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Structure – function analysis of factor VII activating protease (FSAP): Sequence determinants for heparin binding and cellular functions

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ABSTRACT

Factor VII activating protease (FSAP) is associated with cardiovascular diseases and liver fibrosis. To understand the regulation of its proteolytic activity we have characterized recombinant FSAP-mutants over-expressed in HEK-293 cells. The secreted FSAP-protein concentration correlated inversely with the enzymatic activity of the FSAP-mutants. Over-expression of enzymatically active FSAP decreased cell viability, whereas inactive variants were expressed and secreted in adequate amounts. The naturally occurring G534E-variant exhibited reduced proteolytic activity. The ΔEGF-3 mutant showed diminished binding to and activation by heparin. Hence, regulation of FSAP activity is dependent on its EGF-3 domain and over-expression of active variants induces cell death.

1. Introduction

The serine protease FVII activating protease (FSAP) consists of three EGF-domains, a kringle domain and a serine protease domain and was first purified based on its binding to hyaluronic acid [1,2]. FSAP is predominantly produced in the liver and circulates as an inactive zymogen in plasma. It is activated by an auto-catalytic mechanism which is amplified by polyanions such as heparin or RNA [3,4]. FSAP can activate FVII and pro-urokinase (uPA) [4] via cleavage of the respective pro-forms. Its activity can be efficiently inhibited by serine protease inhibitors (SERPINs) such as plasminogen activator inhibitor (PAI)-1 [5]. Platelet-derived growth factor (PDGF)-BB, which plays an important role in atherosclerosis and liver fibrosis [6,7], can be cleaved and inactivated by FSAP.

Two naturally occurring single nucleotide polymorphisms (SNPs) in FSAP, termed Marburg I (MI) (G534E) and Marburg II (MII) (E393Q), have been described. MI-FSAP exhibits reduced proteolytic activity [4], is a risk predictor for carotid stenosis [8] and is associated with the severity of liver fibrosis [7]. MI-FSAP, in contrast to wild-type (WT)-FSAP, failed to reduce PDFG-BB-mediated proliferation of vascular smooth muscle cells (VSMC) [9] as well as hepatic stellate cells [7]. Hence, in a reciprocal manner FSAP enzymatic activity correlates with disease status.

To date, all the reported studies on FSAP have been performed with proteins isolated from human plasma. However, for a definitive structure-function analysis it is necessary to produce recombinant FSAP. Through this study we have laid the foundations for the production of recombinant FSAP and have further characterized the influence of different naturally occurring polymorphisms on FSAP functions. Moreover, we have strengthened the hypothesis that the EGF-3 domain is important for the activation of FSAP by polyanions and have found an unexpected function of intracellular FSAP in regulating cell death.

2. Materials and methods

2.1. Materials

FSAP was isolated from human plasma as described before [10]. Unfractionated heparin and heparin–albumin–biotin were from Sigma (Munich, Germany). PAI-1 was generously provided by Dr. Paul Declerk (Department of Pharmaceutical Science, Leuven, Belgium).

2.2. Plasmids and protein expression

Human wild-type FSAP cDNA was derived from human liver RNA and subsequently cloned into the EcoRI site of pIRESpuro3

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(BD Biosciences, Heidelberg, Germany). MI-SNP (G534E), MII-SNP (E393Q), R313G-cleavage site and H362F-active site mutants were generated by site-directed mutagenesis. For the ΔEGF-3 mutant, the sequence encoding amino acids 150 to 188 was removed. Expression vectors were transfected into human embryonic kidney (HEK)-293 cells using Lipofecamine 2000 reagent (Invitrogen, Karlsruhe, Germany). Selection of transformed cells was performed in 96-well plates (5000 cells/well) in the presence of 3 μg/ml puromycin and 10 μg/ml aprotinin (selection medium). FSAP expression was tested by Western blotting using mAB 677 and mAB 1189 (American Diagnostica, Pfungstadt, Germany). Percentages of wells with clones (of 96-well plates) were counted to obtain an index of the efficiency of clone generation.

Conditioned medium was produced with 0.2% FCS and an ELISA was used for the quantification of secreted FSAP, as described before [4].

For inducible FSAP expression, FSAP coding sequence was inserted into T-REx™ expression vector pcDNA4/TO© (Invitrogen), transfected into T-REx™-293 cells (Invitrogen) and cultivated as described above. Expression was induced with 0.5 μg/ml tetracycline (Invitrogen).

2.3. FSAP binding studies

Heparin–albumin–biotin (2 μg/ml), PDGF-BB (1 μg/ml) or polyclonal antibody (pAb) against FSAP (2 μg/ml) were immobilized on microtiter plates in 100 mM sodium carbonate (pH 9.5) over night at 4 °C. Plates were washed with TBS (25 mM Tris–HCl (pH 7.5) and 150 mM NaCl) containing 0.2% (w/v) Tween 20 (TBS-T) and were blocked with TBS containing 3% (w/v) BSA (Sigma). Conditioned media of the different FSAP-isoforms were allowed to bind to the wells. The plates were washed and bound FSAP was detected by the addition of mAb 677 followed by peroxidase-conjugated secondary antibody.

2.4. FSAP enzyme activity assay

MAb 677 (2 μg/ml) was immobilized in microtiter plates and blocked as above. FSAP containing conditioned medium was added to the wells and pro-uPA activation was measured by adding pro-uPA (1 μg/ml; Saruplase, Grünenthal, Stohlberg, Germany), and 0.2 mM of the chromogenic substrate S-2444 (L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilinedihydro-chloride) (Haemochrome, Essen, Germany). Absorbance was followed in a microplate reader EL 808 (BioTek Instruments, Winooski, OR, USA).

2.5. Western blot analysis

To analyze FSAP protein, a mixture of antibodies directed against the N-terminal (mAb 1189) and C-terminal end (mAb 677), was used as described before [10].

2.6. Immunocytochemistry

Cells were washed and fixed with 3.7% (w/v) paraformaldehyde, permeabilized with 0.2% (w/v) Triton-X 100 (Sigma) and blocked with 3% (w/v) BSA (Sigma). Antibodies against FSAP (mAb 1189 or mAb 677) and cleaved PARP (#9541) (Cell signaling, Danvers, MA, USA) [11] as well as secondary antibodies labeled with fluoroscein isothiocyanate (FITC) or Rhodamine (Dianova, Hamburg, Germany) were used. Cells were preserved in Vecta-shield (Linaris Wehrtheim-Bettingen, Germany), containing 4′,6-diamidino-2-phenylindole dehydrate (DAPI) to stain the nuclei and analyzed using a Leica fluorescence microscope (Wetzlar, Germany). The percentage of cells positive for FSAP, cleaved PARP and both antigens were quantified.

2.7. Statistical test

The significance of the results was determined by using an unpaired two tailed "*t*" test.

3. Results

3.1. Expression of recombinant FSAP-isoforms in HEK-293 cells

The highest number of stably transfected clones were obtained with empty vector control, R313G-cleavage site mutant, H362F-active site mutant and MI SNP, whereas the number of clones obtained with WT, ΔEGF-3 and MII was very low (Fig. 1A). Amount of FSAP in the conditioned media was the highest in the case of R313G, H362F and MI, whereas WT, ΔEGF-3 and MII contained very low levels of FSAP protein, and empty vector exhibited no expression of FSAP (Fig. 1B). To standardize these differences in FSAP concentration for further experiments, the amounts were calibrated using ELISA and Western blotting (Fig. 1C). Overall, the number of stable clones generated as well as the amount of protein produced increased with decreasing proteolytic activity of the FSAP variants.

To further understand this phenomenon, we performed FSAP immunocytochemistry on cells transfected with WT-, MI- and R313G-FSAP plasmids. WT-FSAP appeared as a dense irregular structure within the cytoplasm, whereas MI- and R313G-FSAP (Fig. 2A) exhibited a more granular and dispersed localization. Initially, the number of cells transfected with WT-FSAP was similar to those transfected with the other FSAP variants (data not shown). Hence, we reasoned that cells expressing WT-FSAP had a selective disadvantage or died, leading to a decrease in viable cells. To test this issue more systematically an inducible expression system for

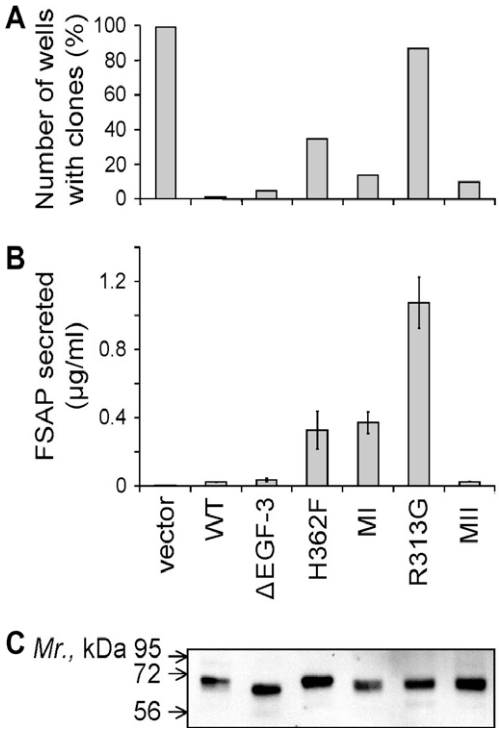

Fig. 1. Expression of FSAP-isoforms in HEK-293 cells. (A) After transfection of cells and transfer into selection medium, the percentage of wells with viable clones was determined. (B) The amount of FSAP in conditioned medium, was determined by ELISA (μg/ml, mean ± S.D., $n = 3$). (C) Supernatants of different FSAP variants, standardized by ELISA, were analyzed by Western blotting performed with a mixture of mAb 677 and mAb 1189.

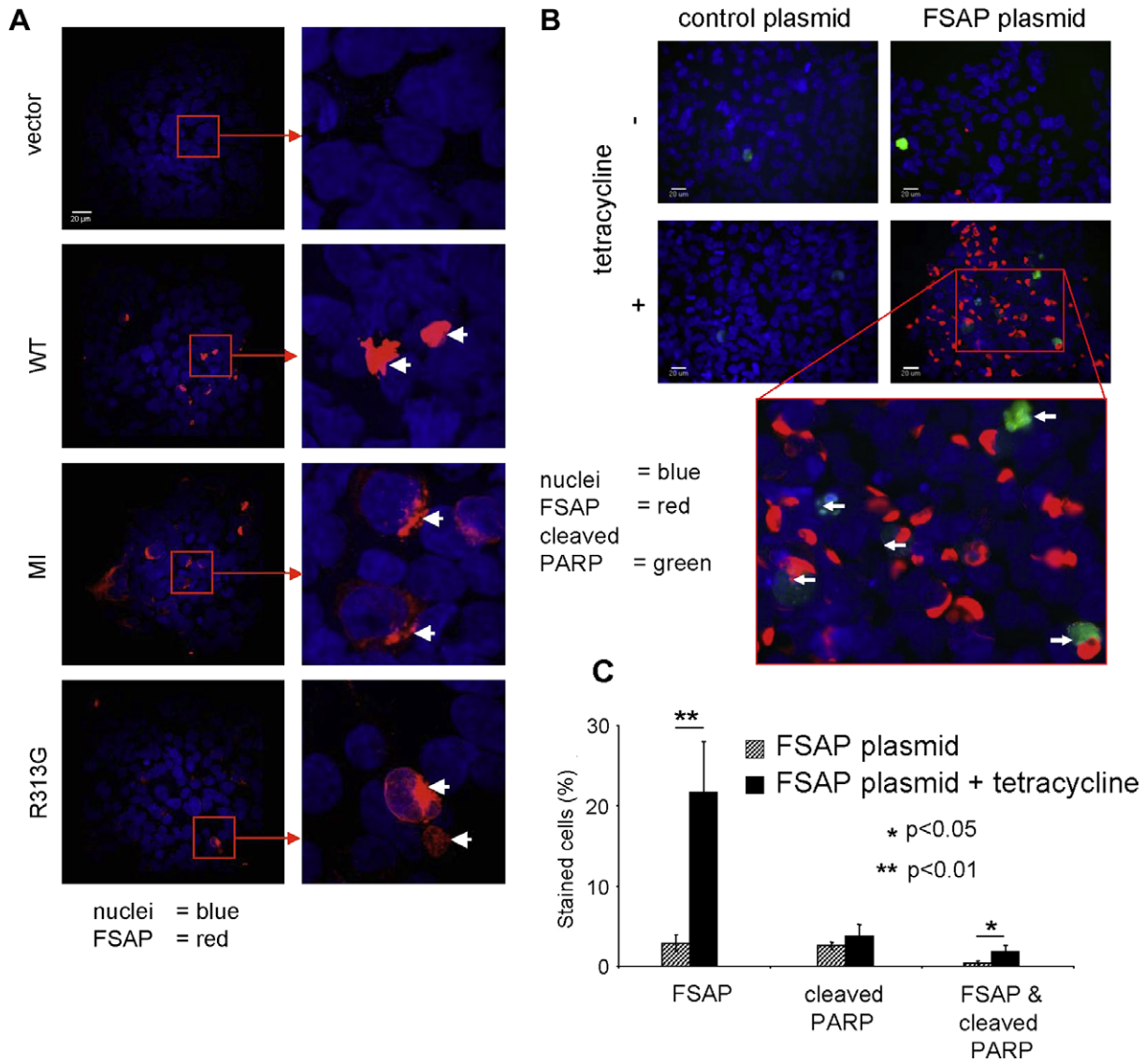

Fig. 2. Influence of FSAP over-expression on cell-phenotype. (A) Confocal microscopy of transiently transfected HEK-293 cells with empty vector, WT-, MI- and R313G-FSAP. FSAP was stained using mAb 1189 (red) and nuclei with DAPI (blue). White arrows highlight staining for FSAP. (B) Transiently transfected T-REx™-293 cells with control vector or vector carrying WT-FSAP, cultivated in growth medium with or without 0.5 μg/ml tetracycline. FSAP is indicated in red, cleaved PARP in green and nuclei in blue. White arrows highlight staining for cleaved PARP. Calibration bars = 20 μm. (C) The staining for FSAP (red), cleaved PARP (green), and co-localized FSAP and cleaved PARP (yellow) was quantified with respect to total cell number in a field. (mean ± S.E.M., $n = 3$). $*$ indicates $P < 0.05$ and $^{**}P < 0.01$, using unpaired two tailed "t" test.

FSAP was used. Without induction, the expression of FSAP and staining for the apoptosis marker, cleaved PARP [11], was low. In contrast, when the induction of FSAP expression was switched on there was a concomitant increase in cleaved PARP staining (Fig. 2B and C). Over-expression of FSAP led to a 15% decrease in cell number after 72 h (data not shown). Addition of tetracycline to cells transfected with the control vector did not show any alterations in FSAP or cleaved PARP staining. As a control, the related protease uPA was efficiently expressed and did not induce apoptosis (data not shown). Finally, addition of exogenous plasma-derived FSAP to cells did not induce any apoptosis (data not shown).

3.2. Interaction of FSAP-isoforms with heparin

Previous studies with limited proteolysis of the FSAP protein have alluded to the role of EGF-3 domain in heparin binding [12]. In accordance, the ΔEGF-3 variant, lacking the EGF-3 domain, clearly exhibited diminished heparin binding (Fig. 3A). In contrast, the recombinant proteins WT-FSAP, MI-FSAP, and R313G-FSAP (Fig. 3A) as well as MII-FSAP and H362F-FSAP (data not shown) bound to a similar extent to immobilized heparin.

PDGF-BB is one of the targets of FSAP in vascular tissue [9]. Without heparin the binding of WT-FSAP, ΔEGF-3-FSAP and R313G-FSAP to PDGF-BB was low, whereas binding to PDGF-BB was increased by heparin for WT-FSAP and R313G-FSAP, but not for ΔEGF-3-FSAP, indicating again the importance of the EGF-3 domain of FSAP for proper substrate interaction (Fig. 3B). As a control the recognition of WT-FSAP, ΔEGF-3-FSAP, MI-FSAP and R313G-FSAP by immobilized pAb against FSAP was similar (Fig. 3C). Using recombinant protein lacking the EGF-3 domain confirmed its importance for binding to heparin.

3.3. Proteolytic activity of FSAP-isoforms

Heparin binding leads to an auto-activation of latent FSAP, which subsequently can activate pro-uPA [4]. Under these conditions, WT-FSAP promoted pro-uPA activation more effectively than ΔEGF-3-FSAP and MI-FSAP (Fig. 4A, filled symbols). In the presence

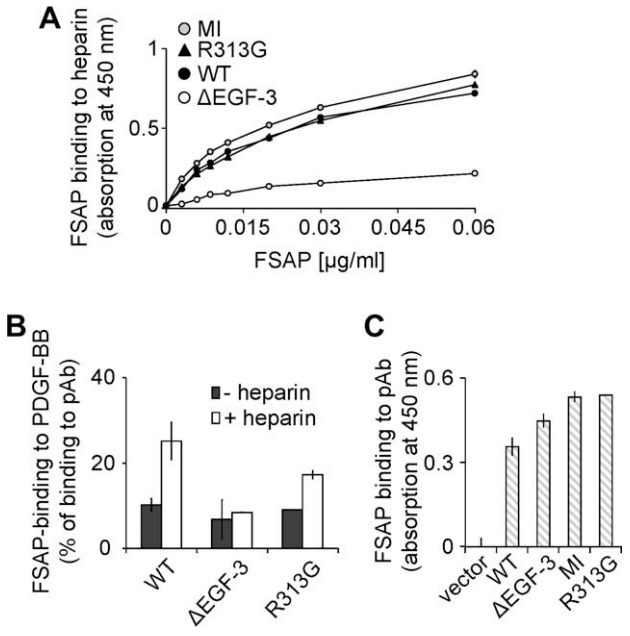

Fig. 3. Binding characteristics of FSAP-isoforms to heparin. (A) WT- (closed circles), ΔEGF-3- (open circles), MI- (gray circles) and R313G-FSAP (closed triangles) (0.003–0.06 μg/ml) were added to immobilized heparin-albumin-biotin. (B) WT-, ΔEGF-3- and R313G-FSAP (0.06 μg/ml) were added to immobilized PDGF-BB. Binding of FSAP-isoforms without (closed columns) or with (open column) heparin (10 μg/ml) was determined. FSAP binding to PDGF-BB was normalized with respect to FSAP binding to anti-FSAP pAb. (C) Binding of recombinant FSAP variants to a pAb against FSAP was tested. Binding was measured with mAb 677 against FSAP, mean ± S.D. (n = 3).

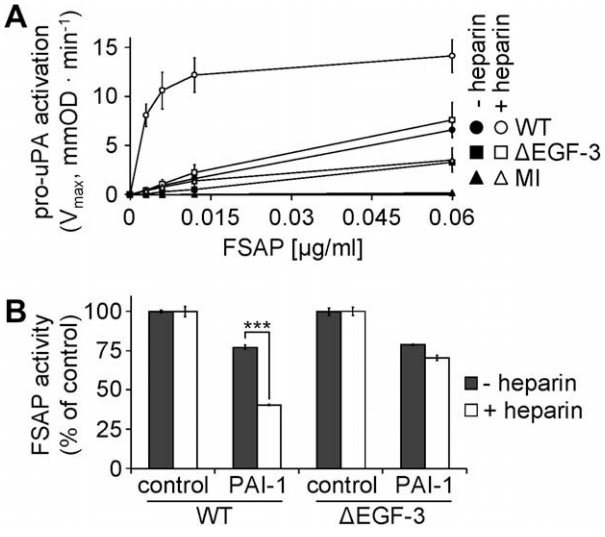

Fig. 4. Enzymatic activity of different FSAP-isoforms. (A) WT- (circles), ΔEGF-3- (squares) and MI-FSAP (triangles) (0.003–0.06 μg/ml) was captured with mAb 677. Pro-uPA (1 μg/ml) activation without (closed symbols) or with (open symbols) heparin (1 μg/ml) was measured by the conversion of S-2444, mean ± S.E.M. (n = 4–6). (B) FSAP inhibition by PAI-1 (0.01 μg/ml), without (closed column) and with (open column) heparin (1 μg/ml) was measured by the conversion of the direct chromogenic substrate S-2288, mean ± S.E.M. (n = 3), *** indicates $P < 0.005$, using unpaired two tailed "t" test.

of heparin the activity of WT-FSAP increased 10-fold, as compared to a 2-fold induction by ΔEGF-3-FSAP (Fig. 4C, open symbols). MII-FSAP exhibited a comparable activity to WT-FSAP, whereas R313G- and H362F-FSAP were inactive towards pro-uPA as would be predicted (data not shown).

WT-FSAP and ΔEGF-3-FSAP demonstrated a similar amidolytic activity in contrast to MI-FSAP and R313G-FSAP, both of which were inactive towards these substrates. In the presence of heparin the activity of WT-FSAP and MI-FSAP was increased, whereas the activity of ΔEGF-3-FSAP remained unchanged (data not shown). PAI-1 is known to inhibit FSAP efficiently in the presence of a polyanionic co-factor [5]. PAI-1-dependent inhibition of WT-FSAP, but not ΔEGF-3-FSAP, was increased by heparin (Fig. 4B). Hence, the EGF-3 domain of FSAP is not only important for heparin binding but contributes largely to the heparin-mediated regulation of FSAP activity.

4. Discussion

Initial attempts to produce FSAP in heterologous expression systems such as in *Escherichia coli* and *Saccharoymces cerevisiae* resulted in none or very low expression rates. In a eukaryotic system (HEK-293) we were able to produce very low amounts of WT-FSAP. Expression of WT-FSAP was associated with an irregular cellular staining pattern for FSAP and concomitant cell death. Indeed FSAP expressing cells showed signs of apoptosis and the number of viable clones generated was extremely low. This was not a cell specific problem, because we observed a similar pattern in CHO cells. Addition of aprotinin, a serine protease inhibitor that efficiently inhibits FSAP, in the medium, did not alter this pattern, indicating that FSAP was acting intracellularly. Mutants with reduced enzymatic activity exhibited a well dispersed granular pattern within cells, high extracellular protein levels and high number of viable clones. This pattern was repeated with mouse WT-FSAP and mouse inactive mutants indicating that there was no species difference (data not shown). In spite of these difficulties, enough FSAP could be produced to pursue structure function studies. In all our test systems the recombinant WT-FSAP behaved similarly to plasma-derived FSAP. The reason for its intracellular toxicity may be related to its ability to bind nucleic acids [3]. This is confirmed by a recent study showing that FSAP can proteolyse nucleosomes in apoptotic cells [13].

Using recombinant MI-FSAP (G534E) we could confirm our original observations with human plasma-derived material that this variant has low enzymatic activity [2,9]. In comparison the MII-FSAP (E393Q), which also exhibits an amino-acid exchange in the protease domain, showed no difference in activity. As expected, the cleavage site and active site mutants were completely inactive.

Previous data generated with proteolytic fragments of native WT enzyme revealed that the EGF-3 domain plays an important role in the binding of FSAP to negatively charged polyanions such as heparin or nucleic acids [12]. The EGF-domain, especially in members of the coagulation system, is important for protein-protein or protein-cell surface interactions [14]. Here we now demonstrate that a recombinant variant of FSAP lacking the EGF-3 domain (ΔEGF-3) indeed exhibited very low ability to bind to immobilized heparin, or to undergo a heparin-dependent increase in auto-activation. This domain is also required for PAI-1-dependent inhibition of FSAP in the presence of heparin. Hence, we conclude that, similar to other members of the coagulation system [14], the EGF-3 domain plays a central role in the regulation of FSAP activity.

Genetic analysis of large populations with respect to FSAP polymorphisms and animal model studies indicate that FSAP has a crucial role in atherosclerosis and liver fibrosis [7,9]. We have, for the first time, produced recombinant FSAP and its variants and have laid the foundation for its more efficient expression through protein engineering [15]. Its enzymatic activity precludes its efficient synthesis and secretion and induces apoptosis in eukaryotic cells. The EGF-3 domain is vital for interaction with heparin and the subsequent activation of latent FSAP.

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