Factor Seven Activating Protease: in Search of a Physiological Role

Fabian Stavenuiter

Lay out: Youri Stavenuiter

ISBN: 978-90-8570-523-9
Printed by Wöhrmann Print Service, Zutphen, The Netherlands.

The research described in this thesis was performed at the Department of Plasma Proteins, Sanquin Research as part of the Van Creveld Laboratory of UMC-Utrecht and Sanquin, Amsterdam, The Netherlands.

Factor Seven Activating Protease: in Search of a Physiological Role

Factor VII Activerend Protease:
op zoek naar een fysiologische rol
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J. C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 23 juni 2010 des middags te 12.45 uur

door

Fabian Stavenuiter
geboren op 16 juli 1975, te Heemskerk

Promotor
Prof. dr. K. Mertens

Co-promotor
Dr. A.B. Meijer

The research described in this thesis was financially supported by the Netherlands Thrombosis Foundation (TSN) grant no. 2005-4.

Financial support by the Netherlands Thrombosis Foundation and the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

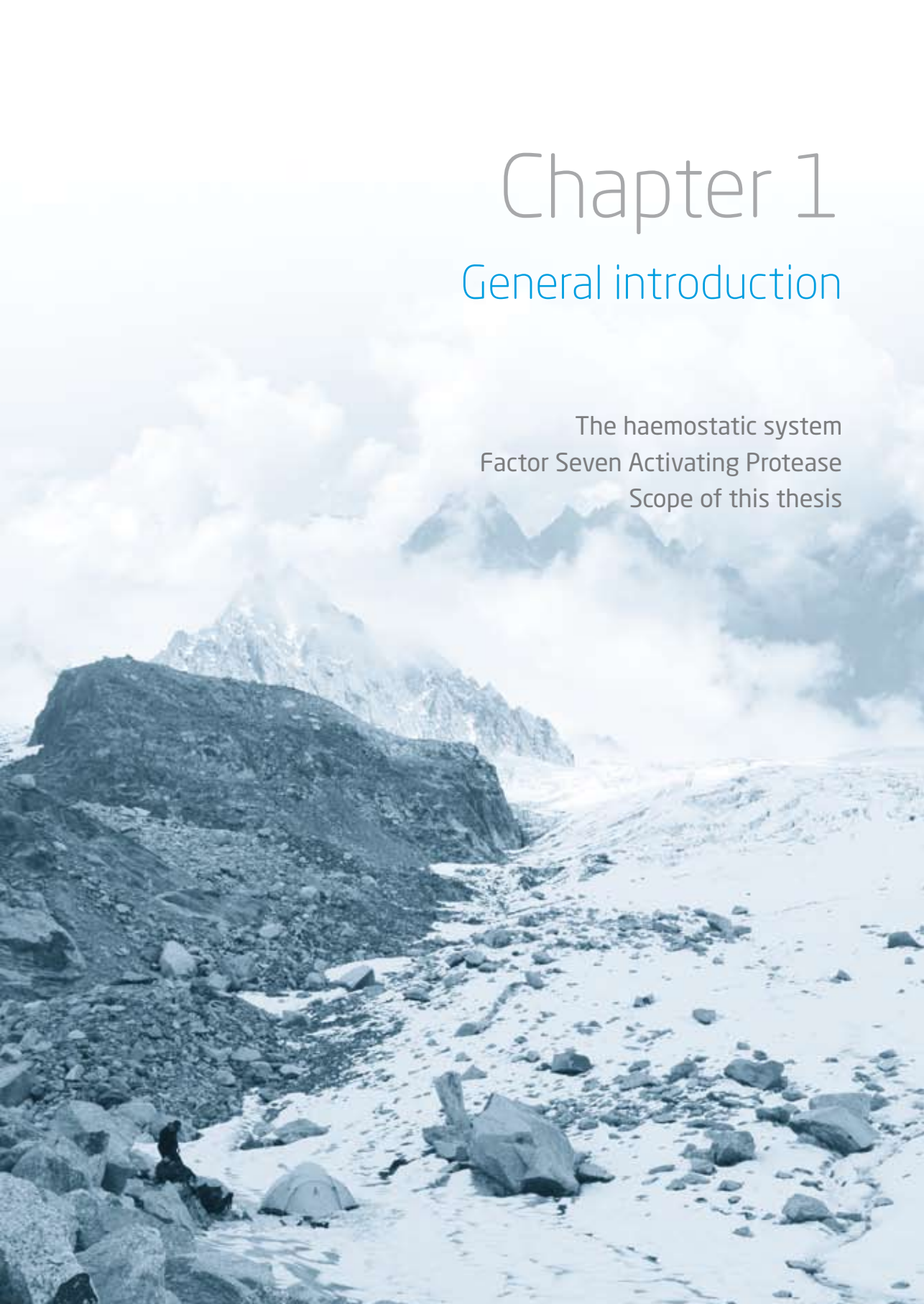
The author is also grateful for the financial support provided by Sanquin Research, J.E. Juriaanse Stichting, Roche Diagnostics B.V. and TAC Trade and Converting B.V.

Contents

Chapter 1

General introduction

The haemostatic system
Factor Seven Activating Protease
Scope of this thesis



Outline of this thesis

After vessel injury a series of sequential events takes place that leads to formation of a blood clot occluding the vessel wall at sites of injury. This process is known as haemostasis. During the first phase, platelets adhere to macromolecules in the subendothelial tissues and subsequently aggregate to form the primary haemostatic plug. During the second phase, local plasma coagulation factors are activated leading to the generation of a fibrin clot that reinforces the platelet plug. Once the blood flow is secured and the tissue is repaired, the fibrin clot is degraded.

Although the major components of the haemostatic system have been known since a few decades, occasionally 'new' plasma proteins are discovered that require a reappraisal of our established views. One prime example is the in 1996 described human Plasma Hyaluronan Binding Protease (PHBP) (1), which was later called Factor VII-activating protease (FSAP) due to its capability to activate coagulation factor VII in vitro (2). This suggested a role for the protease in coagulation. Previously, FSAP was described as a potent activator of single-chain pro-urokinase-type plasminogen activator (scuPA) in vitro. scuPA is a major component of the fybrinolytic pathway, suggesting a role for FSAP in fibrinolysis as well (3). Since its discovery, numerous of additional functions for FSAP have been proposed, including stimulation of endothelial and smooth muscle cells (4, 5). With its decreasing specificity, the physiological role of FSAP has become increasingly unclear. About 5-10 % of apparently healthy individuals were described to carry a FSAP variant known as Marburg-1 FSAP which contains a glycine to glutamine substitution located in the catalytic domain (6). This FSAP variant is associated with a 50-80 % impaired activation of scuPA in vitro, thus underscoring the suggested role for FSAP in fibrinolysis (6).

The aim of this thesis was to assess FSAPs role in the haemostatic system using intact recombinant human FSAP. Further, the pathophysiological implication of the Marburg-1 FSAP polymorphism for FSAP biological activity was addressed. In order to fully appreciate the questions addressed in this thesis, this general introduction provides an overview of the literature regarding FSAP and the haemostatic system.

Blood coagulation

Coagulation can be initiated via two different routes, the "intrinsic" and "extrinsic" pathway (Fig. 1). In vitro, coagulation via the intrinsic pathway is initiated when factor XII (FXII), prekallikrein, and high-molecular weight kininogen (HMWK) bind to artificial surfaces as e.g. kaolin and glass (7, 8). Once bound, autocatalytic cleavage of FXII and kallikrein occurs. FXIIa triggers fibrin clot formation via the sequential activation of factors XI (FXI), IX (FIX), X (FX), and prothrombin (FII). The relevance of this pathway is questioned since patients with deficiency of FXII, prekallikrein, or HMWK do not display an increased bleeding tendency (9). However, patients with factor FXI deficiency tend to have a mild bleeding disorder, implying a role for FXI in haemostasis. A revised model of coagulation has been proposed in which factor XI is activated by thrombin (10, 11). Moreover, the general view has now been established that feedback activation of FXI by thrombin in plasma results in a major mechanism of additional thrombin formation (12).

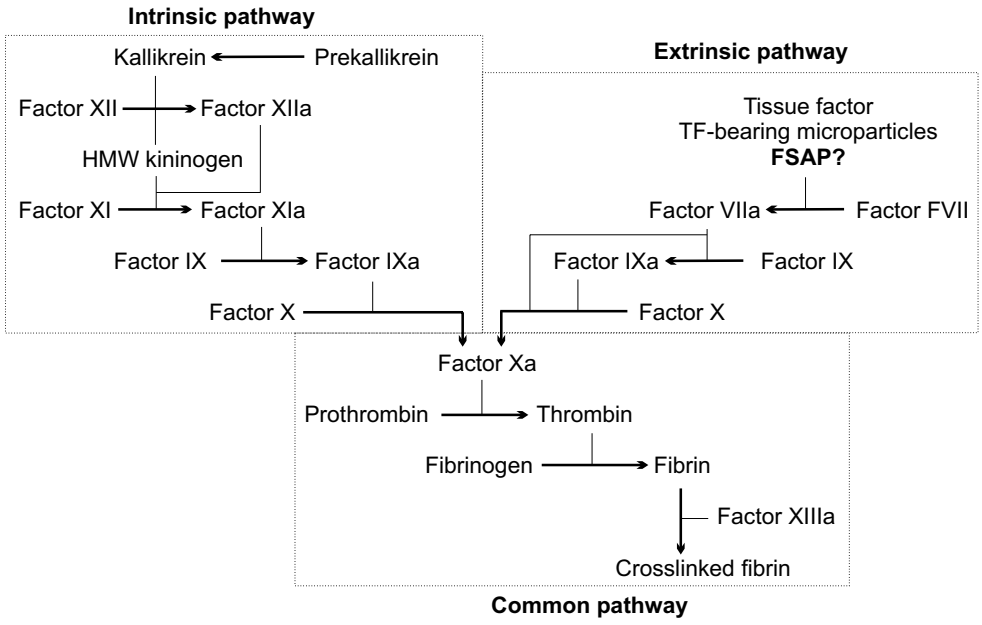


Figure 1. Schematic representation of the intrinsic and extrinsic coagulation pathways.

The physiological initiator of the extrinsic coagulation pathway is the tissue factor (TF). TF is a non-enzymatic lipoprotein constitutively expressed on the surface of cells that are normally not in contact with plasma (e.g., fibroblasts and macrophages). Exposure of plasma to TF exposed on the surface of these cells initiates subendothelial coagulation. Endothelial cells only express TF when stimulated by endotoxin, tumor necrosis factor, or interleukin-1, and may be involved in thrombus formation under pathologic conditions. TF-bearing membrane-derived microparticles in the blood stream, released from e.g. platelets and apoptotic endothelial cells, potentially play a role in thrombosis (13). TF binds activated factor VIIa (FVIIa) and accelerates FX activation about 30,000-fold (14-20). Although FVII is activated by its product, FXa, a trace amount of FVIIa (1-2% of total FVII) appears to be available in plasma at all times to interact with TF (21). The potential contribution of 'newly discovered' activators of FVII to haemostasis, like FSAP, might be of major importance (22). FVIIa also activates FIX (14, 15, 17-19) in the presence of TF. Factors IXa and Xa assemble with their activated non-enzymatic protein cofactors VIIIa and Va, respectively, on the surface of PS exposing platelets aggregated at sites of vessel injury. This leads to local generation of large amounts of FXa and thrombin, followed by conversion of fibrinogen to fibrin (23-25). Covalent cross-linking of fibrin polymers by activated factor XIII (FXIIIa) is required for adequate clot strength and normal wound healing. The zymogen form of FXIII is converted to an active enzyme by thrombin.

The catalytic nature of coagulation reactions allows tremendous amplification of the initial stimulus. Each FVIIa/TF complex can produce many FXa molecules, which subsequently produce an even greater

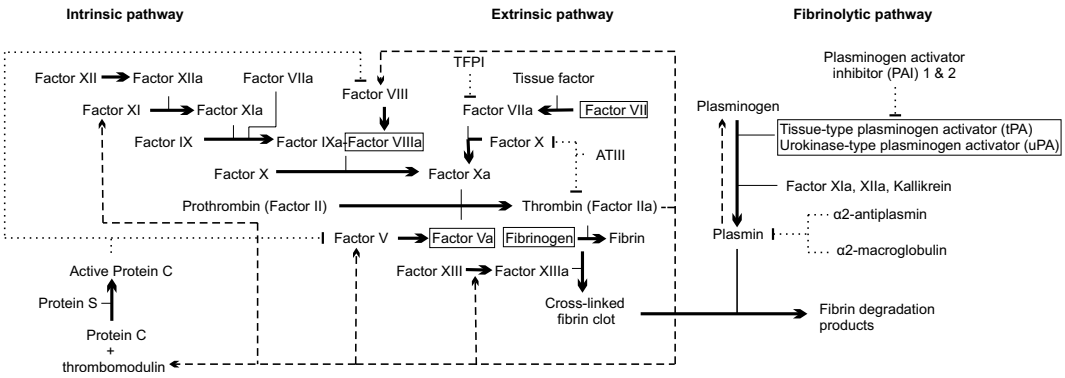


Figure 2. Schematic overview of the regulation of the coagulation and fibrinolytic pathways. Striped lines; feedback loops involved in the amplification of the initial stimulus. Dotted lines; pathways and components involved in the inhibition of the initiated coagulation or fibrinolysis. Boxed factors; potential substrates of FSAP.

amount of thrombin. Amplification also results from positive feedback reactions. These include activation of FV, FVIII, and FXI by thrombin, as well as activation of FVII by FXa. Thrombin cleaves FV and FVIII to yield activated cofactors (FVa and FVIIIa) that increase the proteolytic efficiency of FXa and FIXa, respectively (Fig. 2).

The coagulation system is tightly regulated by the stoichiometric inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin III (AT) and by the dynamic anticoagulant process of protein C activation (26, 27). TFPI is a protein associated with plasma lipoproteins and with the vascular endothelium. It binds to the FXa/TF/FVIIa complex and limits the production of FXa through the formation of a quaternary complex (28). AT is a major inhibitor of thrombin but also inhibits most of the other procoagulant serine proteases, such as FIXa, FXa and FXIa (29). Activated proteins C (APC) together with its cofactor protein S, proteolyticaly inactivate FVa and FVIIIa thereby down regulating the activity of their enzymatic counterparts FXIa and FXa. Therefore, the cofactor function of FVa and FVIIIa is disrupted and thrombin formation is terminated (30). Protein C is activated at the vessel wall by thrombomodulin bound thrombin.

Fibrinolysis

Once the damaged vessel is repaired the clot or thrombus is no longer needed and has to be degraded. This is achieved by the fibrinolytic pathway (31) (Fig.2). The end product of this pathway is the enzyme plasmin, a potent proteolytic enzyme with a broad spectrum of specificity. Plasmin is formed by activation of the proenzyme (plasminogen) by activators either originating from plasma (intrinsic) or tissue (extrinsic). The best characterized activators of plasminogen are tissue- type plasminogen activator (tPA) and plasma-urokinase-type plasminogen activator (uPA). Plasmin inhibitors which can control plasmin activity include

α1-antitrypsin, α2-antiplasmin, C1-esterase inhibitor, and antithrombin III (32-34). Plasmin attacks fibrin and fibrinogen at a number of different sites, reducing its size such that it no longer has haemostatic activity.

Triggering of fibrinolysis occurs when the plasminogen activator, plasminogen, and fibrin are all in close proximity. Both plasminogen and its activator bind firmly to fibrin as the clot forms. This close association prevents inhibition of plasmin activity by inhibitors, and allows proteolysis of the fibrin to proceed (35-37).

The haemostatic balance

An imbalance in the haemostatic system is associated with bleeding or thrombosis. Inherited deficiency of e.g. FVIII or FIX causes a severe bleeding tendency, known as hemophilia A & B, respectively (38, 39). On the other hand, an inherited deficiency of Protein C or Protein S may induce spontaneous clot formation (40). Not only a quantitative deficiency but also inherited mutations in coagulation factors can cause haemostatic imbalance. For example, a variant of FV known as FV-Leiden, displays impaired response to the inhibitory effect of Protein C (41). At the other hand, TF-bearing membrane-derived microparticles in the bloodstream, released from e.g. platelets, apoptotic endothelial cells, and tumours are associated with uncontrolled activation of FVII and subsequent blood clot formation in an intact vessel (13). Since an imbalance in the haemostatic system predisposes clotting abnormalities, knowledge of all factors that contribute to the haemostatic system is crucial for the treatment of patients with haemostatic disorders. Therefore, research on the contribution of occasionally discovered 'new' plasma proteins, like FSAP, to the haemostatic system is of outmost importance.

Factor VII-activating protease (FSAP)

In 1996 Choi-Miura *et al.* (1) identified a new human plasma protein. This was named Plasma Hyaluronan Binding Protease (PHBP) as it eluted during hyaluronic acid affinity chromatography. Subsequently, German investigators identified a 'new' serine protease that was co-purified with FIX and other coagulation factors which they called Hyaluronic Acid Binding Protease (HABP) (42). Due to its capability of hydrolysing a thrombin-specific chromogenic substrate HABP was designated as 'thrombin-like'. However, the specific thrombin inhibitor hirudin did not inhibit the catalytic activity of HABP, whereas the broad spectrum serine protease inhibitor aprotinin did (42). HABP proved identical with the previously discovered PHBP. PHBP revealed partial structural homology with 'Hepatocyte Growth Factor Activator' (HGFA). Römisch *et al.* (2) demonstrated that the isolated PHBP could activate FVII independent of TF and therefore called the protease Factor VII-Activating Protease (FSAP).

Subsequently, FSAP was indicated as a potent activator of single-chain pro-urokinase-type plasminogen activator (scuPA) in vitro (2, 43). Although a poorer substrate for activated FSAP compared to scuPA, FSAP was shown to activate tissue-type plasminogen activator (tPA) in vitro as well (3). The biological role of FSAP in plasma however is unclear. The reported activation of coagulation FVII and scuPA in vitro

suggests a pro-coagulant and pro-fibrinolytic role of FSAP. A pro-coagulant role for FSAP was further suggested by the observation in vitro that FSAP is able to cleave HMW-Kininogen (HK) and LMW-kininogen (LK) thereby releasing bradykinin (44). Kininogen belongs to the intrinsic activation pathway which suggested that FSAP can trigger the coagulation independent of FVII as well. On the other hand, bradykinin is involved in fibrinolysis and is a major contributor to inflammation and acute phase reactions (45). Therefore, FSAP may play a function in initiating inflammatory responses.

Beside a pro-coagulant and pro-fybrinolytic effect, an anticoagulant effect of FSAP through the inactivation of FVa and FVIIIa was reported by Romisch *et al.* (2). Further, fibrinogen and fibronectin were identified as potential substrates for FSAP (43). The relevance of this observation is not clear since the latter did not initiate fibrin clot formation or fibrinolysis either. The putative role of FSAP in the haemostatic system is summarized in Fig. 2.

As FSAP seems a broad spectrum protease it seems likely that its catalytic activity is tightly regulated. In vitro studies identified the plasma serpins C_1-esterase-inhibitor, α2-antiplasmin, antithrombin, and plasminogen-activating inhibitor-1, as well as the Kunitz-Type-Inhibitor Inter-α-trypsin Inhibitor as potential inhibitors of FSAP in plasma (4). To date, however, it is unclear whether these inhibitors are physiological regulators of FSAP catalytic activity.

FSAP may also affect processes other than coagulation. In vitro studies e.g. revealed that FSAP affects the proliferation and migration of human umbilical vein endothelial cells (HUVECs) (4) and vascular smooth muscle cells (VSMC) (5) through cleavage of Platelet-Derived Growth Factor BB (PDGF-BB). This suggests an anti-angiogenic action of FSAP (46). More recently, FSAP was found to be present in unstable atherosclerotic lesions suggesting a potential inflammatory role for FSAP (47).

It thus appears that FSAP is a broad spectrum serine protease with multiple interactions with the haemostatic system and its cellular environment. Therefore, the specific biological function of FSAP has become increasingly unclear.

Structure of FSAP

The human FSAP gene is localized at chromosome 10 and has a size of approximately 35 kb containing 13 exons and 12 introns (48). On homology with the exon/inton-organisation of other serine proteases, FSAP show high similarities with FXII, tPA, uPA, and Hepatocyte Growth Factor Activator (HGFA) (49). FSAP is a 560 amino acids containing protease, including a signal peptide of 23 amino acids at its N-terminal side (1). On basis of homology with other coagulation proteases the secondary structure of FSAP was predicted. A schematic representation of FSAP is shown in Fig. 3.

The signal peptide is followed by three epidermal growth factor domains (EGF 1-3) and a kringle domain, together representing the heavy chain (45-50 kDa). The light chain (25-30 kDa) consists of a catalytic serine protease domain, with the conserved catalytic triad (His^{362}, Asp^{411}, Ser^{509}). FSAP is thought to be

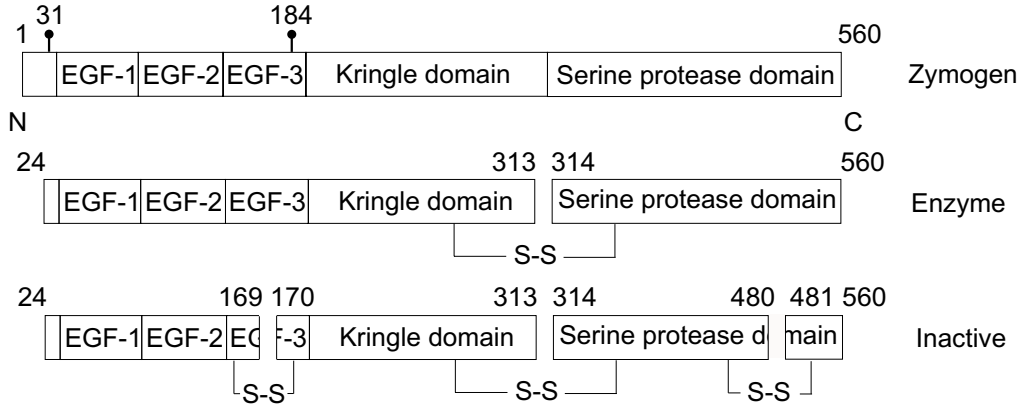


Figure 3. Structure of Factor VII-Activating Protease (FSAP). FSAP consists of 560 amino acids representing a 23 amino acid signal peptide followed by three epidermal growth factor domains (EGF 1-3), a kringle domain, and a catalytic serine protease domain. It is activated through cleavage of the Arg^{313}–Ile^{314} bound located in front of the serine protease domain resulting in a 50 kDa heavy chain and 25 kDa light chain. Both domains are held together by a disulfide bridge. The enzyme is inactivated through two extra cleavages, one located in the EGF-3 domain (between Lys^{169} and Arg^{170}) and one located in the serine protease domain (Arg^{480}). The heavy chain is cleaved in two fragments of 25 kDa whereas the light chain is cleaved into fragments of 18 and 8 kDa. At positions 31 and 184 N-glycosylation sites are predicted.

predominantly synthesized in the liver and circulates as an inactive zymogen in plasma at a concentration of ca. 12 µg/ml (~ 170 nM) (1, 3). It is activated through cleavage of an Arg^{313}-Ile^{314} bond at the N-terminal region of the catalytic serine protease domain (1, 3). The heavy chain and light chain are held together by a disulfide bridge (1, 49, 50). FSAP was found to be very sensitive to cleavage during purification resulting in a protein that is partially activated and degraded due to autocalytic cleavage (1, 50-53). The autocatalytic activity is mediated through the interactions with negatively charged surfaces such as acidic glycosaminoglycans (5, 50, 53). In vitro, the autocatalytic activation is followed by rapid degradation due cleavage at two additional sites (50, 51). One inactivation site is located in the EGF-3 like domain (between Lys^{169} and Arg^{170}) whereas the other is located in the serine protease domain (Arg^{480}). FSAP is a glycoprotein as it contains two conserved N-glycosylation sites (Asn^{31} and Asn^{184}). FSAP has an isoelectric point between 4.9 and 5.5 (50).

Marburg-1 and Marburg-2 FSAP variants

About 5-10 % of apparently healthy individuals were described to be heterozygous for either one or two Single-Nucleotide-Polymorphisms (SNP) in the FSAP gene, also known as Marburg-1 (M1) and Marburg-2 (M2) (6). The M1 SNP results in a glycine by glutamic acid substitution at position 534 (1601G>A; Gly534Glu), whereas the M2 SNP is responsible for a glutamic acid to glutamine exchange at position 393 (1177G>C; Glu393Gln). Both SNPs are located within the FSAP catalytic domain. Whereas no implications

of the M2 polymorphism are reported, the M1 polymorphism seems to be associated with a 50-80 % impaired activation of scuPA (6). Both polymorphisms do not affect FSAPs catalytic activity towards FVII (28).

It has been generally established that in serine proteases the chymotrypsin-like backbone is highly conserved. There are eight surface loops that vary between individual serine protease family members. Of those surface loops, six are known to be located at the border of the substrate binding groove at the reactive centre (54). In Fig. 4A those loops are indicated as c30, c60, c70, c90, c140, and c170 (chymotrypsin numbering). For example, in thrombin and FIX most of these loops have been found to contribute to macromolecular substrate interaction (54-57). The remaining two loops, indicated as c180 and c220, are located more distant from the catalytic site, at the bottom of the protease. In terms of chymotrypsin numbering, the M1 polymorphism is located at position c221 in the c220 loop whereas the M2 mutation is located in the back-bone structure at position c86 (Fig. 4A).

No functional implications for the replacement of residue c86 in serine proteases homologous to FSAP are reported. This strengthens the suggestion that the M2 mutation is no functional mutation. In contrast, residue c221 has been described as highly important in serine proteases. For thrombin Asp221Glu substitution has been associated with a severe defect in fibrinogen clotting (58). For FIX, patients who are hemizygous for a c221 substitution (Ala221Val) suffer from haemophilia B due to a defect in macromolecular substrate binding. This affects FIX reactivity towards its substrates FX and FVII, and towards its inhibitor antithrombin (59). Similarly, individuals that are heterozygous for a mutation in position c221 (Gly221Arg) display impaired protein C biological activity (60). In general, in Na^+-dependent serine proteases like Protein C, FVII, FIX, FX, and thrombin, residue c221 contribute to the binding of Na^+ which is needed for optimal activity and substrate specificity (61-64). This opens the possibility that the M1 polymorphism is a functional mutation that affects FSAP activity towards macromolecular substrates.

Indeed, the M1 polymorphism has been proposed to be a strong and independent risk factor for carotid stenosis (65) and cardiovascular disease (66). Sedding *et al.* (67) related the increase in cardiovasculare risk due the inability of M1-FSAP to inhibit vascular smooth cell accumulation resulting in an increase in neoinitima formation. Later, Hoppe *et al.* (68) associated this polymorphism with idiopathic venous thromboembolism whereas this was not found by others (25, 54, 69, 70). Therefore, the contribution of the M1 polymorphism as a risk factor for thrombosis remains highly controversial. More recently, due to its reduced proteolytic activity towards Platelet-Derived Growth Factor BB (PDGF-BB), the M1-variant was significantly associated with severe hepatitis C infection-induced liver fibrosis (71).

Questions addressed in this thesis

As described above, multiple interactions of FSAP with the haemostatic system and its cellular environment have been proposed. As a result the biological function of FSAP and the functional consequences of the Gly221Glu substitution remain unclear. Do FVII and scuPA really represent physiologically relevant substrates for FSAP? Does FSAP need cofactors for its function? What are the exact implications of the

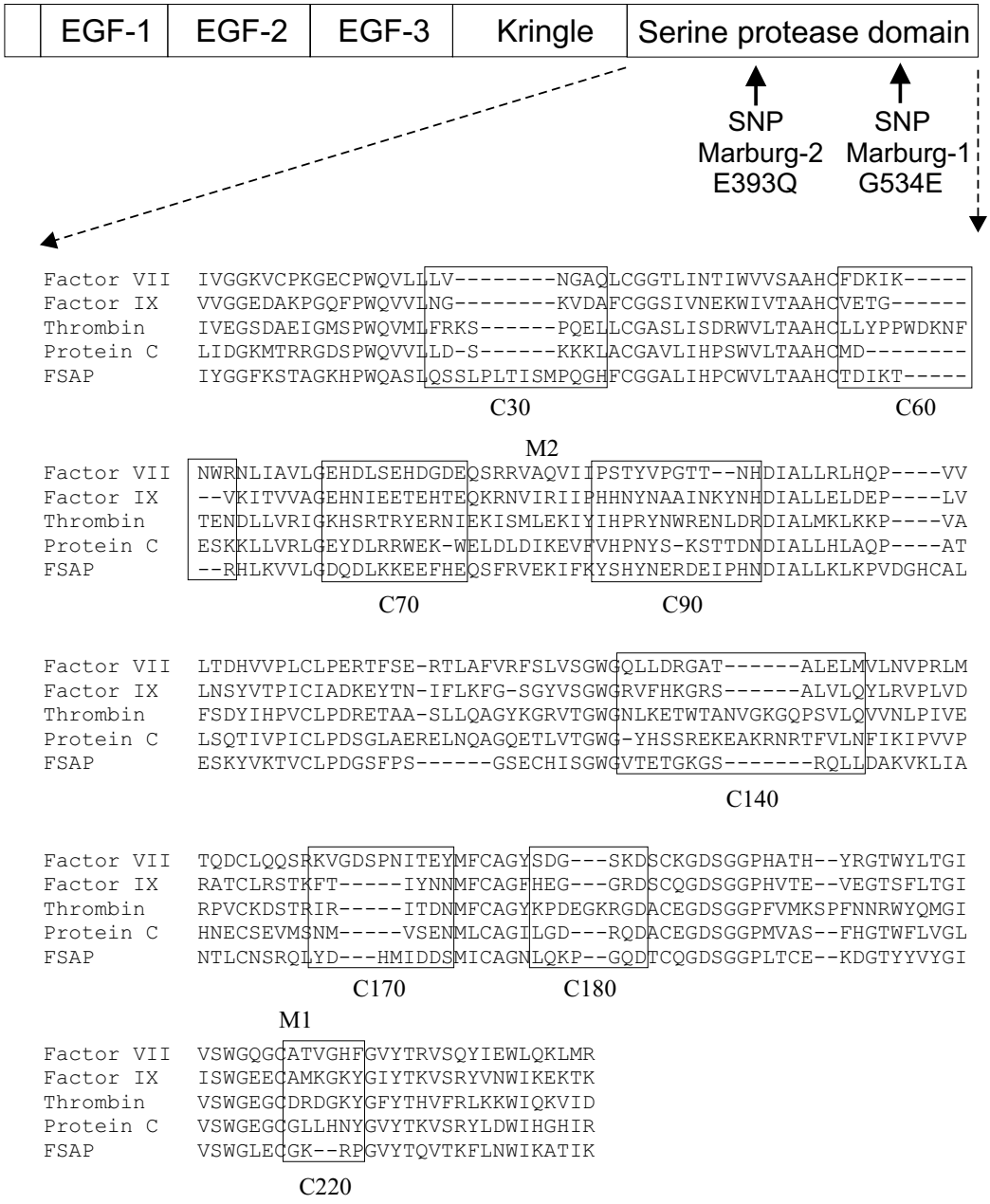


Figure 4. Location of the Marburg-1 and Marburg-2 mutations. (A) Sequence alignment of the serine protease domains of factor VII, factor IX, thrombin, protein C, and FSAP. Six surface loops are located at the border of the substrate binding groove at the reactive centre (His^{57}-Asp^{102}-Ser^{195}). In chymotrypsin numbering, these loops are c30, c60, c70, c90, c140, and c170. The two other loops, c180 and c220, are located more distant from the reactive centre, at the bottom of the protease. The latter contains the "Marburg-1" mutation at position c221. The "Marburg-2" mutation is located at position c86 within the serine protease backbone between surface loops c70 and c90.

G534E mutation for FSAP activation, catalytic activity, and specificity? What are the mechanisms underlying the reduced catalytic activity of this FSAP variant? Does FSAP act freely in circulating in plasma or in interaction with the vasculature?

These intriguing questions will be explored in the following chapters. In order to exclude that the very divergent effects of FSAP are due to contaminations present in plasma-derived FSAP, recombinant human FSAP was used. In chapter 2, the first successful construction of functional recombinant human FSAP is described. By employing kinetic experiments we established that scuPA indeed acts as a substrate for recombinant FSAP, whereas FVII appears surprisingly resistant to activation by recombinant FSAP. In chapter 3, we identified sodium as a cofactor for FSAP catalytic activity whereas the Gly221Glu mutation dramatically reduces this cofactor function. Further, we confirmed the reduced catalytic activity of $FSAP^{G221E}$ towards scuPA whereas a complete lack of activity towards FVII was found. The mechanisms underlying the reduced catalytic activity and specificity induced by the Gly221Glu mutation was unraveled in chapter 4 using a subset of FSAP Gly^{221} substitution variants. As other coagulation factors circulating in blood, FSAP stays in direct contact with the endothelial cells of the blood vessel wall. The vasculature forms a barrier that regulates a number of physiological processes, including extravasation of leukocytes to the underlying tissues. Endothelial cells contain Weibel-Palade bodies (WPBs), specific storage granules containing components such as von Willebrand factor (VWF) and P-selectin, which are released upon activation and support leukocyte rolling, platelet adhesion, and aggregation. In chapter 5 we report that FSAP acts as an agonist of WPB exocytosis from endothelial cells. This observation implies that there still might be a procoagulant role for FSAP in haemostasis unrelated to FVII. Finally, in chapter 6 the functional implications of these findings are further discussed.

References

1 Choi-Miura NH, Tobe T, Sumiya J, Nakano Y, Sano Y, Mazda T, Tomita M. (1996) J Biochem. 119(6):1157-65.
2 Römisch J, Feussner A, Vermöhlen S, Stöhr HA. (1999) Blood Coagul Fibrinolysis. 10(8):471-9.
3 Römisch J, Vermöhlen S, Feussner A, Stöhr H. (1999) Haemostasis. 29(5):292-9.
4 Etscheid M, Beer N, Kress JA, Seitz R, Dodt J. (2004) Eur J Cell Biol. 82(12):597-604.
5 Kannemeier C, Al-Fakhri N, Preissner KT, Kanse SM. (2004) FASEB J. 18(6):728-30.
6 Römisch J, Feussner A, Stöhr HA. (2001) Blood Coagul Fibrinolysis. 12(5):375-83.
7 Gailani D, Broze GJ. (1991) Science. 253: 909-912.
8 Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. (1993) Blood. 81:734-744.
9 Mann KG, Krishnaswamy S, Lawson JH. (1992) Semin. Hematol. 29: 213-226.
10 Gailani D, Broze GJ Jr. (1991) Science. 253(5022):909-12.
11 Von dem Borne PA, Koppelman SJ, Bouma BN, Meijers JC. (1994) Thromb Haemost. 72(3):397-402.
12 Von dem Borne PA, Meijers JC, Bouma BN. (1995) Blood. 86(8):3035-42.
13 Lechner D, Weltermann A. (2008) Thromb Res. 122 Suppl 1:S47-54.
14 Bom VJ, Bertina RM. (1990) Biochem. J. 265:327-336.
15 Jesty J., Silverberg SA. (1979) J. Biol. Chem. 254:12337-12345.
16 Komiyama Y, Pedersen AH, Kisiel W. (1990) Biochemistry. 29:9418-9425.
17 Krishnaswamy S. (1992) J. Biol. Chem. 267:23696-23706.
18 Baglia FA, Walsh PN. (1998) Biochemistry. 37:2271–2281.
19 Naito K, Fujikawa K. (1991) J. Biol. Chem. 266:7353-7358.
20 Osterud B, Rapaport SI. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:5260-5264.
21 Morrison SA, Jesty J. (1984) Blood. 63:1338-1347.
22 Mann KG. (2003) Circulation. 107:654-5.
23 Brummel KE, Paradis SG, Butenas S, Mann KG. (2002) Blood. 100:148-152.
24 Butenas S, van 't Veer C, Mann KG. (1997) J. Biol. Chem. 272:21527-21533.
25 Pieters J, Lindhout T, Hemker HC. (1989) Blood. 74:1021-1024.
26 Monroe DM, Key NS. (2007) J Thromb Haemost. 1097-105. Review.
27 van 't Veer C, Golden NJ, Kalafatis M, Mann KG. (1997) J. Biol. Chem. 272:7983-7994.
28 Bajaj MS, Birktoft JJ, Steer SA, Bajaj SP. (2001) Thromb. Haemost. 86:959-972.
29 Butenas S, Mann KG.(2002) Biochemistry 67(1):3-12. Review.
30 Dalhback B, Villoutreix BO. (2003) J. Thromb. Haemost. 1:1525-1534.
31 Collen D. (1980) Thromb. Haemost. 43:77-89.
32 Aoki N, Harpel PC. (1984) Semin Thromb. Haemost. 10:24.
33 Randby M, Brandstrom A. (1988) Enzyme. 40:130-143.
34 Vassalli JD, Sappino AP, Belin D. (1991) J. Clin. Invest. 88:1067-1072.
35 Kluft C, Dooijewaard G, Emeis JJ. (1987) Thromb Haemost. 13:50-68.
36 Rao LV, Rapaport SI. (1987) Blood. 69:645-651.
37 Van 't Veer C, Mann KG. (1997) J. Biol. Chem. 272:4367-4377.
38 Rosner F. (1969) Ann. Intern. Med. 70:833-837.
39 Sadler JE, Davie EW. (1987) The molecular basis of blood diseases. pp 575-630.
40 De Stefano V, Simioni P, Rossi E, Tormene D, Za T, Pagnan A, Leone G. Haematologica. (2006) 91(5):695-8.

41 Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. (1994) Nature. 369(6475):64-7.
42 Hunfeld A, Etscheid M, König H, Seitz R, Dodt J. (1999) FEBS Lett. 456(2):290-4.
43 Choi-Miura NH, Yoda M, Saito K, Takahashi K, Tomita M. (2001) Biol Pharm Bull. 24(2):140-3.
44 Etscheid M, Beer N, Fink E, Seitz R, Johannes D. (2002) Biol Chem. 383(10):1633-43.
45 Isordia-Salas I, Pixley RA, Sáinz IM, Martínez-Murillo C, Colman RW. (2005) Arch Med Res. 36(1):87-95.
46 Jeon JW, Song HS, Moon EJ, Park SY, Son MJ, Jung SY, Kim JT, Nam DH, Choi-Miura NH, Kim KW, Kim YJ. (2006) Int J Oncol. 29(1):209-15.
47 Parahuleva MS, Kanse SM, Parviz B, Barth A, Tillmanns H, Bohle RM, Sedding DG, Hölschermann H. (2008) Atherosclerosis. 196(1):164-71.
48 Hashimoto K, Tobe T, Sumiya J, Saguchi K, Sano Y, Nakano Y, Choi-Miura NH, Tomita M. (1997) Biol Pharm Bull. 20(11):1127-30.
49 Sumiya J, Asakawa S, Tobe T, Hashimoto K, Saguchi K, Choi-Miura NH, Shimizu Y, Minoshima S, Shimizu N, Tomita M. (1997) J Biochem. 122(5):983-90.
50 Kannemeier C, Feussner A, Stöhr HA, Weisse J, Preissner KT, Römisch J. (2001) Eur J Biochem. 268(13):3789-96.
51 Choi-Miura NH, Takahashi K, Yoda M, Saito K, Mazda T, Tomita M. (2001) Biol Pharm Bull. 24(5):448-52.
52 Choi-Miura NH, Saito K, Takahashi K, Yoda M, Tomita M. (2001) Biol Pharm Bull. 24(3):221-5.
53 Etscheid M, Hunfeld A, König H, Seitz R, Dodt J. (2000) Biol Chem. 381(12):1223-31.
54 Kolkman JA, Christophe OD, Lenting PJ, Mertens K. (1999) J Biol Chem. 274(41):29087-93.
55 Kolkman JA, Mertens K. (2000) Biochemistry. 39(25):7398-405.
56 Kolkman JA, Mertens K. (2000) Biochem J. 350 Pt 3:701-7.
57 Rohlena J, Kolkman JA, Boertjes RC, Mertens K, Lenting PJ. (2003) J Biol Chem. 278(11):9394-401.
58 Rouy S, Vidaud D, Alessandri JL, Dautzenberg MD, Venisse L, Guillin MC, Bezeaud A. (2006) Br J Haematol. 132(6):770-3.
59 Spitzer SG, Pendurthi UR, Kasper CK, Bajaj SP. (1988) J Biol Chem. 263(22):10545-8.
60 Miyata T, Zheng YZ, Sakata T, Tsushima N, Kato H. (1994) Thromb Haemost. 71(1):32-7.
61 Gopalakrishna K, Rezaie AR. (2006) Thromb Haemost. 95(6):936-41.
62 Pineda AO, Carrell CJ, Bush LA, Prasad S, Caccia S, Chen ZW, Mathews FS, Di Cera E. (2004) J Biol Chem. 279(30):31842-53.
63 Schmidt AE, Padmanabhan K, Underwood MC, Bode W, Mather T, Bajaj SP. (2002) J Biol Chem. 277(32): 28987-95.
64 Underwood MC, Zhong D, Mathur A, Heyduk T, Bajaj SP. (2000) J Biol Chem. 275(47):36876-84.
65 Weisbach V, Ruppel R, Eckstein R. (2007) Thromb. Haemost. 97:870-872.
66 Ireland H, Miller GJ, Webb KE, Cooper JA, Humphries SE. (2004) Thromb Haemost. 92(5):986-92.
67 Sedding D, Daniel JM, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM. (2006) J Exp Med. 203(13):2801-7.
68 Hoppe B, Tolou F, Radtke H, Kiesewetter H, Dörner T, Salama A. (2005) Blood. 105(4):1549-51.
69 Gulesserian T, Hron G, Endler G, Eichinger S, Wagner O, Kyrle PA. (2006) Thromb Haemost. 95(1):65-7.
70 Sidelmann JJ, Vitzthum F, Funding E, Münster AM, Gram J, Jespersen J. (2008) Thromb Res. 122(6):848-53.
71 Wasmuth HE, Tag CG, Van de Leur E, Hellerbrand C, Mueller T, Berg T, Puhl G, Neuhaus P, Samuel D, Trautwein C, Kanse SM, Weiskirchen R. (2009) Hepatology. 49(3):775-80.

Chapter 2

Factor Seven Activating Protease (FSAP): does it activate Factor VII?

F. Stavenuiter*†, M.G. Boon-Spijker*, A.B. Meijer* and K. Mertens*†‡

*Department of Plasma Proteins, Sanquin Research, Amsterdam; †Van Creveld Laboratory of UMC-Utrecht and Sanquin, Amsterdam; and ‡Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Abstract

Background: Factor Seven Activating Protease (FSAP) was initially reported as an activator of single-chain urokinase-type plasminogen activator (scuPA) and Factor VII (FVII), suggesting a key role in haemostasis and thrombosis. Subsequently, numerous additional substrates have been identified, and a variety of other biological effects has been reported. Due to the apparent lack of specificity, the physiological role of FSAP has become increasingly unclear. Rigorous studies have been limited by the difficulty of obtaining intact FSAP from blood or recombinant sources.

Objective: Our aim was to produce recombinant human FSAP suitable for functional studies, and to assess its role as a trigger of coagulation and fibrinolysis.

Results: Expression of wild-type FSAP in various mammalian cells invariably resulted in the accumulation of degraded FSAP due to autoactivation and degradation. To overcome this problem, we constructed a variant in which Arg^{313} at the natural activation site was replaced by Gln, creating a cleavage site for the bacterial protease thermolysin. HEK293 cells produced FSAP-R313Q in its intact form. Thermolysin-activated FSAP displayed the same reactivity toward chromogenic peptide substrates as plasma-derived FSAP, and retained its capability to activate scuPA. Polyanions like polyphosphate and heparin increased V_{max} by 2-3 fold, without affecting K_m (62 nM) of scuPA activation. Surprisingly, FSAP proved incapable of cleaving purified FVII, even in the presence of calcium-ions and lipid vesicles comprising negatively charged phospholipids. On membranes of 100% cardiolipin FVII cleavage did occur, but this resulted in transient activation and rapid degradation.

Conclusion: While FSAP indeed activates scuPA, FVII appears remarkably resistant to activation. Whether FVII cleavage on cardiolipin, being an intracellular lipid, has any physiological significance remains unclear.

Introduction

Factor Seven Activating Protease (FSAP) is the precursor of a serine protease that has several putative interactions with the haemostatic system. FSAP was first described in 1996 as Plasma Hyaluronan Binding Protein (PHBP), a novel serine protease with the capability to activate single-chain pro-urokinase (scuPA) (1, 2). Subsequently, others have purified a so far unknown factor VII (FVII) activating protease, designated FSAP, which later proved identical to PHBP (3). These findings suggested that FSAP, being a trigger of both coagulation and fibinolysis, might play a key role in haemostasis and thrombosis. Apart from FVII and scuPA, FSAP has been reported to cleave multiple other substrates, including high molecular weight kininogen (4), and the activated coagulation factors VIIIa and Va (5). More recently, FSAP was localized within atherosclerotic lesions and attributed an inhibitory role in vascular smooth muscle proliferation and migration in vitro (6-9). More than a decade since its discovery, FSAP now seems a broad-spectrum protease with a multitude of substrates in a variety of cross-linked biological pathways. With its decreasing specificity, the 'true' physiological role of FSAP has become increasingly unclear.

FSAP circulates in plasma at a concentration of about 12 $\mu g.mL^{-1}$ (1, 10). FSAP is notoriously difficult to isolate in its single-chain form, because it is highly sensitive to proteolysis (1, 3, 11, 12). FSAP displays a discrete domain structure, and comprises three epidermal growth factor domains (EGF1-3), one kringle domain and a serine protease domain, representing a total molecular mass of approximately 70 kDa (1). Like other serine proteases, FSAP is activated by limited proteolysis. This involves cleavage between Arg^{313} and Ile^{314} at the N-terminal region of the catalytic domain, resulting in a ~ 45 kDa heavy chain and ~ 25 kDa light chain held together by a disulfide bond (1, 11, 13). The activation into two-chain FSAP has been suggested to be the result of autocatalytic cleavage, which is stimulated by interaction with polyanions like glycosaminoglycans (5) and RNA (13-14) and by polyamine (16). However, it has been impossible so far to exclude the possibility that contaminating enzymes that are co-purified from plasma contribute to the apparently disparate roles of FSAP.

The aim of the present study was to produce recombinant FSAP suitable for functional studies, and to re-assess its role as a trigger of coagulation and fibrinolysis. To overcome the problem of autoactivation and subsequent degradation, we constructed a FSAP variant in which Arg^{313} at the natural activation site was replaced by Gln, creating a cleavage site for the bacterial protease thermolysin. This approach allowed us to isolate recombinant FSAP in its intact, single chain form, which then could be activated under controlled conditions by thermolysin. Kinetic studies using purified proteins confirmed that activated FSAP indeed activates scuPA. In contrast, we found no evidence in favor of a role in FVII activation. These data indicate the need for a reappraisal of the putative role of FSAP in haemostasis.

Materials

CNBr-Sepharose 4B, DEAE-Sephadex A-50, Q-Sepharose FF and Heparin-Fractogel were from Amersham Pharmacia Biotech Nederland (Roosendaal, The Netherlands). Optimem-I medium, penicillin/streptomycin, and DNA restriction enzymes were obtained from Life Technologies (Breda, The Netherlands). Microtiter plates and cell culture flasks were purchased from Nunc (Roskilde, Denmark). Oligonucleotide primers, 293 Freestyle® cell culture system, Insulin-Transferrin-Selenium-X (ITS) supplement, restriction enzymes, pcDNA3.1(-) vector, DMRIE-C, Geneticin (G418), and phosphoramidon disodium salt were supplied by Invitrogen (Breda, The Netherlands). Fetal calf serum (fcs) was from Thermo Fisher Scientific (Breda, The Netherlands). The synthetic substrates H-(D)-Ile-Pro-Arg-pNA (S-2288) and pyroGlu-Gly-Arg-pNA (S-2444) from Chromogenix (Milan, Italy). Brain phosphatidylserine (PS), brain phosphatidylethanolamine (PE), and phosphatidylcholine (PC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Thermolysin from Bacillus thermoproteolyticus, cardiolipin disodium salt from bovine heart, polyphosphate type 65 (Poly-P65), Soybean trypsin inhibitor and benzamidine were from Sigma-Aldrich (Zwijndrecht, The Netherlands). Serine-protease inhibitors Aprotinin (Trasylol) and Pefabloc were from Roche Diagnostics (Almere, The Netherlands). Fut-175 (Futhan) was from BD Biosciences (Breda, The Netherlands), PPACK from Bachem (Weil am Rhein, Germany), and C1-inhibitor from Sanquin Plasma Products (Amsterdam, The Netherlands). Heparin was from LEO Pharmaceuticals (Weesp, The Netherlands). Purified human scuPA was obtained from Kordia Life Sciences (Leiden, The Netherlands).

Plasma-derived human FSAP

Human FSAP was purified from cryoprecipitate-depleted plasma (Sanquin Plasma Products, Amsterdam, The Netherlands). Plasma was supplied with benzamidine to a final concentration of 10 mM, and then incubated batch-wise with pre-swollen DEAE-Sephadex A-50 (30 ml per L plasma) for 90 minutes at room temperature. The matrix was then collected in a column, and extensively washed with 125 mM NaCl, 10 mM benzamidine, 10 mM trisodium citrate, pH 7.0. The column was extensively washed using the same buffer containing 0.5 M NaCl, and bound protein was eluted by washing buffer containing 2 M NaCl. FSAP containing fractions were pooled, dialyzed against 50 mM NaCl, 10 mM benzamidine, 10% (v/v) glycerol, 20 mM Hepes (pH 6.0) and loaded onto a Heparin-Fractogel column (29 mL) equilibrated in the same buffer. The column was eluted using a gradient from 0.05 to 2 M NaCl in 10 mM benzamidine, 20 mM Hepes, pH 6.0, and FSAP containing fractions were pooled en concentrated on a Q-Sepharose FF column (2 mL) by elution with 2 M NaCl, 10 mM benzamidine, 50 mM Tris-HCl (pH 8.0). Concentrated FSAP was dialyzed against 250 mM NaCl, 50% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0) and stored at -20 °C.

Recombinant FSAP variants

The complete open reading frame of human wild-type FSAP was produced by PCR from human liver quick-clone cDNA (BD Biosciences, Breda, The Netherlands) using the oligonucleotide primers (a) 5'-TTAGGATCCGCAAAGATGTTTGCCAGGATGTCTGATCTC-3' (sense) and (b) 5'-ATTGGTACCGAAGACAG-TACCTTAGAAGCCACT-3' (antisense), including a BamH1 and KpnI restriction site, respectively. (underlined in primer sequences). The resulting PCR fragment was digested with BamH1 and KpnI and then ligated

into the pcDNA3.1(-) expression vector. This wild-type FSAP plasmid was used as the template for site-directed mutagenesis with the QuickChange® kit (Stratagene, La Jolla, USA) to construct plasmids encoding $FSAP^{S509A}$ and $FSAP^{R313Q}$. The oligonucleotide primers 5'-GACACCTGCCAGGGTGACGCTGGAG-GCCCCCTGACCTGT-3' (sense) and 5'-ACAGGTCAGGGGGCCTCCAGCGTCACCCTGGCAGGTGTC-3' (antisense) were used for the construction of $FSAP^{S509A}$ and 5'-GCAGAGAGGAAGATCAAGCAAATCTATGGAGGCTT-TAAG-3' (sense) and 5'-CTTAAAGCCTCCATAGATTTGCTTGATCTTCCTCTCTGC-3' (antisense) for the $FSAP^{R313Q}$ variant. All constructs were verified by sequence analysis. Plasmids encoding for wild-type FSAP were transfected into LoVo-, HEK293-, and CHO-cells using DRMIE-C, and positive clones were expanded in DMEM-F12 with 10% fetal calf serum (v/v) and 500 µg/ml G418. Stable cell lines were maintained on DMEM-F12 containing 10% fetal calf serum (v/v) or 1% (v/v) ITS. $FSAP^{S509A}$ and $FSAP^{R313Q}$ were transiently expressed in 293-F cells the Freestyle® system according to the manufacturers instructions.

Recombinant FSAP was purified by immuno-affinity chromatography using anti-FSAP4 (17) coupled to CNBr-Sepharose 4B (5 mg per mL gel). Briefly, after loading the column was washed with a buffer containing 1 M NaCl, 50 mM Tris-HCl, pH 8.0, followed by a wash with a buffer containing 100 mM NaCl, 15% ethylene glycol, 50 mM Tris-HCl, pH 8.0. Bound FSAP was eluted with a buffer containing 150 mM NaCl, 55% ethylene glycol, 10% glycerol and 25 mM lysine, pH 11 and immediately neutralized using 1.5 M Imidazole (pH 7.0). Remaining contaminants were removed on a Q-Sepharose FF column, employing a gradient from 0.15 to 1 M NaCl in 5% glycerol, 50 mM Tris-HCl, pH 8.0. FSAP fractions were pooled and concentrated on a second Q-Sepharose FF column (1 mL) by elution with 1 M NaCl, 5% glycerol, 50 mM Tris-HCl, pH 8.0. Concentrated $FSAP^{S509A}$ was dialyzed against 150 mM NaCl, 50 mM Tris-HCl, pH 8.0. $FSAP^{R313Q}$ was dialyzed against 150 mM NaCl, 5% glycerol, 10 mM MES, pH 5.0. Purified recombinant FSAP was stored in aliquots at -20°C.

For all experiments $FSAP^{R313Q}$ (~700 nM) was freshly activated by incubation with thermolysin (~3 nM) for 2 hours at 30°C in 150 mM NaCl, 5% (v/v) glycerol, 0.01% (v/v) Tween 80, 100 µM $CaCl_2$, 50 nM $ZnCl_2$, 10 mM MES, pH 5.0. The reaction was stopped by the addition of phosphoramidon disodium salt (10 µM), and activation was checked by amidolytic activity and SDS/PAGE. Activated $FSAP^{R313Q}$ was stored on ice and used within 30 min.

Other methods

Human FVII was isolated from plasma as previously described for FVIIa (18), with the exception that after cryoprecipitation and the DEAE-Sephadex and Heparin-Sepharose chromatography steps were replaced by a single immunoaffinity chromatography step using antibody 2G3 (19). FVII was stored in 150 mM NaCl, 5% (v/v) glycerol, 10 mM MES (pH 5.0) at -20°C. Rabbit polyclonal anti-FSAP antibodies were produced by Biogenes GmbH (Berlin, Germany) using purified plasma-derived FSAP (see above). The monoclonal antibody anti-FSAP4 has been previously described (17). FSAP antigen was determined by standard ELISA techniques, using monoclonal anti-FSAP4 IgG (1 µg per well) as primary antibody, and peroxidase-labelled polyclonal anti-FSAP-IgG (0.2 $\mu g.mL^{-1}$) for detection. Concentrations of purified proteins were measured by the Bradford method (20) using human albumin as a reference. Molar concentrations were

calculated from protein values using a molecular size of 70 kDa for FSAP, and 55 kDa for FVII. Concentrations of uPA were expressed in International Units based on the International Standard for HMW uPA, encoded 87/594 (NIBSC, South Mimms, UK). Lipid vesicles were prepared by sonification followed by centrifugation as described (21), and lipid concentrations were determined by phosphate analysis (22). SDS/PAGE was performed using 10% or precast 4-12% gradient gels (Invitrogen, Breda, The Netherlands). Protein was reduced using 20 mM 1,4 dithiotreitol for 5 min at 100°C. Protein bands of reduced FSAP were excised and subjected to N-terminal sequencing (Eurosequence, Groningen, The Netherlands). For detecting FVII proteolysis, immunoblotting was performed using rabbit polyclonal antibodies against human FVIIa (18). Bands were visualized by electro-chemiluminescence (ECL, Roche Diagnostics, Mannheim, Germany).

Results

Plasma-derived human FSAP

Given its sensitivity to autoactivation and autodegradation (1, 11-13, 23), it was not surprising that purified FSAP was not obtained as a single chain polypeptide. The reduced protein (Fig. 1A) displayed more constituents than originally reported (1). Therefore, the identity of the individual fragments was verified by N-terminal sequencing. The various sequences are given in Fig. 1B. Ser^{28} was found as the N-terminus of the fragments of approx. 45 kDa, 25 kDa and 18 kDa (Fig. 1B). The 25 kDa band further comprised a second fragment, starting with Arg^{171}, apparently due to cleavage in the N-terminal section of FSAP (the heavy chain). The same N-termini were found in the 18 kDa band, together with Ile^{314} and Ser^{320}, which represent the N-terminus of the protease domain and a truncated form thereof missing 6

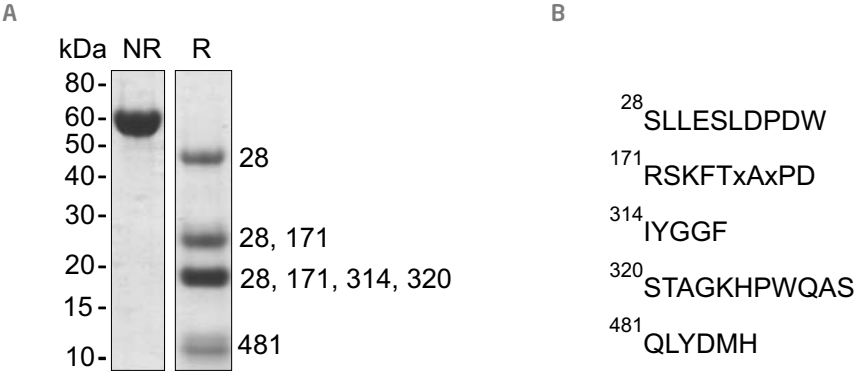


Figure 1. Characterization of purified plasma-derived FSAP. (A) Purified human FSAP (5 µg/lane) was analysed by 10% SDS/PAGE under non-reducing (NR) and reducing (R) conditions, and protein was visualized by Coomassie Blue staining. Individual bands were cut out and subjected to N-terminal sequencing. For each band the found N-termini are indicated as the residue number. Numbering includes the 23 amino acid signal peptide (1). (B) Results of N-terminal sequencing of FSAP fragments recovered in the individual bands indicated in panel A.

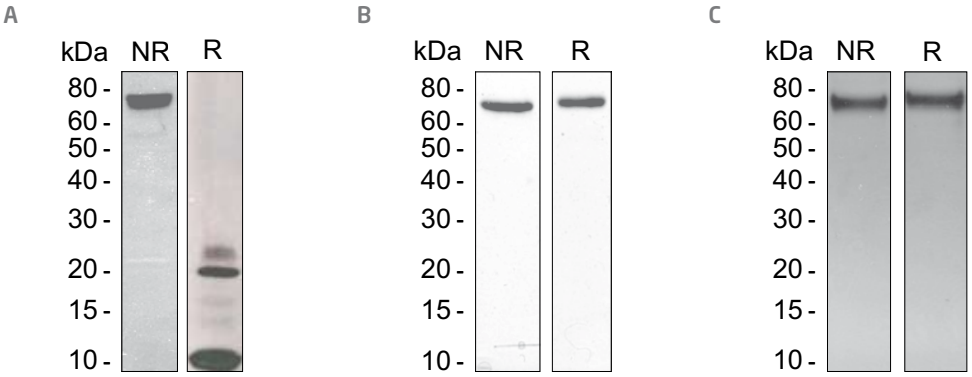


Figure 2. Characterization of purified recombinant FSAP derivatives. (A) Wild-type FSAP was produced in CHO cells and purified as described in Materials and Methods. (B) FSAP^{S509A} and (C) FSAP^{R313Q} were produced in 293-F cells and purified by the same method. Proteins (1 µg/lane) were analysed by 10% SDS/PAGE under non-reducing (NR) and reducing (R) conditions, and protein was visualized by Silver staining.

residues. Finally, the 11 kDa band was identified as a C-terminal fragment from the protease domain, starting at Gln^{481}. Apparently, our purification procedure had resulted in pure, but cleaved human FSAP. This material was suitable for immunisation purposes (see Materials and Methods), but not for any rigorous functional studies.

Recombinant human FSAP

In an attempt to overcome the heterogeneity of plasma-derived FSAP, we expressed recombinant FSAP in a variety of mammalian cell. These were varying from low to high expression of furin-like endoprotease activity (24), and included human colon carcinoma line (LoVo cells), Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) cells, and Human Embryonic Kidney cells (HEK293). By ELISA very low antigen levels were detected in medium from BHK and LoVo cells, while CHO and HEK293 cells showed relatively high expression (in range µg/mL per day). Recombinant human FSAP could be easily purified using immunoaffinity chromatography (see Material and Methods). Wild-type human FSAP was obtained as a 70 kDa species that was even more degraded than FSAP from human plasma (Fig. 2A). Degradation could not be prevented by adding to the medium a variety of serine protease inhibitors, including benzamidine, PPACK, aprotinin, Pefabloc, Soy Bean Trypsin Inhibitor, Fut-175, and C1-inhibitor (data not shown). In contrast, no degradation was observed upon expression of FSAP with a replacement of Ser^{509} to Ala in its active site (Fig. 2B). This identifies autodegradation as the cause of FSAP degradation during expression in mammalian cells. At the same time, this implies that production of wild-type human recombinant FSAP in its intact form would remain difficult, if possible at all. Previous studies have identified the Arg^{313}-Ile^{314} bond in as the target for converting the single chain FSAP zymogen into the active two-chain serine protease (1). We therefore introduced a Arg^{313} to Gln substitution, which renders this bond resistant to cleavage by serine proteases. Indeed, FSAP^{R313Q} could be successfully purified in its intact, single-chain form (Fig. 2C). The observation that FSAP degradation requires both Ser^{509} in the active site

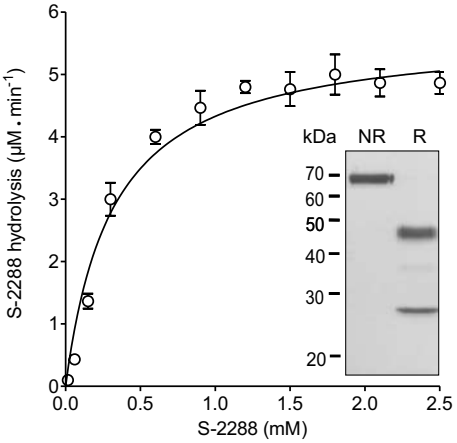


Figure 3. Amidolytic activity of activated FSAP^{R313Q}. Purified FSAP^{R313Q} was activated using thermolysin as described in Materials and Methods. Amidolytic activity was measured by incubating activated FSAP^{R313Q} (10 nM) with substrate S-2288 (50-2500 nM) at 37°C in a buffer containing 0.2 % (w/v) BSA, 150 mM NaCl, 5 mM CaCl2, 50 mM Tris-HCl, pH 8.0. Initial rates of S-2288 cleavage were quantified by monitoring absorbance. Data represent mean ± SD of 3-4 experiments. The curve was fitted to the Michaelis-Menten equation using GraphPad Prism® software. The inset shows 10% SDS/PAGE (1 μg/lane, silver staining) of activated FSAP^{R313Q}, demonstrating that the reduced (R) protein consisted of the ~45 kDa heavy chain and the ~25 kDa light chain.

and Arg^{313} at the scissile bond involved in zymogen activation confirms that autocatalysis greatly contributes to the heterogeneity observed (Figs 1A and 2A).

Activation of FSAP^{R313Q} by thermolysin

The Arg to Gln substitution created a suitable target site (Gln^{313}-Ile^{314}) for the bacterial metalloprotease thermolysin. In order to suppress the intrinsic instability of FSAP^{R313Q} once activated, activation was performed at pH 5, and the protein was kept at low pH until further experiments. Each series of experiments required FSAP^{R313Q} to be freshly activated. As shown in Fig. 3 (see inset) activation by thermolysin resulted in a two-chain species with a heavy chain of approximately 45 kDa and a light chain of the expected molecular size of 25 kDa. The recombinant enzyme efficiently cleaved the chromogenic substrate S-2288, with a K_m of 0.36 ± 0.06 mM (mean ± SD), and a V_{max} of 5.7 ± 0.1 μM.min^{-1}. Plasma-derived FSAP displayed a similar K_m value, but much lower activity (data not shown), presumably because it was largely degraded. These data demonstrate that thermolysin converts FSAP^{R313Q} into the two-chain enzymatic form, and that S-2288 is a suitable substrate to quantify the enzyme concentration in individual preparations of activated FSAP^{R313Q}.

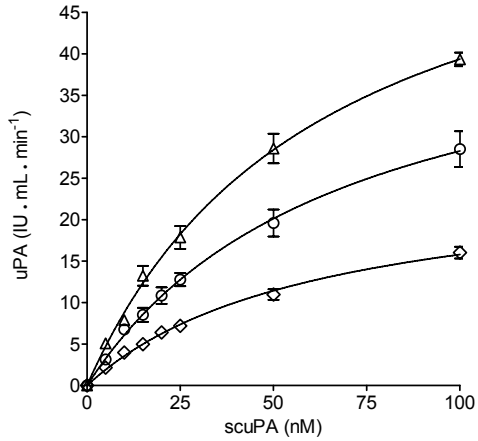


Figure 4. Activation of scuPA by activated FSAP^{R313Q}. Varying amounts of scuPA (5-100 nM) were incubated with activated FSAP^{R313Q} (0.25 nM) in a final volume of 75 µL buffer containing 100 mM NaCl, 0.1% BSA, 0.01% (v/v) Tween-80, 5 mM CaCl2, 20 mM Tris-HCl (pH 7.5) at 37°C. Incubations were performed in the absence of cofactors (diamonds), and in the presence of 10 IU.mL^{-1} of heparin (triangles) or 5 µM of PolyP65 (circles). After 10 min of incubation, reactions were stopped by adding 25 µL of aprotinin (1 mM final concentration) in the same buffer, and uPA formation was quantified as described in Materials and Methods. Data are given as mean ± SD from at least 4 independent experiments. Kinetic parameters were calculated using GraphPad Prism® software (GraphPad, La Jolla, USA).

Activation of scuPA by activated FSAP^{R313Q}

As various studies reported that plasma-derived FSAP activates scuPA (11, 25, 26), we first assessed the reactivity of activated FSAP^{R313Q} towards this macromolecular substrate. As shown in Fig. 4, two-chain FSAP^{R313Q} readily activated scuPA. The K_m for scuPA activation was estimated to be 62 ± 7 nM (mean ± SD), and V_{max} was 24 ± 2 IU.mL^{-1}.min^{-1}. Activation was stimulated 2-3 fold by polyanions like polyphosphate and heparin (Fig. 4). These cofactors did not affect K_m, but increased the calculated V_{max} to 48 and 64 IU.mL^{-1}.min^{-1}, respectively. The presence of 100 µM phospholipid mebranes consisting of PS/PE/PC (20/20/60 mol %) or 100% cardiolipin did not accelerate scuPA activation to any appreciable extent (data not shown). These data demonstrate that recombinant thermolysin-activated, two-chain FSAP^{R313Q} does display reactivity towards scuPA as previously reported for plasma-derived FSAP.

Activated FSAP^{R313Q} is a poor FVII activator

It seems obvious to expect that activated FSAP, as its name suggests, should activate FVII. Assessment of this activity is complicated by the fact that FVII itself is subject to autoactivation (27-29), which becomes especially apparent in the presence of tissue factor, as used in clotting assays or thrombin generation systems. We therefore assessed FVII activation by using purified FVII, and monitored proteolysis by immunoblotting employing a polyclonal antibody against the FVIIa heavy chain. Surprisingly, FVII proved remarkably resistant against cleavage by two-chain FSAP^{R313Q} (Fig. 5). In the absence of phospholipids no FVII cleavage was observed, even not at two-chain FSAP^{R313Q} concentration far exceeding the FVII

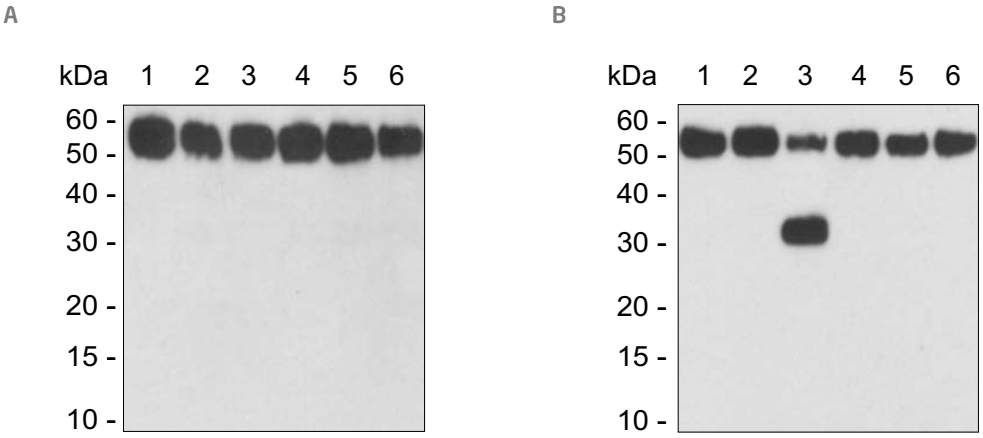


Figure 5. Human FVII is a poor substrate for activated FSAP^{R313Q}. Purified FVII (50 nM) was incubated in a buffer containing 5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4) in the absence (A) and presence (B) of 100 μM of PS/PE/PC vesicles at 37°C. The additions were: FSAP activation buffer (lane 1), 400 nM non-activated FSAP^{R313Q} (lane 2), 2 nM factor Xa (lane 3), and activated FSAP^{R313Q} in concentrations of 40, 200 and 400 nM (lanes 4, 5 and 6, respectively). After 30 min reactions were stopped by the addition of SDS/PAGE sample buffer, and samples were analysed under reducing conditions on 4-12 % gradient gels. FVII was visualized by immunoblotting using an antibody against the FVIIa heavy chain.

concentration (up to 400 nM, see Fig. 5A). In the presence of phospholipids FVII activation was observed for the positive control, FXa at low concentration (2nM). In contrast, no FVII proteolysis occurred for activated FSAP^{R313Q} (Fig. 5B). The lipid membranes used in the experiments of Fig. 5B consisted of PS/PE/PC (20/20/60 mol %) vesicles, which should provide an appropriate surface for most lipid-dependent steps in the coagulation cascade. The absence of FVII proteolysis under these conditions leads us to conclude that activated FSAP is a surprisingly poor FVII activator.

Cardiolipin promotes FVII cleavage by activated FSAP^{R313Q}

Because polyanions support the activity of two-chain FSAP towards scuPA, we examined whether these would support FVII activation as well. Addition of heparin or polyphosphate at concentrations that accelerated scuPA activation (Fig. 4) did not yield any FVII cleavage, neither alone, nor in combination with PS/PE/PC vesicles (data not shown). Replacing PS by another anionic lipid, cardiolipin (up to 40 mol %) did not support FVII cleavage either. Finally, we used membranes consisting of 100 % cardiolipin in order to provide a fully anionic surface. Under these conditions FVII cleavage did occur (Fig. 6A). The initial product was a 30 kDa derivative, which was further degraded into smaller fragments of 23, 16 and 15 kDa, until almost no intact FVII was left after 30 min (lane 8 in Fig. 6A). The extent of FVII cleavage on cardiolipin membranes suggested that activated FSAP^{R313Q} should also cleave FVII at much lower concentration than the 40 nM used in Fig. 5A. Indeed, upon varying the concentration of two-chain FSAP^{R313Q}, substantial formation of the 30 kDa derivative was observed at enzyme concentrations as low as 4-8 nM (lanes 3

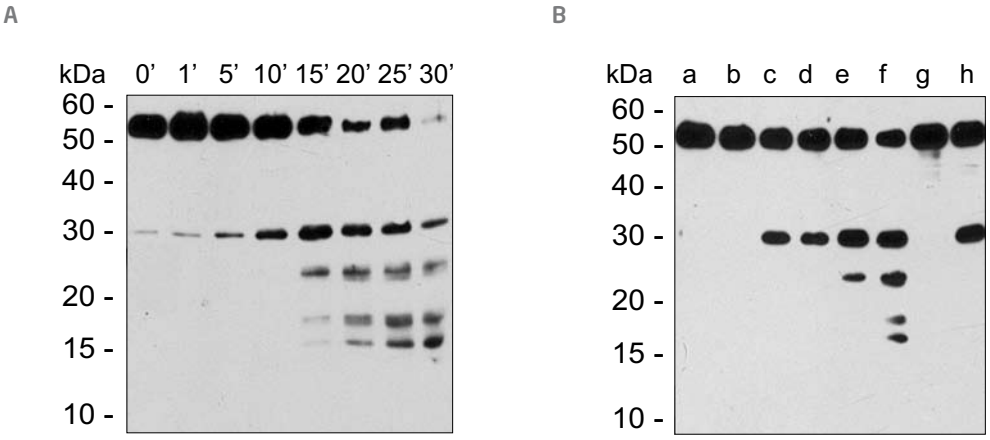


Figure 6. FVII proteolysis by activated FSAP^{R313Q} in the presence of cardiolipin. (A) Purified FVII (50 nM) was incubated with activated FSAP^{R313Q} (40 nM) in a buffer containing 5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4) in the presence of cardiolipin (100 µM). After varying time points (1-30 min) reactions were stopped by the addition of SDS/PAGE sample buffer, and samples were analysed under reducing conditions on 4-12% gradient gels. (B) FVII (50 nM) was incubated with activated FSAP^{R313Q} in concentrations of 1.3, 2, 4, 8, 20 and 40 nM (lanes a, b, c, d, e, and f, respectively) for 30 min under the conditions of panel A. Controls were FSAP activation buffer (lane g) and 2 nM of factor Xa (lane h). The latter control contained 100 µM of PS/PE/PC instead of cardiolipin.

and 4 in Fig. 6B). The 30 kDa fragment has the same mobility as the FXa-cleaved FVII heavy chain (lane 8 in Fig. 6B), and most likely represents FVIIa. It seems evident however, that FSAP also cleaves several additional sites in the FVII heavy chain, resulting in FVII degradation. These data suggest that FVIIa may occur as an intermediate product of FVII degradation by FSAP on membranes consisting of cardiolipin.

Discussion

Since its discovery in 1996 (1), the physiological role of FSAP has remained difficult to establish. One limitation has been that the function of FSAP has been explored using heterogeneous preparations that comprise multiple peptides (1, 23, 30). In this respect, our plasma-derived human FSAP was no exception (Fig. 1). We identified five different N-termini, at positions 28, 171, 314, 320 and 481 (numbering including the 23 residue signal peptide). The same N-termini were also observed in the original reports by Choi Miura et al. (1, 23), and were also reported by Hunfeld et al. (31). The non-reduced protein was 60 kDa instead of the expected 70 kDa (Fig. 1A), suggesting that one or more fragments had been further truncated from the C-terminal end. Using low pH (< 5.5), FSAP can be obtained from plasma in its single-chain form, but this is rapidly activated and further degraded at more physiologic pH (13, 17, 23). This is consistent with autocatalytic degradation occurring upon contact between FSAP and chromatographic matrices within the typical serine protease pH range (6-9), as has also been reported for FXII and FVII (32, 33). The implication thereof is that also the production of the recombinant protein would suffer

from the same problem (34). Indeed, human FSAP produced by mammalian cells was obtained as a 70 kDa species that upon reduction proved consistently degraded (Fig. 2A). The finding that the active site substitution variant FSAP^{S509A} was obtained in its single-chain form (Fig. 2B) implies that cleavage is indeed due to autocatalysis, and not to an endogenous furin-like endopeptidase cleaving in the basic sequence RKIKR^{313}-I^{314} at the activation site (35). Apparently, autocatalytis indeed limits the production of single-chain FSAP.

The problem of autoactivation and degradation was overcome by the Arg^{313} to Gln substitution, which makes the Arg^{313}-Ile^{314} scissile bond uncleavable by serine proteases, and at the same time creates a cleavage site for the bacterial metalloprotease thermolysin. The same strategy has previously been used for the expression of complement factor C1r, another serine protease that is subject to autoactivation (36). The advantage of this strategy is that it allows for the expression and isolation of single-chain FSAP^{R313Q}, which then can be activated by using thermolysin. The disadvantage is that, due to its autocatalytic activity, activated FSAP^{R313Q} is inherently instable. This requires separate activation for each series of experiments. Although we monitored each individual preparation by amidolytic activity and SDS/PAGE, this may nevertheless introduce some variability between experiments. Once activated, FSAP^{R313Q} comprises the wild-type serine protease domain, and a heavy chain ending with Gln instead of Arg as the C-terminus.

Activated FSAP^{R313Q} proved to be an extremely poor FVII activator (Fig. 5), even at very high enzyme concentrations and in the presence of PS-containing membranes that support all lipid-dependent steps in the coagulation cascade. This is surprising, because virtually all papers on FSAP published during the past decade refer to its FVII activating properties and its potential role as tissue factor-independent initiator of the FVII-dependent coagulation pathway (see for instance 37, 38). In retrospect, this notion seems to be based on limited experimental evidence. One paper reported activation of purified FVII added to factor X-deficient plasma, as monitored by a commercial clotting assay using recombinant soluble tissue factor (39). While other papers have generally referred to this finding, we are not aware of any experimental confirmation thereof by others. Our studies differed from those of Römisch in several respects. First, we used intact two-chain FSAP^{R313Q} instead of plasma-derived FSAP with its inherent degradation products and possibly other impurities. Second, we monitored FVII proteolysis rather than activity, because the latter would be easily complicated by FVII autoactivation. Finally, we studied the activation of purified FVII in the presence of potential cofactors, but not in plasma milieu in order to avoid reciprocal FVII activation by other activated coagulation factors. We cannot exclude that plasma contains a cofactor for activated FSAP that was missing in our purified system. Further studies will be needed to substantiate the role of FSAP in the coagulation cascade. In absence of such data, we conclude that FSAP is a strikingly poor FVII activator, in particular when compared with FXa under the same conditions (Fig. 5). An intriguing observation is that two-chain FSAP^{R313Q} does cleave FVII on cardiolipin membranes (Fig. 6). The relevance of this finding remains unclear. Cardiolipin is an anionic phospholipid that is present in the inner mitochondrial membrane, although it may also occur in small amounts in plasma in association with lipoproteins (41). In our experiments, membranes needed to contain more than 40 mol % cardiolipin in order to support FVII cleavage. This makes a role of cardiolipin-supported FVII cleavage in plasma not very likely. It seems

conceivable, however, that suitable cardiolipin-rich membranes may be exposed upon cell damage or apoptotis. Recently, FSAP has been attributed a role in association with apoptotic cells (17). Whether or not there is a role for FVII under such conditions remains an open question.

In agreement with earlier studies (3, 25), activated FSAP proved an effective activator of scuPA (Fig. 4). The kinetic parameters suggest that FSAP would be equally effective as established scuPA activators such as tissue-type plasminogen activator (42). A putative role in the formation of uPA would be compatible with a role of FSAP in fibrinolysis and/or vascular biology. If the observed activation of scuPA occurs under physiological conditions, FSAP could contribute to uPA generation and signalling through the urokinase-type plasminogen activator receptor (uPAR), and as such to cell adhesion, migration and proliferation (43). A role in this area would explain a variety of recent observations (44-47), and supports involvement in pathological conditions other than the initially suggested role in thrombosis (48-50). In this regard, the term 'Factor Seven Activating Protease' seems misleading, as other activities may better reflect its physiological role. In anticipation of an established function, it seems appropriate to refer to this protein by its original name 'plasma hyaluronan-binding protein' (PHBP).

Acknowledgements

We thank Erica Sellink and Jacqueline Klein-Gebbinck for expert technical assistance, and Patrick P.F.M. Clijsters for initiating this study. This work was supported by the Trombosestichting Nederland, grant number 2005-4.

Disclosure of conflict of interest

The authors state that they have no conflict of interest.

References

1 Choi-Miura NH, Tobe T, Sumiya J, Nakano Y, Sano Y, Mazda T, Tomita M. (1996) J Biochem.(Tokyo) 119, 1157-1165

2 Romisch J, Vermohlen S, Feussner A, Stohr H. (1999) Haemostasis 29, 292-299.

3 Romisch J, Feussner A, Vermohlen S, Stohr HA. (1999) Blood Coagul.Fibrinolysis 10, 471-479.

4 Etscheid M, Beer N, Fink E, Seitz R, Johannes D. (2002) Biol.Chem 383, 1633-1643.

5 Romisch J. (2002) Biol.Chem 383, 1119-1124.

6 Jeon JW, Song HS, Moon EJ, Park SY, Son MJ, Jung SY, Kim JT, Nam DH, Choi-Miura NH, Kim KW, Kim YJ. (2006) Int. J Oncol. 29, 209-215.

7 Kannemeier C, Al Fakhri N, Preissner KT, Kanse SM. (2004) FASEB J 18, 728-730.

8 Kress JA, Seitz R, Dodt J, Etscheid M. (2006) Biol.Chem 387, 1275-1283.

9 Shibamiya A, Muhl L, Tannert-Otto S, Preissner KT, Kanse SM. (2007) Biochem.J 404, 45-50.

10 Römisch J, Vermöhlen S, Feussner A, Stöhr H. (1999) Haemostasis. 29(5):292-9.

11 Choi-Miura NH, Takahashi K, Yoda M, Saito K, Mazda T, Tomita M. (2001) Biol.Pharm.Bull. 24, 448-452.

12 Etscheid M, Hunfeld A, Konig H, Seitz R, Dodt J. (2000) Biol.Chem 381, 1223-1231.

13 Kannemeier C, Feussner A, Stohr HA, Weisse J, Preissner KT, Romisch J. (2001) Eur.J Biochem. 268, 3789-3796.

14 Nakazawa F, Kannemeier C, Shibamiya A, Song Y, Tzima E, Schubert U, Koyama T, Niepmann M, Trusheim H, Engelmann B, Preissner KT. (2005) Biochem J. 385(Pt 3):831-8.

15 Muhl L, Galuska SP, Oörni K, Hernández-Ruiz L, Andrei-Selmer LC, Geyer R, Preissner KT, Ruiz FA, Kovanen PT, Kanse SM. (2009) FEBS J. 276(17):4828-39.

16 Yamamichi S, Nishitani M, Nishimura N, Matsushita Y, Hasumi K. (2009) J Thromb Haemost.

17 Zeerleder S, Zwart B, te Velthuis H, Stephan F, Manoe R, Rensink I, Aarden LA. (2008) FASEB J. 22(12).

18 Mertens K, Briët E, Giles AR. (1990) Thromb Haemost. 64(1):138-44.

19 Van 't Veer C, Hackeng TM, Delahaye C, Sixma JJ, Bouma BN. (1994) Blood. 84(4):1132-42.

20 Bradford MM. (1976) Anal. Biochem. 72:248-254.

21 Mertens K., Cuypers R., Wijngaarden A. van en Bertina R.M. (1984) Biochem J. 223, 599-605.

22 Böttcher CJF, van Gent CM, Pries C. (1961) Anal.Chim.Acta. 24, 203-204.

23 Choi-Miura NH, Saito K, Takahashi K, Yoda M, Tomita M. (2001) Biol Pharm Bull. 24(3):221-5.

24 Bennett BD, Denis P, Haniu M, Teplow HD, Kahn S, Louis J-C, Citron M, Vassar R. (2000) J. Biochem. Chem. 275(48):37712-17.

25 Choi-Miura NH, Yoda M, Saito K, Takahashi K, Tomita M. (2001) Biol Pharm Bull. 24(2):140-3.

26 Römisch J, Feussner A, Vermöhlen S, Stöhr HA. (1999) Blood Coagul Fibrinolysis. 10(8):471-9.

27 Pedersen AH, Lund-Hansen T, Bisgaard-Frantzen H, Olsen F, Petersen LC. (1989) Biochemistry. 28(24):9331-6.

28 Nakagaki T, Foster DC, Berkner KL, Kisiel W. (1991) Biochemistry. 30(45):10819-24.

29 Nemerson Y. (1988) Blood. 71(1):1-8. Review.

30 Römisch J, Feussner A, Vermöhlen S, Stöhr HA. (1999) Blood Coagul Fibrinolysis. 10(8):471-9.

31 Hunfeld A, Etscheid M, König H, Seitz R, Dodt J. (1999) FEBS Lett. 456(2):290-4.

32 Tankersley DL, Finlayson JS. (1984) Biochemistry. 23(2):273-9.

33 Pedersen AH, Lund-Hansen T, Bisgaard-Frantzen H, Olsen F, Petersen LC. (1989) Biochemistry. 28(24):9331-6.

34 Muhl L, Hersemeyer K, Preissner KT, Weimer T, Kanse SM. (2009) FEBS Lett. 583(12):1994-8.
35 Duckert P, Brunak S, Blom N. (2004) Prot. Engineering, Design & Selec. 17(1):107-112.
36 Lacroix M, Ebel C, Kardos J, Dobó J, Gál P, Závodszky P, Arlaud GJ, Thielens NM. J (2001) Biol Chem. 276(39):36233-40.
37 Mann KG. (2003) Circulation. 107:654-5.
38 Römisch J. (2002) Biol Chem. 383(7-8):1119-24. Review.
39 Römisch J, Feussner A, Vermöhlen S, Stöhr HA. (1999) Blood Coagul Fibrinolysis. 10(8):471-9.
40 Römisch J, Vermöhlen S, Feussner A, Stöhr H. (1999) Haemostasis. 29(5):292-9.
41 Deguchi H, Fernandez JA, Hackeng TM, Banka CL, Griffin JH. (2000) Proc Natl Acad Sci U S A. 97(4):1743-8.
42 Hoylaerts M, Rijken DC, Lijnen HR, Collen D. (1982) J Biol Chem. 25;257(6):2912-9.
43 Blasi F, Carmeliet P. (2002) Nat Rev Mol Cell Biol. 3(12):932-43.
44 Kanse SM, Parahuleva M, Muhl L, Kemkes-Matthes B, Sedding D, Preissner KT (2008) Thromb Haemost. 99(2):286-9.
45 Etscheid M, Beer N, Kress JA, Seitz R, Dodt J. (2004) Eur J Cell Biol. 82(12):597-604.
46 Kannemeier C, Al-Fakhri N, Preissner KT, Kanse SM. (2004) FASEB J. 18(6):728-30.
47 Jeon JW, Song HS, Moon EJ, Park SY, Son MJ, Jung SY, Kim JT, Nam DH, Choi-Miura NH, Kim KW, Kim YJ. (2006) Int. J Oncol. 29(1):209-15.
48 Weisbach V, Ruppel R, Eckstein R. (2007) Thromb. Haemost. 97:870-872.
49 Ireland H, Miller GJ, Webb KE, Cooper JA, Humphries SE. (2004) Thromb Haemost. 92(5):986-92.
50 Hoppe B, Tolou F, Radtke H, Kiesewetter H, Dörner T, Salama A. (2005) Blood. 105(4):1549-51.

Chapter 3

Functional implications of the Marburg-1 polymorphism in Factor Seven Activating Protease

F. Stavenuiter*†, E. Sellink*, A.B. Meijer* and K. Mertens*†‡

*Department of Plasma Proteins, Sanquin Research, Amsterdam; †Van Creveld Laboratory of UMC-Utrecht and Sanquin, Amsterdam; and ‡Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Abstract

The Marburg-1 polymorphism in Factor Seven Activating Protease (FSAP) encodes a G534E substitution which is located in the serine protease domain. This mutation has been associated with idiopathic venous thromboembolism, carotid stenosis, and cardiovascular disease. Epidemiological studies, however, have remained inconsistent with regard to the implications of this polymorphism. This report addresses the functional impact of this mutation. The G543E substitution is located at position c221 in chymotrypsin, in a surface loop that contributes to Na^+ binding in related serine proteases. In comparison with normal recombinant FSAP, the $FSAP^{G534E}$ variant displayed reduced reactivity towards both the synthetic substrate S-2288 and single-chain urokinase-type plasminogen activator (scuPA), while it completely lacked reactivity towards coagulation factor VII. Cleavage of S-2288 proved Na^+-dependent, but $FSAP^{G534E}$ required higher Na^+ concentrations for maximal activity. In comparison with normal FSAP, scuPA activation by $FSAP^{G534E}$ was negligible, except in the presence of the cofactors heparin and polyphosphate. We conclude that the G534E substitution, being associated with decreased response to Na^+ and increased dependence on polyanions, introduces a major functional defect.

Introduction

Factor Seven Activating Protease (FSAP) is the zymogen of a serine protease that has been discovered in 1996 as Plasma Hyaluronan Binding Protein (PHBP) (1). This protein was found to be an activator of single-chain urokinase-type plasminogen activator (scuPA), and factor VII (FVII), suggesting a key role in haemostasis and thrombosis (2-4). Subsequently, numerous additional functions for FSAP have been proposed, including inhibition of vascular smooth muscle cell proliferation and migration, suggesting a role in vascular biology as well (5, 6). The apparently pleiotropic role of FSAP has created confusion with regard to its main biological function. In this regard, polymorphisms in the FSAP gene may contribute insight into the role of this protein. Two polymorphisms have been described, called Marburg-1 and Marburg-2 (7). The former encodes a Gly to Glu substitution at position 534 in the FSAP protease domain, and has been proposed to be associated with venous thromboembolism, and cardiovascular disease (8-10). Because others have challenged this view (11-13), the role of this polymorphism has remained controversial. In order to better understand the implications of the polymorphism, we now have assessed the effect of the G534E mutation by in vitro studies employing recombinant proteins.

Materials and Methods

Normal FSAP was constructed and expressed as the $FSAP^{R313Q}$ variant, which can be activated under control of the bacterial metalloprotease thermolysin (14). The Marburg-1 G534E substitution was introduced in the wild-type sequence using the QuickChange® mutagenesis kit and oligonucleotide primers 5'AGCTGGGGCCTGGAGTGTGAGAAGAGGCCAGGGGTCTAC3' (sense) and 5'GTAGACCCCTGGCCTCTTCTCACACTCCAGGCCCCAGCT3' (antisense). Expression and purification, as well as functional characterization was performed using materials and methods described in full detail elsewhere (14). Purified $FSAP^{G534E}$ proved spontaneously activated and was fully processed into the two-chain form (Fig. 1B, inset). N-terminal sequencing (Eurosequence, Groningen, The Netherlands) identified Ile^{314} as the N-terminus of the 25 kDa light chain, demonstrating that two-chain $FSAP^{G534E}$ had been cleaved at the authentic activation site.

Results and discussion

At present, no crystal structure of FSAP is available that can assist in elucidating the impact of the G534E substitution for FSAP function. Therefore, a 3-dimensional model of the FSAP protease domain was built by comparative homology modeling using as a template the structure of the protease domain of tissue-type plasminogen activator (tPA), which is most related to that of FSAP (Fig. 1A). This model revealed that the G534E substitution is located in one of the surface loops of the protease domain. This loop is known as the "c220-loop" in terms of the chymotrypsin numbering as commonly used for serine proteases. The inset of Fig. 1A shows a primary sequence alignment of the c220 loop of FSAP with that of factor IX, thrombin, protein C and factor X. For these four proteins, it has been suggested that this surface loop contributes to Na^+ binding, which directly influences the enzymatic activity of these proteins (15). In this respect, the residue at position c221 has been recognized as being of critial importance in several coagulation enzymes, including factor IX and protein C (16, 17). This raises the possibility

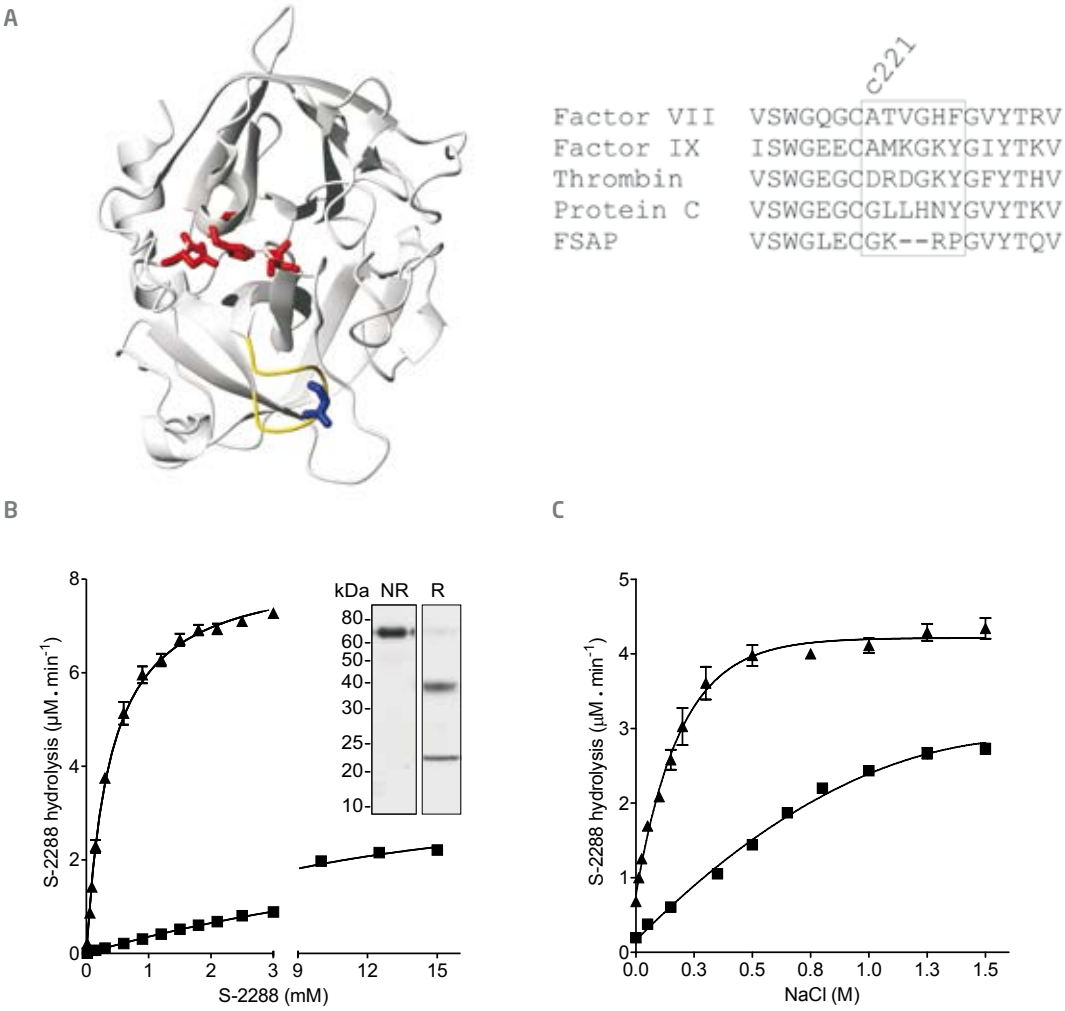


Figure 1. The putative Na^+-binding loop in FSAP and its involvement in amidolytic activity. (A) Molecular model of the catalytic domain of FSAP constructed with comparative homology modeling using the protease domain of tPA as a template (see, ref (19)). Indicated in red are the residues of the catalytic triad and in yellow the putitative Na^+-binding c220 loop. Gly^{534} is shown in blue. The inset shows a primary sequence alignment of the c220 loop with that of the indicated serine proteases. (B) Amidolytic activity of 10 nM activated FSAP (*triangles*) and 25 nM $FSAP^{G534E}$ (*squares*). Both proteins were incubated with the substrate S-2288 at 37 °C in a buffer containing 0.2 % (w/v) BSA, 150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl (pH 8.0). Initial rates of S-2288 cleavage were quantified by monitoring absorbance. Data represent the mean ±SD of 3-4 experiments. The inset shows 10% SDS/PAGE (1 µg/lane, silver staining) of purified $FSAP^{G534E}$ demonstrating that the reduced (R) protein is a two-chain molecule. (C) Na^+-dependence of the catalytic activity of 17.5 nM FSAP (*triangles*) and 350 nM $FSAP^{G534E}$ (*squares*) against S-2288. Substrate cleavage was quantified at 37 °C in a buffer containing NaCl (0-1.5 M), 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 0.1% (v/v) Tween-80. Choline chloride was added to the reaction mixture to keep the ion concentration constant at 1.5 M.

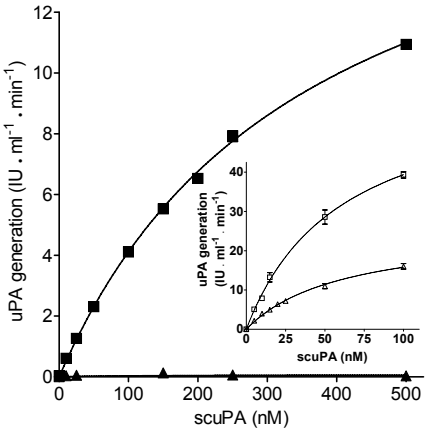
that Gly^{543} could play a similar role in FSAP, and that the Marburg-1 mutation could represent a severe functional defect.

We first assessed the effect of the G534E substitution on amidolytic activity towards the peptide substrate S-2288. As shown in Fig. 1B, the activity of two-chain $FSAP^{G534E}$ was severely reduced. The calculated kinetic parameters indicated a 24-fold increase in K_m, and a 7-fold decrease in V_{max}. This apparent defect was further analyzed by varying the Na^+ concentration. As is evident from Fig. 1C, normal FSAP displayed a marked Na^+-dependence. Response to Na^+ was also observed for $FSAP^{G534E}$, but much higher Na^+ concentrations were needed for maximal activity. The impaired response to Na^+ as resulting from the G534E mutation seems compatible with its location in the putative Na^+-binding c220-loop (Fig. 1A). It should be noted that FSAP is not a typical Na^+-binding serine protease because it has Pro in position c225, while this is believed to be incompatible with Na^+ coordination (18). Moreover, FSAP seems atypical in that the prominent Na^+-dependence is predominantly observed in the absence of Ca^{2+} (Fig. 1C), but not in its presence (not shown, see (19)). Regardless of these differences from other Na^+-binding serine proteases, it seems evident that the Marburg-1 mutation introduces an enzymatic defect that is associated with an impaired response to Na^+-ions. As has been suggested for factor IX and protein C (16, 17, 20), this may reflect a defect in the allosteric regulation of enzymatic activity. The mechanism by which Gly^{534} may contribute to allosteric FSAP regulation will be further explored elsewhere (19).

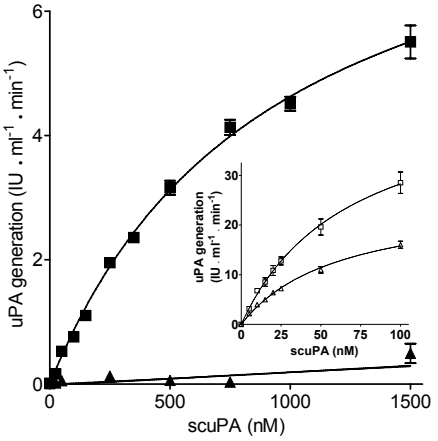
To investigate the effect of G534E substitution on FSAP activity towards a macromolecular, natural substrate, we addressed the activation of scuPA. In comparison with normal FSAP (Fig. 2A, inset), two-chain $FSAP^{G534E}$ displayed a major defect in scuPA activation, as it failed to display any reactivity in the absence of anionic polymers. This was also apparent at scuPA concentration as high as 1500 nM (Fig. 2B). This demonstrates that the activity of $FSAP^{G534E}$ is severely impaired due to the mutation in the putative Na^+-binding loop. It has previously been demonstrated that the catalytic activity of FSAP is enhanced by negatively charged polymers, such as heparin and polyphosphate (21, 22). We have previously shown that these cofactors stimulate scuPA activation by normal two-chain FSAP by increasing V_{max} by 2-3 fold, while leaving K_m (62 nM) unaffected (14) (see also inset of Figs 2A and 2B). In view of these moderate cofactor effects on the activity of normal FSAP, it seems surprising that the reactivity of $FSAP^{G534E}$ proved fully cofactor-dependent. Apparently, the enzymatic defect of $FSAP^{G534E}$ is partially rescued by interaction with the anionic polymers. Nevertheless, scuPA activation remains greatly defective. Calculation of kinetic parameters from the data of Figs 2A and 2B revealed that the estimated V_{max} was 2-3 fold lower than for normal FSAP. While this seems a relatively minor defect, it should be noted that the G534E substitution has a major effect on K_m, with an estimated value of 360 nM in the presence of heparin, and around 900 nM in the presence of polyphosphate. This 6-15 fold increase in K_m for scuPA activation that results from the G534E mutation underscores that the Marburg-1 polymorphism is in fact a mutation at a critical site in the protease domain.

We previously reported that FSAP, in spite of what its name suggests, is a strikingly poor FVII activator (14). Activation and subsequent degradation of purified factor VII was only observed in the presence of

A



B



C

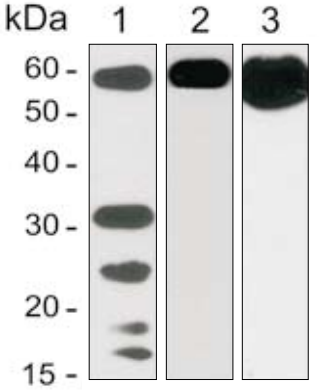


Figure 2. Proteolytic activity of normal FSAP and FSAP^{G534E}. (A) Activation of scuPA by 0.25 nM FSAP^{G534E} was assessed in the presence (*squares*) or absence (*triangles*) of 10 IU.mL^{-1} heparin at 37 °C in a buffer containing 100 mM NaCl, 0.1% BSA, 0.01% (v/v) Tween-80, 5 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.5) as described (13, 19). Data represent the means ± S.D. of 3-4 experiments. The inset shows activation of scuPA by 0.25 nM activated FSAP in the presence (*squares*) and absence (*triangles*) of 10 IU.mL^{-1} heparin employing the same conditions (data derived from Stavenuiter et al. (13) (B) Activation of scuPA by 0.25 nM FSAP^{G534E} was assessed in the presence (*squares*) or absence (*triangles*) of 5 μM $PolyP_{65}$ as indicated under A. The inset shows activation of scuPA by 0.25 nM activated FSAP in the presence (*squares*) or absence (*triangles*) of 5 μM $PolyP_{65}$ at 37 °C (data derived from Stavenuiter et al. (13). (C) 50 nM FVII was incubated for 30 minutes at 37 °C with 50 nM activated FSAP and 100 μM cardiolipin (lane 1), 50 nM FSAP^{G534E} and 100 μM cardiolipin (lane 2), or 50 nM FSAP^{G534E}, 100 μM cardiolipin and 10 IU.mL^{-1} heparin (lane 3). The incubation buffer comprised 5 mM $CaCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4).

fully anionic membranes consisting of cardiolipin (lane 1 in Fig. 2C). Under the same conditions, two-chain FSAP^{G534E} failed to cleave FVII to any appreciable extent, even in the combined presence of cardiolipin and heparin (Fig. 2C, lanes 2 and 3, respectively). It should be noted that studies on the G534E polymorphism generally refer to FVII activation being unaffected by this mutation (8-10, 23). This seems only true in the sense that FVII activation is negligible anyway, irrespective of the presence of the G534E substitution. We propose that future studies on the physiological implications of the Marburg-1 polymorphism should focus on its reduced capacity to activate scuPA, rather than on its earlier proposed role as tissue factor-independent trigger of the coagulation cascade.

References

1 Choi-Miura NH, Tobe T, Sumiya J, Nakano Y, Sano Y, Mazda T, Tomita M. (1996) J Biochem. 119(6):1157-65.
2 Römisch J, Feussner A, Vermöhlen S, Stöhr HA. (1999) Blood Coagul Fibrinolysis. 10(8):471-9.
3 Römisch J, Vermöhlen S, Feussner A, Stöhr H. (1999) Haemostasis. 29(5):292-9.
4 Choi-Miura NH, Yoda M, Saito K, Takahashi K, Tomita M. (2001) Biol Pharm Bull. 24(2):140-3.
5 Etscheid M, Beer N, Kress JA, Seitz R, Dodt J. (2004) Eur J Cell Biol. 82(12):597-604.
6 Kannemeier C, Al-Fakhri N, Preissner KT, Kanse SM. (2004) FASEB J. 18(6):728-30.
7 Römisch J, Feussner A, Stöhr HA. (2001) Blood Coagul Fibrinolysis. 12(5):375-83.
8 Willeit J, Kiechl S, Weimer T, Mair A, Santer P, Wiedermann CJ, Roemisch J. (2003) Circulation. 107(5):667-70.
9 Sedding D, Daniel JM, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM. (2006) J Exp Med. 203(13):2801-7.
10 Hoppe B, Tolou F, Radtke H, Kiesewetter H, Dörner T, Salama A. (2005) Blood. 105(4):1549-51.
11 Van Minkelen R, de Visser MC, Vos HL, Bertina RM, Rosendaal FR. (2005) Blood. 105(12):4898.
12 Gulesserian T, Hron G, Endler G, Eichinger S, Wagner O, Kyrle PA. (2006) Thromb Haemost. 95(1):65-7.
13 Franchi F, Martinelli I, Biguzzi E, Bucciarelli P, Mannucci PM. (2006) Blood. 107(4):1731.
14 Stavenuiter F. et al. (2010) This thesis chapter 2.
15 Page MJ, Macgillivray RT, Di Cera E. (2005) J Thromb Haemost, 3: 2401–2408.
16 Spitzer SG, Pendurthi UR, Kasper CK, Bajaj SP. (1988) J Biol Chem. 263(22):10545-8.
17 Miyata T, Zheng YZ, Sakata T, Tsushima N, Kato H. (1994) Thromb Haemost. 71(1):32-7.
18 Wasmuth HE, Tag CG, Van de Leur E, Hellerbrand C, Mueller T, Berg T, Puhl G, Neuhaus P, Samuel D, Trautwein C, Kanse SM, Weiskirchen R. (2009) Hepatology 49(3):775-80
19 Stavenuiter F. et al. (2010) This thesis chapter 4.
20 Rouy S, Vidaud D, Alessandri JL, Dautzenberg MD, Venisse L, Guillin MC, Bezeaud A. (2006) Br J Haematol. 132:770-773.
21 Kannemeier C, Feussner A, Stöhr HA, Weisse J, Preissner KT, Römisch J. (2001) Eur J Biochem. 268(13):3789-96.
22 Muhl L, Galuska SP, Oörni K, Hernández-Ruiz L, Andrei-Selmer LC, Geyer R, Preissner KT, Ruiz FA, Kovanen PT, Kanse SM. (2009) FEBS J. 276(17):4828-39.
23 Römisch J. (2002) Biol Chem. 383(7-8):1119-24. Review.

Chapter 4

Glycine 221 is indispensable for allosteric regulation of the catalytic activity of Factor Seven Activating Protease

F. Stavenuiter*†, K. Mertens*†‡ and A.B. Meijer*

*Department of Plasma Proteins, Sanquin Research, Amsterdam; †Van Creveld Laboratory of UMC-Utrecht and Sanquin, Amsterdam; and ‡Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Abstract

Replacement of Gly^{221} (chymotrypsin numbering) for a glutamic acid in the Na^+-binding loop of Factor Seven Activating Protease (FSAP) markedly reduces its catalytic activity towards single-chain urokinase plasminogen activator (scuPA). We now addressed the role of Gly^{221} for FSAP function employing kinetic analysis of FSAP derivatives in which Gly^{221} is replaced by alanine, serine or glutamic acid. Although the G221A and G221S variants still demonstrated a reduced activity towards scuPA, the K_m values were 3-fold decreased as compared to the K_m obtained for WT-FSAP. This implies that Gly^{221} is not directly involved in scuPA binding. Employing the substrate S-2288, the G221E, G221S and G221A variant displayed a 24-fold, a 9-fold and a 3-fold increase in K_m relative to WT-FSAP. The results further showed that the presence of a polar residue at position 221 markedly impaired the ability of Na^+ to potentiate the activity of FSAP. Analysis of a homology model of FSAP revealed that Gly^{221} may allosterically stabilize the salt bridge between the N-terminal residue Ile^{16} and Asp^{194} via the residues Asp^{189} and Tyr^{17}. This salt bridge is indispensable for optimal alignment of the catalytic residues, and is formed after insertion of Ile^{16} in the protease domain during activation of FSAP. The model further showed that mutation of Gly^{221} into a polar residue will lead to unfavorable interaction with the hydrophobic side chain of Tyr^{17}, and may therefore prevent the insertion of Ile^{16} in the protease domain. Mass spectrometry studies confirmed this hypothesis, and demonstrated that Ile^{16} of the G221E variant is significantly less protected from chemical modification than Ile^{16} of WT-FSAP. The resulting suboptimal alignment of the catalytic residues was corroborated employing competitive inhibition studies. The findings together demonstrate that Gly^{221} is of critical importance for allosteric regulation of the catalytic activity of FSAP.

Introduction

Factor VII activating protease (FSAP) is a Ca^{2+}-dependent protease that belongs to the family of peptidases bearing a catalytic domain with a chymotrypsin fold. Next to the catalytic domain, which is referred to as the light chain, FSAP contains a heavy chain comprising three EGF-domains and a kringle domain (1, 2). Contrary to what could be inferred from its name, FSAP is a poor activator of factor VII. To activate factor VII, FSAP requires the presence of phospholipid membranes consisting of cardiolipin (3). Others and we have found that FSAP does effectively proteolytically activate single-chain urokinase-type plasminogen activator (scuPA) (3-5). FSAP has further been suggested to regulate cellular activation via cleavage of platelet-derived growth factor BB (6).

A well-known variant of FSAP carries a glycine to glutamic acid substitution at position 221 (chymotrypsin numbering will be used throughout this chapter) (7). This variant has previously been designated as the Marburg-1 polymorphism, and has been associated with an increased risk for cardiovascular disease (8, 9). We have recently shown that this substitution is not merely a polymorphism but a mutation in a critical part of the protease domain (10). This finding demonstrates that Gly^{221} is of critical importance for FSAP function. The reason thereof is unclear, and is the subject of the present paper.

The catalytic domains of serine proteases, like FSAP, have a-two antiparallel beta-barrel architecture with in the center the catalytic triad comprising the residues Ser^{195}, His^{57} and Asp^{102} (11, 12). Catalysis proceeds through hydrogen bond formation between Asp^{102} and His^{57} leading to a transfer of the proton from the hydroxyl group of Ser^{195} to His^{57}. This facilitates a nucleophilic attack of Ser^{195} on the carbonyl group of a peptide substrate. As a result, a tetrahedral oxyanion intermediate is formed that represents a critical step for subsequent substrate cleavage. Substrate specificity is established via the so-called specificity pockets. Asp^{189} at the base of the primary S1 pocket of FSAP renders this protease specific to cleave after positively charged residues. Many serine proteases require proteolytic processing to turn the inactive zymogen into the active protease. Proteolytic cleavage generates a new N-terminus that forms a salt bridge with Asp^{194} leading to a correct positioning and stabilization of Ser^{195} in the catalytic center and of Asp^{189} in the primary S1 pocket.

A characteristic feature of the serine proteases is that the catalytic center is surrounded by eight unique surface loops (11, 12). These loops are involved in substrate recognition and allosteric regulation of the catalytic activity. The so-called "70–loop" of FSAP is most likely involved in binding the Ca^{2+}-ion that is indispensable for allosteric regulation of the catalytic activity of this protease (2, 13). It has been proposed that Ca^{2+} fulfills this role in factor IX by facilitating salt bridge formation between Ile^{16} and Asp^{194} (14). For this and other serine proteases, it has further been established that the 220 loop contributes to a correct formation of the S1 specificity pocket. In thrombin, protein C, factor X, and factor IX this loop is involved in binding a Na^{+}-ion which stabilizes Asp^{189} at the base of the S1 pocket via a bridging water molecule (14-18). For these enzymes, it has been suggested that the presence of a tyrosine or a phenylalanine at position 225 is indispensable for Na^{+}-induced allosteric regulation of their catalytic activity (19). Since FSAP contains a proline at position 225, a Na^{+}-binding role of the 220 loop would seem unlikely for this protease. We have recently shown, however, that the 220 loop in FSAP is unique

in that it does bind a Na^+-ion. Binding of this ion markedly enhances the catalytic activity of FSAP but only in the absence of Ca^{2+} (10).

The amino acid residue at position 221 is of critical importance for the catalytic activity of serine proteases. Substitution of this residue, however, has a differential effect. For thrombin, mutation of the aspartic acid into a glutamic acid, which is highly similar in characteristics to the former amino acid residue, already leads to a severe defect in fibrinogen clotting (20). In contrast, alanine to aspartic acid exchange in coagulation factor IX does not affect its clotting activity or its binding to macromolecular substrates at all (21). Then again, A221V mutation in this protein is associated with haemophilia B due to a defect in its macromolecular substrate binding site (22). Similarly, G221R mutation in proteins C leads to an impaired biological activity of this protease (23). These findings show that the role of the amino acid at position 221 for proper functioning of the serine proteases is not fully understood.

In the present study, we assess the role of Gly^{221} for the catalytic activity of FSAP. Questions that will be addressed are: (I) Is this residue important to stabilize Asp^{189} in the S1 pocket? (II) Does this residue allosterically regulate a proper alignment of the residues of the catalytic triad? (III) Does gly^{221} contribute to scuPA binding, and finally, (IV) is this residue involved in Na^+ binding?

Materials and Methods

Chemicals

All chemical were from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless otherwise stated. Sulpho-NHS-LC-biotin was from QB Perbio (Tattenhall, Cheshire, UK). Acetonitrile and ultra-pure water, which were employed for mass spectrometry, were obtained from Biosolve B.V. (Valkenswaard, The Netherlands).

Recombinant FSAP derivatives and their activation by thermolysin

Recombinant $FSAP^{R15Q}$ and $FSAP^{G221E}$ (i.e. $FSAP^{R313Q}$ and $FSAP^{G534E}$) are described in Stavenuiter et al. (3, 10). The thermolysin FSAP variants carrying amino acid substitution at position 221 were constructed employing QuikChange mutagenesis (Stratagen, La Jolla, CA) using $FSAP^{R15Q}$ DNA as a template. Recombinant proteins were produced, purified, activated and quantified as described (3). The FSAP variants were activated by incubating 0.7 mM of the protein with 3 nM thermolysin for 2 hours at 30 °C in buffer containing 10 mM MES (pH 5.0), 150 mM NaCl, 5% (v/v) glycerol, 0.01% (v/v) Tween-80, 100 µM $CaCl_2$, and 50 nM $ZnCl_2$. Thermolysin was inhibited by the addition of phosphoramidon disodium salt to a final concentration of 10 µM.

Hydrolysis of S-2288 by FSAP derivatives and the determination of uPA concentration

The initial rates of p-nitroalinide release from S-2288 were measured by following the absorbance at 405 nm using a Rosys-Anthos Lucy 3 photometer (Anthos Labtec Instruments GmbH, Wals, Austria). An extinction coefficient of 9.9 $mM^{-1}\,cm^{-1}$ was used to calculate the concentration of p-nitroalinide. The concentration of uPA obtained from scuPA cleavage by the FSAP derivatives was assessed by adding

S-2444 to the reaction mixture (final concentration 375 µM) (3). The initial rates of p-nitroalinide release were assessed by monitoring the absorbance at 405 nm in time as described above. The results were corrected for the background levels of p-nitroalinide generated in the absence of the FSAP derivatives. The concentration of uPA was determined using a calibration curve of the HMW uPA international standard 87/594 (NIBSC, South Mimms, England).

Determination of steady states kinetic constants and inhibition kinetics

Kinetic constants of S-2288 hydrolysis and scuPA activation by the FSAP derivatives were assessed by assuming Michaelis-Menten-like behavior for the enzymatic reactions (24). Graphpad software was employed to fit the data to the Michaelis-Menten equation employing non-linear regression. The inhibition constant (K_i) for the interaction of para-aminobenzamidine (*p*AB) with FSAP were assessed using non-linear regression to equation 1 (25, 26).

(1) $K_i = IC_{50} / (1 + ([S]/K_m))$

Where IC_{50} is the concentrations of *p*AB required for 50% inhibition of the $FSAP^{R15Q}$ and $FSAP^{G221E}$, and [S] is the concentration S-2288. K_m is the Michealis-Menten constant of S-2288 hydrolysis by the FSAP derivatives.

Biotinylation of the free amino group of Ile[16]

350 nM of activated $FSAP^{R15Q}$ or $FSAP^{G221E}$ was incubated with 25 mM Sulpho-NHS-biotin in 100 mM NaCl, 1 mM $CaCl_2$, 0.02% (v/v) Tween-80, 50 mM HEPES (pH 7.5). At different time points the reactions were stopped by the addition of lysine (125 mM). Proteins were subsequently separated on precast NuPage 4-12% Bis-Tris gels (Invitrogen, Breda, The Netherlands) under reducing conditions. The protein bands were subsequently visualized employing Coomassie Brilliant Blue (CBB) staining. Bands of interest were excised and processed for in-gel digestion according to the method of Shevchenko (27). Briefly, bands were washed with a buffer containing 50 mM ammonium bicarbonate (pH 7.9) followed by a wash with 50% (v/v) acetonitrile. This step was repeated three-times. Cysteine bonds were subsequently reduced with 10 mM dithiotreitol for 1 h at 56 °C, and alkylated with 50 mM iodoacetamide for 45 min at RT in the dark. After two subsequent wash/dehydration cycles the bands were dried for 10 min in a vacuum centrifuge (Thermo Fisher Scientific Inc., Bremen, Germany), and incubated overnight with 0.06 µg/µl trypsin at 37 °C. Peptides were eluded from the gel with 1% (v/v) formic acid and twice with 50% (v/v) acetonitrile, 5% (v/v) formic acid. The three elution fractions were pooled and concentrated to 20 µl in a vacuum centrifuge.

Mass spectrometry and data analysis

The above-described peptides were separated using a reversed-phase C18 column (50 µm x 40 cm, 5 µm particles) (Nanoseparations, Nieuwkoop, The Netherlands) running at 100 nl/min with a one hour gradient from 0% to 35% (v/v) acetonitrile with 0.1% (v/v) HAc. The peptides were sprayed directly from the column into the LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Bremen, Germany) using a nanoelectrospray source with a spray voltage of 1.9 kV. The LTQ was operated in a data-dependent

mode by performing collision induced dissociation in the ion-trap (35% normalized collision energy) for the five most intense precursor ions selected from each full scan in the Orbitrap (350-2000 m/z, resolving power 30.000). An isolation width of 2 Da was used for the selected ions (charge ≥ 2) and an activation time of 30 ms. Dynamic exclusion was activated for the MS/MS scan with a repeat count of 1 and exclusion duration of 30 s. Peptides were identified employing a Sequest search against the human entries in the NCBI database utilizing Proteome Discoverer 1.0 software (Thermo Scientific, Bremen, Germany). The same software was used to obtain the peak area of the reconstructed ion chromatograms of the peptide precursor ions for quantification purposes. Direct comparison of the tryptic digests obtained from gel is not possible due to a differential loss of the peptides during the peptide purification procedure. Therefore, the areas of the reconstructed ion chromatograms of control peptides present in each sample were used as a reference to correct for peptide loss.

Results

Construction of FSAP derivatives

To elucidate the role of residue 221 for FSAP function, we initially constructed three FSAP variants in a WT-FSAP background. In these variants, Gly^{221} was replaced by either the small apolar alanine, the small polar serine, or by the negatively charged glutamic acid. Protein expression studies revealed that $FSAP^{G221A}$ and $FSAP^{G221S}$ were, like WT-FSAP, highly sensitive to autocatalytic inactivation (data not shown). As we have previously described, $FSAP^{G221E}$ was obtained in its two-chain constitutively active form (10). We have recently demonstrated that activation of FSAP can be controlled by mutating the natural site of activation into a cleavage site for the protease thermolysin ($FSAP^{R15Q}$) (3). We therefore constructed thermolysin cleavage sites in all the FSAP variants. After introduction of this mutation, all variants were purified as a single-chain molecule. There was, however, a differential effect of thermolysin on the FSAP derivatives. Whereas $FSAP^{R15Q}$ was fully activated by thermolysin within 90 minutes, $FSAP^{R15Q/G221A}$ and $FSAP^{R15Q/G221S}$ required a prolonged incubation time with thermolysin to obtain the two-chain molecule. An even longer incubation with thermolysin was required to proteolytically process $FSAP^{R15Q/G221E}$. However, we were unable to obtain fully activated, two-chain $FSAP^{R15Q/G221E}$ because cleavage by thermolysin was too slow (Fig. 1). These observations show that the nature of the amino acid at position 221 affects the activation of the FSAP variants by thermolysin. To study the role of amino acid substitution at 221, we employed in this study $FSAP^{R15Q/G221S}$, $FSAP^{R15Q/G221A}$, and $FSAP^{R15Q}$ which can be specifically activated by thermolysin, and the constitutively active $FSAP^{G221E}$.

Gly^{221} does not directly contribute to scuPA binding

We first assessed the catalytic efficacy of the FSAP variants towards the macromolecular substrate scuPA. 0.25 nM of each variant was incubated with increasing concentrations of scuPA. The amount of uPA generated after 10 minutes was subsequently assessed employing the uPA specific substrate S-2444. The data revealed that there is no detectable cleavage of scuPA in the presence of $FSAP^{G221E}$ under these conditions (Fig. 2). $FSAP^{R15Q/G221A}$ and $FSAP^{R15Q/G221S}$ were, however, able to activate scuPA with a V_{max} that was respectively 4-fold and 9-fold reduced relative to $FSAP^{R15Q}$. Surprisingly, both

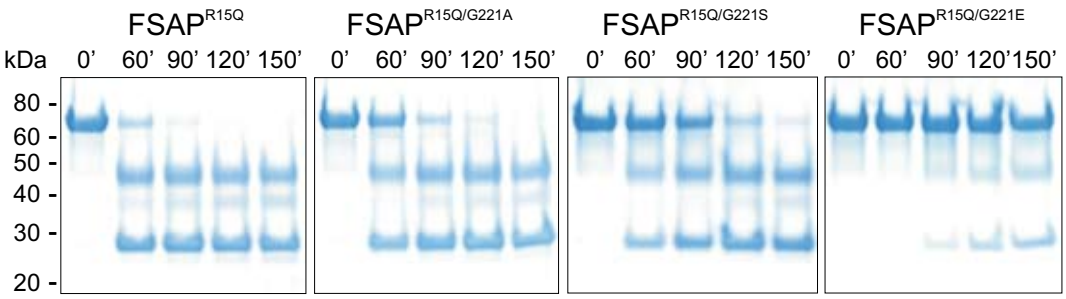
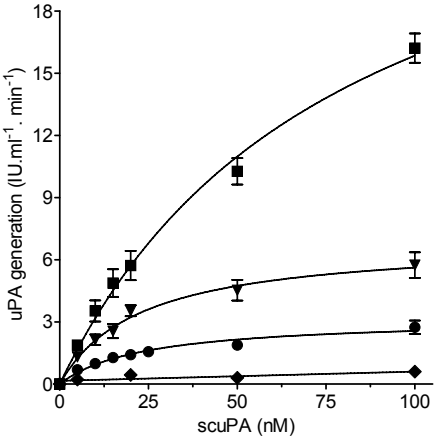


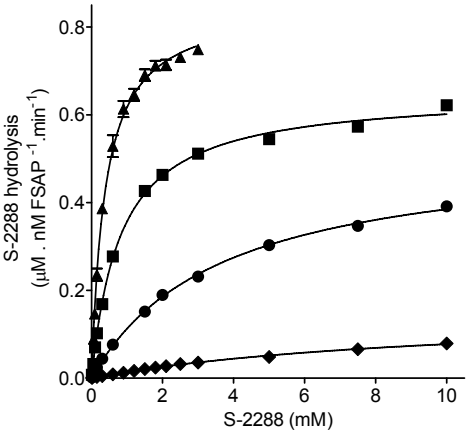
Figure 1. SDS-PAGE analysis of the proteolytic cleavage of the FSAP variants by thermolysin. 0.7 μM FSAP^{R15Q}, FSAP$^{R15Q/G221A}$, and FSAP$^{R15Q/G221S}$, and FSAP$^{R15Q/G221E}$ were incubated with 3 nM thermolysin for the indicated time intervals at 30°C in 10 mM MES (pH 5.0), 150 mM NaCl, 5% (v/v) glycerol, 0.01% (v/v) Tween-80, 100 μM $CaCl_2$, and 50 nM $ZnCl_2$. Aliquots of the samples were separated on 4-12% SDS-PAGE and the protein bands were visualized by CBB staining. The position of the protein bands of the molecular weight marker is shown on the left.



	V_{max} (IU. ml^{-1}. min^{-1})	K_m (nM)
FSAP^{R15Q}	28 ± 3	80 ± 16
FSAP$^{R15Q/G221A}$	6.9 ± 0.6	23 ± 5
FSAP$^{R15Q/G221S}$	3.2 ± 0.3	24 ± 5
FSAP^{G221E}	nd	nd

Figure 2. Activation of scuPA by the FSAP variants. 0.25 nM of activated FSAP^{R15Q} (squares), FSAP$^{R15Q/G221A}$ (triangles), FSAP$^{R15Q/G221S}$ (circles), and FSAP^{G221E} (diamonds), were incubated with scuPA for 10 min at 37 °C in 100 mM NaCl, 0.1% (w/v) BSA, 0.01% (v/v) Tween-80, 5 mM $CaCl_2$, and 20 mM Tris-HCl (pH 7.5). Aprotinin was added to a final concentration of 125 nM to stop scuPA cleavage. The amount of generated uPA was measured employing S-2244 as described in materials and methods. Data represent the means ± S.D. of at least three experiments.

Table I. Kinetic constants for the proteolytic cleavage of scuPA by the FSAP derivatives. The data for scuPA cleavage, shown in Fig. 2, were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} as described in materials and methods.



	K_m x10^{-3} (M)	k_{cat} (s^{-1})	(k_{cat} / K_m) x10^3 (s^{-1} M^{-1})
FSAP^{R15Q}	0,4	14	35
FSAP^{G221E}	9,4	2	0,2
FSAP$^{R15Q/G221A}$	0,8	11	13,8
FSAP$^{R15Q/G221S}$	3,7	9	2,3

Figure 3. Hydrolysis of S-2288 by the FSAP derivatives. 17.5 nM FSAP^{R15Q} (triangles), 17.5 nM FSAP$^{R15Q/G221A}$ (squares), 35 nM FSAP$^{R15Q/G221S}$ (circles), and 70 nM FSAP^{G221E} (diamonds) were incubated with different concentrations of S-2288 at 37 °C in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM $CaCl_2$, and 0.1% (v/v) Tween-80. The amount of cleaved S-2288 was measured as described in materials and methods. Data represent the means ± S.D. of at least three experiments.

Table II. Kinetic constants for S-2288 hydrolysis by FSAP derivatives. Kinetic constants for the S-2288 hydrolysis, shown in Fig. 3, were assessed by fitting the data to the Michaelis-Menten equation as described in materials and methods.

variants showed an about 3-fold lower K_m value as compared to the K_m of FSAP^{R15Q} (Table I). When K_m is assumed to be a measure for the substrate binding affinity, the result implies that the amino acid substitution at position 221 does not directly affect macromolecular binding but rather the catalytic core of the protease.

Amidolytic activity of the FSAP derivatives

To gain further insight into the role of position 221 for FSAP function, we next evaluated the catalytic efficiency of the variants towards the small substrate S-2288. In agreement with our previous report, the data revealed that the activity of FSAP^{G221E} was markedly impaired as compared to that of FSAP^{R15Q} (Fig. 3) (10). The result showed that both K_m and k_{cat} were heavily affected by the introduction of the glutamic acid at position 221 (Table II). A more than 4-fold improvement was obtained for k_{cat} upon changing the glutamic acid into a serine residue. There was, however, only a 2-fold decrease in K_m as a consequence of this amino acid substitution. As compared to the kinetic constants obtained for FSAP^{G221E}, both K_m and k_{cat} were markedly improved in the variant comprising an alanine at position 221. These findings together suggest that the presence of (especially) a polar residue at position 221 affects

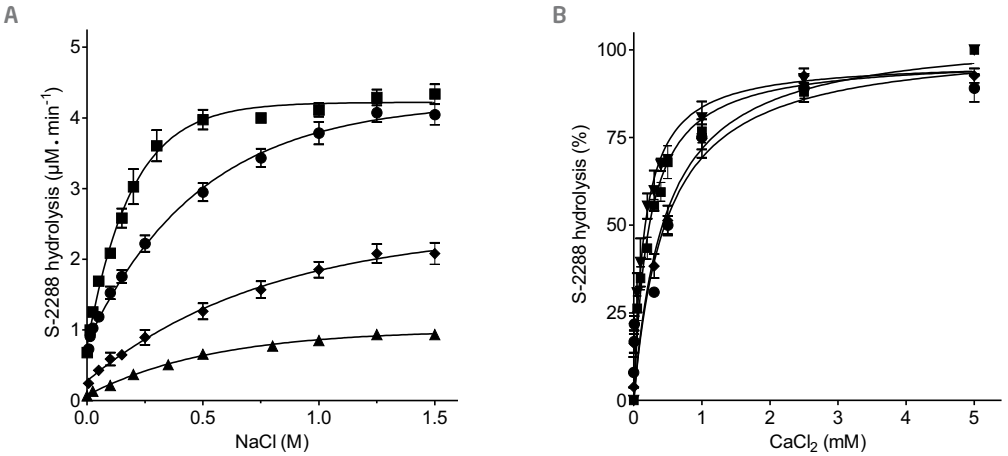


Figure 4. Effects of Na^+ and Ca^{2+} on S-2288 hydrolysis by the FSAP variants. Panel A shows the effect of Na^+ on the activity of the FSAP derivatives. 17.5 nM of activated $FSAP^{R15Q}$ (squares), $FSAP^{R15Q/G221A}$ (circles), $FSAP^{R15Q/G221S}$ (diamonds) and 350 nM of $FSAP^{G221E}$ (triangles) were incubated with varying concentrations of NaCl at 37 °C in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 0.1% (v/v) Tween-80. Choline chloride was added to the reaction mixture to keep the ion concentration constant at 1.5 M. S-2288 was added to a final concentration of 625 μM and its hydrolysis was assessed as described in materials and methods. Panel B shows the effect of Ca^{2+} on the activity of the FSAP variants in a Zn^{2+} displacement assay. 17.5 nM of activated $FSAP^{R15Q}$ (squares), $FSAP^{R15Q/G221A}$ (circles), $FSAP^{R15Q/G221S}$ (diamonds) and 350 nM of $FSAP^{G221E}$ (triangles) were incubated with varying concentrations of $CaCl_2$ in 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% (v/v) Tween-80 and 12.5 μM $ZnCl_2$. S-2288 hydrolysis for each variant is shown as a percentage of its maximum rate of substrate conversion. Data represents the means ± S.D. of at least three experiments.

the apparent binding affinity of the substrate. The presence of a negative charge has an additional effect on substrate turnover.

Substitutions at position 221 affect the Na^+-dependent enhancement of the catalytic activity of FSAP

We have previously established that the 220 loop contains the structural determinants to bind Na^+ (10). We now investigated the effect of the mutations at position 221 on the capacity of Na^+ to enhance the catalytic activity of FSAP in the absence of Ca^{2+} (Fig. 4A). The data showed an increased sensitivity to Na^+ of the variants in the order: $FSAP^{G221E}$ < $FSAP^{R15Q/G221S}$ << $FSAP^{R15Q/G221A}$ < $FSAP^{R15Q}$. From these observations can be deduced that the introduction of a polar residue at position 221 affects Na^+ binding. Alternatively, Na^+ binding is not affected but its ability to allosterically regulate the activity of FSAP is impaired in the presence of a serine or a glutamic acid.

For several serine proteases, it has been suggested that the Na^+-binding loop and the Ca^{2+} binding site are allosterically linked (17-19). We next assessed whether this linkage also exists in FSAP. For this purpose,

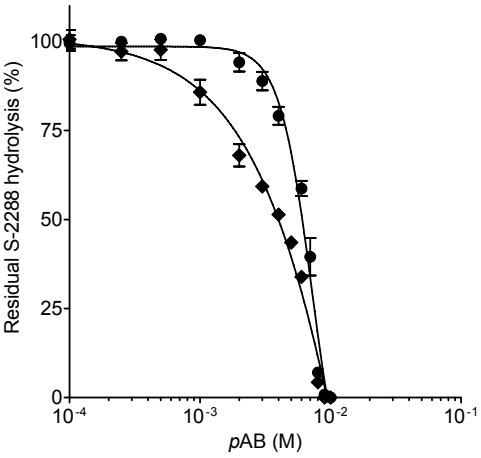


Figure 5. Suboptimal positioning of Asp^{189} in the S1 specificity pocket in FSAP^{G221E}. 17.5 nM of activated FSAP^{R15Q} (circles) and 350 nM of FSAP^{G221E} (diamonds) were incubated with varying concentrations of *p*AB for 15 minutes at 22 °C in 150 mM NaCl, 1 mM $CaCl_2$, 0.01% (w/v) BSA, 50 mM Tris-HCl (pH 8.0). S-2288 was added to a final concentration of 625 µM and the residual S-2288 hydrolysis was assessed as described in materials and methods. Data represent the means ± S.D. of at least three experiments.

we employed a Zn^{2+} displacement assay (28) in which we monitored the efficiency of Ca^{2+} to restore the catalytic activity of the FSAP variants that are inhibited by Zn^{2+}. As shown in Fig. 4B, the Ca^{2+} mediated increase of the catalytic activity was comparable for all FSAP variants. These results indicate that the Gly^{221} substitutions do not affect Ca^{2+} binding. An allosteric link between the Ca^{2+} and Na^+ binding sites in FSAP seems therefore unlikely. These findings together suggest that especially a polar amino acid residue at position 221 may alter the conformation of the 220 loop, which has a direct effect on Na^+-dependent enhancement of the catalytic activity.

Suboptimal positioning of Asp^{189} in the S1 specificity pocket in FSAP^{G221E}

For several serine proteases, it has been demonstrated that the residue at position 221 contributes to the positioning and stabilization of Asp^{189} at the base of the S1 specificity pocket (14-18). To establish whether this role can also be attributed to Gly^{221} of FSAP, we performed inhibition studies with *p*AB, which effectively targets a correctly positioned Asp^{189} (14, 17, 18). We compared the K_i of *p*AB for FSAP^{R15Q} and for FSAP^{G221E} because the latter exhibits the strongest defect in the catalytic activity among the FSAP variants (Fig. 5). The obtained K_i (2 mM) for inhibition of FSAP^{R15Q} was 3-fold lower than the K_i (6 mM) obtained for the inhibition of FSAP^{G221E}. These findings demonstrate that Asp^{189} is suboptimally positioned in FSAP^{G221E} as compared to Asp^{189} in FSAP^{R15Q}. Nevertheless, *p*AB still showed effective inhibition of FSAP^{G221E}. The S1 pocket exhibits, therefore, no major functional defect after replacement of the glycine for the glutamic acid.

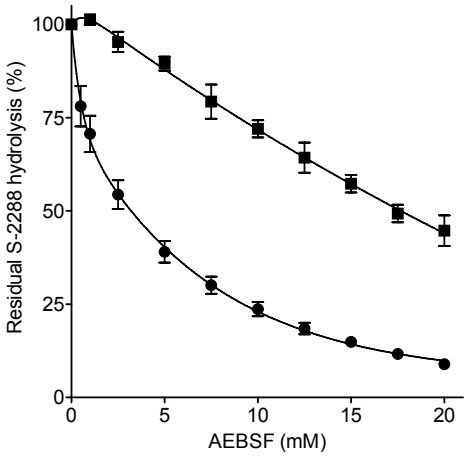


Figure 6. Impaired alignment of the residues of the catalytic triad in FSAP^{G221E}. 17.5 nM of activated FSAP^{R15Q} (circles) and 175 nM of FSAP^{G221E} (squares) were incubated for 15 minutes at 22 °C with different concentrations of AEBSF in 150 mM NaCl, 0.1 % (v/v) Tween-80, and 50 mM Tris-HCl (pH 8.0). S-2288 was added to the reaction mixture (final concentration of 625 µM) to measure residual S-2288 hydrolysis as described in materials and methods. Data represent the means ± S.D. of at least three experiments.

Impaired alignment of the residues of the catalytic triad in FSAP^{G221E}

The transfer of the proton from the side chain of Ser^{195} to His^{57} is a key event for effective attack on the carbonyl group of a substrate. A requirement for effective proton transfer is a proper alignment of the catalytic residues Ser^{195}, His^{57} and Asp^{102} (11, 12). To assess the alignment of these residues, we employed inhibition studies with (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) AEBSF that specifically binds the highly reactive Ser^{195} in serine proteases (29). The results show that AEBSF is a poor inhibitor of FSAP^{G221E} relative to FSAP^{R15Q} (Fig. 6). This finding demonstrates that Ser^{195} in FSAP^{G221E} is markedly less reactive than Ser^{195} in FSAP^{R15Q}. From this notion can be deduced that the residues of the catalytic triad are not optimally aligned in the FSAP variant in which Gly^{221} is replaced by a glutamic acid.

Ineffective insertion of Ile^{16} in the protease domain of FSAP^{G221E}

After proteolytic activation of FSAP, the newly formed N-terminus of Ile^{16} forms a salt bridge with Asp^{194}. This salt bridge is indispensable for the correct positioning of Ser^{195} in the catalytic center thereby facilitating effective interaction with the other residues of the catalytic triad (11, 12). Incomplete insertion of Ile^{16} in the protease domain of FSAP^{G221E} may therefore explain the poor inhibition of its activity by AEBSF. To probe for incorrectly inserted N-terminal isoleucine residues, we incubated FSAP^{G221E} and FSAP^{R15Q} for fixed periods of time with sulpho-NHS-LC-biotin that specifically reacts with free solvent exposed amino groups (30, 31). The biotinylated proteins were proteolytically processed with trypsin, and the peptides were separated on a reversed-phase C18 nano-LC column. The peptide sequence and the

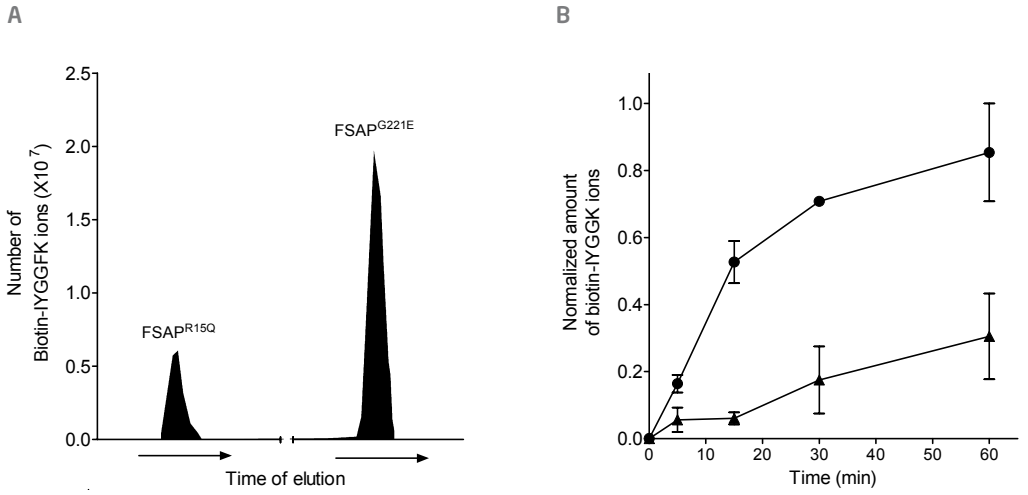


Figure 7. Reduced protection from chemical modification of the amino group of Ile^{16} in FSAP^{G221E}. FSAP^{R15Q} and FSAP^{G221E} were incubated with Sulpho-NHS-LC-biotin for different periods of time. Peptides of the FSAP derivatives were obtained and separated on a C18 liquid chromatography column which was connected to a mass spectrometer as described in materials and methods. (A) shows the reconstructed ion chromatograms of the eluted peptide representing the modified N-terminus of FSAP^{R15Q} and of FSAP^{G221E} after 15 minutes of incubation with sulpho-NHS-LC-biotin. (B) shows the total number of modified N-terminal peptide ions normalized to the maximum number of modified ions that were obtained for FSAP^{G221E} after 60 minutes of incubation with sulpho-NHS-LC-biotin (see materials and methods). The data show the average of two independent experiments. The error bars show the deviation between the two experiments.

biotin modifications thereof were subsequently identified employing mass spectrometry. The data showed that a peptide representing the biotinylated N-terminus (i.e. Biotin-IYGGFK, m/z 512.271) eluted from the C18 column after 45 minutes. After 5 minutes of incubation with sulpho-NHS-LC-biotin, this modified peptide was identified for both proteins suggesting that there are solvent exposed N-terminal isoleucine residues in FSAP^{R15Q} and in FSAP^{G221E}. This observation is in itself not a surprise since the inserted N-terminus and the free N-terminus are in a dynamic equilibrium. However, the area under the curve of the reconstructed ion chromatograms shows that the degree of biotinylation at Ile^{16} was at all time points higher for FSAP^{G221E} than for FSAP^{R15Q} (Fig. 7). These finding demonstrates that the N-terminus of FSAP^{R15Q} is more protected from biotinylation than that of FSAP^{G221E}. This observation suggests that the impaired alignment of the residues of the catalytic triad in FSAP^{G221E} is the result of incomplete insertion of the N-terminus.

Discussion

In the present study, we set out to investigate the role of the glycine at position 221 for the catalytic activity of FSAP. Our findings reveal that the replacement of this amino acid for a small amino acid residue,

like alanine or serine, already has functional implications for the protease. It is therefore not a surprise that that the glycine to glutamic acid change as found in the Marburg-1 variant exhibits a marked defect in its catalytic activity against its substrate scuPA (Fig. 2). Based on the obtained K_m values for the interaction between scuPA and the FSAP variants, a change of the glycine for either an alanine or a serine does not seem to affect the direct binding to scuPA (Table I). This observation suggests that the glycine substitution mainly affects the catalytic core of the protease. The latter is in agreement with the finding that the K_m values do markedly change depending on the nature of the amino acid at position 221 employing the small substrate S-2288 (Table II).

The functional role of the 220 loop has been elucidated for a number of serine proteases, including factor IX, factor X, protein C and thrombin (14,16-18). It has been demonstrated that the 220 loop of these proteases bind a Na^+-ion which mediates the proper orientation of Asp^{189} at the base of the S1 specificity pocket. The 220 loop of FSAP also binds a Na^+-ion but displays no sequence homology with these loops at all. A striking feature is further that the 220 loop of FSAP is markedly shorter than that of any other known mammalian serine protease. Human serine proteases that exhibit the highest primary sequence homology with full-length FSAP are hepatocyte growth factor activator (1) and tissue-type plasminogen activator (tPA). As the tPA catalytic domain shows the highest homology with the catalytic domain of FSAP, we constructed a model of the catalytic domain of FSAP based on that of tPA employing comparative homology modeling (Fig. 8). The model reveals that Gly^{221} is located at the tip of the 220 loop and bends towards Asp^{189}. The close proximity of Gly^{221} and Asp^{189} facilitates a direct interaction via hydrogen bond formation between the backbone amide group of Gly^{221} and the carboxyl group of Asp^{189}. The backbone of Asp^{189}, in turn, shows hydrogen bond interaction with the backbone of Tyr^{17}. The latter interaction has also been observed in, for instance, the crystal structures of Na^+-dependent serine proteases (32-34). However, whereas in these enzymes the Na^+-ion is involved in proper orientation of Asp^{189}, the molecular model clearly suggests that Gly^{221} performs this role in FSAP.

The molecular model of FSAP also provides an explanation for the observed catalytic defects of the FSAP variants. Replacement of Gly^{221} for a serine introduces a polar hydroxyl group in close proximity of the hydrophobic side chain of Tyr^{17} leading to unfavorable interactions. A repellent interaction is even more pronounced after introduction of the negatively charged glutamic acid at position 221 (Fig. 8). These polar residues are therefore expected to create an energy barrier for effective insertion in the protease domain of the free amino group of Ile^{16} after FSAP activation. This will then result in suboptimal positioning of Ser^{195} in the catalytic center impairing effective proton transfer of its side chain to His^{57} during catalysis. This notion is in full agreement with the AEBSF inhibition experiment and mass spectrometry analysis of $FSAP^{G221E}$. These results showed an increased solvent exposure of the amino group of Ile^{16} as well as an impaired inhibition with AEBSF (Figs 6, 7). The unfavorable interaction is expected to be reduced by introduction of the small hydrophobic alanine at position 221 (Fig. 8). This may provide an explanation that $FSAP^{R15Q/G211A}$ is least defective in its catalytic activity towards scuPA and towards the small molecular substrate S-2288 (Figs 2, 3).

Because of the apparent direct interaction between Gly^{221} and Asp^{189}, it would be expected that mutation

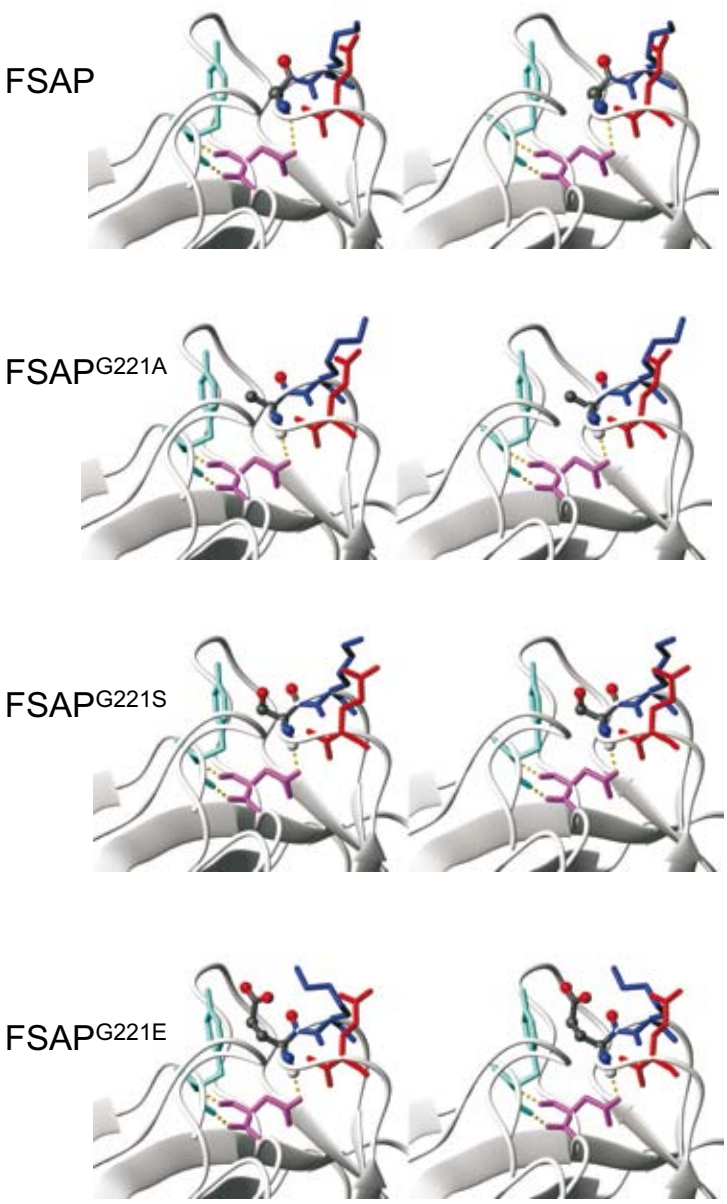


Figure 8. Gly^{221} and Tyr^{17} contribute to stabilization of Asp^{186} at the base of the S1 specificity pocket. Molecular model of FSAP and the variants thereof were constructed with Modeller 9v7 employing the crystal structure of tPA as a template (PDB code: 1rtf.pdb) (36, 37). Indicated on the left is the name of the displayed FSAP variants. Tyr^{17} at the N-terminal end of the protease in indicated in light blue, Asp^{189} at the base of the S1 specificity pocket is shown in purple, the residue at position 221 is shown in a ball and stick representation. The hydrogen bonds between the residues 221, 189 and 17 are indicated by dotted yellow lines. In dark blue is shown Lys^{222} and in red Glu^{219} that interact via a salt bridge. In FSAP^{G221E}, Glu^{221} interacts with Lys^{222} instead of Glu^{219}.

of Gly^{221} has a severe impact on the S1 specificity pocket. Inhibition studies of $FSAP^{G221E}$ with *p*AB revealed, however, no major functional defect of the S1 pocket (Fig. 6). For factor IX, factor X, protein C and thrombin, Na^+ has been proposed to stabilize the S1 pocket via interaction with Asp^{189} (14,16-18). Mutations in the Na^+-binding loop affect then also the catalytic activity of these proteins (20, 22, 23, 35). The observation that not the side chain but the backbone atoms of Gly^{221} interact with Asp^{189} in FSAP may explain the reduced effect on the S1 pocket when a glutamic acid is introduced at this site. Taking the above-mentioned notions into account, it may seem feasible to restore the catalytic activity of $FSAP^{G221E}$ by replacing the tyrosine at position 17 with e.g. an alanine. This would alleviate the unfavorable interaction with the inserted N-terminus, and therefore induce optimal alignment of the residues of the catalytic triad.

It is likely that introduction of the serine and especially the glutamic acid at position 221 has an additional destabilizing effect on the 220 loop. This would provide an explanation for the impaired binding of Na^+ to the 220 loop in these variants (Fig. 4). Residue 221 is flanked by a glutamic acid at position 219 and a lysine at position 222. These residues are fully exposed to the solvent which would facilitate ample interaction with the surrounding water molecules. However, because of their close proximity, these residues of opposing charge may also form a salt bridge thereby supporting an optimal conformation of the 220 loop for effective Na^+ binding. Introduction of the negatively charged glutamic acid at position 221 is likely to compete with Glu^{219} for interaction with Lys^{222} (Fig. 8). A glutamic acid at position 221 may therefore not only prevent effective insertion of the N-terminus but may also destabilize the 220 loop and as such Na^+ binding.

The exact role and physiological significance of the Na^+-ion for FSAP remains unclear. In the absence of Ca^{2+}, Na^+ is capable to partially restore the catalytic activity of FSAP (Fig. 4). For factor IX, it has been proposed that Ca^{2+} stabilizes the N-terminus through allosteric interactions thereby facilitating an optimal positioning of the residues in the catalytic center (14). A similar role of Ca^{2+} is feasible for effective catalysis of FSAP. Apparently, Na^+ can, to some extent, take over this functional role of Ca^{2+}. Possibly, in the absence of Ca^{2+}, Na^+ repositions the 220 loop of FSAP for optimal interaction between Gly^{221} and Asp^{189}. This may in turn facilitate hydrogen bond formation between Asp^{189} and Tyr^{17}, and as such stabilize the inserted N-terminus (Fig. 8). Consequently, in the absence of Ca^{2+}, Na^+ may allosterically regulate the catalytic activity of FSAP via the residues Gly^{221}, Asp^{189} and Tyr^{17}.

The findings of this study show that Gly^{221} is of principle importance for the catalytic activity of FSAP, most likely by mediating direct interaction of the 220 loop with Asp^{189} at the base of the S1 specificity pocket, and by allosterically stabilizing the salt bridge between Ile^{16} and Asp^{194}.

References

1 Choi-Miura NH, Tobe T, Sumiya J, et al. Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a kringle and a serine protease domain, similar to hepatocyte growth factor activator. J Biochem. 1996;119:1157-1165.

2 Hunfeld A, Etscheid M, Konig H, Seitz R, Dodt J. Detection of a novel plasma serine protease during purification of vitamin K-dependent coagulation factors. FEBS Lett. 1999;456:290-294.

3 Stavenuiter F, Boon-Spijker MG, Meijer AB, Mertens K. Factor Seven Activating Protease (FSAP): does it activate Factor VII? Chapter 2 of this thesis. 2010.

4 Choi-Miura NH, Yoda M, Saito K, Takahashi K, Tomita M. Identification of the substrates for plasma hyaluronan binding protein. Biol Pharm Bull. 2001;24:140-143.

5 Romisch J, Vermohlen S, Feussner A, Stohr H. The FVII activating protease cleaves single-chain plasminogen activators. Haemostasis. 1999;29:292-299.

6 Kannemeier C, Al-Fakhri N, Preissner KT, Kanse SM. Factor VII-activating protease (FSAP) inhibits growth factor-mediated cell proliferation and migration of vascular smooth muscle cells. Faseb J. 2004;18:728-730.

7 Romisch J, Feussner A, Stohr HA. Quantitation of the factor VII- and single-chain plasminogen activator-activating protease in plasmas of healthy subjects. Blood Coagul Fibrinolysis. 2001;12:375-383.

8 Ireland H, Miller GJ, Webb KE, Cooper JA, Humphries SE. The factor VII activating protease G511E (Marburg) variant and cardiovascular risk. Thromb Haemost. 2004;92:986-992.

9 Weisbach V, Ruppel R, Eckstein R. The Marburg I polymorphism of factor VII-activating protease and the risk of venous thromboembolism. Thromb Haemost. 2007;97:870-872.

10 Stavenuiter F, Meijer AB, Sellink E, Mertens K. Marburg-1 polymorphysm. Chapter 3 of this thesis. 2010.

11 Di Cera E. Serine proteases. IUBMB Life. 2009;61:510-515.

12 Page MJ, Di Cera E. Serine peptidases: classification, structure and function. Cell Mol Life Sci. 2008;65:1220-1236.

13 Kannemeier C, Feussner A, Stohr HA, Weisse J, Preissner KT, Romisch J. Factor VII and single-chain plasminogen activator-activating protease: activation and autoactivation of the proenzyme. Eur J Biochem. 2001;268:3789-3796.

14 Schmidt AE, Stewart JE, Mathur A, Krishnaswamy S, Bajaj SP. Na+ site in blood coagulation factor IXa: effect on catalysis and factor VIIIa binding. J Mol Biol. 2005;350:78-91.

15 Prasad S, Cantwell AM, Bush LA, Shih P, Xu H, Di Cera E. Residue Asp-189 controls both substrate binding and the monovalent cation specificity of thrombin. J Biol Chem. 2004;279:10103-10108.

16 Pineda AO, Carrell CJ, Bush LA, et al. Molecular dissection of Na+ binding to thrombin. J Biol Chem. 2004;279:31842-31853.

17 Schmidt AE, Padmanabhan K, Underwood MC, Bode W, Mather T, Bajaj SP. Thermodynamic linkage between the S1 site, the Na+ site, and the Ca2+ site in the protease domain of human activated protein C (APC). Sodium ion in the APC crystal structure is coordinated to four carbonyl groups from two separate loops. J Biol Chem. 2002;277:28987-28995.

18 Underwood MC, Zhong D, Mathur A, Heyduk T, Bajaj SP. Thermodynamic linkage between the S1 site, the Na+ site, and the Ca2+ site in the protease domain of human coagulation factor xa. Studies on catalytic efficiency and inhibitor binding. J Biol Chem. 2000;275:36876-36884.

19 Dang QD, Di Cera E. Residue 225 determines the Na(+)-induced allosteric regulation of catalytic activity in serine proteases. Proc Natl Acad Sci U S A. 1996;93:10653-10656.

20 Rouy S, Vidaud D, Alessandri JL, et al. Prothrombin Saint-Denis: a natural variant with a point mutation resulting in Asp to Glu substitution at position 552 in prothrombin. Br J Haematol. 2006;132:770-773.
21 Hamaguchi N, Roberts H, Stafford DW. Mutations in the catalytic domain of factor IX that are related to the subclass hemophilia Bm. Biochemistry. 1993;32:6324-6329.
22 Spitzer SG, Pendurthi UR, Kasper CK, Bajaj SP. Molecular defect in factor IXBm Lake Elsinore. Substitution of Ala390 by Val in the catalytic domain. J Biol Chem. 1988;263:10545-10548.
23 Miyata T, Zheng YZ, Sakata T, Tsushima N, Kato H. Three missense mutations in the protein C heavy chain causing type I and type II protein C deficiency. Thromb Haemost. 1994;71:32-37.
24 Segal IH. Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. John Wiley & Sons, Inc, New York. 1975.
25 Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol. 1973;22:3099-3108.
26 Craig DA. The Cheng-Prusoff relationship: something lost in the translation. Trends Pharmacol Sci. 1993;14:89-91.
27 Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2006;1:2856-2860.
28 Petersen LC, Olsen OH, Nielsen LS, Freskgard PO, Persson E. Binding of Zn2+ to a Ca2+ loop allosterically attenuates the activity of factor VIIa and reduces its affinity for tissue factor. Protein Sci. 2000;9:859-866.
23 Markwardt F, Hoffmann J, and KGrbs E. The influence of synthetic thrombin inhibitors on the thrombin-antithrombin reaction. Thromb. Res. 1973;2:343-348
30 Gabant G, Augier J, Armengaud J. Assessment of solvent residues accessibility using three Sulfo-NHS-biotin reagents in parallel: application to footprint changes of a methyltransferase upon binding its substrate. J Mass Spectrom. 2008;43:360-370.
31 Azim-Zadeh O, Hillebrecht A, Linne U, et al. Use of biotin derivatives to probe conformational changes in proteins. J Biol Chem. 2007;282:21609-21617.
32 Padmanabhan K, Padmanabhan KP, Tulinsky A, et al. Structure of human des(1-45) factor Xa at 2.2 A resolution. J Mol Biol. 1993;232:947-966.
33 Hopfner KP, Lang A, Karcher A, et al. Coagulation factor IXa: the relaxed conformation of Tyr99 blocks substrate binding. Structure. 1999;7:989-996.
34 Mather T, Oganessyan V, Hof P, et al. The 2.8 A crystal structure of Gla-domainless activated protein C. Embo J. 1996;15:6822-6831.
35 Deam S, Srinivasan N, Westby J, Horn EH, Dolan G. F X Nottingham and F X Taunton. Two novel mutations in factor X resulting in loss of functional activity and an interpretation using molecular modelling. Thromb Haemost. 2001;85:265-269.
36 Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol. 1993;234:779-815.
37 Lamba D, Bauer M, Huber R, et al. The 2.3 A crystal structure of the catalytic domain of recombinant two-chain human tissue-type plasminogen activator. J Mol Biol. 1996;258:117-135.

Chapter 5

Factor Seven Activating Protease induces regulated Weibel-Palade body exocytosis from cultured human endothelial cells

F. Stavenuiter*†, E.A.M. Bouwens*‡, A.B. Meijer*, M.G. Rondaij*, K. Mertens*†‡

*Department of Plasma Proteins, Sanquin Research, Amsterdam; †Van Creveld Laboratory of UMC-Utrecht and Sanquin, Amsterdam; and ‡Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Abstract

Factor VII activating protease (FSAP) has been associated with a broad spectrum of functions, including haemostasis, vascular inflammation and atherogenesis. Endothelial cells contain specific organelles known as Weibel-Palade bodies (WPBs). These unique storage granules contain components such as von Willebrand factor (VWF) and P-selectin, which are released upon endothelial activation and support leukocyte rolling, platelet adhesion and aggregation. In this study, we investigated whether FSAP was able to induce the exocytosis of WPBs from human endothelial cells. Stimulation of human endothelial cells with recombinant human FSAP was found to result in the release of WPBs in a dose-dependent manner similarly to thrombin. Surprisingly, WPB release was also induced by the natural occurring Marburg-1 FSAP variant (G534E), which was shown to have reduced catalytic activity and substrate specificity. Moreover, we found that FSAP-mediated WPB release was completely independent of the catalytic activity of FSAP. Interestingly, the FSAP substrate scuPA, to which FSAP is highly homologous, was also found to induce WPB exocytosis. Thrombin-mediated WPB release is dependent on the elevation of intracellular Ca^{2+} levels. In contrast, however, FSAP-induced WPB exocytosis appeared to be independent of the Ca^{2+} pathway suggesting that thrombin and FSAP induce different signalling pathways. Moreover, thrombin-mediated signalling is known to result in major loss of endothelial barrier function, however, no significant loss of endothelial cell barrier function was observed upon FSAP stimulation further indicating that FSAP and thrombin induce endothelial cell activation via different signal transduction pathways. Taken together, these results reveal FSAP as an agonist of WPB exocytosis and suggest a novel mechanism by which FSAP can modulate vascular inflammation and haemostasis.

Introduction

The vascular endothelium plays a central role in multiple physiological processes such as haemostasis and inflammation. Upon activation, endothelial cells acquire a pro-coagulant state and provide an adhesive surface for circulating leukocytes, which allows for their migration to the extravascular space (1). One important activation mechanism of endothelial cells is agonist-induced exocytosis of Weibel-Palade bodies (WPBs) (for review see (2)). Originally defined as an intracellular storage pool for von Willebrand Factor (VWF) and P-selectin, these endothelial cell-specific organelles have been shown to contain an number of other components involved in the divers physiological functions of the endothelium (2). Exocytosis of these WPBs is triggered by a variety of agonists including thrombin, histamine, epinephrine and vasopressin (reviewed in (2)). The divers agonists of WPB exocytosis can be divided into two distinct groups, those that act via an increase in intracellular free Ca^{2+} levels and those that act via a rise in cyclic adenosine 3',5' monophosphate (cAMP) levels in the cell (2). It has been suggested that the different type of agonists can induce exocytosis of distinct subpopulations of WPBs thereby modulating the components released from the endothelial cells resulting in a more specific response (2-4).

Factor VII activating protease (FSAP) is a serine protease that is present in the circulation in its single-chain, zymogen form, scFSAP (~64 kDa) (5, 6). It contains three epidermal growth factor (EGF)-like domains, a kringle domain and a catalytic serine protease domain (5). FSAP can be activated by an autocatalytic cleavage process resulting in the generation of the active two-chain form, tcFSAP (50 and 27 kDa, respectively), linked by a disulfide bond (7-9). Activation is enhanced by binding to negative charged surfaces such as glycosaminoglycans (e.g. heparin and hyaluronic acid) that are found on cell surfaces and in the extracellular matrix (7-9). In vitro, single chain urokinase-type plasminogen activator (scuPA) is a potent substrate for FSAP, which implies a potential role in the haemostatic system (10, 11, this thesis Chapter 2). In addition to its potential role in blood coagulation and fibrinolysis, FSAP may also express activities related to cell migration and proliferation based on its homology to proteases known to display effects on vascular cell functions such as hepatocyte growth factor activator, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (5). Indeed, an anti-atherogenic function has been attributed to FSAP since it was found to inhibit vascular smooth muscle cell (VSMC) migration, proliferation and thus possibly neointima formation through inhibition of platelet-derived growth factor BB (PDGF-BB)-mediated signalling (9, 12). This appears to be dependent on its catalytic activity since the naturally occurring Marburg-1 FSAP (M1 FSAP) variant containing a Gly534Glu substitution was found to be associated with cardiovascular risk due to increased neointima formation (12, 13).

The putative role of FSAP in haemostasis (10, 11) and its proposed involvement in cell-mediated signal transduction (9) led us to investigate the effect of FSAP on endothelial cell-activation and WPB exocytosis. In this study we have found that FSAP induces WPB exocytosis from cultured human endothelial cells. Furthermore, FSAP triggers WPB exocytosis via a mechanism independent of the release of Ca^{2+} from intracellular stores. Interestingly, FSAP-induced WPB release was found to be independent of its catalytic activity. Taken together, these results suggest an additional modulatory role of FSAP in vascular injury, inflammation and haemostasis resulting from the stimulation of WPB exocytosis from endothelial cells.

Materials

Fetal Calf Serum (FCS) was from Hyclone (Logan, UT, USA). RPMI-1640 and M199-Hepes, and BAPTA-AM were obtained from Invitrogen (Breda, the Netherlands). Endothelial Cell Medium-2 (EGM-2) was obtained from Lonza (Walkersville, MD, USA). Phorbol 12-myristate 13-acetate (PMA) and thrombin were from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands). Culture plates and microtiterplates (Maxisorp) were obtained from Nunc (Roskilde, Denmark). Streptomycin and penicillin were from BioWhittacker (Verviers, Belgium). Rat tail-derived collagen type 1 was from BD Biosciences (Upssala, Sweden).

Recombinant, human FSAP variants

The complete open reading frame of human wild type FSAP was produced by PCR from human liver quick-clone cDNA (BD Biosciense, the Netherlands) using the oligonucleotide primers 5'-TTAGGATCCG-CAAAGATGTTTGCCAGGATGTCTGATCTC-3' (sense) and 5'-ATTGGTACCGAAGACAGTACCTTAGAAGCCACT-3' (antisense), including a BamHI and KpnI restriction site, respectively (underlined in primer sequences). The resulting PCR fragment was BamHI/KpnI-digested and subsequently ligated into the pcDNA3.1(-) expression vector. This wt FSAP construct was used as a template for site-directed mutagenesis using the QuikChange® kit (Stratagene, La Jolla, USA) to construct the $FSAP^{R313Q}$ mutant according to the manufacturer's instructions. The R313Q mutation was introduced in the FSAP sequence to prevent autocleavage during the production and purification process. $FSAP^{R313Q}$ was made using oligonucleotide primers 5'-GCAGAGAGGAAGATCAAGCAAATCTATGGAGGCTTTAAG-3' (sense) and 5'-CTTAAAGCCTCCAT-AGATTTGCTTGATCTTCCTCTCTGC-3' (antisense). The Marburg-1 FSAP construct was made using the QuikChange® mutagenesis kit and oligonucleotide primers 5'-AGCTGGGGCCTGGAGTGTGAGAAGAG-GCCAGGGGTCTAC-3' (sense) and 5'-GTAGACCCCTGGCCTCTTCTCACACTCCAGGCCCCAGCT-3' (antisense), introducing the G534E mutation. The $FSAP^{R313Q/S509A}$ mutant, in which the catalytic site is inactivated, was then made using the QuikChange® mutagenesis kit and oligonucleotide primers 5'-GACACCTGCCAG-GGTGACGCTGGAGGCCCCCTGACCTGT-3' (sense) and 5'-ACAGGTCAGGGGGCCTCCAGCGTCACCCTGGCAG-GTGTC3' (antisense). Sequence analysis was performed to verify the sequence of all constructs.

Plasmids encoding $FSAP^{R313Q}$ and Marburg-1 FSAP were introduced into HEK293 cells using DRMIE-C (Invitrogen, Breda, the Netherlands) and stable cell lines were selected with 500 µg/ml G418 and cultured in freestyle medium (Invitrogen, Breda, the Netherlands). $FSAP^{R313Q/S509A}$ was produced transiently using a Freestyle HEK293-cell transfection kit (Invitrogen, Breda, the Netherlands) according to manufacturer's instructions.

Recombinant FSAP was purified by immuno-chromatography with a monoclonal antibody directed to the light chain of FSAP coupled to CNBr-sepharose 4B according to manufacturer's instructions. Briefly, after loading, the column was washed with buffer containing 1 M NaCl, 50 mM Tris-HCl, pH 8.0, followed by a wash with buffer containing 100 mM NaCl, 15% ethylene glycol, 50 mM Tris-HCl, pH 8.0. Bound FSAP was eluted with buffer containing 150 mM NaCl, 55% ethylene glycol, 10% glycerol and 25 mM lysine, pH 11 and directly neutralized to pH 7.4. Remaining contaminants were removed by ion-exchange

chromatography on a Q-sepharose fast flow column employing a gradient from 0.15 to 1 M NaCl in 5% glycerol, 50 mM Tris-HCl, pH 8.0. FSAP fractions were pooled and concentrated on a second Q-sepharose fast flow column by elution with 1 M NaCl, 5% glycerol, 50mM Tris-HCl, pH 8.0. Concentrated $FSAP^{R313Q/S509A}$ was dialyzed against 150 mM NaCl, 50 mM Tris-HCl, pH 8.0. $FSAP^{R313Q}$ was dialyzed against 150 mM NaCl, 5% glycerol, 10 mM MES, pH 5.0, in order to prevent it from degradation after activation by thermolysin. Purified proteins were stored at -20°C.

Activation of $FSAP^{R313Q}$ (~700 nM) was done by incubation with thermolysin (~3 nM) for 2 hours at 30°C in 150 mM NaCl, 5% glycerol, 10 mM MES, pH 5.0. Activation was stopped by the addition of phosphoramidon disodium salt (10 µM).

Cell culture, stimulation of WPB exocytosis and quantification of VWF

Blood outgrowth endothelial cells (BOECs) were isolated from 50 ml venous blood donated by healthy volunteers, essentially as described previously (14). Endothelial cells were isolated from umbilical veins (HUVECs) and cultured as previously described (15). BOECs (passage 5 – 8) and HUVECs (passage 3 - 4) were plated at 1-2 x 10^5 cells/well in collagen or fibronectin-coated 9.6 cm^2 wells, respectively. Medium was refreshed every other day till confluency. Prior to experiments, plates were washed three times with serum-free (SF) medium (50% RPMI-1640 and 50% M199-Hepes) supplemented with 1% (v/v) Human Serum Albumin (HSA; Cealb, Sanquin, Amsterdam, the Netherlands), 0.3 mg/ml L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. After pre-incubation with SF medium for 1 hour, cells were stimulated for the indicated time periods with 1 ml SF medium alone or either 50 ng/ml PMA, 20 nM (1 U/ml) thrombin, 25-100 nM activated catalytically inactive $FSAP^{R313Q/S509A}$ ($tcFSAP^{S509A}$), 25-100 nM activated $FSAP^{R313Q}$ (tcFSAP) or 25-100 nM activated Marburg-1 FSAP (M1 tcFSAP). Medium from the cells was collected after stimulation, supplemented with 10 mM benzamidine, centrifuged for 10 min at 10.000g and stored at -20°C until analysis. The amount of VWF and/or VWF propeptide (VWFpp) released in the medium upon stimulation was measured by ELISA as described previously (14). Cells were subsequently prepared for morphological analysis by immunofluorescence microscopy.

Immunofluorescence microscopy

BOECs were grown till confluency on gelatine- or collagen-coated glass coverslips. After stimulation as described above the cells were fixed in 3.7% (v/v) formaldehyde and permeabilized for 30 minutes with 0.02% saponin in PBS containing 1% BSA. VWF was visualized using mouse monoclonal IgG1 antibody CLB-Rag20 (16) and alexafluor568-conjugated goat-anti-mouse IgG1 as secondary antibody (Invitrogen, Breda, the Netherlands). To visualize the cell membrane, the mouse monoclonal anti-Pecam-1 (anti-CD31, CLB-HEC-75) IgG2a antibody (17) and alexafluor488-conjugated goat-anti-mouse IgG2a secondary antibody (Invitrogen, Breda, the Netherlands) were used. Cells were embedded in Mowiol® 4-88 mounting medium (Polysciences Inc., Eppelheim, Germany) and viewed by confocal microscopy using a Zeiss LSM510 confocal laser microscope (Carl Zeiss, Heidelberg, Germany).

FSAP induces Weibel-Palade Body exocytosis

To study the effect of FSAP on WPB exocytosis, we treated BOECs for 1 hour with increasing concentrations of tcFSAP and measured the concentration of VWF in the medium by ELISA. FSAP was able to activate VWF release from BOECs in a dose-dependent manner (Fig. 1A). Similarly, in HUVECs, tcFSAP stimulation induced WPB exocytosis in a comparable dose-dependent manner as in BOECs (Fig. 1B). Therefore, further experiments were done using BOECs. FSAP-induced VWF release was found to increase in time (Fig. 1C). To exclude the possibility that adding a random compound to the cells could already lead to WPB release, 4 mg/ml BSA was added to the cells. No effect on VWF secretion was observed upon BSA treatment indicating that addition of an aspecific compound was not sufficient to induce WPB release (Fig. 1C). To further support that FSAP activates regulated WPB-secretion from BOECs, we studied the effect of FSAP on the release of VWF propeptide (VWFpp), which is stored together with VWF in WPBs (18). FSAP induced the release of VWFpp from BOECs in a similar fashion as VWF (Fig. 1D) supporting the idea that FSAP stimulation results in the exocytosis of WPBs. Furthermore, the physiological concentration of FSAP in plasma is about 170 nM, which suggests FSAP to be at least as effective in stimulating WPB exocytosis as classical triggers of WPB release such as thrombin.

The natural occurring FSAP variant known as Marburg-1, containing the Gly534Glu substitution has been shown to affect FSAP amidiolytic activity and substrate specificity (11, 19, this thesis Chapter 3). To examine the effect of the Marburg-1 polymorphism on the ability of FSAP to induce VWF release from endothelial cells, BOECs were stimulated with various concentrations of this FSAP variant. Surprisingly, the Marburg-1 FSAP variant induced WPB-exocytosis in a dose-dependent manner similar to that observed for wild-type (wt) FSAP (Fig. 2) indicating that full amidiolytic activity and substrate specificity is not required for FSAP-induced WPB exocytosis.

FSAP induces Weibel-Palade body exocytosis independent of its catalytic activity

Since the Marburg-1 FSAP variant was found to induce WPB exocytosis comparably to wt FSAP, we further explored whether FSAP-induced VWF release is completely independent on its amidiolytic activity. Therefore, we added various doses of an FSAP variant, of which the catalytic centre was inactivated by the Ser509Ala substitution ($FSAP^{S509A}$), to BOECs and stimulated for 1 hour. $FSAP^{S509A}$, in its activated- as well as in its zymogen- form ($tcFSAP^{S509A}$ and $scFSAP^{S509A}$ respectively) was found to stimulate VWF release in a dose-dependent manner comparable to activated wt FSAP (Fig. 3A). These data show that FSAP activates WPB exocytosis independent of its catalytic activity.

FSAP and its substrate scuPA belong to the same family of serine proteases and are highly homology. Therefore, we investigated whether scuPA, like FSAP, was able to induce WPB exocytosis from endothelial cells. Surprisingly, scuPA was found to induce VWF release from confluent monolayers of BOECs in a dose-dependent manner similar to zymogen FSAP (Fig. 3B). To our knowledge, scuPA has not been described before as an agonist for WPB exocytosis.

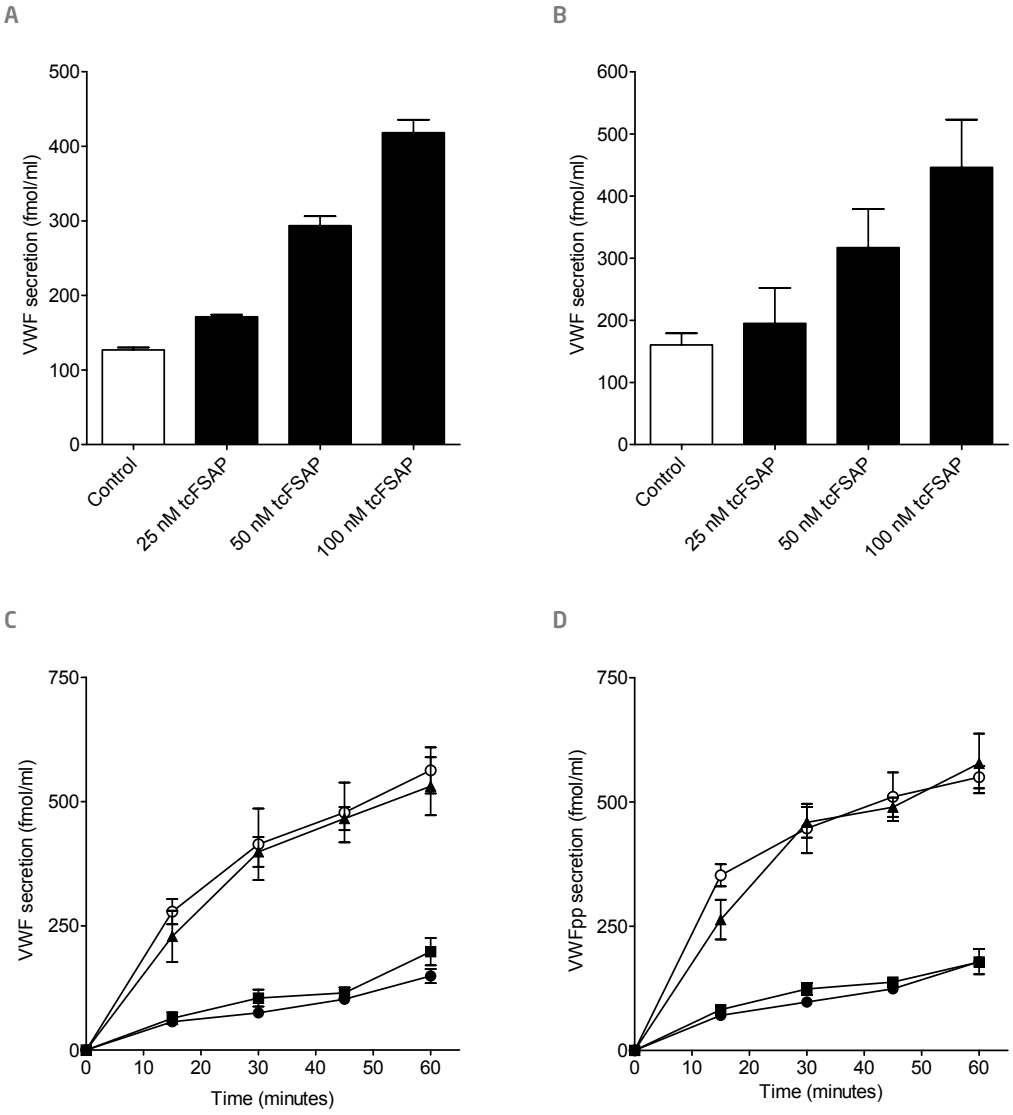


Figure 1. FSAP induces WPB exocytosis from endothelial cells. Cells were grown to confluency in 6-well plates. Prior to experiments, cells were incubated for 1 hour with SF medium and then incubated with 1 U/ml thrombin, various, indicated concentrations of activated FSAP^{R313Q} (tcFSAP) or SF medium alone (control). The concentration of VWF or VWF propeptide (ppVWF) released into the medium was measured by ELISA (A) Dose-dependent VWF secretion from BOECs upon tcFSAP stimulation as measured by ELISA. (B) Dose-dependent VWF release from HUVECs by tcFSAP. Figures shown are representative graphs of at least 3 independent experiments, error bars represent SEM. (C) VWF secretion in time upon stimulation with 1 U/ml thrombin (open circles), 100 nM tcFSAP (triangles), 4 mg/ml BSA (squares), or SF medium alone (control) (closed circles). (D) ppVWF release in time upon stimulation with 1 U/ml thrombin (open circles), 100 nM tcFSAP (triangles), 4 mg/ml BSA (squares), or SF medium alone (control) (closed circles). Results are given as the average of 3 independent experiments and error bars represent SEM.

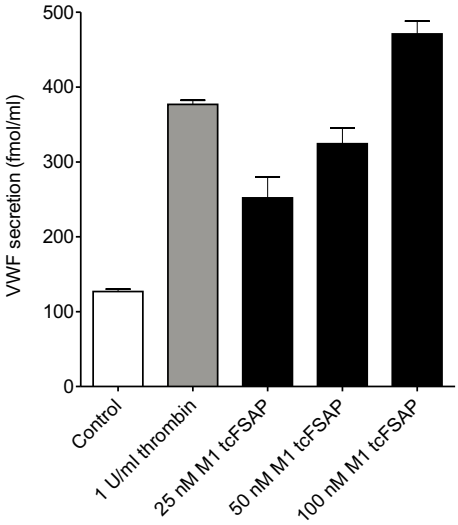


Figure 2. Dose-dependent WPB exocytosis by the Marburg-1 FSAP variant. Increasing amounts of activated Marburg-1 FSAP variant (M1 tcFSAP), 1 U/ml thrombin or SF medium alone (control) were incubated with BOECs for 1 hour and the concentration of VWF released into the medium was measured by ELISA. Figure shows representative graph from 3 independent experiments and error bars represent SEM.

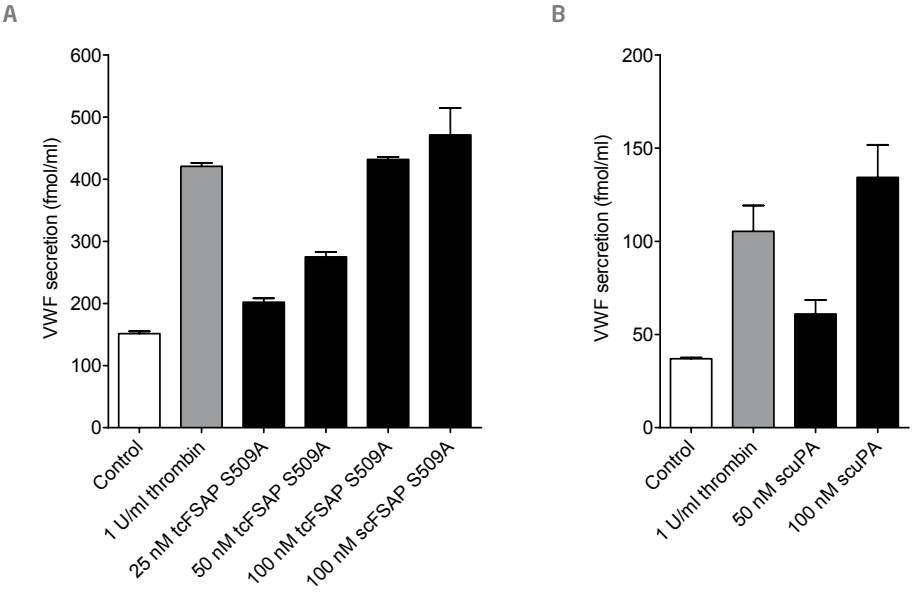


Figure 3. FSAP-induced WPB exocytosis is independent of its catalytic activity. A. BOECs were incubated for 1 hour with 1 U/ml thrombin, increasing amounts of activated FSAPS509A (tcFSAP S509A), 100 nM of its zymogen (scFSAP S509A) or SF medium alone (control). B. BOECs were incubated with 1 U/ml thrombin, 50 nM and 100 nM scuPA or SF medium alone (control). The concentration of VWF released into the medium was measured by ELISA. Figures show representative graphs of 3 independent experiments and error bars represent SEM.

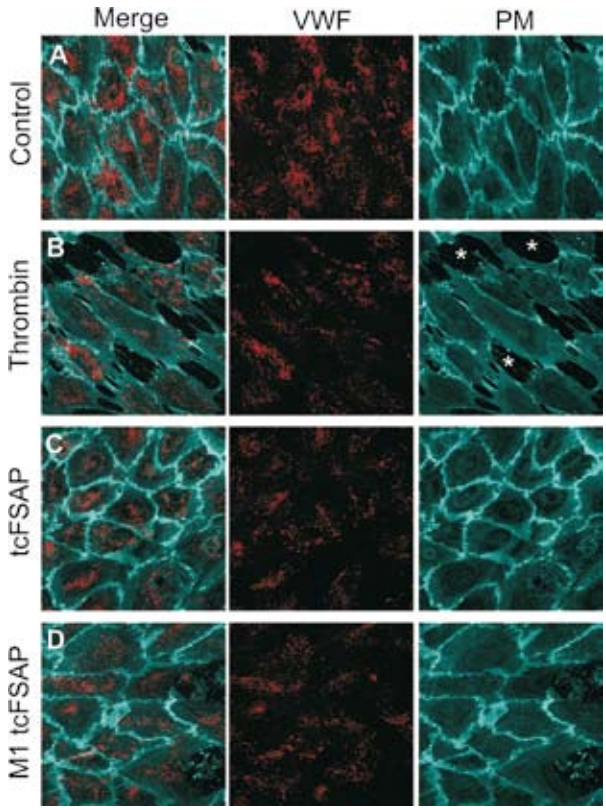


Figure 4. FSAP regulates WPB exocytosis but does not affect endothelial cell morphology. BOECs were grown on gelatine- or collagen-coated glass coverslips till confluency. Cells were incubated with SF medium for 1 hour prior stimulation with A. SF medium (control), B. 1 U/ml thrombin, C. 100 nM tcFSAP or D. 100 nM activated Marburg-1 FSAP variant (M1 tcFSAP). After stimulation, cells were fixed with 3.7% formaldehyde. WPBs were visualized using mouse monoclonal anti-VWF (CLB-RAg-20) antibody and alexafluor568-conjugated goat-anti-mouse IgG1 antibody and the plasma membrane (PM) was stained with mouse monoclonal anti-Pecam-1 (CLB-HEC-75) IgG2a antibody and alexafluor488-conjugated goat-anti-mouse IgG2a secondary antibody as described in Material and Methods. Asterisks indicate gaps between plasma membranes of adjacent endothelial cells resulting from cell contraction upon thrombin stimulation.

FSAP does not affect cell morphology or loss of endothelial barrier function

Both Ca^{2+}-elevating and cAMP-raising agonists display specific patterns of cytoskeletal remodelling that have distinct effects on endothelial cell barrier function (20). Incubation with, for example thrombin or histamine, results in stress fiber formation and loss of endothelial barrier function by disassembly of tight- and adherens- junctions via the RhoA/ROCK-mediated signalling pathway (21, 22). In contrast, cAMP-raising agonists such as epinephrine or vasopressin have been shown to promote VE-cadherin-mediated cell-cell contacts and improve the barrier function of endothelial cells (23). To see whether

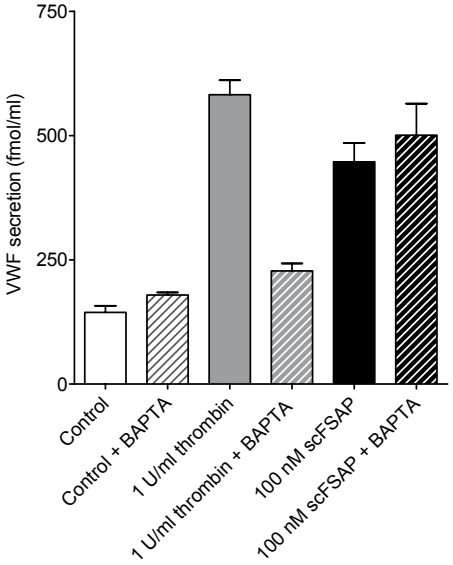


Figure 5. FSAP induces WPB exocytosis in a Ca^{2+} -independent manner. BOECs were pre-treated with SF medium in absence or presence of 15 µM BAPTA-AM for 30 minutes and then incubated with 1 U/ml thrombin, 100 nM tcFSAP or SF medium alone (control) in the absence or presence of 15 µM BAPTA-AM. VWF release in the medium was measured by ELISA. Result shows representative graph of 3 independent experiments and error bars represent SEM.

FSAP can induce cell contraction resulting in loss of endothelial barrier function similarly to thrombin, stimulated and subsequently fixed BOECs were subjected to Laser-scanning confocal (LSC) microscopy. Following fixation, BOECs were co-immunostained for VWF, as a marker for WPBs and PECAM-1 (CD31), as a marker for the plasma membrane. The resulting confocal microscopy images clearly show that both wt tcFSAP and Marburg-1 tcFSAP induce the exocytosis of WPBs similar to thrombin (Fig. 4, compare panel 4B, 4C and 4D with panel 4A). Furthermore, thrombin stimulation visibly results in contraction of the cells, leaving large gaps between plasma membranes of adjacent endothelial cells (Fig. 4B, asterisks) In contrast to thrombin, tcFSAP or Marburg-1 tcFSAP, however, did not cause morphological changes in the endothelial cells suggesting that FSAP and thrombin induce different signalling pathways (Fig. 4C and 4D).

FSAP-induced VWF secretion is independent of Ca^{2+}

Thrombin-induced exocytosis of WPBs depends on the elevation of intracellular Ca^{2+} levels. To determine whether FSAP, like thrombin, induces exocytosis of WPBs in a calcium-dependent manner, we pre-treated BOECs for 30 minutes with 15 µM BAPTA-AM, a cell-permeable intracellular Ca^{2+}-chelator and then incubated the cells for 1 hour with SF medium alone, thrombin or FSAP in the presence of BAPTA-AM. BAPTA-AM alone did not induce WPB exocytosis, whereas thrombin-induced VWF release was almost completely inhibited (Fig. 5). In contrast, however, BAPTA-AM was not able to inhibit FSAP-mediated VWF secretion (Fig. 5). This result is in support with the microscopy data, again indicating that FSAP and thrombin induce WPB exocytosis via different signalling pathways.

Discussion

Regulated exocytosis of Weibel-Palade bodies (WPBs) from endothelial cells has been shown to play an important role in integrating inflammatory- and haemostatic- responses. WPBs undergo exocytosis in response to many different stimuli reflecting both physiological and patho-physiological conditions (for review see (2)). Thrombin, for example, induces a rapid, local response leading to WPB exocytosis, which is mediated by an increase in intracellular free Ca^{2+} levels (24) via activation of protease-activated receptor 1 (PAR1) (25). In addition, coagulation factor Xa is known to induce Ca^{2+} signalling via various PARs on the endothelial surface (26, 27). Factor VIIa is found to induce Ca^{2+} release and MAPK activation in several different cell types via tissue factor (TF) or an, as yet, unidentified PAR (28-30). All these cell-related functions, however, are strongly dependent on the proteolytic activity of these coagulation factors (31).

In the present study, we demonstrated that FSAP is a potent agonist of regulated WPB exocytosis from human endothelial cells. In contrast to thrombin, the FSAP-induced WPB exocytosis was found to be independent of the Ca^{2+} -pathway indicating that thrombin- and FSAP- induced WPB release involves activation of different signalling pathways. This is further supported by our observation that, in contrast to thrombin, FSAP stimulation did not result in major loss of endothelial cell barrier function. Surprisingly, however, both FSAP-stimulated WPB exocytosis and its effect on endothelial barrier function were found to be independent of the catalytic activity of FSAP.

Furthermore, we found that single chain urokinase-type plasminogen activator (scuPA), one of the physiological substrates of FSAP, was also able to induce the release of WPBs from endothelial cells. Both FSAP and scuPA belong to the kringle domain-containing serine protease family and consist of three or one epidermal growth factor (EGF)-like domain(s), respectively, a kringle domain and a catalytic serine protease domain. The receptor for uPA on endothelial cells is the urokinase type plasminogen activator receptor (uPAR) (see for review (32). This receptor does not contain transmembrane domains but is associated with the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor. To activate intracellular signalling, uPAR has been shown to cooperate with transmembrane co-receptors. In the last decade, accumulating evidence suggests that a broad spectrum of integrins may be involved in this cooperative receptor signalling (33). (sc)uPA binds uPAR via its EGF-like domain, whereas its kringle domain stabilizes the interaction through binding to integrins such as αvß3 and αvß5 resulting in activation of cell migration-, adhesion- and proliferation- pathways (34-36).

uPAR is an important regulator of extracellular matrix (ECM) proteolysis, cell-ECM interactions and cell signalling (for review see (32)). It regulates the activity of the plasminogen activation system by localizing both uPA and scuPA, to the cell surface resulting in uPA-mediated plasmin generation, which reciprocally cleaves and activates scuPA (37). Interestingly enough, signalling through uPAR-integrin co-receptor complexes is independent of the proteolytic activity of uPA and involves Ras/MAPK pathway activation (32, 38). Therefore it could be speculated that the observed WPB release upon FSAP and scuPA stimulation is the result of receptor-mediated signalling induced by these proteases, possibly via activation of uPAR-integrin complexes. FSAP added to endothelial cells appears to localize to the plasma membrane, which might correspond to receptor-bound protein (unpublished observations). Previously, it

has been shown that the recombinant kringle domain of uPA displayed anti-angiogenic properties and inhibited migration of endothelial cells (39). In this regard, it is interesting to note that, also for FSAP, a protease domain-independent, kringle domain-dependent anti-angiogenic function has been reported (40). So far, it remains speculative whether uPAR and integrins are involved in FSAP- and scuPA- mediated WPB release. Further study is required to elucidate the mechanism of WPB exocytosis induced by FSAP and scuPA.

In conclusion, our study shows, for the first time, that FSAP and scuPA trigger WPB exocytosis from endothelial cells. Additional studies have to be performed to identify the molecular mechanism behind FSAP-mediated WPB exocytosis. Nonetheless, these results suggest a novel role for FSAP in modulating vascular inflammatory- and haemostatic- responses.

Acknowledgement

This work was supported by the Trombosestichting Nederland, grant number 2005-4.

References

1 Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman Ga, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. (1998) Blood. (91):3527-3561.

2 Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J. (2006) Arterioscler Thromb Vasc Biol. (26):1002-1007.

3 Vischer UM, Barth H, Wollheim CB. (2000) Arterioscler Thromb Vasc Biol. 20(3):883-91.

4 Vischer UM, Wollheim CB. (1997) Thromb Haemost. 77(6):1182-8.

5 Choi-Miura NH, Tobe T, Sumiya J, Nakano Y, Sano Y, Mazda T, Tomita M. (1996) J Biochem. 119(6):1157-65

6 Römisch J, Vermöhlen S, Feussner A, Stöhr H. (1999) Haemostasis. 29(5):292-9

7 Etscheid M, Hunfeld A, König H, Seitz R, Dodt J. (2000) Biol Chem. 381(12):1223-31

8 Kannemeier C, Feussner A, Stöhr HA, Weisse J, Preissner KT, Römisch J. (2001) Eur J Biochem. 268(13):3789-96.

9 Kannemeier C, Al-Fakhri N, Preissner KT, Kanse SM. (2004) FASEB J. 18(6):728-30.

10 Römisch J, Feussner A, Vermöhlen S, Stöhr HA. (1999) Blood Coagul Fibrinolysis. 10(8):471-9.

11 Römisch J. (2002) Biol Chem. 383(7-8):1119-24. Review.

12 Kanse SM, Parahuleva M, Muhl L, Kemkes-Matthes B, Sedding D, Preissner KT. (2008) Thromb Haemost. 99(2):286-9. Review.

13 Sedding D, Daniel JM, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM. (2006) J Exp Med. 25;203(13):2801-7.

14 Van den Biggelaar M, Bouwens EA, Kootstra NA, Hebbel RP, Voorberg J, Mertens K. (2009) Haematologica. (5):670-8.

15 Brinkman HJ, Mertens K, Holthuis J, Zwart-Huinink LA, Grijm K, van Mourik JA. (1994) Br J Haematol. (2):332-42.

16 Stel HV, Sakariassen KS, Scholte BJ, Veerman EC, van der Kwast TH, de Groot PG, Sixma JJ, van Mourik JA. (1984) Blood. (6):1408-15.

17 Van Mourik JA, Leeksma OC, Reinders JH, de Groot PG, Zandbergen-Spaargaren J. (1985) J Biol Chem. (20):11300-6.

18 Wagner DD, Olmsted JB, Marder VJ. (1982) J Cell Biol. (95): 355-360.

19 Römisch J, Feussner A, Stöhr HA. (2001) Blood Coagul Fibrinolysis. 12(5):375-83.

20 Vischer UM, Barth H, Wollheim CB. (2000) Arterioscler Thromb Vasc Biol. (3):883-91.

21 Van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsbergh VW. (2000) Circ Res. (4):335-40.

22 Bader MF, Doussau F, Chasserot-Golaz S, Vitale N, Gasman S. (2004) Biochim Biophys Acta. 1742(1-3):37-49. Review.

23 Wójciak-Stothard B, Potempa S, Eichholtz T, Ridley AJ. (2001) J Cell Sci. (Pt 7):1343-55.

24 Birch KA, Pober JS, Zavoico GB, Means AR, Ewenstein BM. (1992) J Cell Biol. 118(6):1501-10.

25 Klarenbach SW, Chipiuk A, Nelson RC, Hollenberg MD, Murray AG. (2003) Circ Res. 21;92(3):272-8.

26 Coughlin SR. (2001) Thromb Haemost. 86(1):298-307. Review.

27 Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. (2001) Pharmacol Rev. 53(2):245-82. Review.

28 Røttingen JA, Enden T, Camerer E, Iversen JG, Prydz H. (1995) J Biol Chem. 3;270(9):4650-60.

29 Poulsen LK, Jacobsen N, Sørensen BB, Bergenhem NC, Kelly JD, Foster DC, Thastrup O, Ezban M, Petersen LC. (1998) J Biol Chem. 13;273(11):6228-32.

30 Sørensen BB, Freskgård PO, Nielsen LS, Rao LV, Ezban M, Petersen LC. (1999) J Biol Chem. 23;274(30): 21349-54.
31 Camerer E, Pringle S, Skartlien AH, Wiiger M, Prydz K, Kolstø AB, Prydz H. (1996) Blood. 1996 Aug 15;88(4):1339-49.
32 Smith HW, Marshall CJ. (2010) Nat Rev Mol Cell Biol. (1):23-36
33 Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV, Chapman HA. (1996) Science. 273(5281):1551-5.
34 Bdeir K, Kuo A, Sachais BS, Rux AH, Bdeir Y, Mazar A, Higazi AA, Cines DB. (2003) Blood. 102(10):3600-8.
35 Franco P, Vocca I, Carriero MV, Alfano D, Cito L, Longanesi-Cattani I, Grieco P, Ossowski L, Stoppelli MP. (2006) J Cell Sci. 119(Pt 16):3424-34.
36 Tarui T, Akakura N, Majumdar M, Andronicos N, Takagi J, Mazar AP, Bdeir K, Kuo A, Yarovoi SV, Cines DB, Takada Y. (2006) Thromb Haemost. 95(3):524-34.
37 Nielsen LS, Hansen JG, Skriver L, Wilson EL, Kaltoft K, Zeuthen J, Danø K. (1982) Biochemistry. 7;21(25):6410-5.
38 Nusrat AR, Chapman HA Jr. (1991) J Clin Invest. 87(3):1091-7.
39 Kim KS, Hong YK, Joe YA, Lee Y, Shin JY, Park HE, Lee IH, Lee SY, Kang DK, Chang SI, Chung SI. (2003) J Biol Chem. 278(13):11449-56.
40 Jeon JW, Song HS, Moon EJ, Park SY, Son MJ, Jung SY, Kim JT, Nam DH, Choi-Miura NH, Kim KW, Kim YJ. (2006) Int J Oncol. 29(1):209-15.

Chapter 6

General discussion

Inactivation of coagulation factor VII by FSAP
Role of FSAP in haemostasis
Cellular and inflammatory activities of FSAP
Regulation of FSAP amidolytic activity and substrate specificity

F. Stavenuiter

A total of 170 human serine proteases have currently been identified in the human genome (www.ensembl.org) which are involved in diverse biological processes that include complement-fixation, food digestion and blood coagulation. Factor seven activating protease is part of a small subfamily that is comprised of 5 serine proteases. FSAP is most homologous to hepatocyte growth factor activator (HGFA) (1). HGFA is involved in the activation of hepatocyte growth factor (HGF) which has been implicated in a variety of biologic responses through binding to the tyrosine kinase receptor MET (2). Three other members of this subfamily, blood coagulation factor XII (FXII), single-chain urokinase plasminogen activator (scuPA) and tissue-type plasminogen activator (tPA) are involved in blood coagulation or fibrinolysis. In this thesis we addressed several key questions concerning the potential physiological role of FSAP. Does coagulation factor VII (FVII) and single chain urokinase-type plasminogen activator (scuPA) act as physiological relevant substrates for FSAP? If so, does FSAP affect haemostasis in a plasma system? Does FSAP need cofactors for substrate cleavage? A polymorphic site in the FSAP gene has been associated with cardiovascular risk (3). We addressed the implications of the naturally occurring G534E substitution for FSAP activation, amidolytic activity, and specificity. The above issues were addressed using single-chain, recombinant human FSAP and variants thereof.

Inactivation of coagulation factor VII by FSAP

In chapter 2 we explored whether recombinant human FSAP is capable of activating coagulation factor VII (FVII) in vitro. Serine proteases involved in blood coagulation cleave their substrates in the presence of PS, PE, and PC containing membranes and $CaCl_2$. In Chapter 2 we show that FSAP does not cleave FVII under these experimental conditions. Unexpectedly, FSAP was capable of cleaving FVII in the presence of membranes consisting of cardiolipin (CL). Moreover, this resulted in rapid inactivation of FVII rather than in its activation. We propose that the observed inactivation of FVII by activated FSAP in presence of CL serves a physiological function. CL is an anionic phospholipid that differs from PS, PE, and PC in that it contains two phosphate groups and four fatty acid chains instead of the usually single phosphate group and two fatty acid chains. CL is abundantly exposed in membranes of (mainly gram-positive) bacteria (4), several yeast species (5), and cells undergoing apoptosis (6, 7). Circulating low density lipoproteins particles also contain CL although a functional role for the presence of CL in these particles has not been defined (8). In vitro studies show that CL enhances protein S-dependent inactivation of factor Va (FVa) by activated protein C (9) thereby down-regulating blood coagulation. The CL-induced inactivation of FVII by FSAP may provide an additional anti-coagulant mechanism.

As mentioned previously, CL is a major component of the bacterial membrane, especially gram positive bacteria as e.g. Staphylococcus aureus and Streptococcus pneumoniae (4). Interestingly, performing facsscan-analysis with an antibody against FSAP our preliminary findings have indicated that FSAP indeed binds to gram-positive bacteria, yeast, and apoptotic cells but not to gram-negative bacteria and non-apoptotic cells (data not shown). As yet we do not know whether binding of FSAP to gram positive bacteria proceeds in a CL-dependent manner. Recently, FSAP has been reported to function in cellular homeostasis by catalysing the release of nucleosomes from late apoptotic, secondary necrotic cells (10). This finding suggests a role of FSAP in the context of autoimmune disease, and implies that single-chain

FSAP might be activated upon binding to secondary necrotic cells (10). The precise mechanism by which FSAP interacts with apoptotic cells remains to be further clarified. We propose that interaction of FSAP with bacteria and apoptotic cells serves to limit activation of blood coagulation during clearance of bacteria and apoptotic cells from the circulation. A number of studies have suggested that FSAP can interact with poly-anionic surfaces like hyaluronan, heparin, RNA, DNA, and polyphosphate (1, 11-13). Recently, activated neutrophils have been shown to release their nuclear contents into extracellular space forming extracellular traps (NETs) that bind and kill pathogens (14). Exposure of chromatin, histones and DNA within NETs may provide anchoring sites for FSAP and other circulating plasma proteins. Further studies are needed to address whether FSAP interacts with NETs and whether it contributes to the elimination of invading pathogens.

Role of FSAP in haemostasis

The findings reported in Chapter 2 show that FSAP cannot be considered as a physiological activator of FVII. In view of the strong homology with FXII, uPA and tPA a role for FSAP in blood coagulation or fibrinolysis cannot be excluded. This prompted us to investigate whether FSAP affects blood coagulation through FVII-independent mechanisms. To explore this issue we performed thrombin generation experiments in FVII-deficient plasma.

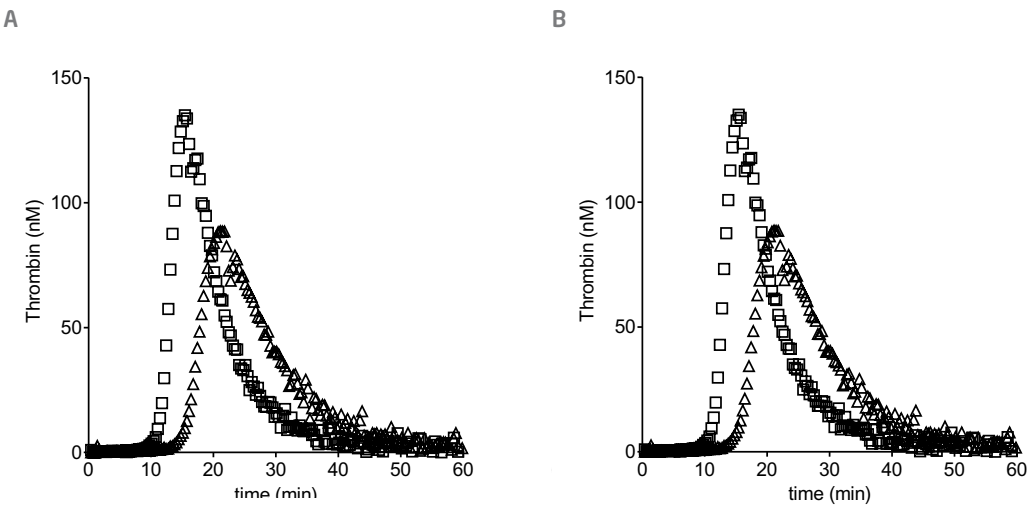


Figure 1. Effect of FSAP on the generation of thrombin. (A) Thrombin generation in FVII-deficient plasma initiated by FVIIa (2 nM) (inverted triangles) or activated FSAP (85 nM) and FVIIa (2 nM) (squares) in the presence of 1 pM TF and 4 μM phospholipids. (B) The same experiment as described in figure A but FVIIa was replaced by FXa (1 nM). Shown are the results of single experiments representative for data obtained from at least 3 independent experiments. Experiments were performed in collaboration with Dr. Herm-Jan Brinkman and Erica Sellink.

Small amounts of tissue factor and activated FVII were added and thrombin-formation was monitored using a small fluorogenic substrate. Under conditions of low TF/FVIIa thrombin generation was maximal at 20 minutes (Figure 1A; inverted triangles). Upon addition of activated FSAP thrombin formation was accelerated at peaked at 11 minutes; also enhanced levels of thrombin were generated (Figure 1A; open squares). These findings clearly show that activated FSAP accelerates thrombin-generation and that this is independent of its ability to activate FVII. We next initiated thrombin generation in the presence of factor Xa. Thrombin generation was maximal at 20 minutes under these experimental conditions (Figure 1B; inverted triangles). Addition of activated FSAP resulted in enhanced thrombin formation (Figure 1B; open squares). These results show that activated FSAP can accelerate thrombin generation and that this process is independent of its proposed role in the activation of FVII. Our findings suggest that FSAP may activate other coagulation factors thereby enhancing thrombin generation. Alternatively, activated FSAP may bind and neutralize inhibitors of blood coagulation such as antithrombin or heparin cofactor II. In this respect it is interesting that hepatocyte growth factor activator is inhibited by protein C inhibitor (15).

Cellular and inflammatory activities of FSAP

Endothelial cells lining the vasculature comprise a barrier that prevents leakage of blood components to the underlying tissues. Endothelial cells are not an inert barrier since they regulate a number of physiological processes, including extravasation of leukocytes to the underlying tissues, neovascularization, vascular tone and haemostasis (16). A number of blood coagulation factors that include thrombin and activated protein C have been shown to interact with specific receptors on the surface of endothelial cells (17). Glycosaminoglycans like heparin and hyaluronic acid have also been suggested to interact with serine proteases present in the circulation (18). In chapter 5 we observed that activated FSAP and its zymogen form can induce release of storage organelles, so-called Weibel-Palade bodies (WPB), from endothelial cells. Originally defined as an intracellular storage pool for von Willebrand Factor (VWF), an increasing number of other components, including P-selectin, interleukin 8 (IL-8), eotaxin-3, endothelin-1, tissue-type-plasminogen activator (tPA), and angiopoietin-2 (Ang-2), are present within this subcellular organelle (see for review (19)). Those components may initiate haemostasis (VWF), induce vasoconstriction (endothelin-1), regulate inflammatory responses (P-selectin, IL-8, Ang-2), or direct fibrinolysis (tPA).

Weibel-Palade bodies (WPBs) are released from endothelial cells in response to a large number of agonists such as thrombin, histamine, complements components C5a and C5b-9, epinephrine, vasopressin, and vascular endothelial growth factor (VEGF) (see for review 19). These agonists can be divided into two distinct groups, those that act by elevating intracellular Ca^{2+} levels and those that act by elevating raising cAMP levels in the cell. Examples of agonists that act by elevating intracellular Ca^{2+} levels are thrombin, histamine, complement components, and VEGF, whereas epinephrine and vasopressin elevate cAMP levels. Ca^{2+} elevating agonist activate G protein-coupled receptors (GPCRs) of the G_q-type whereas agonists interacting with receptors coupled to the G_s protein elevate cAMP levels. GPCRs are receptors which are activated by binding of their agonists. In contrast, e.g. protease-activated receptors (PARs), a subfamily of 4 receptors (PAR1-4) of the GPCRs, are activated by cleavage of their extracellular domain by serine

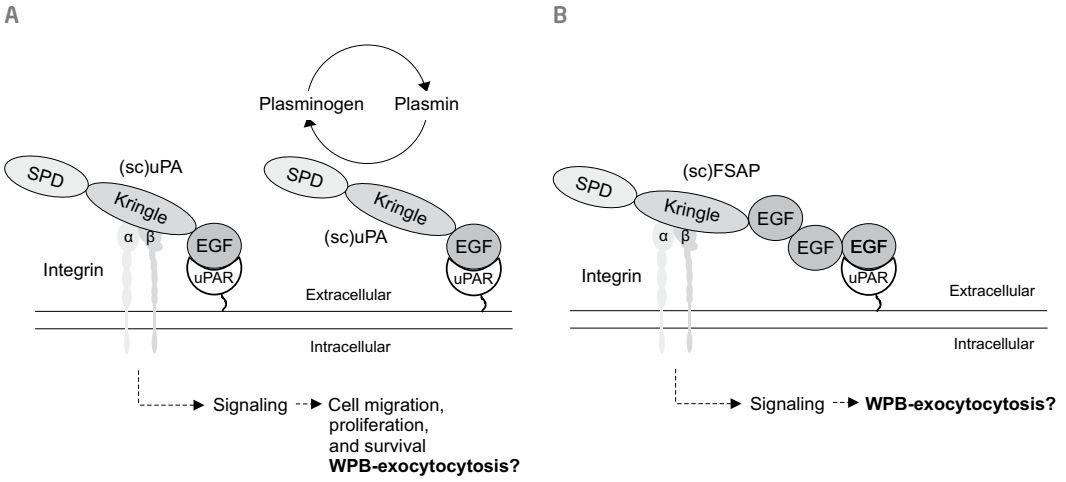


Figure 2. Proposed model for FSAP-induced Weibel-Palade body exocytosis through binding to uPAR and integrins. (A) The urokinase-type plasminogen activator receptor (uPAR) regulates the activity of the plasminogen activation system by binding uPA and its zymogen form. Activated uPA cleaves the zymogen plasminogen, generating the protease plasmin, which in turn cleaves and activates scuPA. To activate intracellular signalling, which is independent of uPA proteolytic activity, uPAR cooperate with transmembrane co-receptors like integrins. (sc)uPA binds uPAR via its EGF-like domain whereas its kringle domain stabilizes the interaction through binding to integrins as αvß3 and αvß5, affecting cell migration, adhesion, and proliferation. Whether the in this thesis observed scuPA-induced Weibel-Palade body exocytosis is triggered by scuPA-uPAR-integrin complex formation still remains speculative. (B) Proposed model of FSAP interaction with uPAR and integrins on the endothelium leading to intracellular signalling and induction of Weibel-Palade body exocytosis.

proteases such as thrombin (PAR-1, -3, and -4) and trypsin (PAR2) (20). Thrombin triggers WPB-exocytosis via PAR-1 (21). As the observed FSAP induced WPBs exocytosis was independent of its amidolytic activity (chapter 5) the involvement of PARs seems unlikely. This is supported by our observation that BAPTA-AM, an intracellular Ca^{2+}-chelator did not inhibit the FSAP induced secretion of WPBs whereas it completely inhibited the action of thrombin.

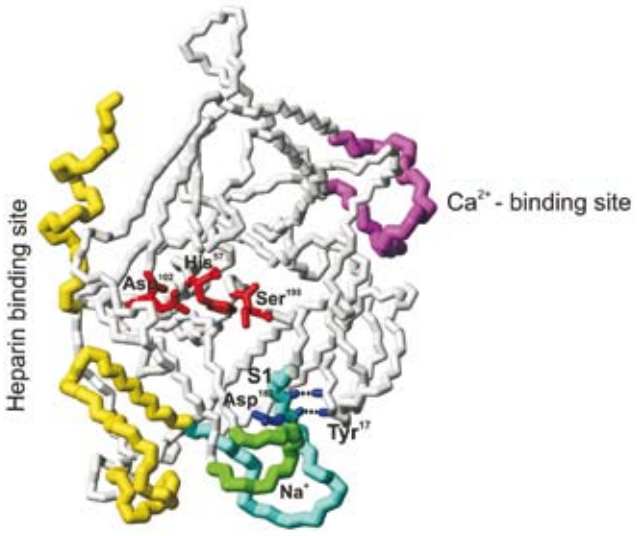
In chapter 5 we show that scuPA, triggers WPBs exocytosis as effective as FSAP. So far, scuPA has not been described as an agonist that induces exocytosis of this subcellular organelle. ScuPA and FSAP are highly homologous; scuPA contains a single epidermal growth factor (EGF) like domain, a kringle domain, and a catalytic serine protease domain. FSAP differs from scuPA as it contains 3 EGF-like domains proximal to its kringle domain. In both proteases the EGF domain(s) and kringle domain comprise the heavy chain which is separated from the light chain upon activation. The EGF-like domains of human FSAP show a 24% (EGF-1), 27% (EGF-2), and 37% (EGF-3) homology to that of human scuPA whereas both kringle domains are 37% homologous. The receptor for uPA on endothelial cells is the urokinase type plasminogen activator receptor (uPAR) (see for review (22)). uPAR does not contain a transmembrane domain and it

associates with the membrane through a glycosyl phosphatidylinostitol (GPI) anchor. uPAR is an important regulator of extracellular matrix (ECM) proteolysis, cell-ECM interactions and cell signalling. Signalling through uPAR, which is independent of uPA proteolytic activity, can activate different pathways as e.g. the Ras-mitogen-activated protein kinase (MAPK) pathway and downstream mediators, such as the extracellular signal-regulated kinases (ERK) 1 & 2 (23-26). In this regard, it is interesting to note that MAPK and ERK1/2 are also activated following incubation of endothelial cells with FSAP (27). At present we do not know whether FSAP mediated activation of these pathways is required for release of WPBs. Both scFSAP and scuPA can induce release of WPBs from endothelial cells (see Chapter 5). The structural requirements on scFSAP and scuPA have not been established. The three-dimensional structure of an amino-terminal fragment of uPA in complex with uPAR has been solved (28). Binding of uPA to uPAR is mediated by its EGF and kringle domains (22, 29, 30). Several studies show that αvß3 and αvß5 integrins are crucial co-receptors for uPAR-mediated signalling (22). Interestingly, FXII has also been reported to interact with uPAR on endothelial cell membranes through its amino-terminus (31). Based on this finding it is attractive to speculate that FSAP mediated release of WPBs also proceeds via uPAR (see figure 2).

Factor VII-activating protease; regulation of its amidolytic activity and substrate specificity

Serine proteases display a number of distinct structural features that allow for proteolytic conversion of their substrates. The catalytic triad of chymotrypsin-like serine proteases comprises Ser^{195}, His^{57} and Asp^{102} that through an inventive charge-relay system mediates nucleophilic attack of the carbonyl group of a susceptible peptide bond in the substrate (see for review (32)). Correct positioning of these active sites residues is required in order to allow for efficient conversion of their substrates. Many serine proteases circulate as zymogens that only reach their full activity once they are activated by proteolytic cleavage. Exposure of a novel amino-terminus induces a number of changes in the catalytic domain. The newly formed amino-terminus directly interacts with Asp^{189} located within the primary substrate pocket (S1). This coincides with a conformational change in the protease domain that shapes the S1 pocket (see Figure 3). In addition, the so-called oxyanion hole is formed that stabilizes the tetrahedral oxyanion intermediate that is formed following nucleophilic attack of the active site serine to the carbonyl group of the substrate (32). Conversion of the zymogen into an active serine protease is required in order to develop full activity.

In chapter 2 we show that FSAP can only convert substrates upon cleavage at Arg^{313}. The protease domain of FSAP only obtains its catalytically active conformation following insertion of the newly formed amino-terminus. In chapter 4 we provide evidence for a limited accessibility of the newly formed amino-group for chemical modification (Chapter 4; figure 7). This observation suggests that the newly formed amino-group rapidly enters the active site. A naturally occurring variant of FSAP known as the "Marburg-1" variant (G543E) is found in 5-10% of the normal population (33). Gly^{543} is present in close proximity of a Na^+-binding site (Figure 3). In this thesis we show that $FSAP^{G534E}$ display reduced hydrolysis of small chromogenic substrates as well as scuPA (Chapter 3). In addition, $FSAP^{G534E}$ displays less sensitivity towards the active site inhibitor 4-(2-aminoethyl) bezenesulfonyl fluoride hydrochloride



Heparin-binding sequences

FXIa	158	CQ**KR-YR**GHKITHKMIC	177	226 EYVDWILEKTQ 236
Thrombin	173	CKDS--T**RIR**ITDNMFC	187	245 **R**L**KK**WIQ**K**VID 255
FXa	156	CKLS--SSFIITQNMFC	170	223 -FL**K**WID**R**SM**K** 233
FIXa	177	CL**RS**--T**K**FTIYNNMFC	191	244 RYVNWI**K**EKT**K** 254
FSAP	164	CNS**R**QLYDHMIDDGMIC	180	232 **K**FLNWI**K**ATI**K** 242

Figure 3. Model of FSAP catalytic domain based on the highly homologous catalytic domain of tPA employing comparative homology modelling. Depicted are the potentially heparin binding site (exosite II) (yellow), the Ca^{2+}-binding site (exosite I: FSAP residues 64-74) (purple), the catalytic triad (red), Asp^{189} that interacts through its backbone with Tyr^{17} thereby stabilizing the inserted amino-terminus into the S1-pocket (S1) (see chapter 4) (dark blue), and the Na+-binding c180- & c220-loops (light blue and green respectively). (Alignment) Two regions have been established in FXIa, Thrombin, FXa and FIXa as important for heparin binding (34). These regions are compared to FSAP. Amino acids indentified as part of heparin-binding regions in FXIa, thrombin, FXa and FIXa are represented in bold. The potentially important residues in FSAP are also in bold. Alignment has been modified from Badellino. et al. (34).

(AEBSF) when compared to FSAP (Chapter 4; Figure 6). These results suggest that the active site function is impaired in $FSAP^{G534E}$. In agreement with these findings we were able to show that the newly formed amino-terminal group in $FSAP^{G534E}$ remained accessible for chemical modification by NH2-targeting reagents (Chapter 4; Figure 7). As argued in chapter 4 these data are consistent with impaired formation of a catalytically active serine protease domain in $FSAP^{G534E}$ resulting in a reduced activity towards scuPA and small substrates. This endows $FSAP^{G534E}$ with zymogen-like properties.

Recently, activated factor X (FXa) variants with zymogen-like properties have been designed by site-directed modification of the amino acid residues at its newly formed amino-terminus (35). Also for these FXa variants an impaired conversion of small substrates was observed. Interestingly, the reduced catalytic activity of these amino-terminal FXa variants could be rescued by the addition of high concen-

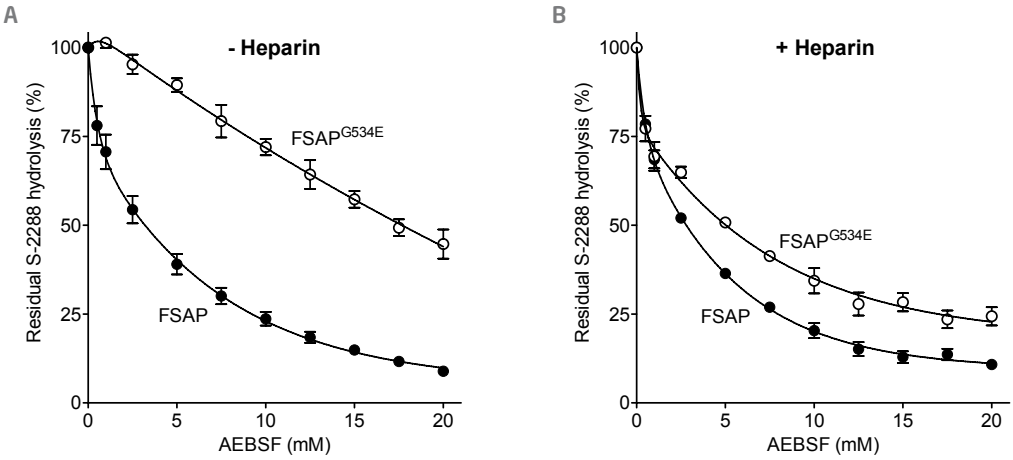


Figure. 4 Inhibition of activated wtFSAP and FSAP^{G221E} by AEBSF. (A) Each reaction mixture contained activated wtFSAP (filled circles) or FSAP^{G534E} (open circles) with different AEBSF concentrations in the absence (A) or presence (B) of 1.0 IU/ml heparin. Data represent the means ± S.D. of at least three experiments. See chapter 4 for experimental procedures.

trations of its cofactor factor V (35) suggesting that occupancy of other interactive surfaces (so-called exosites) in the protease domain could partially restore the active-site conformation of these FXa variants. Based on homology with related serine proteases two exosites can be defined on FSAP (see figure 3). Exosite I encompasses residues 64-74; whereas exosite II comprises amino acids 164-180 and 232-242. FSAP has been reported previously to bind to negatively charged surfaces like heparin, hyaluronan, polyphosphate and a number of other negatively charged polymeric structures (1, 11-13). Exosite II has been implicated in heparin-binding for e.g. FXIa, thrombin, FIXa, and FXa (33). Inspection of the primary sequence of exosite II reveals a cluster of positively charged amino acids that is conserved in other serine proteases like FIXa and FXa (see inset Figure 3).

We explored whether heparin could restore the conformation of the active site in FSAP^{G534E}. Incubation of activated FSAP with AEBSF resulted in a rapid decline of the catalytic activity towards a small chromogenic substrate (Figure 4A; closed cirles). Addition of heparin did not further accelerate modification of the active site of FSAP by the inhibitor (Fig 4B; closed circles). Incubation of FSAP^{G534E} with AEBSF resulted in a slow decline in the catalytic activity of this variant for the small substrate when compared to wtFSAP (Figure 4A; open circles). Strikingly, addition of heparin greatly facilitates inhibition of FSAP^{G534E} by AEBSF (Figure 4B; open circles) indicating that the conformation of the active site of FSAP^{G534E} is partially restored in the presence of heparin. As outlined previously the G534E polymorphism has been associated with cardiovascular risk (3) and venous thrombosis (36). These findings could however not be confirmed in other studies (37-39). Therefore the reported contribution as a risk factor for thrombosis and other vascular diseases remains highly controversial. Our biochemical studies on FSAP^{G534E} show that this variant has an impaired capacity to convert small substrates and to activate

scuPA in vitro. We propose that in vivo the zymogen-like conformation of FSAP^{G534E} is rescued by the presence of physiological cofactors such glycosaminoglycans that bind to exosites on the protease domain. This mechanism most likely explains the limited risk for vascular disease associated with this common polymorphism.

References

1 Choi-Miura NH, Tobe T, Sumiya J, Nakano Y, Sano Y, Mazda T, Tomita M. (1996) J Biochem. 119(6):1157-65.

2 Zhou HY, Pon YL, Wong AS. (2008) Curr Mol Med. 8(6):469-80. Review.

3 Sedding D, Daniel JM, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM. (2006) J Exp Med. 25;203(13):2801-7.

4 Matsumoto K, Kusaka J, Nishibori A, Hara H. (2006) Mol Microbiol. 61(5):1110-7. Review.

5 Li G, Chen S, Thompson MN, Greenberg ML. (2007) Biochim Biophys Acta. 1771(3):432-41. Review.

6 Sorice M, Circella A, Misasi R, Pittoni V, Garofalo T, Cirelli A, Pavan A, Pontieri GM, Valesini G. Clin Exp (2000) Immunol. 122(2):277-84.

7 Sorice M, Circella A, Cristea IM, Garofalo T, Di Renzo L, Alessandri C, Valesini G, Esposti MD. (2004) Cell Death Differ. 11(10):1133-45.

8 Deguchi H, Fernandez JA, Hackeng TM, Banka CL, Griffin JH. (2000) Proc Natl Acad Sci U S A. 97(4):1743-8.

9 Fernández JA, Kojima K, Petäjä J, Hackeng TM, Griffin JH. (2000) Blood Cells Mol Dis. 26(2):115-23.

10 Zeerleder S, Zwart B, te Velthuis H, Stephan F, Manoe R, Rensink I, and Aarden L.A. (2008) FASEB J. 22(12), 4077–4084.

11 Kannemeier C, Feussner A, Stöhr HA, Weisse J, Preissner KT, Römisch J. (2001) Eur J Biochem. 268(13):3789-96.

12 Nakazawa F, Kannemeier C, Shibamiya A, Song Y, Tzima E, Schubert U, Koyama T, Niepmann M, Trusheim H, Engelmann B, Preissner KT. (2005) Biochem J. 385(Pt 3):831-8.

13 Muhl L, Galuska SP, Oörni K, Hernández-Ruiz L, Andrei-Selmer LC, Geyer R, Preissner KT, Ruiz FA, Kovanen PT, Kanse SM. (2009) FEBS J. 276(17):4828-39.

14 Papayannopoulos V, Zychlinsky A. (2009) Trends Immunol. 30(11):513-21. Epub 2009 Aug 21. Review.

15 Hayashi T, Nishioka J, Nakagawa N, Kamada H, Gabazza EC, Kobayashi T, Hattori A, Suzuki K. (2007) J Thromb Haemost. 5(7):1477-85.

16 Aird WC. (2005) J Thromb Haemost. 3(7):1392-406. Review.

17 Krueger T, Westenfeld R, Schurgers L, Brandenburg V. (2009) Int J Artif Organs. Feb;32(2):67-74. Review.

18 Bloom AL. (1990) Haemostasis. Suppl 1:14-29. Review.

19 Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J. (2006) Arterioscler Thromb Vasc Biol. (5):1002-7. Review.

20 Coughlin SR. (2005) J Thromb Haemost. 3(8):1800-14. Review.

21 Cleator JH, Zhu WQ, Vaughan DE, Hamm HE. (2006) Blood. 107(7):2736-44.

22 Smith HW, Marshall CJ. (2010) Nat Rev Mol Cell Biol. (1):23-36. Review.

23 Aguirre Ghiso JA. (2002) Oncogene. 21(16):2513-24.

24 Vial E, Sahai E, Marshall CJ. (2003) Cancer Cell. 4(1):67-79.

25 Liu D, Aguirre Ghiso J, Estrada Y, Ossowski L. (2002) Cancer Cell. 2002 1(5):445-57.

26 Kjøller L, Hall A. (2001) J Cell Biol. 152(6):1145-57.

27 Etscheid M, Beer N, Dodt J. (2005) Cell Signal. 17(12):1486-94.

28 Llinas P, Le Du MH, Gårdsvoll H, Danø K, Ploug M, Gilquin B, Stura EA, Ménez A. (2005) EMBO J. 24(9):1655-63.

29 Bdeir K, Kuo A, Sachais BS, Rux AH, Bdeir Y, Mazar A, Higazi AA, Cines DB. (2003) Blood. 102(10):3600-8.

30 Huai Q, Mazar AP, Kuo A, Parry GC, Shaw DE, Callahan J, Li Y, Yuan C, Bian C, Chen L, Furie B, Furie BC, Cines DB, Huang M. (2006) Science. 3;311(5761):656-9.

31 Mahdi F, Madar ZS, Figueroa CD, Schmaier AH. (2002) Blood. 99(10):3585-96.

32 Polgár L. (2005) Cell Mol Life Sci. 62(19-20):2161-72. Review.
33 Römisch J, Feussner A, Stöhr HA. (2001) Blood Coagul Fibrinolysis. 12(5):375-83.
34 Badellino KO, Walsh PN. (2001) Biochemistry. 40(25):7569-80.
35 Toso R, Zhu H, Camire RM. (2008) J Biol Chem. 4;283(27):18627-35.
36 Hoppe B, Tolou F, Radtke H, Kiesewetter H, Dörner T, Salama A.(2005) Blood. 105(4):1549-51.
37 Van Minkelen R, de Visser MC, Vos HL, Bertina RM, Rosendaal FR. (2005) Blood. 105(12):4898.
38 Gulesserian T, Hron G, Endler G, Eichinger S, Wagner O, Kyrle PA. (2006) Thromb Haemost. 95(1):65-7.
39 Sidelmann JJ, Vitzthum F, Funding E, Münster AM, Gram J, Jespersen J. (2008) Thromb Res. 122(6):848-53.

Summary
Samenvatting

Summary

Since its discovery in 1996, numerous functions have been proposed for Factor VII-activating protease (FSAP) including interactions with the haemostatic system. These include activation of the single chain urokinase-type plasminogen activator (scuPA) and coagulation factor VII (FVII), and interaction with the endothelium and smooth muscle cells. With its decreasing specificity, the physiological role of FSAP has become increasingly unclear. About 5-10 % of apparently healthy individuals carry a FSAP variant (known as "Marburg-1") which contains a glycine (G) to glutamate (E) substitution located in the catalytic domain. This FSAP variant is associated with a 50-80 % impaired activation of scuPA in vitro, suggesting an effect on fibrinolysis. Epidemiological studies however, vary strongly in their conclusions concerning the implications of this polymorphism.

The studies presented in this thesis were performed to assess the role of FSAP in the haemostatic system and the potential pathophysiological implications of the Marburg-1 FSAP polymorphism (G534E) for FSAP biological activity. A detailed overview of the current knowledge regarding the structure of FSAP, its suggested physiological functions, and the potential consequences of the Marburg-1 polymorphism is given in chapter 1.

Rigorous studies have been limited by the difficulty of obtaining intact FSAP from blood or recombinant sources. In chapter 2, we describe the first successful construction and purification of human recombinant human FSAP. By employing kinetic experiments we confirmed that scuPA is efficiently cleaved by recombinant FSAP. In contrast, we found that recombinant FSAP shows only minor capability, if any, to activate coagulation factor VII (FVII). Interestingly, FVII was observed to be cleaved by activated FSAP only in the presence of membranes consisting of cardiolipin. However, this resulted in rapid inactivation of FVII rather than activation. Therefore we propose that FSAP, in contrast to what its name suggests, does not play a critical role in mediating haemostasis through activation of FVII.

The G534E substitution is located in the protease domain, at position c221 in the commonly used chymotrypsin numbering. In chapter 3 we demonstrate that this mutation is not a neutral polymorphism but is located in a surface loop (known as the "c220-loop") which is of critical importance in mediating catalytic activity and substrate specificity. Moreover, this loop is part of a Na^+-binding site in those serine proteases that need Na^+ for optimal function. We found that in absence of $CaCl_2$ FSAP catalytic activity indeed was dependent on Na^+. Compatible with the location of the G221E mutation, this FSAP variant displays a striking decrease in sensitivity to Na^+. Further, we demonstrated that recombinant $FSAP^{G221E}$ exhibits a marked reduction in catalytic activity towards scuPA. In contrast to normal FSAP this activation was completely dependent on the presents of heparin. This suggests that the G221E substitution makes FSAP more sensitive to the presence of polyanions.

The mechanisms underlying the reduced catalytic activity induced by the G221E mutation was investigated in chapter 4 using a subset of FSAP Gly^{221} substitution variants. Substitution of Gly^{221} by either

the small polar Ser or the negatively charged Glu resulted in a significant loss of catalytic activity towards a peptide substrate. A variant comprising the small non-polar Ala at position 221 was least defective in its catalytic activity towards this substrate. All three Gly^{221} substitutions further showed a marked defect in scuPA activation. Therefore, we proposed that Gly^{221} itself contributes to allosteric regulation of the protease. Indeed, we demonstrated that the G221E substitution affects the formation of the primary substrate binding pocket. In addition, competitive inhibition experiments revealed a defective alignment of the catalytic triad residues in $FSAP^{G221E}$. Optimal alignment of these residues usually requires formation of a salt bridge between Asp^{194} and the newly formed N-terminus that inserts into the protease domain during activation of the zymogen. Employing mass spectrometry, we demonstrated that the N-terminus of $FSAP^{G221E}$ is more accessible for chemical modification than that of activated FSAP. These data suggest that a reduced N-terminal insertion is the cause of the impaired alignment of the catalytic residues in $FSAP^{G221E}$. Modelling studies showed that a Glu at position 221 may sterically hinder insertion of the N-terminus. Together, our data contribute to a better understanding of the role of Gly^{221} for the catalytic activity of FSAP.

As other coagulation factors circulating in blood, FSAP stays in direct contact with the endothelial cells of the blood vessel wall. The vasculature forms a barrier that regulates a number of physiological processes, including extravasation of leukocytes to the underlying tissues. FSAP has been associated with inflammatory responses and atherogenesis. Endothelial cells contain Weibel-Palade bodies (WPBs), specific storage granules containing components such as von Willebrand factor (VWF) and P-selectin, which are released upon activation and support leukocyte rolling, platelet adhesion, and aggregation. In chapter 5 we demonstrate that activated FSAP induces VWF release from WPBs at least as effective as classical triggers of WPB-exocytosis such as thrombin. Thrombin-induced WPB exocytosis is mediated by an increase in intracellular free calcium upon cleavage of the protease-activated receptor 1 (PAR-1). In contrast, the FSAP-induced WPB exocytosis was found to be independent of the Ca^{2+}-pathway, which makes the involvement of PARs unlikely. This is further supported by the observation that the zymogen form of FSAP induced VWF release from endothelial cells as well. Furthermore, we found that single chain urokinase-type plasminogen activator (scuPA), of which the primary structure displays high sequence homology with FSAP triggers WPB exocytosis as effective as FSAP. So far, scuPA has not been described as an agonist inducing exocytosis of this subcellular organelle. One specific receptor for scuPA on endothelial cells is the urokinase type plasminogen activator receptor (uPAR) which activates many intracellular signalling pathways. To activate intracellular signalling uPAR cooperate with transmembrane co-receptors, including specific integrins. This opens the possibility that the uPAR pathway contributes to WPB exocytosis as mediated by scuPA and FSAP.

Finally, in chapter 6 the functional implications of our findings are further discussed in combination with data collected by other investigators. Additionally to new insights into the potential interactions of FSAP with the haemostatic system, we also propose a few models in which FSAP may act as a mediator during vascular inflammation.

Samenvatting

Factor VII activerend protease (FSAP) werd voor het eerst beschreven in 1996. Sindsdien zijn er verschillende potentiële functies voor FSAP gerapporteerd, waaronder interacties met de bloedstollings cascade door de activatie van stollingfactor VII (FVII) en urokinase-type plasminogeen activator (scuPA). Verder zou FSAP interacties aangaan met endotheelcellen en gladde spiercellen. Met de afnemende specificiteit en het toenemende aantal functies is de fysiologische rol van FSAP steeds onduidelijker geworden. Ongeveer 5-10% van de gezonde individuen zijn drager van een FSAP variant (bekent als "Marburg-1") met een Gly (G) naar Glu (E) mutatie in het katalytische domein. Deze FSAP variant wordt geassocieerd met een 50-80% gereduceerde activiteit ten opzichte van scuPA in vitro en impliceert daarmee een nadelig effect op de fibrinolyse. Echter, de conclusies van de tot nu toe gerapporteerde klinische studies variëren sterk voor wat betreft de implicaties van deze mutatie als risicofactor voor trombose.

Het onderzoek zoals beschreven in dit proefschrift is uitgevoerd met als doel de potentiële rol van FSAP binnen de hemostase nauwkeuriger te bepalen. Daarnaast is gekeken naar de mogelijke pathofysiologische implicaties van het Marburg-1 FSAP polymorfisme (G534E) op de biologische activiteit van FSAP. Een gedetailleerd overzicht wat betreft de bestaande kennis over de structuur van FSAP, de mogelijke fysiologische functies ervan en de eventuele gevolgen van de Marburg-1 mutatie zijn beschreven in hoofdstuk 1.

Veel van het tot nu toe in de literatuur beschreven FSAP onderzoek werd belemmerd doordat FSAP moeilijk in zijn intacte vorm te isoleren is vanuit bloed of recombinante bronnen. In hoofdstuk 2 beschrijven we de succesvolle constructie en zuivering van humaan recombinant FSAP. Door het uitvoeren van kinetische experimenten konden we bevestigen dat scuPA efficiënt geactiveerd kan worden door recombinant FSAP. Daarentegen vonden we dat recombinant FSAP slecht in staat is om FVII te activeren. Interessant is de waarneming dat FVII alleen door FSAP geknipt wordt in aanwezigheid van cardiolipine. Echter, het knippen van FVII door FSAP onder deze condities resulteert eerder in de degradatie dan activatie van FVII. Cardiolipine is een fosfolipide dat hoofdzakelijk intracellulair gelokaliseerd is. Daarom concluderen wij dat FSAP, in tegenstelling tot wat de naam impliceert, geen belangrijke rol speelt binnen de hemostase door het activeren van FVII.

In termen van chymotrypsine nummering is de G534E mutatie (Marburg-1) gelokaliseerd op positie c221. In hoofdstuk 3 laten wij zien dat deze mutatie niet zomaar een polymorfisme is maar dat het gelokaliseerd is in een oppervlakte loop (bekend als de "c220-loop") die van kritisch belang is voor de regulatie van de katalytische activiteit en substraat specificiteit. In homologe eiwitten die Na^+ nodig hebben om optimaal te kunnen functioneren maakt deze "loop" juist onderdeel uit van de Na^+-bindende plaats. Wij vonden dat de katalytische activiteit van FSAP in afwezigheid van Ca^{2+} sterk afhankelijk is van de aanwezigheid van Na^+. Overeenkomend met de locatie van de G221E mutatie vonden we dat deze FSAP variant inderdaad een sterk verlaagde gevoeligheid heeft voor Na^+. Verder hebben we

kunnen bevestigen dat recombinant FSAP^{G221E} sterk gereduceerd is in zijn activiteit ten opzichte van scuPA. In tegenstelling tot normaal FSAP was deze activatie geheel afhankelijk van de aanwezigheid van heparine. Dit suggereert dat de G221E mutatie FSAP gevoeliger maakt voor de aanwezigheid van polyanionen.

De mechanismen die verantwoordelijk zijn voor de gereduceerde katalytische activiteit en substraat-specificiteit geïnduceerd door de G221E mutatie zijn onderzocht in hoofdstuk 4 door gebruik te maken van een set FSAP G221 substitutie varianten. Vervanging van Gly^{221} door een klein, polair Ser- of een groot, polair Glu-residu resulteerde in een drastische verlaging in katalytische activiteit voor een klein synthetisch substraat. Een variant met een klein, apolair Ala residu op positie 221 was het minste defect in zijn katalytische activiteit ten opzichte van dit substraat. In tegenstelling hiermee vertoonden alle drie de mutanten een sterk gereduceerde activiteit voor scuPA. Daarom veronderstellen we dat G221 betrokken is bij de allostere regulatie van het protease domein. We hebben inderdaad aangetoond dat de G221E mutatie de formatie van de primaire substraat-bindingsplaats beïnvloedt. Competitieve remmingsexperimenten lieten een defect zien in de uitlijning van de residuen van de catalytische triade in FSAP^{G221E}. Voor de optimale oriëntatie van deze residuen is het noodzakelijk dat er na activatie van het zymogeen een zoutbrug wordt gevormd tussen Asp^{194} en de amino groep van de nieuw gevormde N-terminus. Door gebruik te maken van een nano-LC-massa spectrometer hebben we kunnen aantonen dat de N-terminus van FSAP^{G221E} toegankelijker is voor modificatie door biotine dan die van geactiveerd, normaal FSAP. Deze gegevens suggereren dat een gereduceerde N-terminale insertie verantwoordelijk is voor gebrekkige ruimtelijke rangschikking van de residuen van het katalytische centrum in FSAP^{G221E}. Modelling studies toonden aan dat de zijketen van een Glu residu op positie 221 insertie van de N-terminus sterisch kan hinderen. Samengenomen, dragen onze bevindingen bij tot een beter begrip van de rol die G221 heeft binnen de regulatie van de katalytische activiteit van FSAP.

Zoals de andere in bloed circulerende stollingsfactoren bevindt FSAP zich in direct contact met de endotheelcellen van de vaatwand. De vaatwand bestaat uit onder meer endotheelcellen die samen de barrière vormen tussen het bloed en het onderliggende weefsel. Endotheelcellen zijn betrokken bij verschillende fysiologische processen zoals het reguleren van ontstekingsreacties en hemostase. FSAP is geassocieerd met ontstekingreacties en bloedvatvorming. Endotheelcellen bevatten speciale opslagorganellen, Weibel-Palade bodies (WPBs) genaamd, die kunnen worden uitgescheiden na activatie van het endotheel. WPBs bevatten verschillende componenten zoals von Willebrand Factor (VWF) en P-selectine die de adhesie van leukocyten, bloedplaatjes -adhesie en -aggregatie ondersteunen. In hoofdstuk 5 laten we zien dat geactiveerd FSAP de secretie van VWF door WPBs induceert. Dit proces is minstens zo effectief als regulatie van WPB-exocytose door klassieke agonisten zoals trombine. Trombine-geïnduceerde secretie van WPBs wordt gereguleerd door een toename in de vrije, intracellulaire calcium concentratie na het knippen van de protease-geactiveerde receptor 1 (PAR-1). In tegenspraak hiermee bleek de FSAP-geïnduceerde WPB-exocytose onafhankelijk te zijn van de Ca^{2+}-route. Dit wordt verder ondersteund door de observatie dat de zymogeen vorm van FSAP ook in staat is om endotheel cellen aan te zetten tot gereguleerde secretie van VWF. Verder vonden we dat zymogeen urokinase-type plasminogeen activator (scuPA), waarvan de primaire structuur grotendeels

overeen komt met dat van FSAP, een net zo effectieve stimulator van WPB-exocytose is als FSAP. Tot nu toe is scuPA niet gerapporteerd als een regulator van WPB-exocytose. Een specifieke receptor voor scuPA op het oppervlak van endotheelcellen is de urokinase-type plasminogeen activator receptor (uPAR) die veel verschillende intracellulaire signaalroutes kan activeren. Voor de activatie van de intracellulaire signalering werkt uPAR samen met transmembraan co-receptoren waaronder specifieke integrines. Dit opent de mogelijkheid dat de uPAR route bijdraagt aan WPB-exocytose door scuPA en FSAP.

Uiteindelijk wordt in hoofdstuk 6 de functionele gevolgen van onze resultaten verder bediscussieerd in het licht van de gegevens gerapporteerd door andere onderzoekers. Naast de nieuwe inzichten in de mogelijke associaties van FSAP met het hemostatisch systeem komen we met een aantal modellen waarin FSAP als een regulator zou kunnen dienen gedurende vasculaire ontsteking.

Curriculum Vitae

Fabian Stavenuiter werd op 16 juli 1975 geboren te Heemskerk. In augustus 1996 begon hij aan de studie Medische biologie aan de Hogeschool Leiden. Tijdens deze studie liep hij stage bij de afdeling Moleculaire & Cellulaire Neurobiologie van de Vrije Universiteit in Amsterdam onder leiding van Drs. P. van Nierop en Dr. A.B. Smith. Na het behalen van het bachelor examen in 2000 begon hij in september van dat jaar aan de studie Medische biologie aan de Vrije Universiteit te Amsterdam. Tijdens deze studie liep hij stage bij de afdeling Moleculaire Celbiologie en Immunologie van de Vrije Universiteit te Amsterdam. Na het behalen van het doctoraal examen in 2002 begon hij in augustus van dat jaar als analist binnen het Interuniversitair Oogheelkundig Instituut van het Academisch Medisch Centrum te Amsterdam. Vanaf oktober 2003 was hij werkzaam als analist bij de afdeling Plasma Eiwitten van Sanquin Research te Amsterdam. In april 2005 begon hij aan zijn promotieonderzoek bij deze afdeling. Onder begeleiding van Prof. Dr. Koen Mertens werd onderzoek gedaan naar de potentiële rol van het Factor Seven Activerend Protease (FSAP) binnen de hemostase. De resultaten van het onderzoek zijn beschreven in dit proefschrift.

The research described in this thesis has been presented at the following international meetings:

December 2009
Poster presentation at the 51th American Society of Haematology Annual Meeting, New Orleans, Louisiana, USA.

July 2009
Oral presentation at the XXIIst International Society of Thrombosis and Haemostsis Congress, Boston, USA.

July 2008
Poster presentation at the Gordon Research Conference on Haemostsis, Waterville Valley, New Hampshire, USA.

August 2007
Oral presentation at the XXIst International Society of Thrombosis and Haemostsis Congress, Geneva.

Published peer reviewed abstracts

F. Stavenuiter, A.B. Meijer, E. Sellink, K. Mertens. Factor VII activating protease (FSAP): functional implications of the "Marburg-1" polymorphism. Blood (ASH annual meeting abstracts), Blood (ASH annual meeting abstracts), Dec 2009; 110: 765.

F. Stavenuiter, E. Sellink, H.J.M. Brinkman, A.B. Meijer, K. Mertens. Factor FVII-Activating Protease (FSAP): does it activate Factor VII? Journal of Thrombosis and Haemostasis 2009; Volume 7, Supplement 2: OC-WE-084.

F. Stavenuiter, M. Boon-Spijker, P. Clijsters, K. Mertens. The Marburg-1 mutation reduces the autocatalytic activity of Factor seven activating protease (FSAP). Journal of Thrombosis and Haemostasis 2007; Volume 5, Supplement 1: O-W-088.

Manuscripts in preparation

Factor Seven Activating Protease (FSAP): does it activate FVII? F. Stavenuiter, M.G. Boon-Spijker, A.B. Meijer and K. Mertens

Functional implications of the Marburg-1 polymorphism in Factor Seven Activating Protease. F. Stavenuiter, E. Sellink, A.B. Meijer and K. Mertens

Glycine 221 is indispensable for allosteric regulation of the catalytic activity of Factor Seven Activating Protease. F. Stavenuiter, K. Mertens and A.B. Meijer.

Human Factor Seven Activating Protease (FSAP) induces regulated Weibel-Palade body exocytosis from cultured human endothelial cells. F. Stavenuiter, E.A.M. Bouwens, A.B. Meijer, M.G. Rondaij, K. Mertens.

Activation of Factor Seven Activating protease (FSAP) does not induce coagulation or fibrinolysis in plasma. F. Stephan, F. Stavenuiter, I. Bulder, K. Mertens, L. Aarden, S. Zeerleder.

Dankwoord

Het succesvol volbrengen van een promotietraject doe je niet alleen. Het einde van dit proefschrift is dan ook gereserveerd voor het bedanken van verschillende mensen die hierin een rol hebben gespeeld.

Naar mijn mening valt een promotietraject te vergelijken met het beklimmen van een hoge berg in 'de expeditiestijl'. Tot de kenmerken van 'de expeditiestijl' behoren: het opzetten van vaste kampen langs de beklimmingroute en het voorzien van de moeilijke of gevaarlijke delen van de route met vaste touwen, inlandse dragers voor het transporteren van lasten van kamp naar kamp en in sommige gevallen zuurstof-apparatuur om de ijle lucht te compenseren.

Koen, jij was de expeditieleider van het project. Bedankt voor je vertrouwen en de kans die je me gegeven hebt om als AIO te werken aan dit project. In tegenstelling tot wat we eerst verwachtten bleek de 'berg' technisch gezien toch wat lastiger te zijn om te beklimmen. Gedurende het verstrijken van de tijd en het toenemen van de druk om de onderneming te doen slagen is er even overwogen om een naast gelegen berg te gaan beklimmen waarvan de route al deels was voorzien van vaste touwen. Uiteindelijk hebben we toch de voorgenomen berg weten te bedwingen. De beklimming was zeer leerzaam en het uiteindelijke uitzicht meer dan de moeite waard. Bedankt.

Sander, jouw bijdrage werd steeds groter naarmate de top dichterbij kwam en was zeer waardevol voor het daadwerkelijk bereiken ervan. Bedankt voor je wetenschappelijke input en actieve bijdrage om van het geheel een succes te maken. Jan, mijn waardering voor je bijdrage aan het project en de afronding van het manuscript.

Erica, jij hebt een deel van de route voorzien van vaste touwen en een aantal kampen op de berg voorzien van proviand waaruit ik in een later stadium kon putten. Zelf neem je inmiddels met plezier deel aan een volgende uitdaging. Leuk dat je een van mijn paranimfen wilt zijn en bedankt voor alles.

Herm-Jan, bedankt voor je input in het project en het aanhoren van de nodige frustraties gedurende het laatste gedeelte van de beklimming. Mariska, Mariëtte, Patrick, en Jaqueline, jullie actieve bijdrage tot het doen slagen van de hele onderneming wordt zeer gewaardeerd! Timo en Michel, het lijkt er op dat een nieuwe berg beklommen kan gaan worden. Ik hoop dat het een succesvolle beklimming wordt. Bedankt voor jullie bijdrage en enthousiasme. Wouter Sparreboom, als student heb je een waardevolle bijdrage geleverd aan het bereiken van een hoger op de berg gelegen kamp. Veel succes met je carrière. Lucien, Sacha, en Femke, bedankt voor jullie voortdurende belangstelling voor dit project.

Gedurende het verstrijken van de tijd zijn er zowel veel 'bekende' gezichten verdwenen als 'nieuwe' verschenen in het basiskamp (laboratorium). Hierbij wil ik een ieder die hier niet bij naam wordt genoemd bedanken voor hun bijdrage en gezelligheid in de afgelopen 5 jaar.

During the passage of time, many 'familiar faces' have disappeared from base camp (laboratory) but also a lot of 'new faces' have arrived. I would like to thank everyone who is not mentioned by name for their contribution and conviviality during the past 5 years.

('Schoon'-)familie en vrienden, jullie hebben me zo nu en dan (veelal onopgemerkt) voorzien van de benodigde zuurstofapparatuur om de ijle lucht te compenseren. Dank jullie wel.

Martine, de weg naar de top hebben we nagenoeg geheel samen afgelegd. Uiteindelijk hebben we hem niet samen mogen bereiken. De goede tijd die we samen hebben gehad zal ik me altijd blijven herinneren en ik wens je alle geluk voor de toekomst.

Youri, jou wil ik als laatste noemen. Woorden doen afbreuk aan wat je voor mij betekent en zijn ook overbodig. Uiteraard ben je een van mijn paranimfen. We maken er een mooie dag van!

De opgedane ervaringen gedurende deze beklimming maken de weg vrij om een volgende berg in de 'alpine stijl' te gaan beklimmen: met minder vaste steunpunten en touwen, minder of géén dragers boven het basiskamp en zonder zuurstofflessen.

Bedankt allemaal!

Fabian