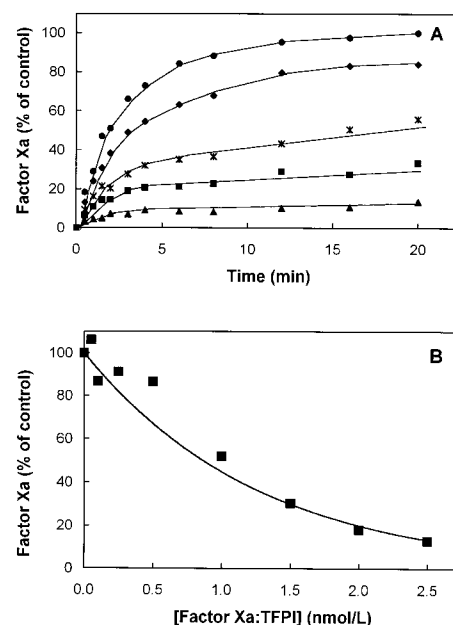


Fig. 6 Effect of preformed factor Xa : TFPI complex on tissue factor : factor VIIa catalyzed factor X activation. Panel A) Factor X (100 nmol/l) was incubated with tissue factor (0.1 nmol/l), 10  $\mu$ mol/l PSPC and 1 nmol/l factor VIIa in the absence (●) or presence of varying amounts (0-2.5 nmol/l) factor Xa:TFPI. Shown are: 0.5 nmol/l (◆), 1.0 nmol/l (★), 1.5 nmol/l (■), 2.5 nmol/l (▲). Samples were removed at indicated time points and assayed for factor Xa. Panel B) Averaged amounts of factor Xa measured between 12 and 20 min versus the factor Xa : TFPI complex concentration

Arg<sup>199</sup> and Ala<sup>200</sup> in Kunitz-3 and the peptide bond Lys<sup>86</sup>-Thr<sup>87</sup> between Kunitz-1 and Kunitz-2. We also found a cleavage at Lys<sup>249</sup>-Gly<sup>250</sup> and/or Arg<sup>246</sup>-Ile<sup>247</sup>, but the rates of these cleavages were relatively slow compared to the cleavage at Arg<sup>199</sup>-Ala<sup>200</sup>.

*The effect of proteolysis on the anticoagulant activity of TFPI.* To assess the loss of anticoagulant activity of TFPI upon factor Xa-dependent proteolysis, we used a dilute tissue factor clotting assay in which the tissue factor dilution was such that normal plasma has a clotting

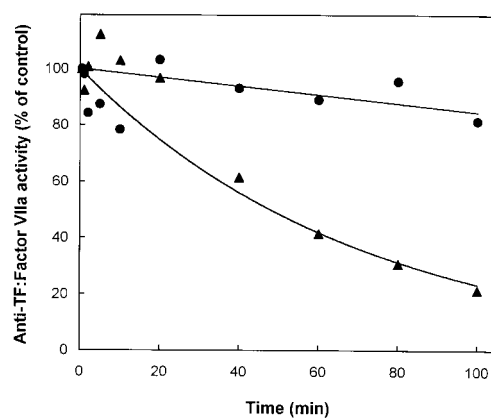


Fig. 7 Effect of factor Xa cleavage on the anti-tissue factor : factor VIIa activity of TFPI. Timed samples from the PSC (▲) or PC (●) incubations of Fig. 3 were added to the factor X activation mixtures of Fig. 6. Plotted are the residual TFPI activities (calculated from the plateau values of factor Xa and Fig. 6B) as a percentage of the control (factor Xa : TFPI incubated in the absence of PSC vesicles) versus the incubation time of the factor Xa, TFPI and phospholipid mixtures

time of 200 s. Fig. 4 shows that the coagulation time increases linearly with increasing concentrations of (truncated-)TFPI whether or not in a preformed complex with factor Xa. Table 2 summarizes the prolongation of the clotting time in seconds when 1 nmol/l (truncated-)TFPI or preformed factor Xa : (truncated-)TFPI complex is added to the assay. Noteworthy is the negligible anticoagulant effect of TFPI alone, compared to its complex with factor Xa. It is apparent that the anticoagulant activities of preformed factor Xa : TFPI<sub>1-161</sub> and factor Xa : TFPI<sub>1-250</sub> complexes are 2-3 fold lower than of the complex of factor Xa with full length TFPI.

When preformed factor Xa : TFPI complex (50 nmol/l TFPI plus 75 nmol/l factor Xa) was incubated with PSPC (5 µmol/l) in Tris-buffer containing 5 mmol/l CaCl<sub>2</sub>, the measured anticoagulant activity in timed samples shows a biphasic disappearance (Fig. 5). An initial rapid decrease of about 50% within 5 min is followed by a continuing decline, eventually leading to complete loss of anticoagulant activity. As expected, when the negatively charged phospholipid vesicles in the incubation mixture were replaced by PC vesicles, the anticoagulant activity of the factor Xa : TFPI complex remained unchanged. Immunoblot analysis of reduced samples taken from the incubation mixture containing PC vesicles confirmed that TFPI was not cleaved at the peptide bond Arg<sup>199</sup>-Ala<sup>200</sup>, Lys<sup>86</sup>-Thr<sup>87</sup>, Lys<sup>249</sup>-Gly<sup>250</sup> and/or Arg<sup>246</sup>-Ile<sup>247</sup> (data not shown).

A comparison between the time dependency of the loss of anticoagulant activity (Fig. 5) and the cleavage patterns shown in Figs. 2 and 3, makes it possible to associate the loss of inhibitory activity of the preformed factor Xa : TFPI complex in the dilute tissue factor assay with specific peptide bond cleavages. The rapid cleavage of the Arg<sup>199</sup>-Ala<sup>200</sup> peptide bond in the Kunitz-3 domain results in 50-70% loss of the anticoagulant activity of the preformed factor Xa : TFPI complex. Complete loss of anticoagulant activity could be attributed to the ensuing, slowly progressing cleavage of the peptide bond Lys<sup>86</sup>-Thr<sup>87</sup> between the Kunitz-1 and Kunitz-2 domain. We thus clearly demonstrated that cleavage of Arg<sup>199</sup>-Ala<sup>200</sup> also reduces the anticoagulant activity of the preformed factor Xa : TFPI complex. This in addition to the common knowledge that loss of anticoagulant activity of TFPI is associated with truncation of TFPI at the C-terminus. Interestingly, the residual activity of this factor Xa : two-chain TFPI complex is similar to that of factor Xa in complex with C-terminal truncated variants of TFPI (Table 2).

*The effect of proteolysis on the anti-tissue factor : factor VIIa activity.* We extended our investigation on the neutralization of the inhibitory activity of factor Xa : TFPI upon proteolysis with an assay on the inhibition of the TF : factor VIIa catalyzed activation of factor X by the preformed factor Xa : TFPI complexes. Fig. 6A shows the generation of factor Xa, both in the absence and presence of various concentrations of preformed factor Xa : TFPI complex, as a function of the incubation time. It is shown that the plateau value of factor Xa formed, decreases with increasing concentrations of factor Xa : TFPI complex. Plateau values were measured in a range from 0 to 2.5 nmol/l factor Xa : TFPI and were replotted in Fig. 6B which shows the relationship between the extent of inhibition of factor X activation and the concentration of preformed factor Xa : TFPI complex.

Incubation of preformed complex (50 nmol/l TFPI plus 75 nmol/l factor Xa) with PSPC (5 µmol/l) in Tris-buffer containing 5 mmol/l CaCl<sub>2</sub> resulted in a decrease of factor Xa : TFPI inhibitory activity towards TF : factor VIIa (Fig. 7). It is evident that the factor Xa : TFPI complex retains its inhibitory activity when incubated with PC vesicles. A comparison of the time dependency of the inhibition of TF : factor VIIa activity (Fig. 7) with the Western blot data of Figs. 3A and B,

indicates that the cleavage in the Kunitz-3 domain has no effect on the anti TF : factor VIIa activity. This in contrast to the effect the Arg<sup>199</sup> to Ala<sup>200</sup> cleavage exerts on the anticoagulant activity of TFPI as observed in Fig. 5. In fact, the gradual decline of the anti TF : factor VIIa activity seems to be related to the cleavage of the peptide bond Lys<sup>86</sup>-Thr<sup>87</sup> between the Kunitz-1 and Kunitz-2 domain. This observation confirms earlier reports that showed that a cleavage between the Kunitz-1 and Kunitz-2 domain destroys the anti TF : factor VIIa inhibitory activity of TFPI (18, 19).

*Proteolysis of TFPI and its anti-factor Xa activity.* The loss of anticoagulant activity and anti-TF : factor VIIa activity after cleavage of a peptide bond between the first and second Kunitz domain could also result from dissociation of factor Xa from the cleaved TFPI as reported by other investigators (18, 19). We, however, observed that during the incubation of factor Xa (75 nmol/l) and TFPI (50 nmol/l) in the presence of PSPC, as in Fig. 5, the factor Xa activity dropped within 5 min to a residual activity that corresponds with 25 nmol/l free factor Xa. Upon prolonged incubation (120 min) only a slight increase of 5% free factor Xa was measured. Thus in contrast with others we found that the active site of factor Xa remains blocked by TFPI in spite of the cleavage of TFPI between the Kunitz-1 and Kunitz-2 domain.

## Discussion

Here, we report studies on the factor Xa mediated proteolysis of TFPI in complex with factor Xa and the concomitant loss of the inhibitory activity of this complex. It was shown that the rate of proteolysis is proportional to the free factor Xa concentration and that it is greatly enhanced by the presence of a negatively charged phospholipid surface. Proteolysis of TFPI abolished the ability of TFPI to inhibit TF : factor VIIa mediated factor X activation as well as the anticoagulant activity of the factor Xa : TFPI complex in a dilute tissue factor clotting assay.

Three cleavage sites were identified, namely between the Kunitz-1 and Kunitz-2 domain (Lys<sup>86</sup>-Thr<sup>87</sup>), in the C-terminus at Lys<sup>249</sup>-Gly<sup>250</sup> and/or Arg<sup>246</sup>-Ile<sup>247</sup> and within the Kunitz-3 domain (Arg<sup>199</sup>-Ala<sup>200</sup>). The Lys<sup>86</sup>-Thr<sup>87</sup> cleavage has also been described very recently in relation to thrombin-mediated proteolysis of TFPI (30) and differs only one amino acid with the Thr<sup>87</sup>-Thr<sup>88</sup> cleavage in TFPI reported for human leukocyte elastase (18, 19). The Arg<sup>199</sup>-Ala<sup>200</sup> cleavage site is reported here for the first time and is of special interest because the Arg<sup>199</sup> is the so called P<sub>1</sub> residue of the third Kunitz type protease inhibitor domain (31) of which the physiological role is not known. The cleavage of the Arg<sup>199</sup>-Ala<sup>200</sup> peptide bond appeared to be much faster than that of the Lys<sup>86</sup>-Thr<sup>87</sup> bond. It remains however to be established whether the cleavages Arg<sup>199</sup>-Ala<sup>200</sup> and Lys<sup>86</sup>-Thr<sup>87</sup> occur at random with different rates or in a sequential order. In this respect it should be noted that Ohkura et al. (30) report three thrombin cleavage sites in TFPI; Lys<sup>254</sup>-Thr<sup>255</sup>, Arg<sup>107</sup>-Gly<sup>108</sup> and Lys<sup>86</sup>-Thr<sup>87</sup> which are claimed to occur in that order.

Cleavage of TFPI within the Kunitz-3 domain at Arg<sup>199</sup>-Ala<sup>200</sup> results in a two-chain form of TFPI as established by N-terminal sequencing. Remarkably though is the size reduction from 36 to 30 kDa, observed on the Western blot under non-reducing conditions using antibodies against TFPI<sub>1-161</sub> (Fig. 3B). This observation suggests a further cleavage in the C-terminal tail. However, the Western blots in which an antibody against the C-terminal tail was used, did not reveal a C-terminal truncation in the same time course of the abundant appearance of the 30 kDa peptide. Therefore we assume that the ~30 kDa fragment is in fact full length TFPI but with an altered electro-

phoretic mobility. A close look at Fig. 2B reveals a faint doublet in the 36 kDa region which, taking into account the lower resolution for gradient SDS-PAGE, might point at the same two-chain form of TFPI, also migrating with a different mobility compared to the uncleaved TFPI. This observed abnormality in electrophoretic mobility could be due to a strongly altered protein conformation induced by cleavage of the Arg<sup>199</sup>-Ala<sup>200</sup> bond. We note that a shift in electrophoretic mobility on SDS-PAGE under non-reducing conditions due to conformational changes has been demonstrated for a number of proteins (32-34). A dramatic conformational change upon cleavage of the peptide bond in the Kunitz-3 domain could also explain why the two-chain TFPI is hardly visible on the blot that used antibodies against the C-terminal region of TFPI. Again, hampering of epitope recognition upon a conformational change has been reported previously (33). It is tempting to speculate that the conformational change as a result of the cleavage within the Kunitz-3 domain enables an interaction between the negatively charged N-terminal and positively charged C-terminal regions.

It is of interest to see that cleavage of the Arg<sup>199</sup>-Ala<sup>200</sup> peptide bond in TFPI also causes functional changes in the molecule. We previously reported that the phospholipid-dependent and factor Xa-mediated partial proteolysis of TFPI caused a dramatic reduction in the affinity of the factor Xa : TFPI complex for negatively charged phospholipids (17). The present study has demonstrated that this loss in binding affinity is due to the cleavage of the Arg<sup>199</sup>-Ala<sup>200</sup> bond. Speculating about the mechanism that causes this loss in binding affinity we suggest that the aforementioned cleavage-induced conformational change in TFPI prohibits the C-terminal chain of TFPI from interacting with the negatively charged phospholipid membrane. Recent work reported by Kazama et al. (35) suggests that negatively charged phospholipids are essential to the anticoagulant activity of the factor Xa : TFPI complex. This phospholipid requirement provides an interesting explanation for the partial loss of anticoagulant activity that was associated with the cleavage of the Arg<sup>199</sup>-Ala<sup>200</sup> bond in TFPI. In this respect it is also interesting to see that the anticoagulant activity of preformed complexes of factor Xa with C-terminal truncated TFPI (TFPI<sub>1-161</sub> and TFPI<sub>1-250</sub>), having a low affinity for negatively charged phospholipids (17), showed the same reduction in anticoagulant activity as the Arg<sup>199</sup> to Ala<sup>200</sup> cleaved TFPI in complex with factor Xa. These observations are the more intriguing because cleavage at Arg<sup>199</sup>-Ala<sup>200</sup> seems to leave the inhibitory activity towards TF : factor VIIa activity in a purified system unaltered. Other studies have shown that, unlike free TFPI, preformed factor Xa : TFPI complexes inhibit the TF : factor VIIa activity in a purified system with the same rate irrespective of the presence of the Kunitz-3 domain and the positively charged C-terminus (14, 15). Thus, once the complex between factor Xa and TFPI is formed, structural alterations in the C-terminal region like truncation or cleavage at Arg<sup>199</sup>-Ala<sup>200</sup>, have no effect on the inhibitory activity towards TF : factor VIIa. At present we have no explanation for the apparent lack of correlation between anti-TF : factor VIIa activity in a purified system and anticoagulant activity. However, it appears that in a plasma environment the interaction between negatively charged phospholipids and factor Xa : TFPI is more important for the inactivation of TF : factor VIIa activity than in a buffer system with purified proteins.

Cleavage between the Kunitz-1 and Kunitz-2 domain at the Lys<sup>86</sup> to Thr<sup>87</sup> peptide bond dissociates the factor Xa binding region from the factor VIIa binding region. Since the simultaneous interaction of TFPI with both factor Xa and factor VIIa is essential to inhibit TF : factor VIIa activity, it is not surprising that this cleavage results in a complete

annihilation of the anticoagulant activity of the factor Xa : TFPI complex (18, 19, 30). Furthermore, proteolysis of TFPI in complex with factor Xa at the Lys<sup>86</sup>-Thr<sup>87</sup> peptide bond does not result in recovery of factor Xa activity, suggesting that factor Xa remains complexed with two-chain TFPI that lacks the Kunitz-1 domain. Omission of the Kunitz-1 domain is obviously sufficient to abrogate the inhibitory activity of the factor Xa : TFPI complex.

Our findings suggest that factor Xa might have a dual function in its own, TFPI-controlled, generation. That is, factor Xa in complex with TFPI, rapidly inactivates TF : factor VIIa catalyzed factor X activation. But at the same time and at the same membrane, the anticoagulant activity of the factor Xa : TFPI complex is rapidly neutralized by factor Xa that is not inactivated by TFPI.

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