Factor VII–Activating Protease Promotes the Proteolysis and Inhibition of Tissue Factor Pathway Inhibitor

Sandip M. Kanse, Paul J. Declerck, Wolfram Ruf, George Broze, Michael Etscheid

Objective—Factor VII–activating protease (FSAP) activates both factor VII and pro-urokinase and inhibits platelet-derived growth factor-BB, thus regulating hemostasis- and remodeling-associated processes in the vasculature. A genetic variant of FSAP (Marburg I polymorphism) results in low enzymatic activity and is associated with an enhanced risk of carotid stenosis and stroke. We postulate that there are additional substrates for FSAP that will help to explain its role in vascular biology and have searched for such a substrate.

Methods and Results—Using screening procedures to determine the influence of FSAP on various hemostasis-related processes on endothelial cells, we discovered that FSAP inhibited tissue factor pathway inhibitor (TFPI), a major anticoagulant secreted by these cells. Proteolytic degradation of TFPI by FSAP could also be demonstrated by Western blotting, and the exact cleavage sites were determined by N-terminal sequencing. The Marburg I variant of FSAP had a diminished ability to inhibit TFPI. A monoclonal antibody to FSAP that specifically inhibited FSAP binding to TFPI reversed the inhibitory effect of FSAP on TFPI.

Conclusion—The identification of TFPI as a sensitive substrate for FSAP increases our understanding of its role in regulating hemostasis and proliferative remodeling events in the vasculature. (Arterioscler Thromb Vasc Biol. 2012;32:427-433.)

Key Words: atherosclerosis • hemostasis • thrombosis • vascular biology

Factor VII activating protease (FSAP) is a protease in human plasma with a broad substrate specificity which includes hemostasis-related proteins like factor VII (FVII) and prourokinase.1,2 The addition of exogenous FSAP to plasma and whole blood influences coagulation and fibrinolysis.1,2 In vitro, the activation of FSAP is mediated by its binding to positively charged polyamines,3 as well as negatively charged polyanions, such as heparin, RNA, and polyphosphates, resulting in a bimolecular (auto-) activation.4 Activation of FSAP in plasma can be induced by postapoptotic/dead cells that presumably release nucleic acids, histones and nucleosomes.5,6 Hence, tissue damage releases nucleic acids/nucleosomes/polymamines, and platelet activation releases polyamines7 which together can contribute to FSAP activation.

About 5% of the white population are carriers of a single-nucleotide polymorphism (SNP) in the FSAP gene (official name, Hyaluronic acid binding protein-2) HABP2 rs800536 that results in an exchange of a single amino acid in the protease domain (Gly534Glu).8 The Gly534Glu polymorphism (Marburg I [MI]) is only a weak activator of prourokinase, but its ability to activate FVII is reportedly unchanged.8 Hence, the presence of this SNP may shift the activity profile of FSAP toward a more thrombotic phenotype. This has prompted many investigations into its linkage to venous thrombosis, but with the exception of one study that did find an association,9 the others came to an opposite conclusion.10–14 Other SNPs found in the HABP2 gene have a predictive value in venous thromboembolism in the elderly.15 The MI-SNP is also linked to a higher incidence of carotid stenosis,16 cardiovascular disease in general,17 stroke,18 and liver fibrosis,19 indicating a probable role for FSAP in hemostasis, as well as remodeling processes.

We have previously shown that MI-FSAP has a lower proteolytic activity towards chromogenic substrates, prourokinase and platelet-derived growth factor-BB compared with wild-type (WT)–FSAP and that MI-FSAP did not inhibit neo-intima formation in vivo.20 In contrast to previous reports that WT and MI-FSAP activate FVII equally well,8 we found that FVII was an extremely poor substrate for WT-FSAP and not activated at all by MI-FSAP (Supplemental Data I, available online at http://atvb.ahajournals.org). These observations led us to hypothesize that there are probably other substrates for FSAP that can explain its role in vascular biology.

The primary inhibitor of the tissue factor (TF)/activated factor X (FXa)/activated FVII (FVIIa) complex is tissue factor pathway inhibitor (TFPI).21 TFPI is produced by many cells in the vasculature, and its activity in the vessel wall is regulated in numerous ways. It is bound to the vasculature via glycosami...
glycans and can be released by heparin from intracellular stores. It is also found in platelets and bound to lipoproteins, whereby the latter have a major influence on the levels of circulating TFPI. TFPI consists of 3 Kunitz domains that allow it to specifically inhibit FXa and FVIIa and dampen the initiation of coagulation. The C-terminal Kunitz domain has strong propensity to bind to heparin. Multiple transcriptional start sites and alternative splicing lead to expression of different forms of TFPI. There is a strong species difference in the expression of these forms in that in adult humans, the predominant form is the 3-domain form called TFPI-α, whereas a truncated transcript coding for TFPI-β, lacking the C-terminal Kunitz domain, is expressed mainly in the adult mouse. TFPI can be proteolytically inactivated by proteases such as plasmin, thrombin, and elastase, as well as activated protein C. Apart from influencing the activity of the extrinsic pathway, TFPI also regulates vascular smooth muscle proliferation and has a role in innate immunity. Our studies with isolated proteins and with endothelial cells show that TFPI is indeed an excellent substrate for FSAP, and this could account for the effects of FSAP in vascular as well as the extravascular compartment.

**Materials and Methods**

**Materials**

WT- and MI-FSAP, as well as Phe-Pro-Arg-chloromethylketone (PPACK)-FSAP, were isolated, prepared, and characterized as described before. Single-chain FSAP zymogen is rapidly converted to the active 2-chain form within minutes at physiological pH. Recombinant full-length TFPI and truncated 2-domain TFPI (1–160) were produced as described before. Anti-FSAP monoclonal antibodies (Mab 1189, 677, 570), anti-human TFPI, anti-TF polyclonal antibodies (Ab), and TFPI-depleted plasmas were from American Diagnostica (Pfungstadt, Germany). Anti-FSAP Mab MA-38C7 was produced in-house. Anti-human TFPI polyclonal Ab SC-28861 was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Tumor necrosis factor α (TNFα) was from R&D Systems (Frankfurt, Germany). Endotoxin levels in FSAP were determined using the limulus amebocyte lysate test from Biowhittaker (Verviers, Belgium).

**Cell Surface FXa Generation**

Cell surface functional TF activity was determined by measuring the generation of FXa from factor X (FX) (Hemochrom Diagnostica, Essen, Germany) using 200 μmol/L chromogenic substrate N-α-benzyloxy carbonyl-d-arginyl-glycyl-l-arginine-p-nitroanilide (S-2765, Hemochrom Diagnostica). After stimulation of cells in 96-well plates with test substances for the indicated times, they were washed with Hepes-buffered saline and incubated for 15 minutes at 37°C. In all solutions, 10 mmol/L aprotinin was present to prevent any proteolytic activity. The mixture was incubated at 37°C. In all solutions, 10 mmol/L aprotinin was present to prevent any proteolytic activity. Furthermore, the activity of FSAP-treated TFPI was measured as diluted prothrombin time. The test solution was diluted 1:10 in TFPI-depleted plasma (containing 10 μmol/L aprotinin), and 20 μL was mixed with 100 μL imidazole and 30 μL of PTT reagent (Thromborel S, prediluted 1:1000 in imidazole). The mixture was incubated for exactly 3 minutes at 37°C. Finally, 50 μL of 20 mmol/L CaCl2 was added, and clot formation was measured at 405 nm for 15 minutes at 37°C. In all solutions, 10 μmol/L aprotinin was present to prevent any FSAP activity during the assay. The diluted prothrombin time is defined as the time when 50% of maximum absorption is reached.

**FSAP Binding to TFPI**

Recombinant full-length TFPI or truncated 2-domain TFPI (1–160) was coated (5 μg/mL) with endothelial basal medium (PromoCell, Heidelberg, Germany), containing hydrocortisone (1 μg/mL), epidermal growth factor (10 ng/mL), basic fibroblast growth factor (10 ng/mL), and fetal calf serum (5% vol/vol). For the complete study, cells from ~25 donors at passages 2 to 4 were used. Cells in 96-well plates were stimulated with test substances in serum-free medium. Cells were stimulated with TNFα (25 ng/mL) for 5 to 6 hours.

**Processing of TFPI by FSAP in Solution**

Recombinant TFPI was incubated with FSAP, and TFPI was analyzed by Western blotting. In other experiments, cleaved TFPI was transferred to polyvinylidene difluoride membranes and subjected to N-terminal sequencing (Applied Biosystems, Darmstadt, Germany) for the determination of the cleavage sites.

**Western Blots**

Cells were lysed in SDS-containing buffer, and separation of polypeptides was performed by SDS-PAGE under reducing (10% vol/vol mercaptoethanol) or nonreducing conditions and electrotransferred onto the polyvinylidene difluoride membrane (GE Healthcare, Munich, Germany). Labeling of the transferred protein by primary antibody was done for 16 hours at 4°C in 5% (wt/vol) skim milk in tris-buffered saline–Tween 20 (0.1% vol/vol). Secondary horseradish peroxidase–conjugated antibody against primary antibody was incubated for 1 hour at room temperature. Detection of the horseradish peroxidase signal was performed by ECL Plus Western Blotting Detection Reagents (GE Healthcare).
Statistical Analysis
Results are shown as mean±SD from triplicate wells. All in vitro data were replicated in at least 3 times, and similar results were obtained in 3 independent experiments. Where indicated, statistical significance was analyzed by ANOVA followed by the Bonferroni post test.

Results

Regulation of FXa Generation On HUVECs by FSAP

We investigated whether FSAP influences TF/FVIIa-mediated FXa formation on activated-endothelial cells using FVIIa. HUVECs were prestimulated with TNFα for 5 hours to upregulate TF and then further treated with 2-chain FSAP for 1 hour. After the cells were washed and any remaining FSAP activity was inhibited with excess aprotinin, FXa formation on the cell surface was determined.

Incubation of TNFα-stimulated cells with FSAP further increased FXa generation (Figure 1A). The fold increase with FSAP was identical in the presence or absence of TNFα (data not shown), but in TNFα-stimulated cells, the absolute amount of FXa generated was higher. The effect of FSAP on TNFα-stimulated cells was apparent after 5 minutes and maximal after 30 minutes. A significant effect was observed already with 15 nmol/L of FSAP (Figure 1B). MI-FSAP had a much weaker effect than WT-FSAP and was in the same range of active site-blocked FSAP, PPACK-FSAP. In different isolates of TNFα-stimulated HUVECs, a 2- to 4-fold increase in FXa generation with FSAP was observed, and this variation was intrinsic to the different isolates of cells. The synergistic effect between FSAP and TNFα could be blocked by an anti-TF, as well as anti-FSAP, blocking antibody, which emphasizes the specificity of this effect (Figure 1C). Measurement of FXa generation required the presence of both FVIIa and FX in the incubation mixture, and omission of either resulted in no hydrolysis of the FXa substrate (data not shown). These controls indicated that FSAP did not directly activate FX or cleave the chromogenic substrate S-2765; moreover, the activation of cell-bound/serum-derived FVII could be blocked by an anti-TF, as well as anti-FSAP, blocking antibody, which emphasizes the specificity of this effect (Figure 1C).

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In TNFα-activated cells, the effect of increasing concentrations of wild-type (WT)-FSAP , Marburg I (MI)-FSAP , and Phe-Pro-Arg-chloromethylketone (PPACK)-FSAP , added for 60 minutes, on FXa generation was measured. B, In TNFα-activated cells, the effect of increasing concentrations of wild-type (WT)-FSAP , Marburg I (MI)-FSAP , and Phe-Pro-Arg-chloromethylketone (PPACK)-FSAP , added for 60 minutes, on FXa generation was measured. C, In TNFα-activated cells, FSAP (150 nmol/L) was added for 60 minutes, and FXa was measured in the presence of an anti-tissue factor (anti-TF) antibody (Ab), a control Ab, an anti-FSAP monoclonal antibody (Mab) (Mab 570), or a control Mab (all 20 μg/mL). In A to C, results are shown as mean±SD (n=3). *P<0.05.

Figure 1. Effect of factor VII–activating protease (FSAP) on activated factor X (FXa) generation on the human umbilical vein endothelial cell (HUVEC) surface. A, Cells were activated for 6 hours with tumor necrosis factor-α (TNFα) (25 ng/mL) and then treated with FSAP (150 nmol/L) (○) or its control buffer (□) for the indicated times, and FXa generation was measured. B, In TNFα-activated cells, the effect of increasing concentrations of wild-type (WT)-FSAP (□), Marburg I (MI)-FSAP (○), and Phe-Pro-Arg-chloromethylketone (PPACK)-FSAP (△), added for 60 minutes, on FXa generation was measured. C, In TNFα-activated cells, FSAP (150 nmol/L) was added for 60 minutes, and FXa was measured in the presence of an anti-tissue factor (anti-TF) antibody (Ab), a control Ab, an anti-FSAP monoclonal antibody (Mab) (Mab 570), or a control Mab (all 20 μg/mL). In A to C, results are shown as mean±SD (n=3). *P<0.05.

conirms that the effect of FSAP is not mediated by endotoxin or any other contaminant but by FSAP itself.

Because the increase in FXa generation was not due to upregulation of TF or procoagulant phospholipids, we considered the possibility that FSAP cleaves and inactivates TFPI. To test this hypothesis, Western blot analysis of cell extracts and supernatants from HUVECs were performed with anti-TFPI Ab. FSAP decreased TFPI levels in a concentration- and time-dependent manner in cell supernatants and extracts (Figure 2A; only data for cell extracts are shown). A significant loss of TFPI was seen with FSAP in a time- and concentration-dependent manner (Figure 2A). PPACK-FSAP did not reduce TFPI levels, whereas MI-FSAP had a weaker effect (Figure 2B).
This reduction in the levels of TFPI in cell extracts of FSAP-treated cells (cf. Figure 2) was not due to FSAP-mediated downregulation of TFPI mRNA expression (Supplemental Data III). Thus, the influence of FSAP on TF-dependent FXa generation on endothelial cells could be correlated with a loss of TFPI protein.

To substantiate this assumption, we investigated whether the loss in TFPI protein leads to the loss of TFPI activity in TFPI-depleted plasma using the Actichrome TFPI chromogenic activity assay. This assay measures the generation of FXa by TF/FVIIa complex in TFPI-depleted plasma relative to a TFPI standard.32 TFPI activity is shown in plasma U/mL (with 1 U/mL corresponding to 55 ng/mL or 1.4 nmol/L). In the conditioned medium of HUVECs, a TFPI activity of 0.3 U/mL was seen after 2 hours and 0.5 U/mL after 6 hours (Figure 2C). Treatment of HUVECs with FSAP for 2 or 6 hours prevented the accumulation of TFPI activity, supporting the hypothesis that TFPI released by the cells is inactivated by FSAP. Because other proteases are known to cleave TFPI, we compared their relative efficacy. On HUVECs, elastase was very effective in cleaving TFPI, followed by activated protein C and FSAP, which was more potent than plasmin, thrombin, and activated factor X (FXa) (0–90 nmol/L), and cell extracts were examined for TFPI by Western blot (WB) analysis with an anti-TFPI antibody.

Characterization of the Interaction Between FSAP and TFPI

Binding studies with isolated proteins were performed to characterize the interaction between FSAP and TFPI. FSAP bound to full-length TFPI but not to the 2-domain form(1–160), which does not have the C-terminal heparin binding domain (Figure 4A). We then compared how the 2 forms of TFPI were inhibited by FSAP. TFPI activity was determined by a 2-step FXa generation assay as described before.31 In a first set of experiments, it was confirmed that recombinant TFPI decreased TF/FVIIa-dependent FXa-activation in a dose-dependent manner. With a TFPI-blocking Ab, the inhibitory effect of TFPI on FXa generation could be reversed, and FX activation was increased (Supplemental Data V). Similarly, in the concomitant presence of FSAP, the inhibitory effect of TFPI was reversed, and an increase in FX activation was observed. In this experiment, FSAP activity was blocked with excess aprotinin so that this did not interfere with the chromogenic substrate assay for FXa. FSAP had no influence on the assay if TFPI was simultaneously neutralized by anti-TFPI Ab. Hence, in a purified system, FSAP could regulate TFPI activity (Supplemental Data V).

In this assay, full-length TFPI was more effective in inhibiting FXa generation than TFPI(1–160) (Figure 4B). When both forms were tested in the presence of FSAP, the full-length TFPI was completely inhibited, whereas the TFPI(1–160) was much less inhibited by FSAP (Figure 4C). Hence, the C-terminal heparin binding domain of TFPI is crucial for binding to FSAP and for inactivation of TFPI by FSAP.

We then screened a panel of Mab to identify ones that (1) inhibited the proteolytic activity of FSAP, and (2) blocked the binding of FSAP to TFPI. We used one Mab (Mab 570) that inhibited the enzymatic activity of FSAP (Figure 4C) and another Mab (MA-38C7) that blocked the binding of FSAP to TFPI but not its enzymatic activity (Figure 4D). Both antibodies could reverse the effect of FSAP on TFPI, indicating that the binding of FSAP to TFPI is important for inhibiting the activity of the latter (Figure 4E).
The proteolytic cleavage of TFPI was then characterized in further detail. Incubation of recombinant TFPI with FSAP led to the degradation of TFPI in a time- and dose-dependent manner, leading to the disappearance of the TFPI band in SDS-PAGE under nonreducing conditions and the appearance of a lower molecular weight band under reducing conditions (Figure 5A). N-terminal sequencing of the smaller peptides arising from the cleavage showed that many of them contained the original N terminus of intact TFPI, indicating multiple sites of proteolytic cleavage (Figure 5B). Peptides exhibiting internal TFPI sequences indicated that the cleavage occurred between kringle domains K1 and K2 (Lys86-Thr87), as well as in the active site of K2 (Arg107-Gly108) and K3 (Arg199-Ala200) domains (numbering refers to mature full-length TFPI protein). These sites are nearly identical to those reported previously for plasmin,24 thrombin,25 and elastase26 (Supplemental Data VI). Therefore, TFPI is cleaved at multiple sites by FSAP, leading to its complete inactivation.

Influence of the MI-SNP on TFPI Anticoagulant Function

Recombinant TFPI was incubated with WT-and MI-FSAP and the residual TFPI activity was measured in TFPI-depleted plasma using a TFPI chromogenic activity assay (Figure 6A). WT-FSAP was much more effective, reducing TFPI activity by 60%, whereas MI-FSAP under the same conditions did not inactivate TFPI. WT-FSAP-treated TFPI, added to TFPI-depleted plasma, accelerated the diluted prothrombin time by 30 to 40 seconds compared with untreated TFPI, providing evidence that FSAP can inhibit TFPI activity in plasma (Figure 6B). As could be expected from the preceding results, the effect of
Figure 6. Effect of wild-type (WT)– and Marburg I (MI)–factor VII–activating protease (FSAP) on tissue factor pathway inhibitor (TFPI) activity and clotting time in human plasma. A, Residual TFPI activity of FSAP-treated TFPI (12.5 nmol/L) (WT-FSAP ○; MI-FSAP ●) was measured with the Actichrome TFPI chromogenic activity assay (American Diagnostica). The result is shown as percentage of residual TFPI activity compared with untreated TFPI. Data are the mean of 3 experiments ± SD. B, The effect of FSAP-treated TFPI (WT-FSAP ○; MI-FSAP ●) on the clotting time of TFPI-depleted plasma was measured in the presence of 1:6600 diluted tissue factor (TF) (Thromborel S) as dilute prothrombin time (dPT). The absolute dPT of untreated TFPI in TFPI-depleted plasma was 185 ± 13 s (n = 6); the dPT of TFPI-depleted plasma alone was 139 ± 18 s (n = 6). Data are the mean of 3 experiments ± SD.

MI-FSAP on TFPI activity in plasma was weaker (Figure 6B). These results demonstrate that the cleavage of TFPI by FSAP also inhibits TFPI function in plasma, leading to accelerated coagulation, and show that the MI variant is much weaker in this respect.

Discussion

About 10 years ago, FVII activation was shown to be a key function of FSAP. However, there have been no further studies confirming these findings. We found a concentration-dependent increase in FVIIa on treatment of purified FVII with the WT-FSAP, but not more than 4% of FVII could be activated, similar to previously published data. Supraphysiological concentrations of FSAP were needed for FVII activation, confirming that FVII is indeed a weak substrate of FSAP. MI-FSAP was even worse and did not activate FVII, in contrast to earlier reports. This pattern was exactly similar to what we had previously reported for prourokinase activation. Our search for another substrate relevant in extrinsic coagulation led to the finding that TFPI is a novel substrate for FSAP. Direct comparison shows that the EC50 for FVII activation by FSAP was in the range 100 nmol/L, whereas the EC50 for TFPI inactivation was ≈3 nmol/L. TFPI is a key factor in determining the threshold for the initiation of the extrinsic pathway of blood coagulation. FSAP can inactivate TFPI, thus altering the balance in favor of FX activation. Although procoagulant effects of FSAP have been mainly attributed to FVII activation in plasma, the effect of FSAP on TFPI degradation likely plays an equally important role in specifically supporting the initiation of the TF pathway of coagulation.

Proteolytic inactivation of TFPI by plasmin, thrombin, elastase, and activated protein C has been reported, and there is evidence for truncated TFPI in the circulation. The generation of smaller TFPI fragments by FSAP with an intact original N terminus indicates truncation in the direction of the C terminus, similar to that described for other proteases. It is likely that cleavage at multiple locations leads to a cumulative loss of TFPI activity. A comparison of these proteases showed that elastase and activated protein C were more effective than FSAP, which in turn was more effective than plasmin, thrombin, and FXa. There was a difference in the sensitivity of TFPI to FSAP in Western blots using purified proteins compared with that of TFPI in activity assays in HUVECs. It is likely that protease inhibitors bound to the HUVEC surface and in the conditioned medium may also account for the inhibition of exogenously added FSAP and increase the effective concentration required for TFPI inactivation. Our observation that FSAP inhibits the full-length 3-domain TFPI efficiently but not the 2-domain form has fundamental implications for future work in this area. Strong species differences in the expression of different TFPI isoforms exist in humans and mice such that adult mice express predominantly the 2-domain TFPI-β and humans the 3-domain TFPI-α. This would suggest that any influence of FSAP on mouse TFPI is not to be expected.

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fine-tuning these noncoagulant aspects of the hemostatic system in vascular remodeling processes.

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Disclosures
None.

References
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Activation of FVII by WT-and MI-FSAP: The concentration of FVIIa generated by incubating 100 nM FVII for 10 min with different concentrations of WT- or MI-FSAP was quantified in a FVIIa-specific coagulation assay in the presence of recombinant soluble tissue factor (rsTF). The data are the mean ± S.D. of 3 experiments. In detail; WT-FSAP and MI-FSAP (400 nM each) were auto-activated in TNC-buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl$_2$, pH 6.0) at 37°C for 10 and 30 min, respectively to achieve comparable level of activated WT- and MI-FSAP. 100 nM FVII (Haemochrom Diagnostica, Essen) was incubated for 10 min at 37°C with 25-200 nM pre-activated FSAP; the reaction was terminated by adjusting to 2 µM aprotinin/10 mM EDTA and further dilution with 50 mM imidazol/100 mM NaCl/1% BSA, pH 7.4 (imidazol/BSA buffer). FVIIa clotting activity was determined as follows; diluted FVII solution was mixed with equal volumes of FVII-deficient plasma (Haemochrom Diagnostica, Essen, Germany), a mixture of rsTF and phospholipids (PTT reagent, Diagnostica Stago, Asnieres, France) and 25 mM CaCl$_2$. Turbidity changes were monitored at 37°C and 405 nm in a Sunrise microplate reader (Tecan, Crailsheim, Germany). In each well the final concentration of total FVII/FVIIa was 5 nM and rsTF was 60 nM. The level of FVIIa generated was quantified relative to the WHO 1st International Standard FVIIa concentrate (1 IU/ml FVIIa corresponds to 20 ng/ml or 0.5 nM).
Effect of FSAP on TF expression in HUVEC: (A) HUVEC were stimulated for 6 h with the indicated concentrations of TNFα (0, 0.25, 2.5, 25 ng/ml) and then with FSAP (150 nM) or control buffer for a further 1 h. Cell lysates were examined by Western blotting for the expression of TF. (B) Same as above except that after TNFα (25 ng/ml) stimulation the cells were then treated with FSAP (150 nM) in the presence or absence of aprotinin (10 μg/ml). Similar results were obtained in 3 independent replicates of this experiment.
Effect of FSAP on mRNA expression in HUVEC: HUVEC were either pre-activated for 5 h with TNFα (25 ng/ml) or left untreated. They were then stimulated for a further 2 or 6 h with FSAP (150 nM) or its buffer and/or heparin (25 μg/ml). Cells were also stimulated with PMA (1.6 μM), plasmin (100 nM) or thrombin (50 nM). RNA was isolated with the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich) and reverse transcription was performed using random hexamer primer and M-MuLV reverse transcriptase (Applied Biosystems) from equal amounts of total RNA. RT-PCR was performed using primers and a predetermined cycle number that amplified the signal in the linear range as described before (Shibamiya et al, BLOOD, 113(3); 714-722, 2009). Similar results were obtained in 3 independent replicates of this experiment.
Effect of FSAP on negatively charged phospholipids on HUVEC:
Cells were treated with or without TNFα (25 ng/ml) for 6 h and then stimulated with FSAP (150 nM) or control Buffer for 1h. As a positive control cells were stimulated with Ionomycin (10μM) for 10 min. Staining for negatively charged phospholipids was performed with FITC-Annexin V (BD Biosciences, Heidelberg, Germany) and flowcytometry was on FACSCalibur (BD Biosciences). Similar results were obtained in 3 independent experiments.
Effect of FSAP on TFPI activity in vitro using purified components: (A) The effect of adding increasing concentrations of recombinant TFPI on FXa formation was determined. (B) The effect of adding increasing concentrations of anti-TFPI Ab in the presence of 5 nM TFPI on FXa formation was measured. A matched control antibody was also tested at 20 µg/ml (*). (C) The influence on FXa formation of pre-treating TFPI with FSAP at the indicated concentrations for 30 min in the presence (●) or absence (O) of anti-TFPI Ab (20 µg/ml) was determined. In panels A-C results are shown as mean ± SD (n=3). Similar results were obtained in 3 independent replicates of these experiments.
**Signal peptide**

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**K2**

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311 VKNM

**Heparin binding region**

- Active site for protease inhibition.
- FSAP cleavage sites; this study.
- Thrombin cleavage sites; Okhura et al, 1997, Blood, 90, 1883-1892.

**Comparison of the cleavage sites in TFPI with various serine proteases:** K1-3 represent the 3 Kunitz domains. The signal peptide as well as the heparin-binding region are indicated.
Activation of FVII by WT-and MI-FSAP: The concentration of FVIIa generated by incubating 100 nM FVII for 10 min with different concentrations of WT- or MI-FSAP was quantified in a FVIIa-specific coagulation assay in the presence of recombinant soluble tissue factor (rsTF). The data are the mean ± S.D. of 3 experiments. In detail; WT-FSAP and MI-FSAP (400 nM each) were auto-activated in TNC-buffer (50 mM Tris, 150 nM NaCl, 10 mM CaCl₂, pH 6.0) at 37°C for 10 and 30 min, respectively to achieve comparable level of activated WT- and MI-FSAP. 100 nM FVII (Haemochrom Diagnostica, Essen) was incubated for 10 min at 37°C with 25-200 nM pre-activated FSAP; the reaction was terminated by adjusting to 2 µM aprotinin/10 mM EDTA and further dilution with 50 mM imidazol/100 mM NaCl/1% BSA, pH 7.4 (imidazol/BSA buffer). FVIIa clotting activity was determined as follows; diluted FVII solution was mixed with equal volumes of FVII-deficient plasma (Haemochrom Diagnostica, Essen, Germany), a mixture of rsTF and phospholipids (PTT reagent, Diagnostica Stago, Asnieres, France) and 25 mM CaCl₂. Turbidity changes were monitored at 37°C and 405 nm in a Sunrise microplate reader (Tecan, Crailsheim, Germany). In each well the final concentration of total FVII/FVIIa was 5 nM and rsTF was 60 nM. The level of FVIIa generated was quantified relative to the WHO 1st International Standard FVIIa concentrate (1 IU/ml FVIIa corresponds to 20 ng/ml or 0.5 nM).
Effect of FSAP on TF expression in HUVEC: (A) HUVEC were stimulated for 6 h with the indicated concentrations of TNFα (0, 0.25, 2.5, 25 ng/ml) and then with FSAP (150 nM) or control buffer for a further 1 h. Cell lysates were examined by Western blotting for the expression of TF. (B) Same as above except that after TNFα (25 ng/ml) stimulation the cells were then treated with FSAP (150 nM) in the presence or absence of aprotinin (10 μg/ml). Similar results were obtained in 3 independent replicates of this experiment.
Effect of FSAP on mRNA expression in HUVEC: HUVEC were either pre-activated for 5 h with TNFα (25 ng/ml) or left untreated. They were then stimulated for a further 2 or 6 h with FSAP (150 nM) or its buffer and/or heparin (25 μg/ml). Cells were also stimulated with PMA (1.6 μM), plasmin (100 nM) or thrombin (50 nM). RNA was isolated with the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich) and reverse transcription was performed using random hexamer primer and M-MuLV reverse transcriptase (Applied Biosystems) from equal amounts of total RNA. RT-PCR was performed using primers and a predetermined cycle number that amplified the signal in the linear range as described before (Shibamiya et al, BLOOD, 113(3); 714-722, 2009). Similar results were obtained in 3 independent replicates of this experiment.
Effect of FSAP on negatively charged phospholipids on HUVEC:
Cells were treated with or without TNFα (25 ng/ml) for 6 h and then stimulated with FSAP (150 nM) or control Buffer for 1 h. As a positive control cells were stimulated with ionomycin (10 μM) for 10 min. Staining for negatively charged phospholipids was performed with FITC-Annexin V (BD Biosciences, Heidelberg, Germany) and flow cytometry was on FACSCalibur (BD Biosciences). Similar results were obtained in 3 independent experiments.
Effect of FSAP on TFPI activity in vitro using purified components: (A) The effect of adding increasing concentrations of recombinant TFPI on FXa formation was determined. (B) The effect of adding increasing concentrations of anti-TFPI Ab in the presence of 5 nM TFPI on FXa formation was measured. A matched control antibody was also tested at 20 µg/ml (*). (C) The influence on FXa formation of pre-treating TFPI with FSAP at the indicated concentrations for 30 min in the presence (●) or absence (○) of anti-TFPI Ab (20 µg/ml) was determined. In panels A-C results are shown as mean ± SD (n=3). Similar results were obtained in 3 independent replicates of these experiments.
**Signal peptide**

MIYTMKKVHA LWASVCLLLN LAPAPLNADS EEDEEHTIIT DTELPPLKLM HSFCAFKADD

GPCKAIMKRF FFNIIFTRQCE EFIIYGGCEGN QNRFESLEECE KKMCTRDNAN RIIKTLQQE

KPDFCFLEED PGICRGYITR YFYNNQTKQC ERFKYGGCLG NMNNFETLEE CKNICEDGPN

GFQVDNYGTQ LNAVNNSLTP QSTKVPSLFE FHGPSWCLTP ADRGLCRANE NRFYYNSVIG

KCRPFKYSGC GNENNFTSK QECLRACKKG FIQRISKGGL IKTKRKRKKQ RVKIAYEEIF

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