Structure and function analysis of Factor VII Activating Protease (FSAP) with respect to vascular pathophysiology

Inauguraldissertation zur Erlangung des Grades eines
Doktors der Naturwissenschaften
im Fachbereich 08; Biologie und Chemie
an der Justus-Liebig Universität Gießen

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Gießen, im Herbst 2008

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Tag der mündlichen Prüfung:_____

Für meine Eltern

Gertrud & Reinhold!

Wozu die Menschen da sind, wozu „der Mensch“ da ist, soll uns gar nicht kümmern:
Aber wozu Du da bist, das frage dich: Und wenn du es nicht erfahren kannst,
nun so stecke Dir selber Ziele, hohe und edle Ziele und gehe an ihnen zu Grunde!
Ich weiss keinen besseren Lebenszweck
als am Großen und Unmöglichen zu Grunde zu gehen…

Friedrich Nietzsche

Abbreviations

(A)PC	(active) protein C
α2-AP	a2-anti plasmin
α2-MG	a2-macroglobulin
ABCs	ATP-binding cassette transporters
apoE	apolipoprotein E
AT	antithrombin III
bFGF	basic fibroblast growth factor
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CD36	cluster of differentiation 36 / thrombospondin receptor
CIAP	calf intestinal alkaline phophatase
CUB	complement subcomponents Clr/Cls, Urchin EGF-like domain
DAPI	4', 6-diamidino-2-phenylindole dehydrate
DMEM	dulbecco's modified eagel's medium
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbant assay
EPCR	endothelial protein C receptor
ERK	extracellular activated kinase
FCS	fetal calf serum
FII(a)	(active) factor II
FITC	fluoroscein isothiocyanate
FIX(a)	(active) factor IX
FPLC	fast performance liquid chromatography
FSAP	factor VII activating protease
FV(a)	(active) factor V
FVII(a)	(active) factor VII
FVIII(a)	(active) factor VIII
FX(a)	(active) factor X
FXI(a)	(active) factor XI
FXII(a)	(active) factor XII
FXIII(a)	(active) factor XIII
Gpi	glycosylphosphatidylinositol
GpIbα	glycoprotein Ibα
Gp IV	Glycoprotein IV
HABP-2	hyaluronic acid binding protein-2
HGF-A	hepatocyte growth factor activator
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IC_{50}	inhibitor concentration to inhibit 50 %
ICAM-1	intracellular cellular sdhesion molecule-1
IMDM	iscov's modified medium
kDa	kilo dalton
KIU	kallikrein inhibiting units
KM	Michaelis-Menten constant
LDL	low-density lipoprotein
LDLR	LDL receptor
LMW	low molecular weight
LPS	lipopolysaccharide
LRP	LDL-receptor related protein
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase

MCP-1	macrophage stimulating protein
M-CSF	macrophage colony stimulating factor
MEF	mouse embryonic fibroblasts
MI / II	Marburg I / II
MMP	matrix metalloproteinase
MW	molecular weight
OD	optical density
p(dI:dC)	polydeoxyinosinic-polydeoxycytidylic acid
p(I:C)	polyinosinic-polycytidylic acid
pAb	polyclonal antibody
PAI-1 /-2	plasminogen activator inhibitor-1 /-2
PAR	protease activated receptor
PARP	poly [ADP-ribose] polymerase 1
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PHBP	plasma hyaluronan binding protein
PHBSP	plasma hyaluronan binding serine protease
PI3K	phosphoinositol 3 kinase
PN-1	protease nexin-1
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
PVDF	polyvinylidene fluoride
RAP	receptor associated protein
RT	room temperature
S-2288	H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilinedihydro-chloride
S-2444	L-pyroglutamyl-glycyl-L-arginine-p-nitroanilinedihydro-chloride
sc	single chain
ScRs	scavenger receptors
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SERPIN	serine protease inhibitor
SNP	single nucleotide polymorphism
TAFI	thrombin activated fibrinolysis inhibitor
TBE	tris borate EDTA
TBS	tris buffered saline
TBS-T	TBS tween20
tc	two chain
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLRs	toll-like receptors
TM	thrombomodulin
TMB	3.3', 5.5'-tetramethylbenzidine
tPA	tissue type plasminogen activator
UH	unfractionated heparin
uPA	urokinase
uPAR	uPA receptor
v/v	volume per volume
VCAM-1	vascular cell adhesion molecule
VLDL	very low-density lipoprotein
VLDLR	VLDL receptor
v_{max}	maxima velocity
VSMC	vascular smooth muscle cells
VWF	von Willebrand factor
w/v	weight per volume
WB	Western blot
WT	wild type

1. Schedules

1.1 Index

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2. Publications resulting from this work

The G534E Polymorphism Of The Gene Encoding The Factor VII-Activating Protease Is Associated With Cardiovascular Risk Due To Increased Neointima Formation
J Exp Med. 2006 Dec 25;203(13):2801-7
Sedding D, Daniel JM, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, Braun-Dullaeus RC, Tillmanns H, Weimer T, Preissner KT, Kanse SM.

Nucleic Acids Potentiate Factor VII-Activating Protease (FSAP)-Mediated Cleavage Of Platelet-Derived Growth Factor-BB And Inhibition Of Vascular Smooth Muscle Cell Proliferation
Biochem J. 2007 May 15;404(1):45-50.
Shibamiya A, Muhl L, Tannert-Otto S, Preissner KT, Kanse SM.

Inhibition Of PDGF-BB By Factor VII-Activating Protease (FSAP) Is Neutralized By Protease Nexin-1, And The FSAP-Inhibitor Complexes Are Internalized Via LRP
Biochem J. 2007 Jun 1;404(2):191-6
Muhl L, Nykjaer A, Wygrecka M, Monard D, Preissner KT, Kanse SM.

High Negative Charge-To-Size Ratio In Polyphosphates And Heparin Regulates Factor VII Activating Protease.
FEBS J. in press (July 2009)
Muhl L, Galuska SP, Öörni K, Hernández-Ruiz L, Andrei-Selmer LC, Geyer R, Preissner KT, Ruiz FA, Kovanen PT, Kanse SM.

Structur-Function Analysis Of Factor VII Activating Protease (FSAP) Sequence Determinants For Heparin Binding And Cellular Functions.
FEBS Lett. 2009 Jun 18;583(12):1994-8.
Muhl L, Weimer T, Preissner KT, Kanse SM.

3. Introduction

The blood circulation is required for the transport and the supply of organs with metabolites, vitamins, hormones and for nutrition. The gas-exchange of oxygen and carbon dioxide, and the maintenance of blood- and body-pressure represent other features of the circulation. Leakage or obstruction of the circulation leads to severe pathological complications. In order to preserve the functionality of the circulation, evolution has developed mechanisms that circumvent extensive liquid-loss and reconstitute the vasculature. These homeostatic mechanisms are characterized as hemostasis, fibrinolysis, vascular wall remodeling and wound healing.

3.1 Hemostasis

Numerous proteases control the activation, regulation and coordination of hemostasis. These mechanisms are subject to tight control. Proteins involved in the coagulation process exhibit a high specificity for their substrates, cofactors and assembly of protein-protein complexes [1]. Damaged or activated cells, platelets and endothelial cells serve as templates for these complex reactions. Blood coagulation on specified cell surfaces prevents the spread of coagulation throughout the whole vascular system [2]. Genetic or physiological defects in these systems can lead to thromboembolic diseases or hemorrhagic disorders. These life-threatening circumstances indicate the importance of a functioning blood-coagulation system [3].

Hemostasis can be classified into two pathways, the intrinsic or contact factor pathway and the extrinsic or tissue factor pathway. The key protease of the blood coagulation system is pro-thrombin (factor II, FII). The activation of pro-thrombin

generates active thrombin (FIIa), which fulfills its very important task in the cleavage of fibrinogen [3].

To maintain circulation, hemostasis has to respond rapidly to vessel injury and prevent excessive blood loss. Subsequent to a disruption of the vascular endothelium, extravascular collagen or tissue factor become exposed to blood components. Vascular smooth muscle cells (VSMC), pericytes and adventitial fibroblasts constitutively express tissue factor (TF). When TF becomes complexed with FVII/FVIIa coagulation is initiated [4]. Deficiencies in TF result in severe complications, e.g., mice lacking TF die during embryonic development, due to vascular and hemostatic defects [5, 6]. No humans with a deficiency in TF have yet been identified [4].

Platelets bound to the injured vessel wall via collagen/glycoprotein (Gp) IV [7] or von Willebrand factor (VWF)/GpIbα serve as a negatively charged surface for proteases of the coagulation cascade and ensure that the process of clot-formation is confined to the damaged site of the vessel wall [3].

Categorizing hemostasis into different phases, three stages can be distinguished. In the initiation phase, TF/FVIIa complexes are formed and initial amounts of activated factor IX (FIXa) and factor X (FXa) are generated. FXa in turn associates with its cofactor factor Va (FVa) and forms a complex on a negatively charged phospholipid membrane. This assembly results in the so-called prothombinase complex. This complex activates FII to FIIa in the presence of Ca^{2+}. This initial phase is followed by the amplification phase, where the basis is laid for the generation of large amounts of thrombin. Activated thrombin activates FV, factor VIII (FVIII) and small amounts of factor XI (FXI) on the surface of activated platelets [2].

The amplification phase is followed by the propagation phase. FIXa binds to activated FVIII (FVIIIa) on the platelet surface. This complex is called the tenase complex. Subsequently, the tenase complex catalyzes the conversion of FX to FXa in

the presence of Ca^{2+}. In situations of severe hemostatic challenge, additional amounts of FIXa are supplied by active FXI (FXIa) [3].

FX activation is restricted to cell surfaces, since it is rapidly inactivated by tissue factor pathway inhibitor (TFPI) or antithrombin III (AT) in the fluid phase. These inhibitors regulate and restrict the activity of FXa to the cell surfaces where it is generated [2]. Formation of the tenase complex on the surface of platelets, in addition to the TF/FVIIa complex located on cells of the damaged vessel wall, represents another more efficient site of FXa generation. Assembly of the tenase complex is accompanied by dissociation of FVIII/VWF complexes, thereby permitting additional platelet aggregation [1, 2]. Generation of the tenase complex accelerates the formation of the prothrombinase complex, which produces sufficient amounts of FIIa to cleave abundant amounts of fibrinogen to fibrin in forming the fibrin clot.

Under normal physiological circumstances the generation of thrombin is located and restricted to the surface of platelets, as illustrated by the tenase and prothrombinase complexes [3]. Due to their importance, these two complexes are putative targets for therapeutical strategies to inhibit blood coagulation [1].

To stabilize the fibrin clot, FIIa activates factor XIII (FXIII) to active FXIII (FXIIIa). Fibrin serves as a cofactor for FIIa-dependent activation of FXIII. When FIIa cleaves fibrinogen it remains bound to the produced fibrin peptides, thereby enabling it to activate FXIII more efficiently. Additionally, this ensures the generation of FXIIIa at the site where it is needed [8]. FXIIIa stabilizes the fibrin clot by covalently cross-linking fibrin [3]. FIIa remains active and promotes FXIIIa-dependent fibrin cross-linkage until the complete platelet clot consists of stabilized fibrin.

The propagation phase is followed by an intrinsic anticoagulation phase, even though the mechanisms characterizing the termination are active throughout the process of blood coagulation [2]. The main components of the termination phase are protein C

(PC), protein S and thrombomodulin (TM). PC becomes activated by FIIa and is converted to active PC (APC). PC activation is promoted when FIIa is bound to TM. Its receptor, the endothelial PC receptor (EPCR), functions as a cofactor for FIIa-dependent PC activation. When TM is located on an endothelial cell membrane together with EPCR, then the activation of PC is further accelerated [9]. APC, together with its cofactor protein S, proteolytically inactivates FVa (prothrombinase complex) and FVIIIa (tenase complex). This process exhibits an even higher efficiency if FVa is located on an endothelial cell membrane rather than on platelets [10]. Apart from the activation of PC, the FIIa/TM complex also activates TFPI, which in turn can rapidly inhibit other proteases. On intact endothelial cell membranes, the presence of TM, TFPI and AT bound to heparan sulfate restricts the coagulatory processes to the sites of vessel injury[2].

AT-dependent inhibition of FIIa is inefficient without cofactors, such as, heparin or heparan sulfate. Both, FIIa and AT bind to these cofactors, which serve as a template. FIIa recognizes AT as a substrate, but is trapped by AT during the proteolysis reaction [3]. Another inhibitor which uses a similar, but more complex mechanism, for the inhibition of FIIa is heparin cofactor II. As in the case of AT, glycosaminoglycans accelerate the efficiency of FIIa inhibition by heparin cofactor II through template-dependent effects. Additionally, a highly acidic part of heparin cofactor II is released during the inhibition process, which binds FIIa at another site, thereby changing FIIa's conformation [11]. However, the contribution of heparin cofactor II in normal hemostasis has been suggested to be only secondary [3]. The FIIa-inhibitor complexes generated during hemostasis are cleared from the circulation by receptor-mediated endocytosis [12].

Whether proteases of the contact factor pathway (or plasma kallikrein/kinin system) belong to hemostasis has not been elucidated so far. Bradykinin is constitutively released during the cleavage of pre-kallikrein and is important for the

maintenance of the vascular homeostasis [13]. The contribution of FXIIa to hemostasis is not clear. FXII becomes activated by negatively charged surfaces, such as, dextran sulfate, nucleic acids [14] or polyphosphate released by activated platelets [15]. At the site of a developing thrombus, FXII may become activated to generate greater amounts of FXIa, thus propagating FIIa-formation [13]. A FXII polymorphism exhibiting reduced proteolytic activity is associated with an increased risk of thrombosis, which might be potentially explained by an FXIIa-dependent reduced fibrinolytic activity. Controversial observations in FXII-deficient mice have revealed reduced thrombus size, which suggests a role of FXII in the development of the thrombus [13].

Proteases of the coagulation cascade also contribute to signaling events, such as, the activation of protease-activated receptors (PARs). Related to hemostatic progression and pro-inflammatory responses, PAR-1 or PAR-2 become activated by FIIa or FXa, respectively [9]. Not only are pro-coagulatory enzymes involved in cellular signaling, but also proteins that contribute to the termination of blood coagulation. APC is able to counteract systemic inflammation, such as in cases of sepsis, via signaling through PARs involving EPCR as a co-receptor [9].

Recent reports provide evidence for the existence of intravascular TF. Leukocytes, platelets, cell-derived microparticels and even endothelial cells, if they become exposed to LPS, express TF [4]. The contribution of intravascular TF to hemostasis and thrombosis has to be elucidated and may present new targets for antithrombotic therapies [4].

Highly specific mechanisms for protein activation and subsequent inhibition are hallmarks of hemostasis. The assembly of protein complexes, the cofactor-dependent changes in substrate specificity and the cofactor-directed inhibition highlight important features of blood coagulation. To sum up, blood coagulation is an extremely tightly regulated system.

3.2 Fibrinolysis

The dissolution the formed clot is denoted by fibrinolysis. The main components of this mechanism are plasminogen and the plasminogen activators, namely, tissue-type plasminogen activator (tPA) and urokinase (uPA). The zymogen plasminogen is cleaved by tPA or uPA to active plasmin, which in turn degrades fibrin. Also important in this process are the plasminogen-activator inhibitors (PAI)-1 and -2 that inhibit the tPA and uPA-dependent generation of plasmin [16]. Serine protease inhibitors (SERPINs) are present at high concentration in plasma. Proteins, such as, α_2-antiplasmin (α_2-AP), α_2-macroglobulin (α_2-MG) and thrombin-activated fibrinolysis inhibitor (TAFI) inhibit plasmin-dependent degradation of fibrin using different mechanisms [16].

Both plasminogen and tPA bind fibrin, which accelerates the generation of plasmin. Other substances, such as, collagen IV, thrombospondin and cell membrane components can serve as cofactors for tPA-dependent plasminogen activation as well, but with lower capability than fibrin [16]. When plasminogen becomes activated a two-chain conformation is formed, with kringle domains in the N-terminal heavy chain and the trypsin-like, serine protease domain in the C-terminal light chain. These kringle domains are vital in plasminogen as well as in plasmin for their interaction with substrates, inhibitors and cell membranes. Interaction with these substances is necessary for plasminogen activation, localization and the regulation of plasmin activity.

Endothelial cells synthesize and secrete tPA in a single-chain conformation. Already in this conformation, tPA is a potent activator of plasminogen. The binding of tPA with fibrin is guided by one of its kringle domains and the finger-like domain (similar to that of fibronectin), both located at the N-terminal part of tPA. Processing to the two-chain conformation of tPA, by plasmin, kallikrein or FXa, increases its activity towards synthetic chromogenic substrates and plasminogen in the absence of fibrin.

Affinity towards its inhibitors is also higher for two-chain tPA [16, 17]. The inhibition of tPA by PAI-1 involves the catalytic domain of tPA, as well as a site located in one of its kringle domains. TPA-PAI-1 complexes exhibit a binding capacity to fibrin, which increases the inhibitory effect of PAI-1 by blocking of putative binding sites on the fibrin clot for free tPA [18].

In contrast to tPA, uPA exhibits no specific binding affinity for fibrin. The uPA concentration in plasma is lower compared to tPA, but nevertheless, an intravenous infusion of single-chain (sc)-uPA increases the lysis of thrombi in a plasminogen-independent fashion [16]. Sc-uPA is cleaved by plasmin, kallikrein, stromelysine-1 or FIIa resulting in different outcomes concerning uPA activity. Plasmin or kallikrein release a two-chain uPA variant that exhibits full proteolytic activity, whereas FIIa forms a variant that is not able to cleave plasminogen *in vitro*, but can induce thrombolysis *in vivo*. This FIIa-generated uPA-variant can be further processed by plasmin to generate active uPA [16]. A unique feature among components of the fibrinolysis system is the existence of a specialized cell-surface receptor for uPA, the uPA receptor (uPAR). This receptor is a cystein-rich glycoprotein that is expressed by many cell types. The uPAR is covalently linked to the cell surface via a glycosylphosphatidylinositol (GPI)-anchor. The epidermal growth factor-like (EGF) domain of uPA is important for its interaction with the N-terminal region of uPAR. This interaction appears to be specific, since other proteins containing EGF domains lack an interaction with uPAR [16]. The interaction of uPA with uPAR has been suggested to regulate pericellular proteolysis by activating plasminogen and initiating intracellular signaling cascades. These processes are important for cellular development [16] and wound-healing [19]. Whereas uPA seems to be more important for cell surface-located plasminogen activation, tPA, with its fibrin-binding feature, is suggested to be important for the lysis of fibrin clots in the circulation [20].

PAI-1 is expressed in almost all cell types including endothelial cells, hepatocytes and adipocytes [19], and inhibits single-chain and two-chain tPA; whereas PAI-2 is expressed by monocytes, macrophages and by the placenta [16], and only inhibits two-chain tPA. Platelet α-granules contain large amounts of PAI-1, which is released upon platelet stimulation and results in a local increase of PAI-1 at sites of thrombus formation. Thereby, PAI-1 increases the resistance of the fibrin matrix to proteolytic degradation [21]. PAI-1 in plasma is spontaneously converted from its active conformation into latent PAI-1. The active conformation of PAI-1 is stabilized by specific binding to vitronectin [16], again increasing local capability to increase thrombus stability.

Inhibitors of plasmin, such as α_2-AP, TAFI and α_2-MG play important roles in regulating the fibrinolytic process. α_2-AP binds to fibrin and is covalently linked to the fibrin clot by FXIIIa. The efficient inhibition of plasmin by α_2-AP is dependent on its binding to fibrin. The generated plasmin which cleaves fibrin in the first instance, thereby creating new high-affinity binding sites for plasminogen and tPA, is directly inhibited by α_2-AP [22]. Inhibition of plasmin by α_2-MG, in comparison to inhibition by α_2-AP, follows a lower rate and does not lead to complete inactivation of plasmin. However, α_2-MG remains an important regulator of plasmin activity because of its very high plasma-level as compared to other inhibitors (α_2-MG = 2.5 mg/ml; α_2-AP = 0.07 mg/ml) [16].

FIIa or plasmin can activate TAFI. The efficiency of TAFI activation by FIIa increases when FIIa is bound to TM, which usually promotes anti-thrombotic activities [9]. The activation rate of TAFI by plasmin is increased when glycosaminoglycans, such as unfractionated heparin are present [16]. TAFI inhibits fibrinolysis by a cleavage of C-terminal Lys- or Arg-residues, those generated during the initial cleavage of fibrin by

plasmin and forming the new high-affinity binding sites for tPA and plasminogen. TAFI-related cleavage of fibrin, therefore, prevents the additional binding of plasminogen and tPA, preserving fibrin stability [23].

Members of the fibrinolysis system are crucial in the development of pathological situations if they are deregulated. PAI-1 levels correlate with a certain risk for thrombotic complications, in-line with statements that PAI-1 is suggested to be the main determinant of fibrinolysis [16]. PAI-1 is also linked to myocardial infarction and obesity [19]. A correlation between the metastatic spread of tumors and cardiac fibrosis with uPA activity has also been found [19]. Additionally, uPAR was found to be related to cancer progression [24], and both proteins seem to be involved in inflammatory responses [25]. TPA is reported to be a regulator of inflammatory processes, especially in the inflammatory response during ischemia reperfusion injury [26] and recently described in stroke [27].

A contribution of hemostatic and fibrinolytic events to pathological situations becomes even more evident in the case of atherosclerosis, where an extensive thrombogenic signal induces thrombus formation causing an eventual occlusion of the blood vessel and is followed by severe complications in the vascular system.

3.3 Atherosclerosis

Cardiovascular diseases, such as myocardial infarction, coronary heart disease, ischemic gangrene, abdominal aortic aneurysm, heart failure and stroke are the major determinants of morbidity and mortality in the Western world [28, 29]. The underlying cause of the above-mentioned pathological situations is atherosclerosis, a slowly progressive disease of the arteries [30]. Risk factors, such as poor diet, low physical

activity, smoking, alcohol and stress contribute to the progression of atherosclerosis [31]. An inflammatory process, in response to lipid retention in the arterial wall, has been suggested to represent a hallmark for the initiation as well as the propagation of atherosclerosis [28, 32].

Cholesterol-rich very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) typify mediators for the initiation of atherosclerosis. As in the case of hypercholesterolemia they enter the vascular vessel wall, if their plasma levels are excessive [28]. In the extravascular space they are usually eliminated, but if their concentration is too high they remain stored in the extracellular matrix [33, 34]. The enzymatic or non-enzymatic oxidation of LDL leads to the generation of bioactive lipids, which in turn can activate endothelial cells. Hemodynamic strain represents another cause of endothelial cell activation, apart from lipid accumulation. In this case, not a high average, but high oscillatory, shear stress is responsible for endothelial cell activation [35].

Activated endothelial cells start to produce and express cell-surface adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1 together with pro-inflammatory mediators [36]. These attract cells originating from the blood, including monocytes and lymphocytes, that roll on the vascular surface at sites of endothelial activation [28]. Subsequently, these blood cells adhere and start to infiltrate the underlying vessel wall. Platelets have been described to adhere to these regions in addition to mononuclear cells [37].

The migration and infiltration by adhered mononuclear cells is regulated through chemotactic stimuli. A variety of chemokines (chemotactic cytokines) are involved in this process. Macrophage-chemotactic protein (MCP/CCL)-1 and its counter-receptor CCR-2 hold crucial roles during the development of atherosclerotic lesions [28]. Macrophage-colony stimulating factor (M-CSF), interleukin 8 and its

receptor CXCR-2 [38], as well as other cytokines and chemokines are involved in leukocyte infiltration and transformation [39]. Infiltrated monocytes will differentiate into macrophages, which is a necessary early step in atherosclerosis. Differentiated macrophages start to express a special pattern of cell-surface receptors, including scavenger receptors (ScRs) and toll-like receptors (TLRs) [40]. ScRs are known to exhibit a broad range of ligands. They internalize bacterial endotoxins, apoptotic cell fragments and oxidized LDL (oxLDL). The excessive uptake of oxLDL leads to a high intracellular accumulation of cholesterol, which cannot be mobilized in a sufficient way and is therefore stored in cytosolic droplets. Besides ScRs, CD36 also contributes to the uptake of modified LDL [41]. The uptake of native LDL by macrophages is regulated by the membrane receptors LDL receptor (LDLR), VLDL receptor (VLDLR) and LDL receptor-related protein (LRP) [41]. Despite its rapid down-regulation due to cellular cholesterol accumulation, LDLR is important in atherosclerotic development. So is VLDLR, which is not down-regulated in response to cholesterol loading, hence, also important during atherosclerotic development. LRP expressed on macrophages which is involved in a variety of physiological processes, contributes to LDL uptake by internalization of apoE-containing LDL [41]. Mobilization and efflux of cholesterol from macrophages is a process dependent on ATP-binding cassette transporters (ABCs). These transmembrane transporters are down-regulated in inflammation, thus augmenting the intracellular deposition of cholesterol. Due to the massive uptake of cholesterol the macrophages become foam cells, the determinant cell of atherosclerosis [40]. The inflammatory response in developing atherosclerotic plaques might be due, additionally in part, dependent on the initiated signaling pathways of TLRs, which lead to further macrophage activation [42].

Macropinocytosis is another endocytotic process described for epithelial cells, fibroblasts, neutrophils and macrophages that occurs constitutively, but slowly [43]. *In*

vivo studies have revealed this mechanism to occur in the spleen, lymph nodes and liver. Its observation in Kupffer cells, resident macrophages of the liver, is especially important. Hence, an occurrence of macropinocytosis in macrophages, transforming them into foam cells of atherosclerotic plaques is plausible [41].

The result of the uptake of lipids by macrophages and their subsequent transformation into foam cells is characterized by fatty streaks [28, 39]. The development of advanced or complicated atherosclerotic lesions from fatty streaks is the next step in atherosclerosis. The fatty streak becomes covered by a fibrous cap consisting of mainly VSMC, VSMC-derived collagen, elastin, proteoglycans and extracellular matrix [44]. This event represents a kind of healing response to injury. VSMC proliferate in response to different growth factors [45, 46] and cover the lipid core. The center of an atherosclerotic plaque is composed of a mixture of leukocytes, lipids and debris, from which conversion into a necrotic core may occur [39]. This highly atherogenic area is a result of lipid accumulation, apoptosis and necrosis of macrophages and mural cells. An atherosclerotic lesion can continuously enlarge at its border, due to uninterrupted infiltration of leukocytes, dependent on MCP-1, M-CSF and oxLDL [39]. In addition, the pericellular localization of uPA, bound to uPAR, may facilitate this migration and invasion, as well as extracellular matrix degradation in close proximity to macrophages[47].

Due to plaque rupture, its lipid or necrotic core becomes exposed to blood components, such as coagulation factors, especially, FVII. TF, which is highly present in the core of an atherosclerotic plaque, forms a complex with FVII and initiates thrombus formation. Subsequently, occlusion of the vessel may occur and lead to ischemic events in the adjacent organs [39].

Plaque rupture usually occurs at its shoulder. The expression rate of matrix-degrading proteases, such as matrix metalloproteinases (MMPs), is a key determinant in

the vulnerability of a plaque [46]. MMPs are expressed by activated macrophages in response to stimulation by immune and inflammatory cytokines [28]. Location of MMP expression is often observed in macrophage-rich areas at the shoulders of atherosclerotic plaques [46]. The latent pro-forms of MMPs are activated by plasmin. Active MMPs degrade extracellular matrix components leading to a weakening of the fibrous cap [28]. Additionally, mast cells respond to inflammatory stimulation, secreting proteases and other substances leading to degradation of matrix or inhibition of fibrous cap formation [28]. Matrix destabilization and reduced fibrous cap formation results in reduced thickness and strength of the plaque, finally causing plaque rupture, due to the inability to withstand the hemodynamic forces of blood flow [32].

There seems to be an important contribution of VSMC to the vulnerability of atherosclerotic plaques. Identification of healed plaques led to the investigation of further factors involved in plaque stability and immediate repair [44]. Apart from proteolytic weakening, VSMC-senescence is suggested to be another crucial determinant in plaque vulnerability. Senescent cells cannot respond to mitogenic stimuli. But proliferation of VSMC is a central event for the repair mechanisms and stable construction of atherosclerotic plaques [44]. Therefore, senescence caused either by exhausting replication or stress in VSMC contributes to plaque instability [44, 48].

LRP expression in the arterial wall is mainly associated with macrophages and VSMC. Besides its role in lipid uptake in macrophages, the LRP-dependent uptake of lipids into VSMC and their subsequent transformation into foam cells was recently described [49]. Lipid uptake in VSMC of atherosclerotic plaques correlates with LRP mRNA levels. LRP gene expression is increased in VSMC during atherosclerotic development and correlates with pro-thrombotic alteration of the vascular wall [50]. These VSMC exhibit a lower proliferative response and do not respond to growth factor stimulation, but this is not related to reduced survival [49]. However, the role of LRP

located on the surface of VSMC seems to play miscellaneous roles during atherosclerotic development. It is not only been involved in lipid uptake, but also in the regulation of growth factor-dependent activation of VSMC and thereby controls the integrity of the vascular wall and protects from atherosclerotic lesion formation [51, 52].

Atherosclerosis is a complex, inflammatory disease involving a variety of mechanisms [53], which lead to a thickening of the arterial wall by accumulation of lipids in macrophages that form foam cells. Subsequently, atherosclerotic lesions with a fibrous cap and a lipid core emerge, that contain highly thrombogenic material. Stable atherosclerotic lesions with a uniformly dense fibrous cap cause cardiovascular complications due to the narrowing of the artery. Unstable atherosclerotic plaques are often non-occlusive and, therefore, difficult to detect via angiography [39]. They cause life-threatening complications dependent on their rupture, followed by a thrombotic response, resulting in severe complications in the effected organ, such as heart, lung or brain [32, 39].

Atherosclerosis can be related to other diseases caused by a chronic inflammatory stimulation, such as cirrhosis of the liver, rheumatoid arthritis in joints, glomerulosclerosis in kidney, pulmonary fibrosis in lung and pancreatitis in pancreas [39]. It also displays the involvement of hemostasis and fibrinolysis, and their deregulation or extensive induction due to non-physiological events, resulting in dramatic complications for the individual.

3.4 Factor VII-activating protease (FSAP)

Proteolysis is an exquisitely regulated event *in vivo* which defines the cleavage of the appropriate substrate at the right time, location and cellular context. Abnormal tuning of endogenous proteolysis causes many life-threatening diseases, such as hemophilia, cancer and heart disease [54].

3.4.1 Origin and structure of FSAP

Like many other proteases that contribute to coagulation or fibrinolysis, FSAP is mainly expressed by the liver. Northern blot analysis revealed that the major expression is related to the liver, but FSAP mRNA could also be detected in kidney, skeletal muscle and pancreas [55]. In mice, FSAP mRNA expression is restricted to liver and kidney [56].

FSAP is a serine protease belonging to the *S1 family* of *Clan PA* (cf.: MEROPS peptide database; http://merops.sanger.ac.uk/). In the first instance, FSAP was denoted as "plasma hyaluronan binding protein" (PHBP), due to its isolation via adsorption to an hyaluronic acid column, from plasma [55]. The FSAP gene is denoted as *hyaluronic acid binding protein* (*habp)-2*. Later it was found in a pro-thrombin-complex and isolated by the ability to bind to the serine protease inhibitor aprotinin. It was demonstrated to activate thrombin specific chromogenic substrates, but was not inhibited by hirudin, a specific thrombin inhibitor, and therefore, termed as "plasma hyaluronan binding serine protease" (PHBSP) [57]. Finally, the observation that FSAP can cleave and activate FVII in a TF-independent mechanism led to the denotation "factor seven activating protease" (FSAP) [58].

FSAP protein consists of 560 amino-acids, including a 23 amino-acid signal peptide. Structural features of FSAP are similar to those of other serine proteases of the

coagulation or fibrinolysis processes. The 237 amino-acid C-terminal region exhibits properties of a trypsin-like serine protease domain, whereas the N-terminal region consists of three epidermal growth factor-like (EGF) domains and a kringle domain [55] (Figure 1).

Similarities of FSAP gene-structure to other proteases of the hemostasis – fibrinolysis system were found and revealed high homology to Factor XII, tPA, uPA and hepatocytes growth factor-activator (HGF-A), which are composed in a similar fashion like FSAP, exhibiting various kringle and / or EGF domains at the N-terminal region and a trypsin-like serine protease domain at the C-terminal end of the amino-acid chain [55, 58-60].

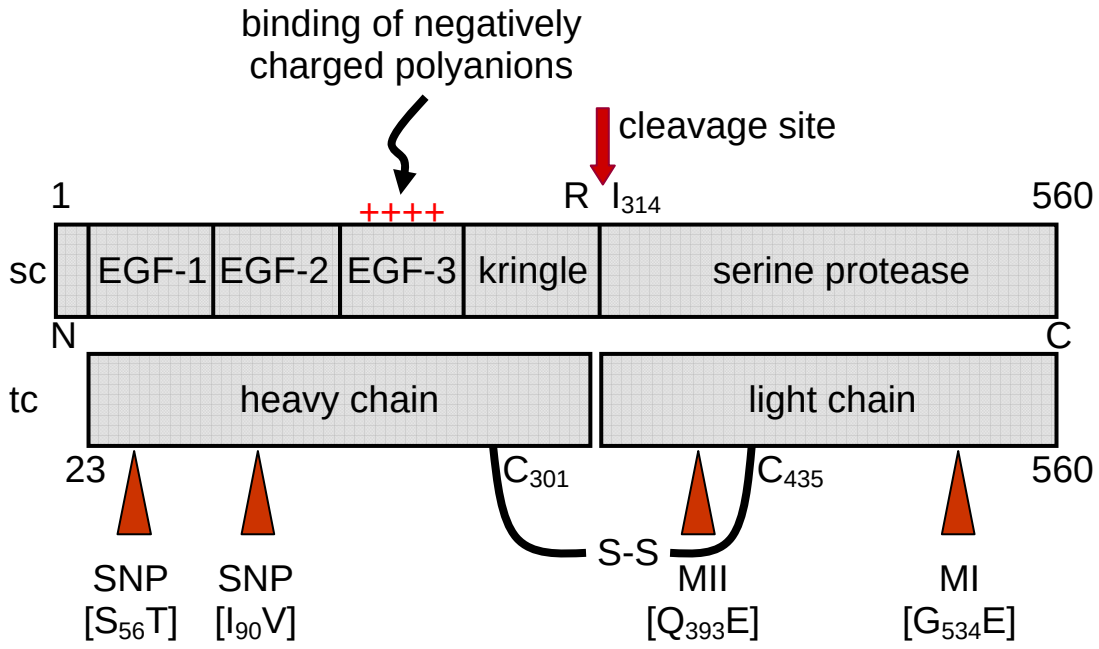


Figure 1: *Schematic structure of FSAP.* Inactive single-chain (sc) conformation (top) and active two-chain (tc) conformation (bottom) of FSAP with EGF-, kringle and serine protease domains indicated. The cleavage site is highlighted by the red arrow, cleavage occurs at $R^{\blacktriangledown}I_{314}$. Red triangles highlight locations of SNP, in either heavy-chain or light-chain. (Modified from [61])

3.4.2 Activation and enzymatic function of FSAP

FSAP is secreted by hepatocytes as an inactive zymogen and circulates in plasma, requiring activation to become a functioning protease [62, 63]. This process is

determined by a specific cleavage at position $R^{\blacktriangledown}I_{314}$, thereby separating the EGF domains and the kringle domain from the serine protease domain. Cleavage of FSAP generates the active two-chain (tc) conformation from the inactive single-chain (sc) conformation [62, 64, 65]. The heavy chain contains the three EGF domains and the kringle domain, therefore, the light chain only contains the serine protease domain. The two chains are held together by a disulfide bridge spanning $C_{301} - C_{435}$ [62-64]. Cleavage at position $R^{\blacktriangledown}I_{314}$ causes a morphological change that opens the access to the active center, which is composed of H_{362}, D_{405} and S_{509} (Figure 1).

Once activated, FSAP is capable of activating other FSAP molecules in an auto-catalytic fashion. For this mechanism, negatively charged polyanions, such as, heparin [63, 65], nucleic acids [66], dextran sulfate [63] and polyphosphatidylethanolamine [64] serve as cofactors, amplifying the auto-activation efficiency. Subsequently to auto-catalytic activation, an auto-degradation occurs, leading to the fragmentation of the FSAP molecule, resulting in inactivation [62].

Known substrates of FSAP are Factor VII [58], pro-uPA [59, 65], fibrinogen [67], high and low molecular weight kininogen [68] and basic fibroblast growth factor (bFGF) [69]. Additionally, FSAP inhibits platelet-derived growth factor (PDGF)-BB-mediated activation VSMC [70].

The counterparts of serine proteases are the serine protease inhibitors (SERPINs), which regulate the activity of their target proteases in a variety of important systems, as demonstrated in the case of hemostasis and fibrinolysis. FSAP is efficiently inhibited by C1-inhibtor, α_2-AP, inter-α-trypsin inhibitor, AT/heparin, which are present in plasma and by aprotinin [71] originating from bovine lunge tissue, formerly used in complex surgeries to prevent bleeding. The main function of this kunitz-type inhibitor was to slow down the fibrinolysis process, by inhibiting kallikrein and plasmin [72]. Furthermore, FSAP is efficiently inhibited in the lung, by PAI-1 [73].

3.4.3 Polymorphisms in the FSAP gene

Two single nucleotide polymorphisms (SNPs) were identified that cause a single amino-acid exchange in the serine protease domain of FSAP. One is called Marburg I (MI), which leads to an exchange from Gly to Glu at position 534 ($G_{534}E$) (Figure 1 and 2) and the other is called Marburg II (MII), which leads to the change of Gln to Glu at position 393 ($Q_{393}E$) [74] (Figure 1). Two other SNPs also lead to an exchange of a single amino-acid, but these SNPs are located in the heavy chain, in the EGF domains. These exchanges are $S_{56}T$ and $I_{90}V$, respectively (Figure 1). Their location in the heavy chain suggests no consequences for proteolytic activity. Until today there exist no reports raising evidence for changed enzymatic properties caused by these two polymorphisms.

About 5 % of the Caucasian populations are carriers of both SNPs, the MI and MII, respectively. MI-FSAP exhibits low activating capacity towards pro-uPA, compared to wild type (WT)-FSAP, but the activation of Factor VII is not changed [74]. This suggests that the $G_{534}E$-genotype of FSAP provokes a shift to a pro-thrombotic status. Moreover, the MI-SNP of FSAP was related to late complications of carotid stenosis, indicating a direct role for FSAP in atherosclerosis [75]. Additionally, MI-FSAP was determined as a risk factor in cardiovascular disease in general [76] and associated with venous thromboembolism [77]. Concerning a relation of MI-FSAP to thromboembolism, the observations have recently been discussed critically [78-82]. The reduction in proteolytic activity, when G is exchanged for E, is most likely due to the change in charge around the active center (Figure 2, lower panel, black arrows). The relation of MII-FSAP to diseases has not yet been reported. This is, most likely due to no changes in proteolytic activity, enzymatic behavior or other functions when MII- is compared to WT-FSAP [74].

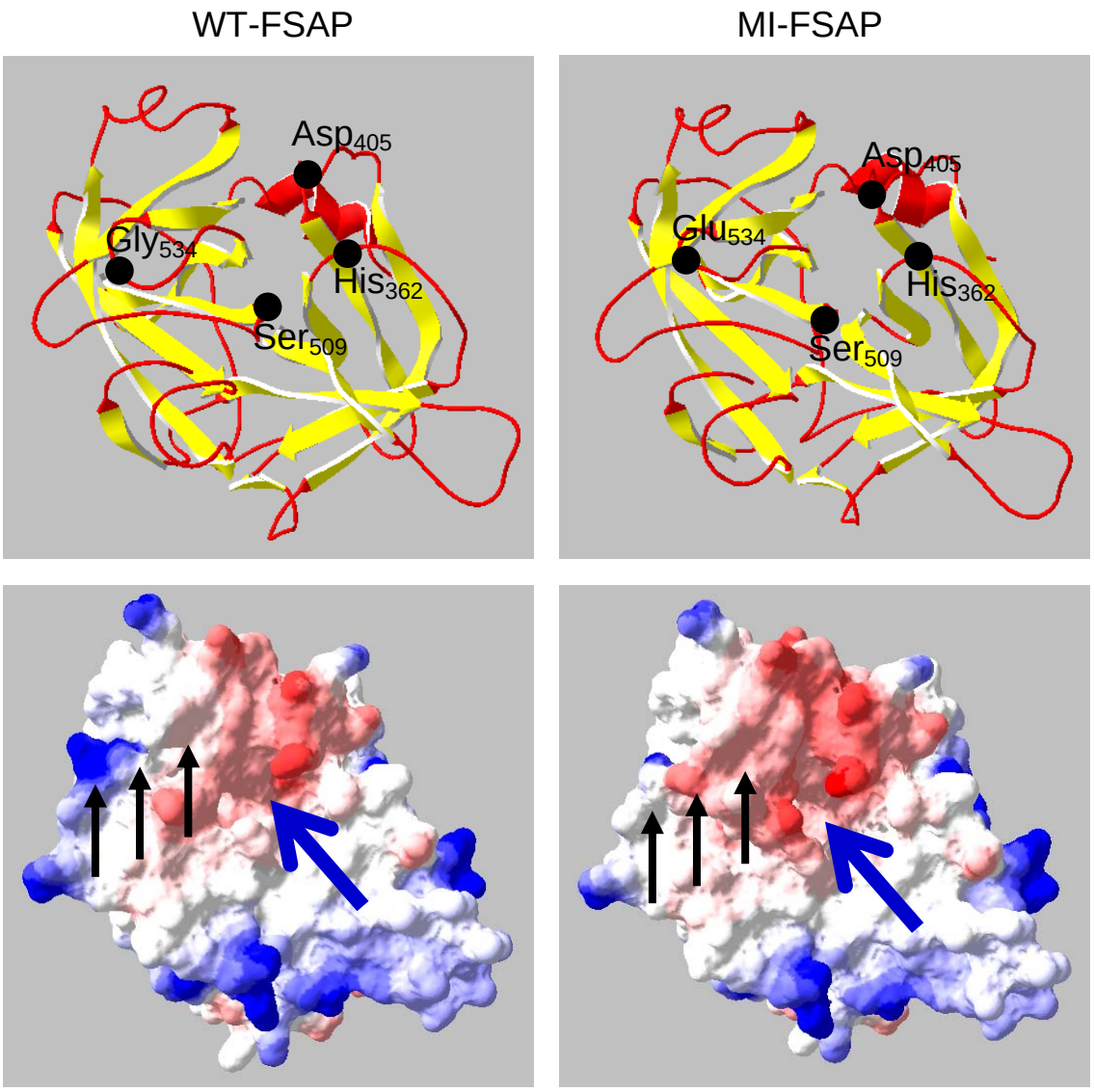


Figure 2: *Model structures of WT- and MI-FSAP light chain (serine protease domain).* The amino-acid sequence from either WT- or MI-FSAP was analyzed using Swiss-model (cf.: http://swissmodel.expasy.org/) "First approach mode". The structure for both FSAP variants was generated using the serine protease domain of two-chain tPA (1a5hA) as a template. Upper panel shows ribbons with catalytic triad (His_{362} – Asp_{405} – Ser_{509}) and the location of MI-SNP (Gly / Glu_{534}) denoted by black spots and labels. Lower panel shows the calculated molecule surface with electrostatic potential (blue = 3; white = 0; red = -6). Changes dependent on the MI-SNP are highlighted by black arrows. Center of the catalytic triad is highlighted by blue arrows.

3.4.4 FSAP and polyanions

The mechanism of FSAP activation has not been fully understood yet. The interaction with negatively charged polyanions, such as, heparin or RNA [66], accelerates the auto-catalytic activation mechanism. The polyanion can bind to latent FSAP and thereby changes the conformation of FSAP that leads to activation. Or the polyanion

serves as a template for FSAP molecules to bring them in closer proximity, therefore promoting the proteolytic activation of latent FSAP. Polyanions exhibit various functions in mammalian organisms.

Especially the polysaccharides, glycosaminoglycans are involved in developmental processes [83, 84] as well as in angiogenesis [85] and cancer [86, 87]. Their interaction with inhibitors of the coagulation cascade, namely, AT and heparin cofactor II, is important for the regulation of hemostasis [88] (see page 7). Glycosaminoglycans are located on the cell surface, stored in intracellular granules or retained within the extracellular matrix, where they can modulate protein functions [89].

Synthesis of glycosaminoglycans occurs in the Golgi. This process is non-template driven and involves numerous enzymes that assemble and sulfate the glycosaminoglycans. The amount of sulfate groups determines the negative charge density of glycosaminoglycans. Several specific patterns of sulfate groups among glycosaminoglycans lead to a discrimination of the molecules. Heparin exhibits a more dense distribution of sulfate groups than heparan sulfate, leading to a higher negative charge density [89]. Expression of the glycosaminoglycans varies with cell type; heparin is expressed in mast cells and stored in intracellular granules, whereas heparan sulfate is ubiquitously found on cell surfaces, but with tissue-specific sulfation patterns.

Although FSAP was first purified via its binding to hyaluronic acid [55], no information is available how hyaluronic acid binds FSAP. Hyaluronic acid exists as high MW and low MW polymers. The discrimination is accomplished by the synthesis and degradation of hyaluronic acid. High MW hyaluronic acid exhibits anti-angiogenic and anti-inflammatory properties, whereas the low MW polymer is pro-angiogenic and pro-inflammatory [90-93]. Furthermore, content, size and turnover of hyaluronic acid are important determinants in atherosclerosis, tissue injury and repair as well as angiogenesis and cancer [94, 95].

Recently, the contribution of polyphosphates to coagulation has been described [15]. Polyphosphate is a linear polymer of phosphate residues found in many cell types [96-101]. Polyphosphate with an approximate chain length of 70 to 75 residues is stored in platelet dense granules [102] and released upon platelet activation. It can activate FV, FXII, TFPI [15, 103] and modulate fibrin clot stability [104]. The contribution of polyphosphate to blood coagulation and its high negative charge density holds evidence for a potential interaction with FSAP, similar to heparin.

Nucleic acids have been described as cofactors for FSAP activation [66], especially RNA, which activates FSAP 10 to 100-fold stronger than DNA. RNA has been additionally reported to be involved in coagulatory processes [14]. At sites of massive cell destruction, intracellular RNA might become released and can modulate protein functions in this proximity.

3.4.5 FSAP and inhibitors

Regulation of FSAP activity can at least partly occur via its inhibition by protease inhibitors [71]. The SERPINs PAI-1 and protease nexin (PN)-1 can inhibit tPA and uPA. PAI-1 is synthesized by many cell types [19, 105] and an important regulator of hemostasis and fibrinolysis (see page 9ff). PAI-1-dependent inhibition of its target proteases occurs via covalently complex formation. Thereby, the morphology of the protease is changed [106]. The contribution of PAI-1 to several diseases, such as atherosclerosis, tumor angiogenesis or fibrosis was revealed by transgenic mouse studies [107].

PN-1 is expressed in the brain during development. A constitutive expression in the vasculature, by smooth muscle cells and endothelial cells implies contribution to vascular homeostasis [108]. In hypertension, the expression of PN-1 is increased [109]. The

inhibition mechanism of PN-1 follows a similar mechanism than that of PAI-1, also reported to be dependent on the presence of cofactors [110, 111]. In complex with their target proteases, PAI-1 and PN-1 can bind to LRP and undergo internalization [112, 113].

The presence of PAI-1 and PN-1 in the vasculature and their ability to bind polyanions suggests possible interactions with FSAP. The release and expression of PAI-1 or PN-1 during pathological situations may indicate a putative regulation of FSAP activity within these events.

3.4.6 FSAP in hemostasis & fibrinolysis

A high homology of FSAP with proteases of the coagulation and fibrinolysis systems, as well as the observation that FSAP activates FVII and pro-uPA suggests a function in blood coagulation [58, 59, 71]. However, no activation of FSAP could be detected subsequently to activation of either the intrinsic or extrinsic coagulation pathway [65]. Exogenously added FSAP shortened the plasma re-calcification and pro-thrombin times, but no changes of the activated partial thromboplastin times could be observed [71]. Fibrinolysis in whole blood is increased via pro-uPA activation by exogenously applied FSAP, whereas the exogenous addition of pro-uPA to increase fibrinolysis was not associated with activation of endogenous FSAP [65]. The activation of pro-uPA by FSAP and thereafter plasminogen conversion to plasmin by uPA or a direct interaction with uPAR appears to be of higher relevance for the regulation of extra-vascular cellular processes and pericellular extracellular matrix remodeling and turnover (see page 10ff).

3.4.7 FSAP in atherosclerosis

Proteases contribute to the microenvironment of atherosclerotic plaques, involved in cellular activities as well as extracellular matrix turnover and remodeling [47]. FSAP antigen has been found in atherosclerotic plaques, particularly detected within unstable plaques [114], but no immunostaining was found in normal vessels. The distribution throughout the plaque was similar to that of uPA. Defining the co-localization of FSAP immunostaining with classified areas within the plaques, an association mainly with macrophages and to a much lower extend with VSMC was observed [114]. Not only FSAP antigen could be detected, but also FSAP mRNA was localized in unstable atherosclerotic plaques, indicating *in situ* expression. Monocyte / macrophages have been shown to be able to express FSAP as a response to stimulation with inflammatory mediators [114]. On-site expression as well as possible protein uptake and deposition from the circulation raises evidence for a contribution of FSAP in atherosclerosis. Although, the association with unstable plaques goes well in-line with a suggested increase in overall proteolytic activity and extracellular matrix remodeling, the association of MI-FSAP, which is characterized by reduced proteolytic activity towards pro-uPA, with cardiovascular disease needs further explanation.

The importance of VSMC activation by growth factors, especially PDGF-BB within the atherosclerotic plaque has been mentioned before (see page 15ff). FSAP-dependent inhibition of PDGF-BB-mediated VSMC proliferation and migration [70] *in vitro*, additionally indicates evidence for a contribution of FSAP to plaque behavior and shape, considering the relation of VSMC to plaque stability and formation of the fibrous cap [44, 115] (see page 15ff).

3.4.8 Interaction of FSAP with growth factors

FSAP was shown to interact specifically with PDGF-BB-dependent activation of VSMC. PDGF-BB is a member of the PDGF-family of growth factors. PDGFs are characterized by disulfide bond homo- and heterodimers, exhibiting a conserved cystein-knot motive [116, 117]. Four gene products form five different members, namely, PDGF-AA, PDGF-BB, PDGF-AB [118, 119], PDGF-CC [120] and PDGF-DD [121]. The two classical members are PDGF-AA and PDGF-BB, which exhibit about 60% sequence homology. PDGF-CC and PDGF-DD are newly identified members, which have a unique two-domain structure, composed of an N-terminal complement subcomponents Clr/Cls, Urchin EGF-like (CUB) domain linked by a hinge region to the conserved C-terminal PDGF / VEGF growth factor domain [122]. The CUB domain determines latency and has to be proteolytically removed by extracellular proteases before the ligand can bind its receptor [120, 121, 123].

Two PDGF-receptors (PDGFR) exist, the PDGFRα and the PDGFRβ. PDGF-AA, PDGF-BB, PDGF-AB and PDGF-CC are ligands for the PDGFRα [119, 120, 124, 125], whereas PDGF-BB, and PDGF-DD are ligands for PDGFRβ [121, 123]. PDGF-PDGFR-signaling is involved in many cellular functions. Embryonic development, wound healing and the regulation of vascular cells are supposed to be its main scopes [126]. PDGF expression is increased in atherosclerotic lesions [45]. The activation of VSMC within atherosclerotic plaques is PDGF-dependent. Further on, the PDGF-system is linked to fibrotic diseases and cancer [127]. FSAP has been demonstrated to inhibit PDGF-BB-dependent activation of VSMC. Thereby, FSAP inhibits PDGF-BB-mediated proliferation as well as migration of VSMC [70], unraveling it as an inhibitor of the pro-atherogenic phenotype of VSMC. These effects of FSAP are dependent on its

enzymatic activity, while either addition of an FSAP-blocking antibody or addition of inhibitors neutralized its inhibitory effect [70].

FSAP also interacts with the bFGF / FGF receptor-1 system which leads to a stimulation of the extracellular regulated kinase (ERK) 1 / 2 and phosphoinositol 3 kinase (PI3K) / Akt signaling pathways in fibroblasts, thereby stimulating their proliferation and migration [128]. By the release of bFGF, bound to heparan sulfate proteoglycans, FSAP induces signaling in human umbilical vein endothelial cells (HUVEC) [129]. The observations that FSAP cleaves and inactivates bFGF as well as allows bFGF-dependent cell activation demonstrate opposing effects of FSAP with respect to the regulation of bFGF activity.

The influence of FSAP towards vascular cells is restricted to its proteolytic activity. This together with the aforementioned association of the MI-polymorphism of FSAP with cardiovascular diseases provides reasonable evidence for a contribution of FSAP to the regulation of mural cells and the remodeling mechanisms of the extracellular matrix during pathophysiological situations.

4. Aims of the study

The work presented herein has been designed to investigate the physiological functions of FSAP within the vascular system. In particular, attempts to shed light on the mechanisms that regulate FSAP-activity have been undertaken. The defined goals were;

1. Characterize in more detail the processes that contribute to FSAP-activation and –inhibition.

2. Analyze the potential interaction of FSAP or FSAP-inhibitor complexes with cell-surface clearance receptors and the consequences thereof.

3. Define the precise mechanism by which PDGF-BB-dependent signaling is inhibited by FSAP.

4. Generate recombinant FSAP-isoforms for structure-function studies.

5. Relate FSAP-function based on these newly obtained findings to different disease states, such as atherosclerosis, fibrosis and cancer.

5. Material and methods

5.1 Materials

FSAP was prepared from human plasma as described previously [130]. In brief, human plasma provided from CSL Behring (Marburg, Germany) was filtered, adjusted to pH 6.0, 150 mM NaCl and supplemented with aprotinin (CSL Behring) to a final concentration of 40 KIU/ml. Subsequently plasma was incubated for 1 h with a mAb 677-conjugated CNBr-sepharose (GE-Healthcare, Uppsala, Sweden), washed and thereafter, bound FSAP was eluted in a single step with the elution buffer (100mM glycin, 0.05% Tween 80, 40 KIE/ml aprotinin, pH 2.5). The fraction obtained from the antibody column then was supplemented with 20 % glycerol and adjusted to pH 4.5, 100 mM NaCl. The primed elution then was applied to an anion-exchange column, Source 15Q (Amersham Pharmacia, Freiburg, Germany), connected to an EKTA FPLC (Amersham Pharmacia). After loading the fraction from the first purification step, the anion-exchange column was washed and finally FSAP was eluted with a buffer containing 5 mM Na-citrate, 20 mM NaCl, 200 arginin and 200 mM lysine, pH 3.2. This buffer was designed to preserve FSAP in its single chain, inactive conformation. Subsequent to the elution, pH was adjusted to pH 4.5, the FSAP solution was filter-sterilized, aliquoted and stored on -80°C. This buffer was used in experiments as a negative control.

Artificial RNA analogue polyinosinic-polycytidylic acid (p(I:C)) and artificial DNA analogue polydeoxyinosinic-polydeoxycytidylic acid (p(dI:dC)) were from Amersham Pharmacia. Polyphosphate 65- (MW ~ 6600 Da) and 15-mer (MW ~ 1500 Da) were from Sigma (Munich, Germany) and 35-mer (MW ~ 3500 Da) was from Roth (Karlsruhe, Germany). Unfractionated heparin (UH) (MW ~ 15000 Da) was from Ratiopharm (Ulm, Germany) or as heparan sulfate, dermatan sulfate, chondroitin sulfate

C, LMW heparin (MW ~ 3000 Da), *N*-acetyl heparin, de-*N*-sulfated heparin and *N*-acetyl-de-*O*-sulfated heparin (all MW ~ 15000 Da) from Sigma, hyaluronic acid (MW ~ 100000 Da) from human placenta or rooster comb and biotinylated heparin-albumin were from Sigma. Polysialic acid (MW ≤ 38000 Da) was separated from oligosialic acid as described before [131] and generously provided by Dr. Sebastian P. Galuska (Institute for Biochemistry, Giessen, Germany). Calf intestinal alkaline phosphatase (CIAP) was from Fermentas (St. Leon-Rot, Germany). Isolated LRP protein from human placenta was generously provided by Dr. Anders Nykjaer (Institute of Medical Biochemistry, University of Aarhus, Denmark) [132]. PAI-1 was generously provided by Dr. Paul Declerck (Katholieke Universiteit, Leuven, Belgium) [133]. PN-1 was generously provided by Dr. Denis Monard (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) [134] and AT was from CSL Behring.

5.2 Isolation of platelet-derived polyphosphate and mast cell-derived macromolecular heparin

Native polyphosphate was extracted with perchloric acid [102] from platelet homogenates obtained in the initial steps of a procedure described to isolate dense granules [135], using the 19000 x g pelleted material before the separation through density gradients and generously provided by Dr. Felix A. Ruiz (Unidad de Investigacion, Hospital Universitario Puerta del Mar, Cadiz, Spain). When native polyphosphate was used as a cofactor for FSAP activation, polyphosphate 65 and native polyphosphate were applied to an OMIX C18 100 µl tip (Varian, Lake Forest, U.S.A.), to further clean it from additional contaminations.

Native macromolecular heparin (MW 750000 Da; range 500000 – 1000000 MW) was purified from granule remnants of rat serosal mast cells, as described before [136]. Briefly, granule remnants were treated with 2 M KCl to release heparin-bound

molecules (notably chymase and other proteases) from heparin proteoglycans and to disintegrate the granule remnants into heparin proteoglycan monomers [137]. The incubation mixture was then applied to a Sepharose 200 column for the isolation and separation of heparin proteoglycans. The residual chymase activity in the heparin proteoglycan fraction was inhibited with PMSF. Mast cell-derived heparin proteoglycan was generously provided by Dr. Petri T. Kovanen (Wihuri Research Institute, Helsinki, Finland).

5.3 Cell culture

Mouse vascular smooth muscle cells (VSMC) were cultured in Iscove's modified medium (IMDM) (Invitrogen, Karlsruhe, Germany) with 10 % (v/v) fetal calf serum (FCS) (HyClone, Logan, U.S.A.), 10 U/ml penicillin, 10 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium-pyruvate (Invitrogen, Karlsruhe, Germany).

WT and $LRP^{-/-}$ mouse embryo fibroblasts (MEF) were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Karlsruhe, Germany) with 10% FCS, 10 U/ml penicillin, 10 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium-pyruvate. Cells were growth arrested in serum-free medium for 18 h prior to experiments.

5.4 Plasmids and protein expression

FSAP MI-SNP ($G_{534}E$), MII-SNP ($E_{393}Q$), ΔEGF-3, $R_{313}G$-cleavage site, $H_{362}F$-active site mutants were generated by site-directed mutagenesis of the human wild-type FSAP cDNA sequence and subsequently inserted into the *EcoRI* site of pIRESpuro3 (BD Biosciences, Heidelberg, Germany). For the ΔEGF-3 mutant, the sequence

encoding amino acids 150 to 188 was removed. Plasmids for FSAP-mutants were generously provided by Dr. Thomas Weimer (CSL Behring).

Expression vectors were transfected into human embryonic kidney (HEK)-293 cells using Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany). Cells were grown in DMEM containing 10 % (v/v) FCS (HyClone), 10 U/ml penicillin, 10 µg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium-pyruvate. 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 (of 96-well plates) were counted to obtain an index of the efficiency of clone generation.

Conditioned medium was prepared with 0.2 % (v/v) FCS and an ELISA was used for the quantification of FSAP present in the conditioned media, as described before [58].

For inducible FSAP expression, FSAP coding sequence was inserted into T-REx™ expression vector pcDNA4/TO© (Invitrogen), also provided by Dr. Thomas Weimer (CSL Behring), transfected into T-REx™-293 cells (Invitrogen) and cultivated as described above. Expression was induced with 0.5 µg/ml Tetracycline (Invitrogen).

5.5 Immunocytochemistry

Cells in 8-well chamber slides (Nunc, Wiesbaden, Germany) were incubated with the test substances for the indicated times, washed and fixed with 3.7% (w/v) paraformaldehyde, permeabilised with 0.2% (w/v) Triton X-100 (Sigma) and blocked with 3% (w/v) BSA (Sigma). After incubation with the primary antibody against FSAP (mAb 1189 or mAb 677) (American Diagnostica), LRP (Rabbit polyclonal #2629

generously provided by Dr. Dudley Strickland, American Red Cross, Rockville, Maryland, U.S.A.), PDGFRβ (Upstate / Millipore, Billerica, MA, U.S.A.) or cleaved PARP (#9541) (Cell signaling, Danvers, MA, U.S.A.) [138], secondary antibodies labeled with fluoroscein isothiocyanate (FITC) or Rhodamine (Dianova, Hamburg, Germany) were used for visualization. Finally, cells were washed and preserved in Vecta-shield (Linaris, Wehrtheim-Bettingen, Germany), containing 4', 6-diamidino-2-phenylindole dehydrate (DAPI) to stain the nuclei. Slides were analyzed using a Leica fluorescence microscope or confocal spectral laser scanning microscope (Wetzlar, Germany) and the images were prepared with the Metamorph software (Visitron, Puchheim, Germany).

5.6 DNA-synthesis assays

VSMC were stimulated in medium containing 0.2 % (v/v) FCS for 36 h with the test substances. During the last 24 h 5-bromo-2-deoxyuridine (BrdU) was added and the cells were processed with a BrdU detection kit (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer.

5.7 Mitogen activated protein kinase (MAPK)-phosphorylation

Test samples were preincubated for 60 min in serum-free medium and the cells were stimulated for 5 to 15 min. Thereafter, SDS sample buffer, containing 1 mM orthovanadate was used to lyse the cells. Phospho-42/44-MAPK antibody (ERK-1/2) (Cell Signaling Technology) and an antibody directed against total MAPK (Upstate) was used for detection by Western blotting.

5.8 Western blot analysis

Proteins were separated on SDS-polyacrylamide gels and were transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare). Western blotting was performed using enhanced chemiluminescence (ECL) plus reagent (GE Healthcare) as described by the manufacturer. For Western blot analysis of FSAP protein a mixture of antibodies, directed against the N-terminal (mAb 1189) and C-terminal end (mAb 677), was used as described before [70].

5.9 FSAP binding to LRP

LRP and the respective control buffer containing 2 µg/ml BSA were immobilized, in TBS (25 mM Tris-HCl (pH 7.4) and 150 mM NaCl) in a Maxisorp plate (Nunc). Thereafter the plate was blocked with 3% (w/v) BSA. Test substances or mixtures were preincubated for 30 min prior to their application to the coated wells for 60 min. FSAP was detected by a monoclonal anti FSAP antibody (mAb 677) followed by a horseradish peroxidase (HRP) conjugated secondary antibody (Dako, Glosptrup, Denmark). The binding to control buffer containing 2 µg/ml BSA coated wells was used as a blank in all experiments and was subtracted to obtain specific binding.

5.10 Expression and purification of receptor associated protein (RAP)

The constructed plasmid containing His-tag, ampicillin (Amp)-resistance and human RAP cDNA was a kind gift from Dr. Willnow (MDC, Berlin, Germany). Extracts were purified over a ProBond^{TM} column (Promega, Mannheim, Germany) and fractions containing the protein were dialyzed against TBS pH 8.0 containing 5 mM

reduced glutathione (GSH) and 1 mM oxidized glutathione (GSSG) (both Sigma) for refolding and finally dialyzed against TBS pH 8.0.

5.11 Gel shift assays to detect polyanion binding to FSAP

6 % to 10 % polyacrylamide – bisacrylamide (37.5:1) native gels were poured in Tris Borate EDTA (TBE) (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3), with or without 6.7 M urea, in a horizontal gel-chamber. 5 µg FSAP was preincubated for 30 min with or without respective polyanions (10 µg), native sample buffer (TBE with sucrose and bromphenol blue) was added and samples loaded on the gel. After separation the gel was stained either with toluidine blue to visualize polyanions (data not shown) or with coomassie brilliant blue to visualize proteins.

5.12 Competition of heparin binding to immobilized FSAP with various polyanions

Microtiter plates (Nunc) were coated with 50 µl of a 10 µg/ml FSAP solution in 100 mM sodium carbonate (pH 9.5) over night (ON) at 4°C. Wells were washed and non-specific binding sites were blocked with TBS containing 3 % (w/v) BSA for 60 min. Biotinylated heparin-albumin (0.5 ng/ml) mixed with dilutions of polyanions was allowed to bind for 60 min at room temperature in TBS containing 0.1 % (w/v) BSA, after which the plates were washed three times with TBS containing 0.1 % (w/v) Tween20 (TBS-T). Bound biotinylated heparin-albumin was detected using HRP-conjugated streptavidin (DAKO) and immunopure TMB substrate kit (Thermo Fischer Scientific, Rockford, U.S.A.).

5.13 Binding studies with recombinant FSAP-variants from conditioned media

Heparin-albumin-biotin (2 µg/ml), PDGF-BB (1 µg/ml) or pAb against FSAP (2 µg/ml) were immobilized on maxisorp plates in 100 mM sodium carbonate (pH 9.5) ON at 4°C. Plates were washed with TBS-T and were blocked with TBS containing 3 % (w/v) BSA. Conditioned media of the different FSAP-isoforms were allowed to bind to heparin-biotin-albumin or PDGF-BB, or pAb. Subsequently plates were washed and bound FSAP was detected by the addition of mAb 677 followed by HRP-conjugated secondary antibody (DAKO).

5.14 FSAP enzyme activity assay

To determine activation and enzymatic activity of FSAP, the hydrolysis of the chromogenic substrate S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilinedihydro-chloride) (Haemochrom, Essen, Germany) at a final concentration of 0.2 mM was measured over a time period of 60 min at 37°C in a microplate reader EL808 (BioTek Instruments, Winooski, Vermont, U.S.A.) at 405 nm. If the inhibitors, PN-1, PAI-1 or aprotinin were used, they were preincubated with FSAP for 10 to 30 min prior to the enzymatic activity assay. When the assays were performed with plasma-derived FSAP, it was used at a final concentration of 1 µg/ml. For the measurement of FSAP activity from conditioned media, mAb 677 (2 µg/ml) was immobilized in maxisorp plates, blocked with TBS containing 3 % (w/v) BSA and washed with TBS-T. The FSAP containing conditioned medium was added to the wells to allow FSAP-capture. Finally, the plates were washed and pro-uPA activation was measured. The standard assay system consisted of TBS, 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). The change in

absorbance was followed over a period of 60 min at 37°C in a microplate reader EL 808 at 405 nm. To test the FSAP activity directly the conversion of the chromogenic substrate S-2288 was determined. Since the direct cleavage of the amidolytic substrate is slow, due to the low amount of FSAP in the conditioned media, incubations were performed for up to 7 h. Polyanions were used at a concentration of 1 µg/ml in this experimental setting. In case of using an inhibitor, it was added to the plates with and without polyanion 10 min before adding the chromogenic substrate.

5.15 PDGF-BB cleavage by FSAP

Cleavage experiments were performed with ^{125}I-PDGF-BB (GE Healthcare) and cleavage was followed by SDS-PAGE under reducing conditions (10% v/v β-mercaptoethanol) and autoradiography. Additionally, recombinant PDGF-BB (R&D Systems, Wiesbaden, Germany) was incubated with FSAP in the absence or presence of cofactors in TBS. For determining the cleavage sites in the PDGF-B chain the gels were blotted onto PVDF membranes and the bands were cut out for further analysis. The amino-terminal sequences of the fragments were determined by automated Edman degradation using an Applied Biosystems 492 pulsed liquid phase sequencer equipped with an on-line 785A phenylthiohydantoin derivative analyzer. Ten cycles of Edman degradation were performed. The obtained amino-acids were aligned with the PDGF-B chain sequence allowing for detection of multiple N-termini in the preparation.

5.16 Statistical test

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

6. Results

6.1 FSAP is activated by heparin and polyphosphate

6.1.1 FSAP binding to polyanions

A unique feature of FSAP is its propensity to bind to negatively charged polyanions. The aim is to define which polyanion is important for the activation of FSAP. Therefore, the ability of different polyanions to bind to FSAP have been tested. This binding represents the first step for the activation of FSAP. The interaction of FSAP with polyanions could be shown by the following experimental setups.

The binding of polyanions to FSAP could alter the electrophoretic mobility of both interacting components. Preincubation of FSAP with unfractionated heparin (UH), low MW (LMW) heparin, polyphosphate 65 and 35 (polyphosphate of different chain-length), induced a shift in the mobility of FSAP in polyacrylamide gels with, or without urea. Heparan sulfate had a very weak effect and other polyanions had no influence at all. When BSA was used as a control, none of the polyanions induced a shift in the BSA band (Figure 3).

To examine whether the different polyanions use the same region in the FSAP molecule for binding, competition binding assays where the binding of biotinylated heparin to FSAP was measured were performed (Figure 4). UH competed with biotinylated heparin for binding to FSAP whereas LMW heparin showed clearly diminished ability. All other heparin derivates as well as chondroitin sulfate, were completely ineffective (Figure 4, upper panel and data not shown). Polyphosphate bound to FSAP in a specific and chain length-dependent manner and competed for the binding of biotinylated heparin to FSAP. Dermatan sulfate, polysialic acid, heparan sulfate, and hyaluronic acid showed no competition, indicating no binding to FSAP

(Figure 4, lower panel and data not shown). Using gel-shift and competition binding assays it could be demonstrated that UH binds strongly, LMW weakly, *N*-acetyl heparin, de-*N*-sulfated heparin, *N*-acetyl-de-*O*-sulfated heparin, chondroitin sulfate, dermatan sulfate, heparan sulfate and polysialic acid did not bind to FSAP. In contrast to earlier studies, hyaluronic acid did not bind to FSAP. Polyphosphate, in a size-dependent manner, bound to FSAP.

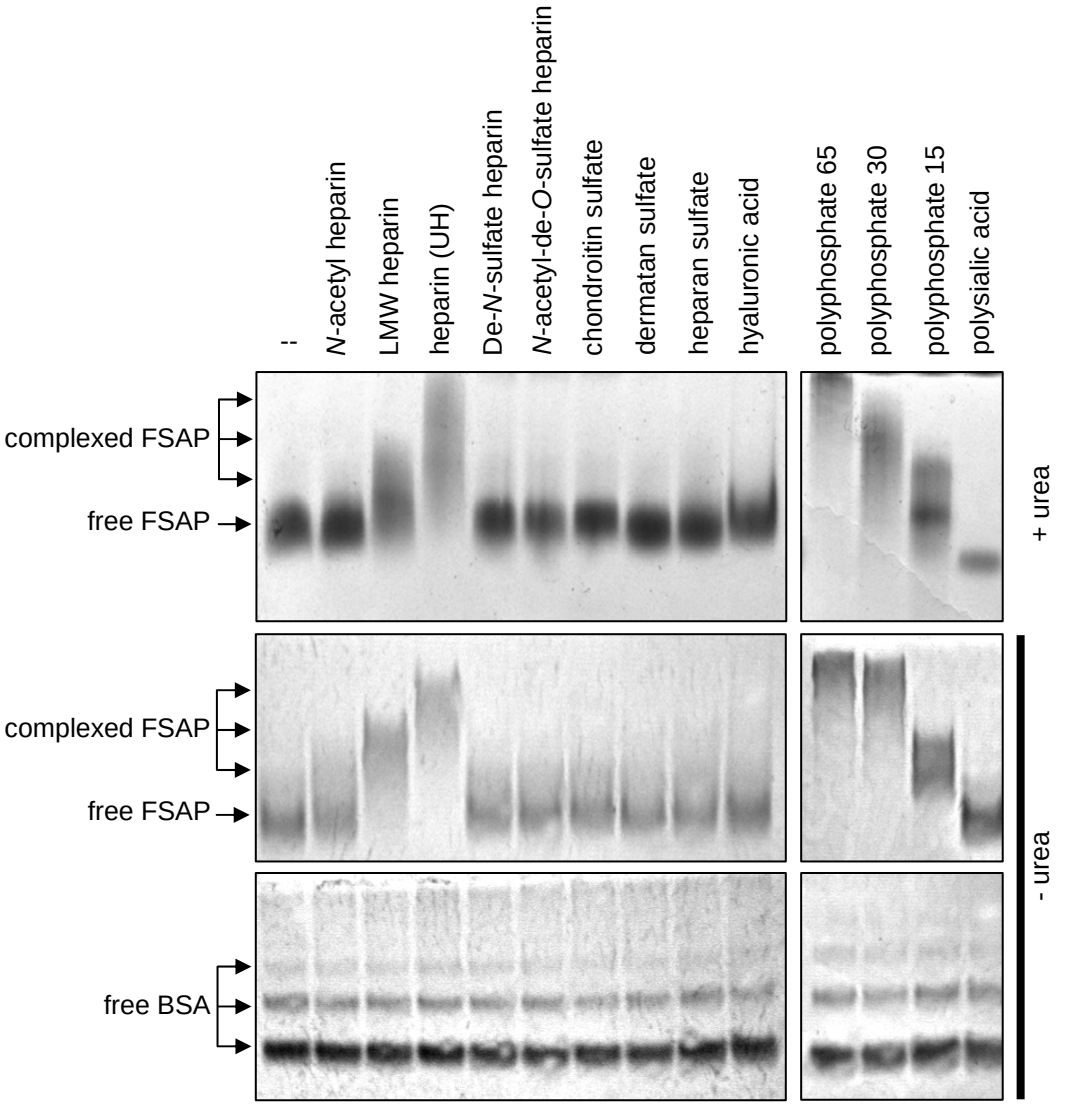


Figure 3: *Binding of polyanions to FSAP, indicated by a change in electrophoretic mobility.* FSAP or BSA (5 µg/lane) were preincubated with the respective polyanion (10 µg/lane) for 30 min at RT. Native sample buffer was added and samples were directly loaded on gels containing urea (upper panel) or native (middle and lower panel) polyacrylamide gels. Shifted bands (complexed FSAP and polyanion) indicate the binding of the particular polyanion to FSAP.

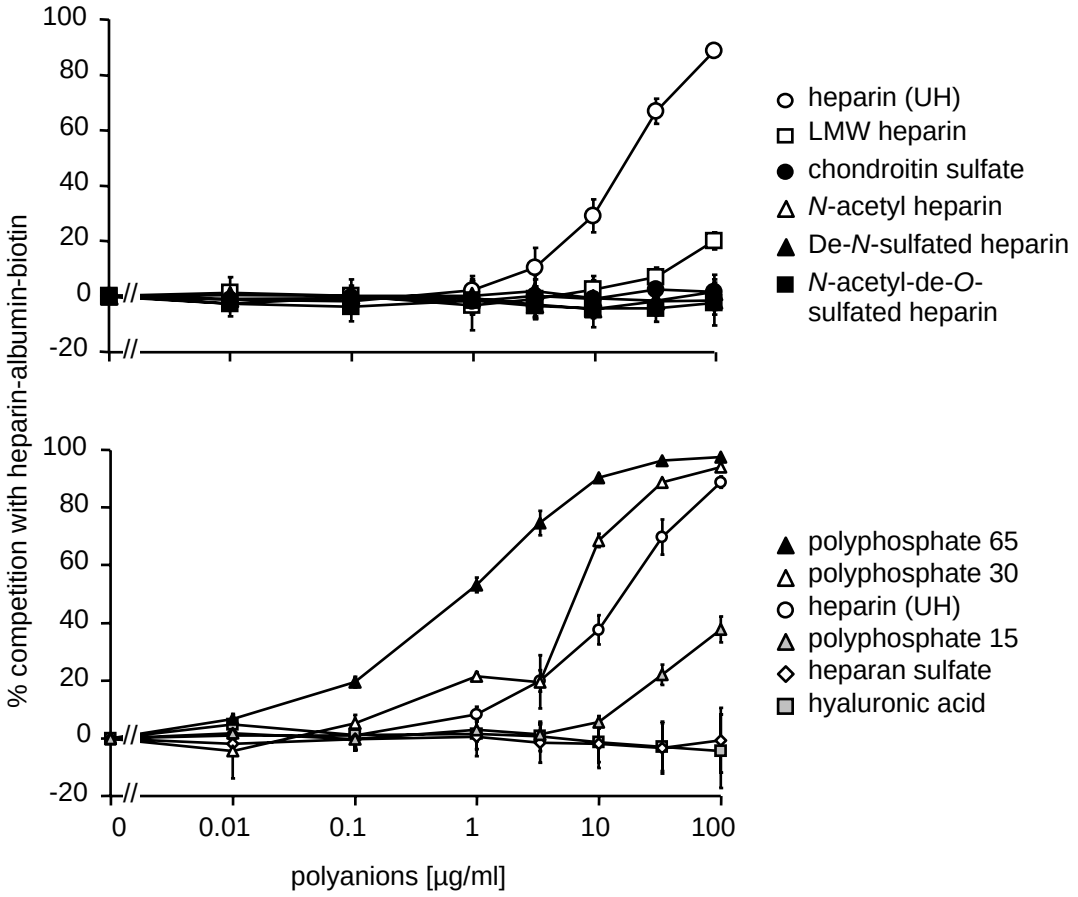


Figure 4: *Binding-competition of different polyanions with heparin-biotin for FSAP.* FSAP (10 µg/ml) was immobilized and heparin-derivates (upper panel) or other polyanions (lower panel) (0 – 100 µg/ml) were mixed with biotinylated heparin-albumin (0.5 ng/ml) and added to the plate. Detection of bound biotinylated heparin-albumin was measured by HRP-conjugated streptavidin and TMB substrate. Data is shown as % competition of the polyanion with biotinylated heparin-albumin (mean ± SEM, n = 4).

6.1.2 Activation of FSAP by different polyanions

Ensuing a binding of polyanions to FSAP, this interaction may increase the auto-catalytic activation of FSAP. Assessing different variations of heparin and other polyanions will specify the requirements for this mechanism. Next all the polyanions described above have been analyzed with respect to their abilities to activate FSAP. UH was a strong activator whereas LMW heparin activated FSAP to a smaller extent, and all other tested heparin-derivates exhibited no activation (Figure 5, upper panel). Polyphosphate showed potent activation of FSAP to the same extent as UH in a chain

length-dependent manner. Heparan sulfate and dermatan sulfate showed weak activation of FSAP at high concentrations (Figure 5, middle panel). Polysialic acid and hyaluronic acid did not activate FSAP (Figure 5, lower panel). To show the specificity of the polyphosphate effect it was degraded with a phosphatase (Figure 6C). The polyphosphate-associated accelerating effect on FSAP-activity was decreased by phosphatase pretreatment in a time- (Figure 6A) and dose-dependent (Figure 6B) manner. As a control, it could be observed that phosphatase treatment did not influence UH-mediated activation of FSAP (Figure 6). These studies showed that FSAP auto-activation is efficiently increased by UH, by LMW heparin to lower extent, while glycosaminoglycans have a weak effect. *N*-acetyl heparin, de-*N*-sulfated heparin, *N*-acetyl-de-*O*-sulfated heparin, polysialic acid and hyaluronic acid, again, totally failed to increase FSAP activity. Polyphosphate accelerated the FSAP auto-activation in a comparable fashion to UH. Overall, the pattern of binding of polyanions to FSAP was identical to the pattern of their ability to activate FSAP.

To quantify the ability of heparin or polyphosphate to activate FSAP, the values for K_M and v_{max} towards the chromogenic substrate S-2288 were measured. The K_M was reduced when heparin or polyphosphate were present, but not significantly, whereas v_{max} was significantly increased by 5-fold when heparin and 6-fold when polyphosphate was present (Figure 7). The difference between heparin and polyphosphate with respect to FSAP activation was not significant, but obvious ($p=0.1031$). Compared to K_M where no difference could be observed (Figure 7).

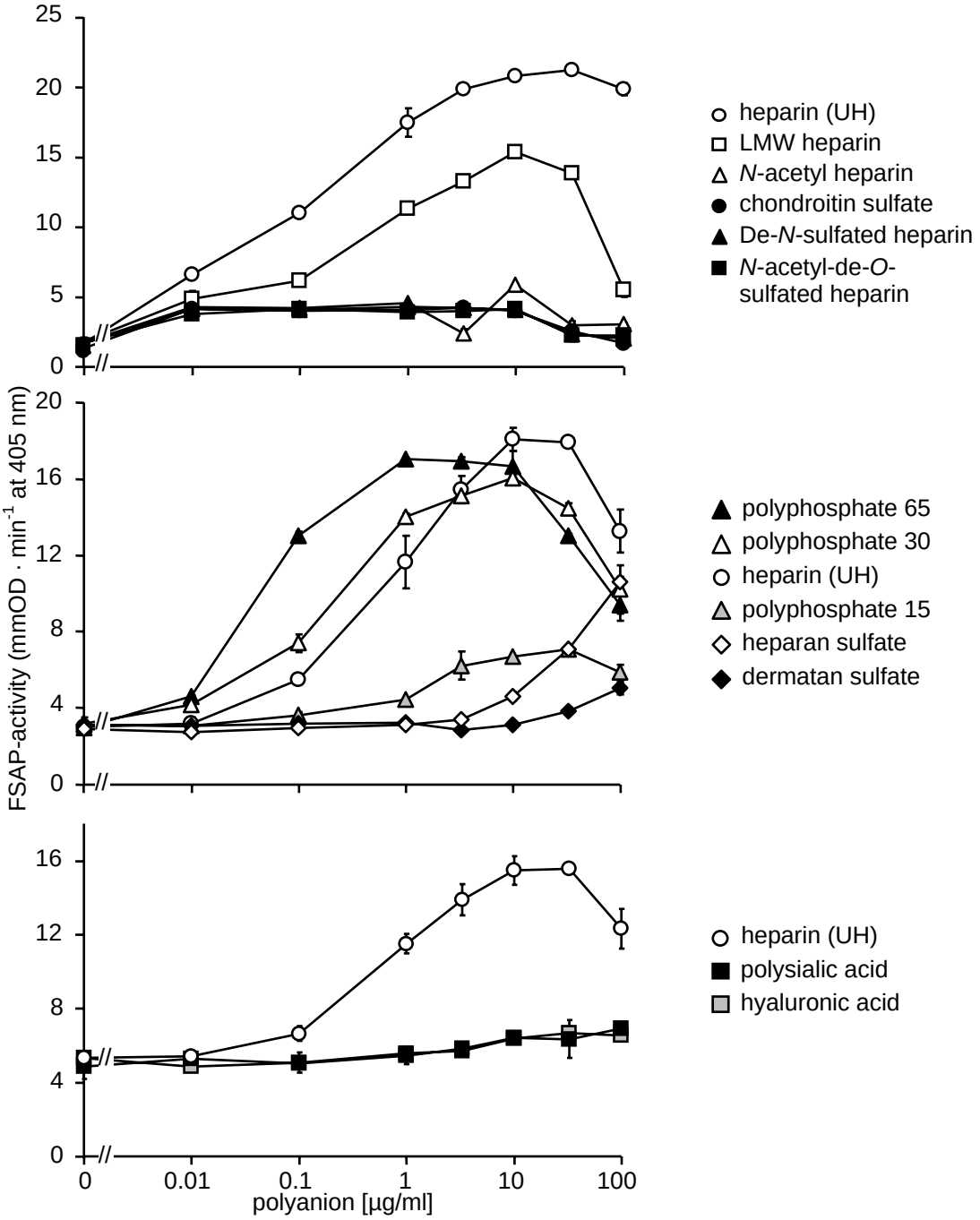


Figure 5: *FSAP activity dependent on the presence of different polyanions.* Polyanions in the range of 0.01 – 100 µg/ml were added to FSAP (1 µg/ml), and FSAP-activity (mmOD · min^{-1}) was determined, with the conversion of the chromogenic substrate S-2288 (mean ± SEM, n = 4).

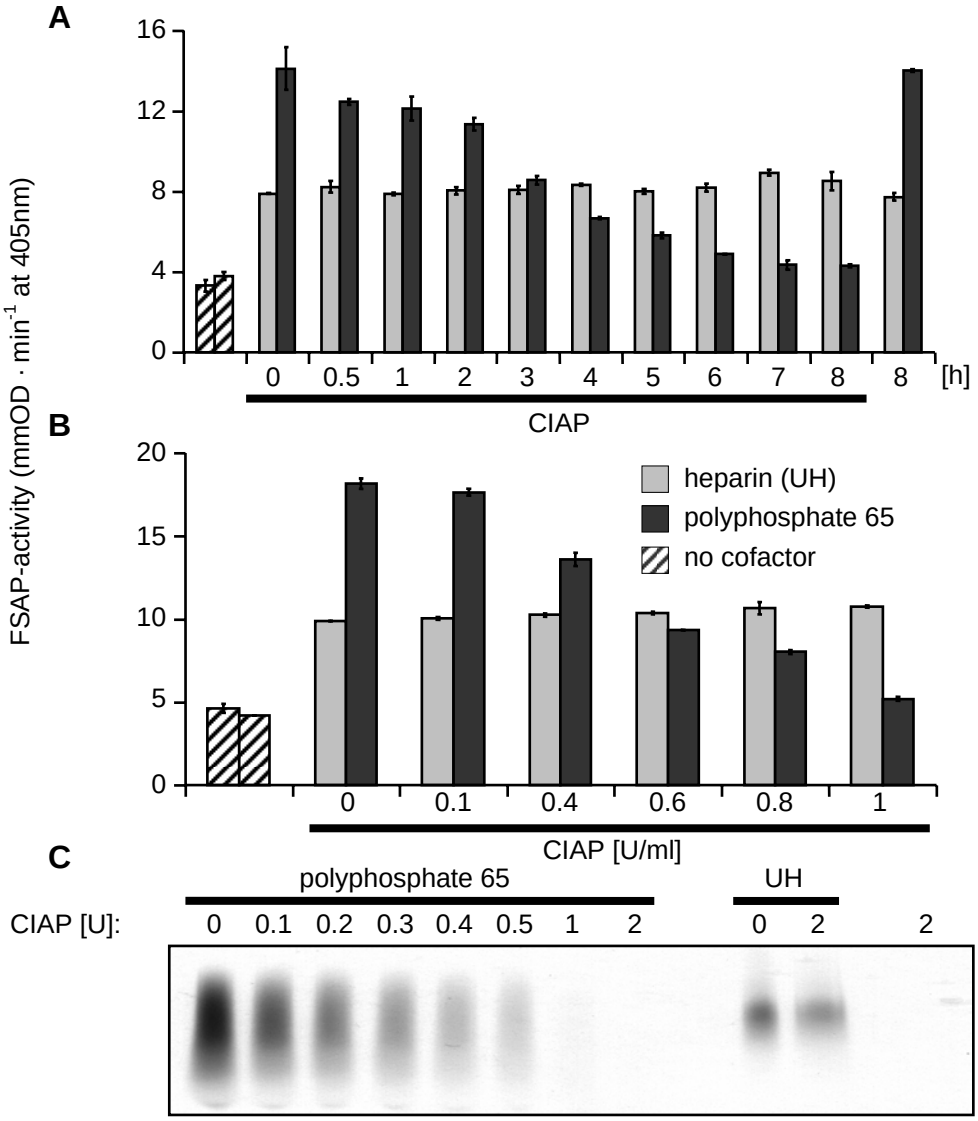


Figure 6: *Effect of phosphatase pretreatment on polyphosphate-dependent activation of FSAP.* (A) Polyphosphate 65 or heparin (1 µg/ml) was incubated in 50 mM Tris (pH 9.5) for 0 – 8 h on 37°C with 1 U/ml CIAP. Then polyphosphate or heparin – CIAP solution was added to FSAP or (B) 1 µg/ml polyphosphate 65 or heparin was incubated with CIAP in the concentration range of 0.1 – 1 U/ml in 50 mM Tris (pH 9.5) for 4 h on 37°C and subsequently added to FSAP. The FSAP-activity was measured with the conversion of chromogenic substrate S-2288 (mean ± SEM, n = 3, similar results have been obtained from 2 independent experiments). (C) To visualize degradation of polyP, 5 µg polyP 65 or heparin was incubated for 4 h on 37°C in 50 mM Tris (pH 9.5) with CIAP (0.1 – 2 U). CIAP-treated polyP and heparin was run on a urea-gel and stained with toluidine blue.

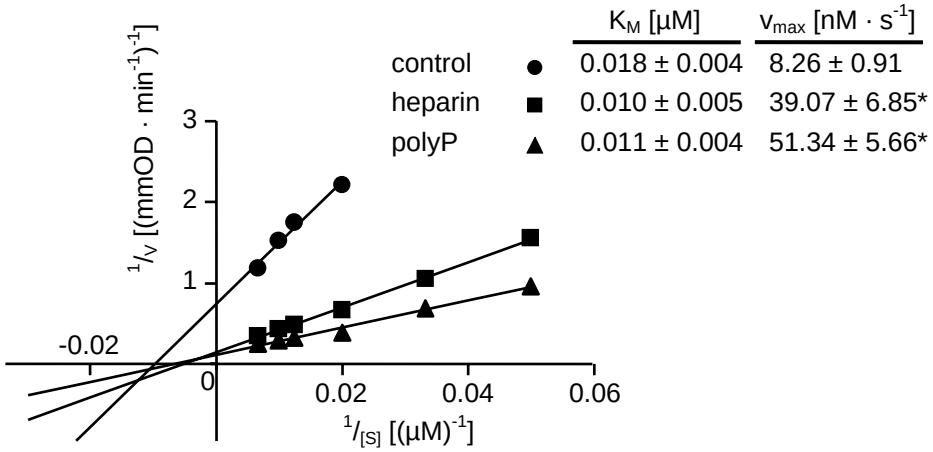


Figure 7: *Lineweaver-Burke-Plot for K_M and v_{max}-determination of FSAP with cofactors.* FSAP activity was measured with a concentration range of the substrate S-2288 (10 – 150 µM) without cofactor (circles), with heparin (squares) or polyphosphate (triangle)(both 1 µg/ml). Values for K_M or v_{max} were determined by linear regression. Figure shows one representative example of 4 experiments (n = 3) with similar results. * indicates $p<0.05$ versus control.

6.1.3 Polyanions as cofactors for the inhibition of FSAP by SERPINs

The regulation of FSAP activity occurs via specific inhibition by inhibitors present in the circulation or adjacent tissue. One class of inhibitors are the SERPINs. A contribution of polyanions to FSAP regulation by this class of inhibitors was investigated. SERPINs exhibit enhanced or altered substrate specificity in the presence of cofactors [139]. PAI-1 and PN-1 neutralized the enzymatic activity of FSAP as measured by the direct chromogenic substrate S-2288 (Figure 8A). Complete inhibition at lower concentrations was achieved with PAI-1 compared to PN-1, whereas heparin was needed as a cofactor in case of PAI-1. The formation of complexes between FSAP and PN-1 or PAI-1 could be observed in a dose dependent manner with Western blotting (Figure 8B). To examine the cofactor function of polyanions with respect to FSAP inhibition, active tc-FSAP was preincubated with PAI-1 or AT with or without different concentrations of polyanions. Inhibition of FSAP by PAI-1 was increased by UH, LMW heparin and to a lower extent by *N*-acetyl heparin (Figure 9A, upper panel). Polyphosphate exhibits a strong cofactor function for the inhibition of FSAP by PAI-1 in a chain length-dependent manner. Heparan sulfate was a cofactor at high

concentrations (Figure 9A, lower panel), dermatan sulfate and polysialic acid at even higher concentrations (data not shown) while hyaluronic acid exhibited no effect at all (Figure 9A, lower panel). In the case of inhibition of FSAP by AT only UH and heparan sulfate were able to serve as cofactors (Figure 9B). Polyphosphate and other tested polyanions showed no cofactor properties for the AT-dependent FSAP inhibition (Figure 9B, and data not shown). LMW heparin, *N*-acetyl heparin (Figure 9B, upper panel) and polyphosphate (Figure 9B, lower panel) increased the activity of FSAP even in the presence of AT. There was a difference in the cofactor effect of polyanions with respect to inhibition of FSAP by PAI-1 and AT.

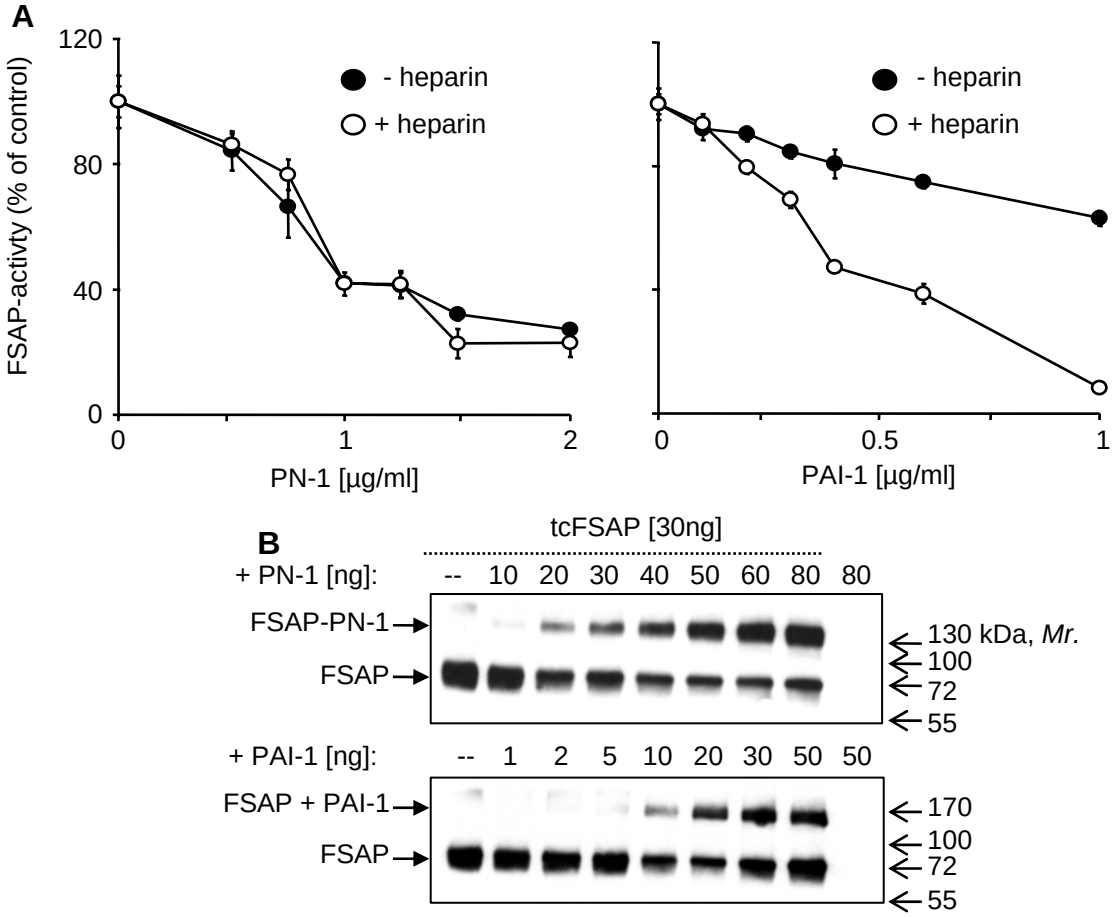


Figure 8: *FSAP interaction with PN-1 or PAI-1.* (A) FSAP (1 µg/ml) was preincubated for 30 min with either PN-1 (0 – 2 µg/ml) (left panel) or PAI-1 (0 – 1 µg/ml) (right panel) without (closed circles) or with (open circles) heparin (10 µg/ml) (mean ± SD, n = 3). Thereafter, FSAP activity was measured via the conversion of S-2288. (B) FSAP (30 ng/lane) was preincubated with PN-1 (upper panel), in the concentration range 10 – 80 ng/lane, or PAI-1 (lower panel) in the concentration range 1 – 50 ng/lane. Western blot analysis was performed using two monoclonal antibodies against FSAP. Single FSAP and FSAP-inhibitor complexes are indicated by arrows.

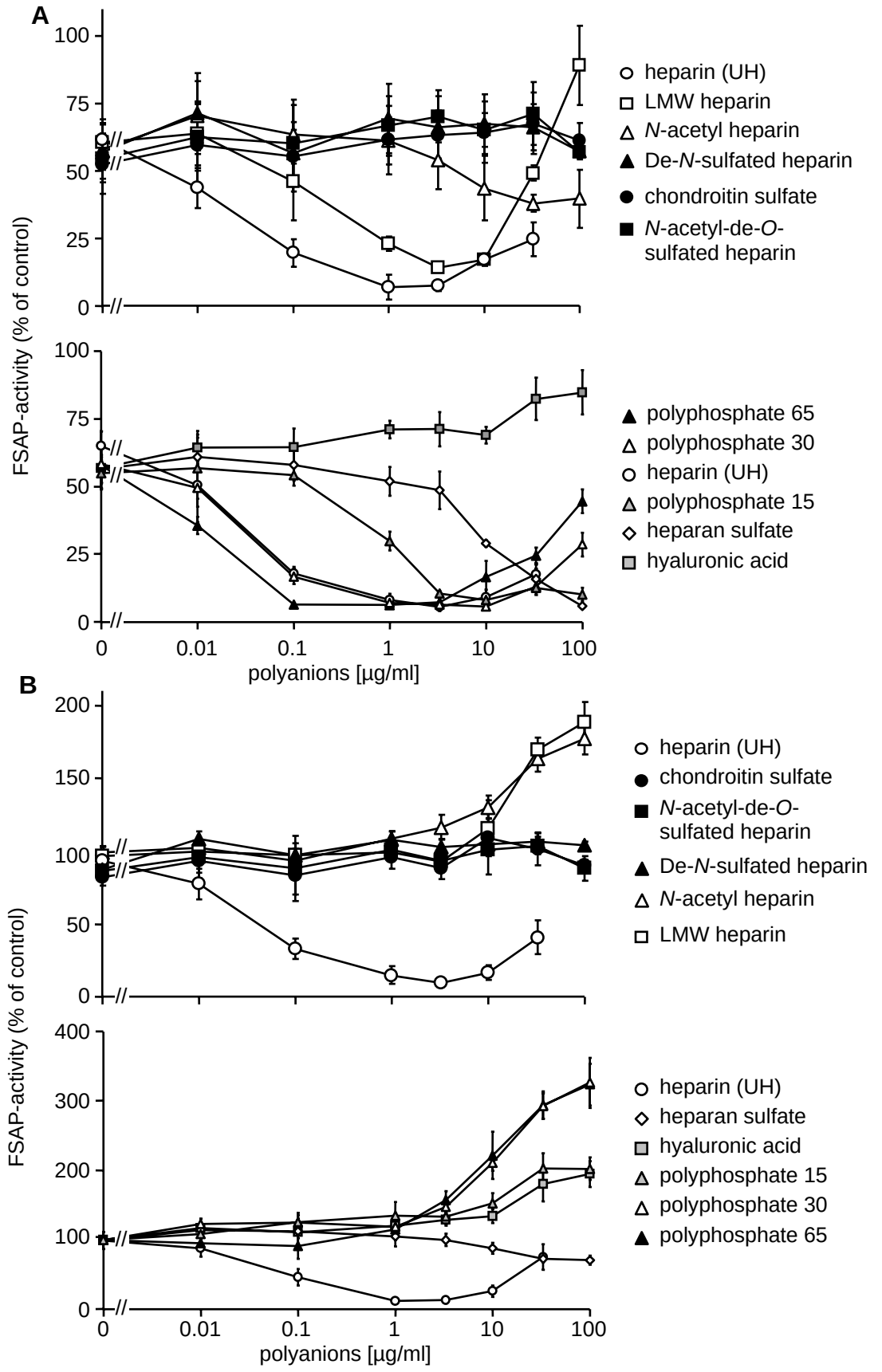


Figure 9: *FSAP inhibition by PAI-1 or AT, dependent on the different polyanions.* FSAP (1 µg/ml) was preincubated either with PAI-1 (1 µg/ml) (A) or with AT (5 µg/ml) (B) for 30 min without, or with heparin-derivates (upper panels) or other polyanions (lower panels) in the concentration range of 0.01 – 100 µg/ml. FSAP-activity (mmOD · min^{-1}) was determined and inhibition was calculated as % to FSAP-activity without inhibitor (mean ± SEM, n = 4).

Only UH and related molecules are cofactors for inhibition with AT, whereas UH and related molecules, as well as polyphosphate, are cofactors for inhibition by PAI-1. The more detailed analysis of PAI-1-dependent inhibition of FSAP with respect to the cofactor function of UH and polyphosphate 65 revealed that the IC_{50} of PAI-1, at the given conditions, was reduced to 50 % by UH and polyphosphate 65 (Figure 10).

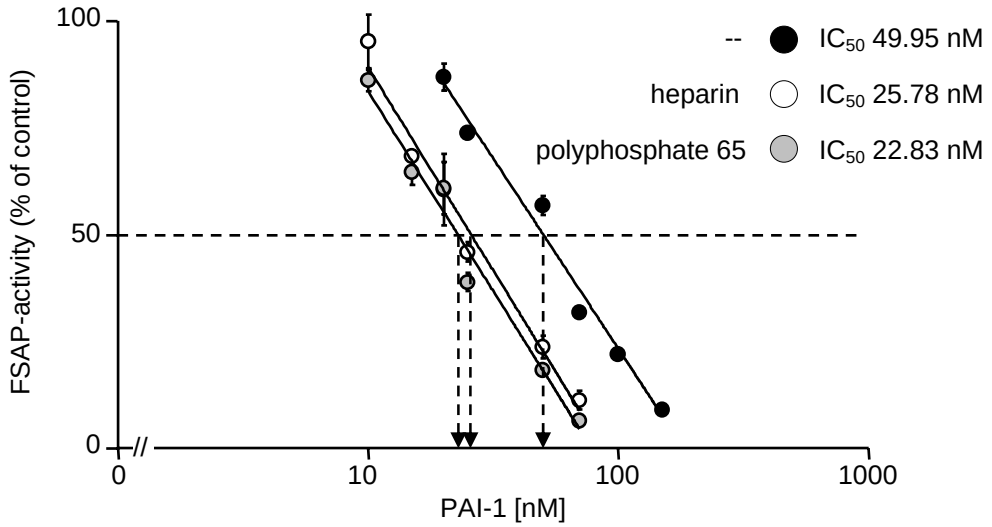


Figure 10: *IC_{50}-values for PAI-1-dependent FSAP inhibition.* FSAP (1 µg/ml ≡ 15 nM) was incubated with PAI-1 in the concentration range of 5 – 150 nM (≡ 0.23 – 7 µg/ml), without co-factor or with heparin or polyphosphate 65, for 30 min at RT. FSAP-activity (mmOD · min^{-1}) was determined and calculated to % of FSAP activity without inhibitor. The inhibitory efficiency of PAI-1 for the inhibition of FSAP is increased by 1.98 ± 0.2 fold in the presence of UH and 2.23 ± 0.28 fold in the presence of polyphosphate 65 (mean ± SEM, n = 3, figure shows one representative of 5 independent experiments with similar results).

6.1.4 Polyphosphate as a cofactor for the FSAP-dependent inhibition of VSMC proliferation

VSMC proliferation is a critical event in numerous diseases [127, 140]. Therefore, a regulation of VSMC activation is of high interest. It was recently shown by us that inhibition of VSMC proliferation by FSAP occurs through a specific proteolytic cleavage of PDGF-BB [70, 141], and that this mechanism is promoted by heparin or RNA [142]. Identification of other cofactors among the investigated polyanions opens new gates for the physiological relevance of FSAP-dependent PDGF-BB inhibition. Now it could

be observed that LMW heparin and heparan sulfate also increased the inhibitory effect of FSAP on VSMC proliferation, but to a lower extent compared to UH. Polyphosphate also promoted the inhibitory effect of FSAP on VSMC proliferation, whereas de-*N*-sulfate heparin and hyaluronic acid were ineffective (Figure 11). The ability of the different polyanions to inhibit cell proliferation matched with the pattern of FSAP binding and activation.

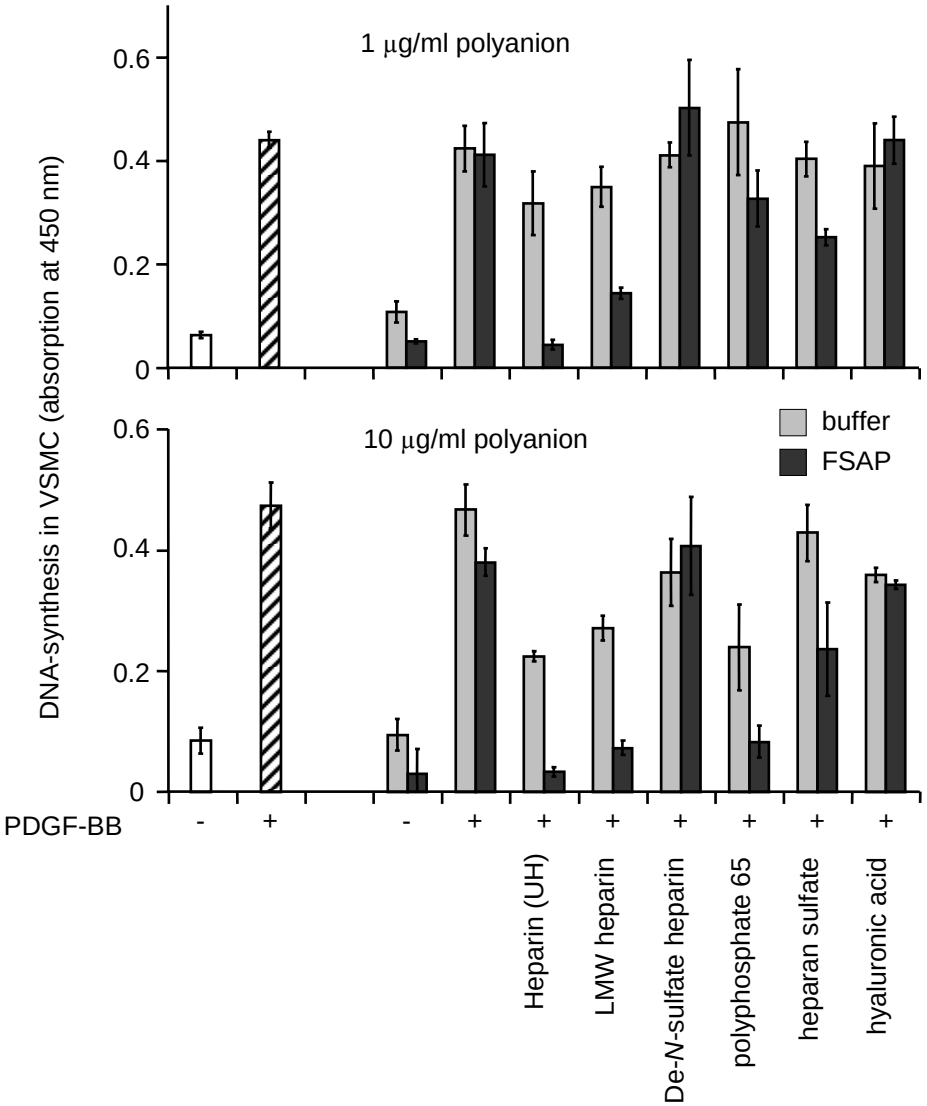


Figure 11: *Cofactor function of polyanions on FSAP-dependent inhibition of PDGF-BB-mediated VSMC activation.* PDGF-BB (20 ng/ml) was preincubated without (grey columns) or with (black columns) FSAP (0.25 µg/ml) and 1 µg/ml (upper level) or 10 µg/ml (lower panel) of the different polyanions for 1 h at 37°C in serum free medium. Subsequently, VSMC were stimulated for 36 h in medium containing 0.2 % FCS. DNA-synthesis was measured (mean ± SD, n = 3) using a kit detecting BrdU incorporation into newly synthesized DNA.

6.1.5 Assessment of native heparin and native polyphosphate as cofactors for FSAP function

UH isolated from skin and synthetic polyphosphate demonstrated the most effective cofactor function among all investigated polyanions. The confirmation of these observations with natural material will strengthen their physiological contribution to FSAP regulation. Mast cell-derived macromolecular heparin and platelet-derived polyphosphate were isolated as native substances and tested for their interaction with FSAP. The mast cell-derived heparin bound to FSAP as indicated by a shift in mobility in native polyacrylamide gel (Figure 12A) and, when compared to UH, native heparin was even more efficient with respect to competition of biotinylated heparin binding to immobilized FSAP (Figure 12B, left panel) and FSAP-activation (Figure 12C, left panel). As shown by the mobility shift assay polyphosphate isolated from platelets also bound FSAP (Figure 12A) and more efficiently competed with biotinylated heparin for the binding to immobilized FSAP, than its synthetic analogue polyphosphate 65 (Figure 12B, right panel). The activation of FSAP by native polyphosphate was much lower when compared to the synthetic material (Figure 12C, right panel). Taken together, mast cell-derived heparin was identical to UH in all aspects investigated, but there were differences between platelet-derived and synthetic polyphosphate.

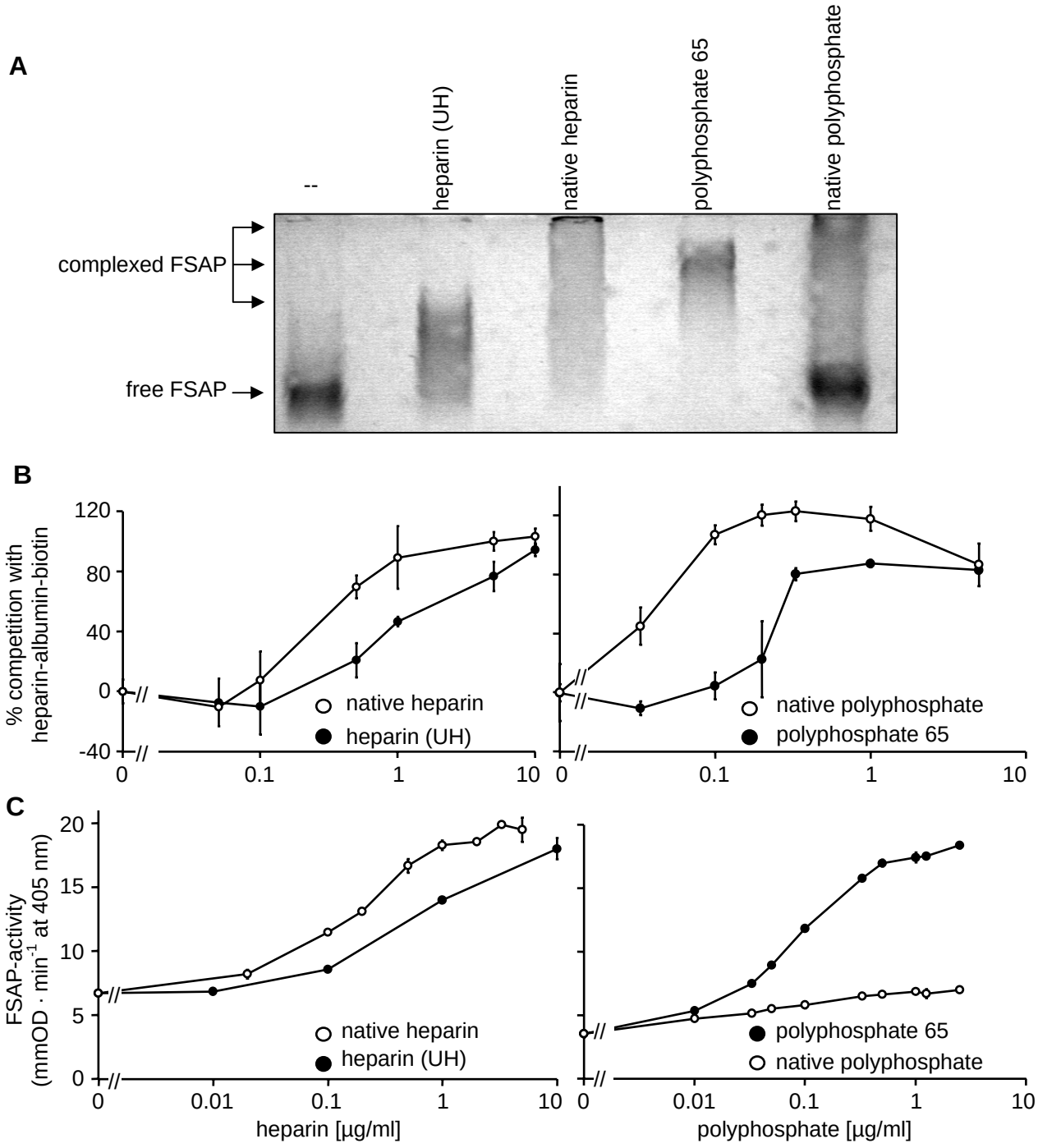


Figure 12: *Assessment of native mast cell-derived heparin or platelet-derived polyphosphate with respect to cofactor function.* (A) FSAP (5 µg/lane) was preincubated with unfractionated heparin (UH), native heparin, polyphosphate 65 or native polyphosphate (each 2 µg/lane) and directly loaded to native polyacrylamide gel. Shifted bands (complexed FSAP) indicate binding of the respective polyanion to FSAP. (B) FSAP (10 µg/ml) was immobilized and synthetic or native heparin (0.05 – 10 µg/ml) (B, left panel) as well as synthetic or native polyphosphate (0.033 – 5 µg/ml) (B, right panel) were mixed with biotinylated heparin-albumin (0.5 ng/ml) and added to the plate. Bound biotinylated heparin-albumin was measured by POD-conjugated streptavidin and TMB substrate (mean ± SD, n = 3). (C, left panel) Unfractionated heparin (0.01 – 10 µg/ml), native heparin (0.02 – 5 µg/ml) or (C, right panel) synthetic or native polyphosphate (0.01 – 2.5 µg/ml) was added to FSAP (1 µg/ml). FSAP-activity (mmOD · min^{-1}) was determined (mean ± SD, n = 3).

6.2 FSAP-inhibitor complexes are internalized via LRP

6.2.1 Binding of the FSAP-inhibitor complexes to LRP

The scavenger and signaling receptor LRP is involved in atherosclerosis and other diseases [50, 51, 143]. The LRP-dependent internalization of protease-protease inhibitor complexes represents an important feature of LRP [112, 113, 144]. Therefore, the interest was high in a potential interaction of either FSAP or FSAP in complex with its inhibitors with LRP. PAI-1 and PN-1 are SERPINs produced locally in the vessel wall. FSAP-PN-1 complex showed a strong specific binding whereas FSAP-PAI-1 complex showed weaker binding and FSAP by itself, or in complex with aprotinin (data not shown), exhibited no binding at all to LRP (Figure 13). Concentration-dependent analysis confirmed that FSAP-PN-1 complex has a higher affinity for LRP compared to the FSAP-PAI-1 complex (Figure 13A). The binding of FSAP-PN-1 complex could be inhibited with either the addition of heparin (Figure 13B) or receptor associated protein (RAP), a 39 kDa LRP antagonist, known to inhibit ligand binding to LRP [145] (Figure 13C). Maximal inhibition (≈ 70%) was observed with 10 µg/ml heparin or RAP (data not shown). Therefore this concentration was used in the following experiments.

6.2.2 Binding of FSAP-PN-1and PAI-1 complexes to LRP on cells

To demonstrate that the binding of FSAP inhibitor complexes, shown with isolated proteins (Figure 13), to LRP holds true also under more physiological conditions LRP-bearing VSMC or WT and $LRP^{-/-}$ mouse embryo fibroblasts (MEF) were used. The binding of FSAP, FSAP-PN-1 or FSAP-PAI-1 complexes to cells was analyzed with immunofluorescence microscopy. Application of FSAP alone to VSMC did not lead to an accumulation of cellular FSAP, whereas FSAP-PN-1 or FSAP-PAI-1 complexes were internalized by VSMC (Figure 14).

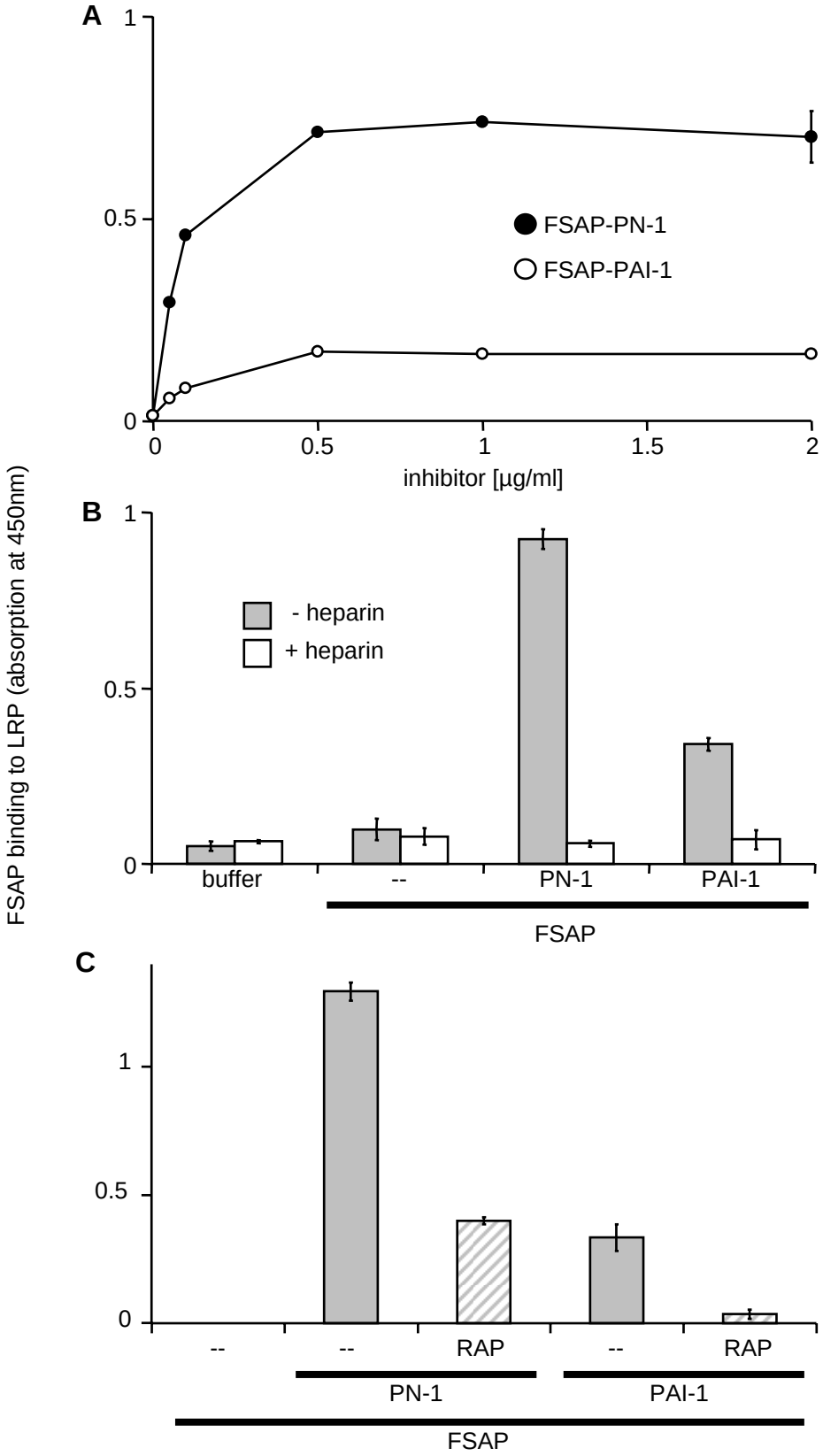


Figure 13: *FSAP-inhibitor complexes binding to LRP (isolated protein).* (A) FSAP (1 µg/ml) was preincubated with PN-1 (closed circles) or PAI-1 (open circles) in the concentration range of 0 – 2 µg/ml for 30 min to allow complex formation and binding of the FSAP-inhibitor complexes to immobilized LRP (1 µg/ml) was measured by specific monoclonal antibody against FSAP. To demonstrate the influence of heparin (B) or RAP (C) (both 10 µg/ml) for FSAP-inhibitor complexes binding to LRP, FSAP binding was measured as before. FSAP was preincubated with PN-1 or PAI-1 (2 µg/ml) in the absence (dark bars) or the presence (white bars) of heparin (B), or without (dark bars) or with RAP (striped bars) (C) (mean ± SD, n = 3).

Co-staining of FSAP (green) and LRP (red) on VSMC shows a co-localization of the ligand and the receptor in the intracellular compartment. Time course analysis showed that FSAP-PN-1 complexes were internalized within minutes (Figure 14A). More FSAP-PN-1 complexes were internalized by VSMC compared to FSAP-PAI-1 complexes in line with the observed binding characteristics to isolated LRP *in vitro* (Figure 14B). Preincubation of VSMC with RAP prior to application of FSAP-PN-1 complex completely inhibited the internalization of the complex and the co-localization of FSAP and LRP (Figure 14C). FSAP-PN-1 complexes but not FSAP alone were internalized by WT-MEF but not by $LRP^{-/-}$ MEF (Figure 14D) and this was observed irrespective of the fixation procedure used (data not shown). FSAP-inhibitor complex binds to LRP, is internalized by the scavenger receptor and is directed to lysosomes. Although FSAP has been efficiently inhibited by PAI-1 or PN-1, differences concerning the binding of the respective FSAP-inhibitor complexes to LRP could be observed. FSAP complexed with PN-1 exhibited stronger binding to LRP than FSAP complexed with PAI-1. Studies on uPA-inhibitor complexes have not shown such differences [144]. However, with respect to leukocyte adhesion, opposing effects of either uPA-PN-1 or uPA-PAI-1 complexes have been demonstrated [146].

The herein shown disparity between FSAP complexed with PN-1 or PAI-1 might be due to the fact that PAI-1 requires cofactors, such as heparin for an efficient inhibition of its targets [139], whereas PN-1 not. Heparin inhibits binding to LRP. Therefore, in our LRP-binding studies no heparin was present. This explains the observed lower binding capability, due to a reduced formation of FSAP-PAI-1 complexes under these conditions.

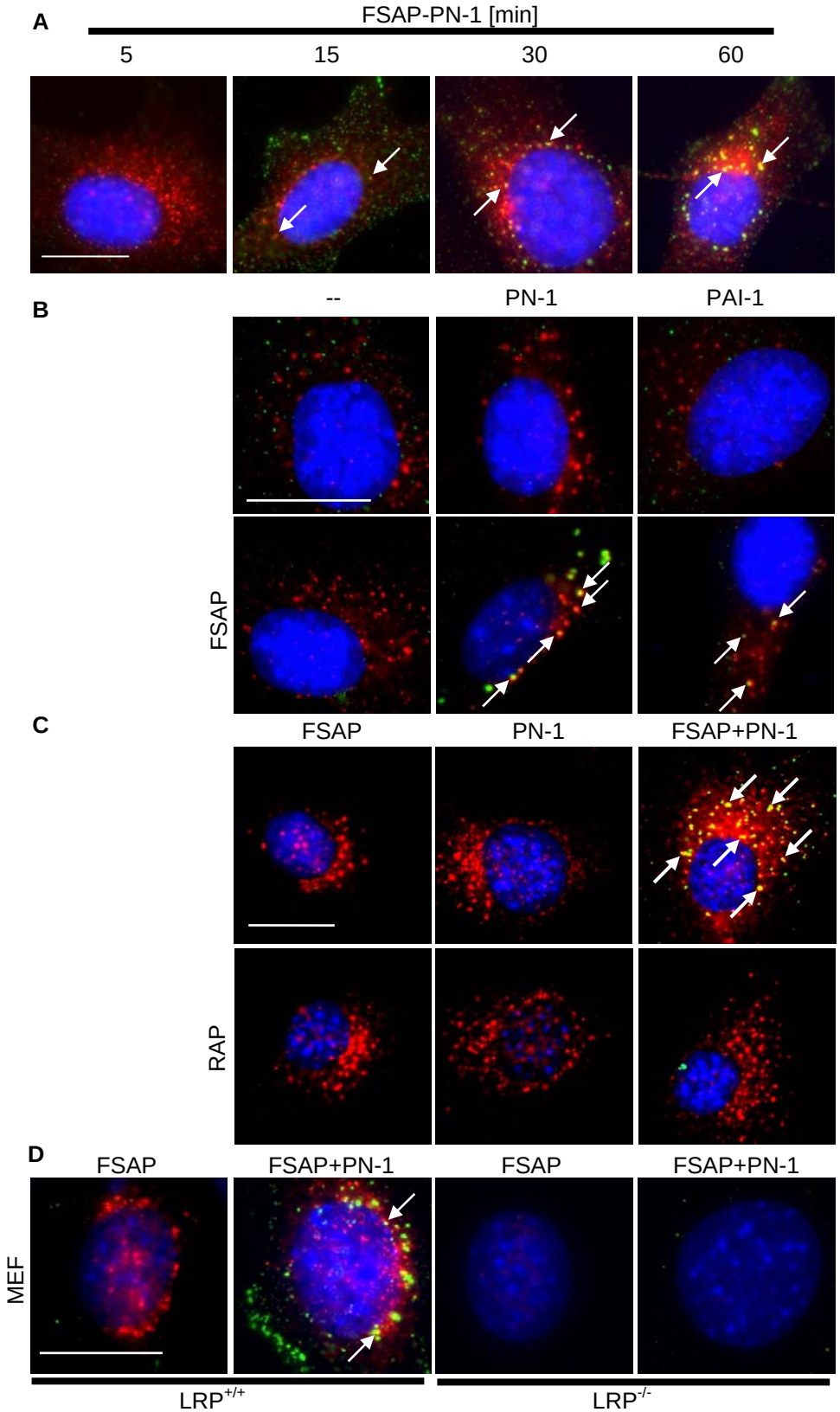


Figure 14: *Intracellular detection of FSAP-inhibitor complexes in VSMC and MEF.* FSAP (1 µg/ml) was preincubated with PN-1 or PAI-1 (2 µg/ml) and applied to VSMC. Internalization of FSAP was detected by immunostaining using a monoclonal antibody (mAb 1189) for FSAP, indicated in green, and LRP, indicated in red, co-localization is indicated by yellow color and highlighted by white arrows. (A) Cells were fixed after the indicated time points (5 – 60 min) and intracellular FSAP was evaluated. (B) The difference between FSAP in complex with PN-1, or PAI-1 was evaluated by internalized FSAP after 60 min. (C) Visualization of the inhibitory effect of RAP towards LRP-dependent internalization of FSAP-PN-1 complexes. (D) FSAP internalization in $LRP^{+/+}$ and $LRP^{-/-}$ MEF. Nuclei were stained with DAPI, scale bars = 20 µm.

6.2.3 Effect of FSAP-PN-1 complex on PDGF-BB-induced receptor activation

Since FSAP is a strong inhibitor of the PDGF-BB signaling pathway and LRP is involved in modulating the same pathway the interactions between FSAP, LRP and PDGFRβ were investigated. With respect to a potential influence of FSAP-PN-1 complexes to LRP-dependent PDGFRβ handling the cellular arrangement of PDGFRβ was investigated. In unstimulated cells PDGFRβ is diffusely distributed over the whole cell membrane and upon stimulation with PDGF-BB internalization of PDGFRβ into lysosomes is observed (Figure 15) as has been described before [147]. This PDGF-BB-induced internalization of PDGFRβ was not influenced by the presence of FSAP-PN-1 complex (Figure 15, upper panel). Hence, FSAP-PN-1 complexes are internalized via LRP but this does not influence the internalization of PDGF-BB-PDGFRβ by LRP even though there was co-localization of FSAP-PN-1 complex (green) with internalized PDGFRβ (red) (Figure 15, lower panel). Because of a diffuse distribution of PDGFRβ, as in unstimulated cells [148], no staining was observed when PDGF-BB was preincubated with FSAP alone due to a cleavage and inactivation of PDGF-BB. Although FSAP-PN-1 complexes as well as the PDGF-BB-PDGFRβ complexes bind to LRP their internalization was completely independent of each other.

6.2.4 Effect of FSAP-PN-1 complex on PDGF-BB-dependent cell activation

No influence of FSAP-PN-1 with respect to an altered cellular trafficking of PDGFRβ was observed. To confirm the suggested independence of FSAP-PN-1 clearance and PDGFRβ internalization by LRP, the potential influence of FSAP-PN-1 complexes on PDGF-BB-mediated signal transduction have been analyzed.

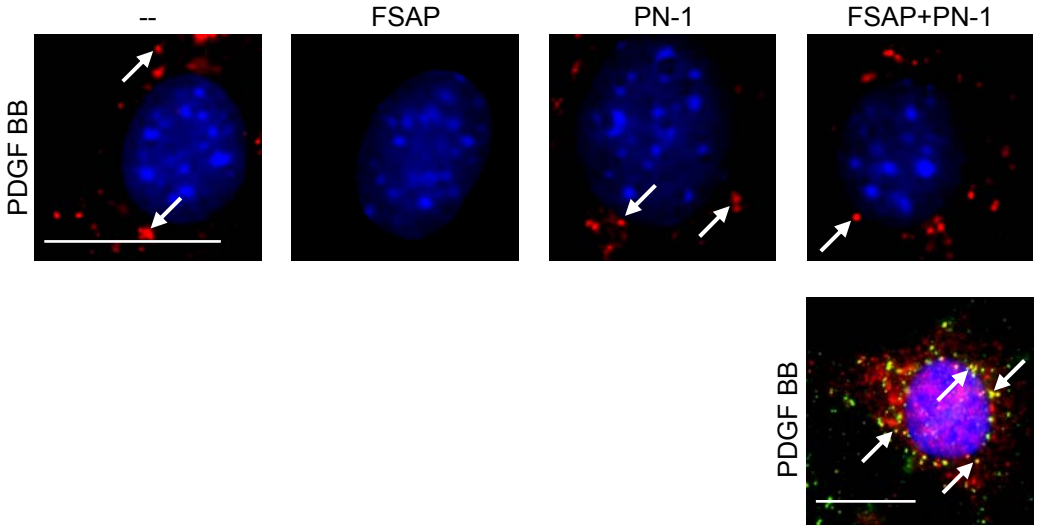


Figure 15: *FSAP-PN-1 complexes co-localize with internalized PDGFRβ.* PDGF-BB was preincubated without, or with FSAP (1 µg/ml), PN-1 (2 µg/ml) or FSAP + PN-1 for 1 h before addition to VSMC. PDGFRβ is indicated in red, internalized FSAP is indicated in green. White arrows highlight activated and subsequently internalized PDGFRβ (upper panel). Yellow color represents co-localization of PDGFRβ and FSAP, highlighted by white arrows (lower panel). Nuclei were stained with DAPI. Scale bars = 20 µm.

Preincubation with FSAP-PN-1 did not alter PDGF-BB-mediated phosphorylation of 42/44-MAPK in VSMC (Figure 16A). Inhibition of the proteolytic activity of FSAP by PN-1 led to neutralization of its ability to inhibit PDGF-BB-mediated DNA synthesis (Figure 16B). Neither RAP nor FSAP-PN-1 complex had any significant influence on PDGF-BB-mediated DNA-synthesis indicating that LRP is not regulatory under these conditions on these cells.

Generally, more PDGFRβ was present in $LRP^{-/-}$ MEF than in WT MEF (Figure 17C) and higher levels of tyrosine phosphorylation were observed in $LRP^{-/-}$ MEF than in WT MEF after PDGF-BB stimulation (Figure 17B). These results confirm the known role of LRP in regulating PDGF-BB activity. No effect of FSAP-PN-1 complex was observed on this pattern of expression or phosphorylation of PDGFRβ.

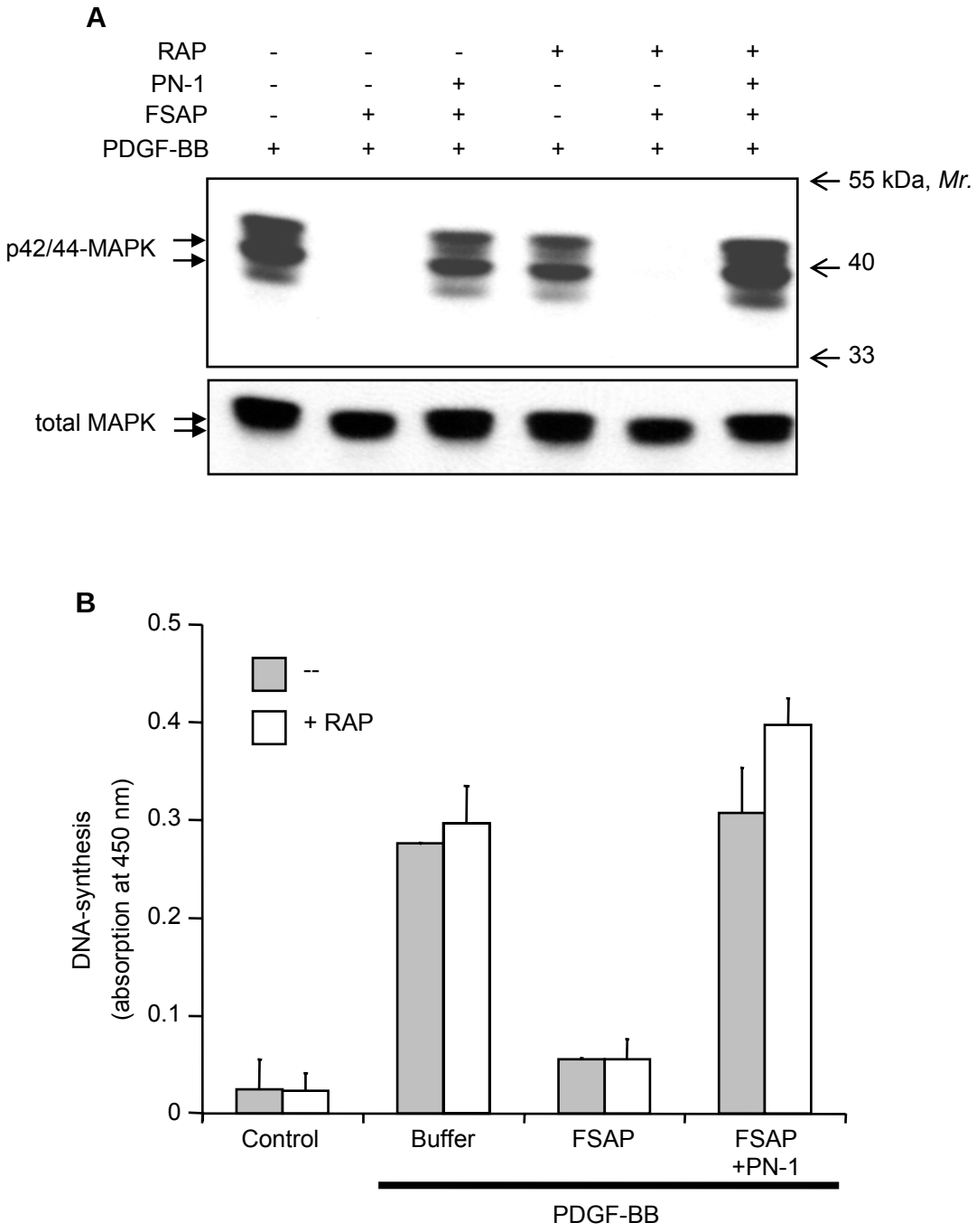


Figure 16: *Influence of FSAP-PN-1 complexes on PDGF-BB-dependent cell activation.* (A) In serum-free medium containing heparin (10 µg/ml) VSMC were stimulated with PDGF-BB (20 ng/ml), PDGF-BB preincubated with FSAP (1 µg/ml) or PDGF-BB preincubated with FSAP-PN-1 complex in the absence or presence of RAP (10 µg/ml) for 15 min. Western blot analysis was performed using a monoclonal antibody against phospho 42/44 MAPK. As a loading control a polyclonal antibody against total MAPK was used on the stripped membrane. (B) As in the above experiment VSMC were stimulated in the absence (gray column) or presence of RAP (10 µg/ml) (open column). DNA-synthesis was measured (mean ± SD, n = 3) using a kit to measure BrdU incorporation into newly synthesized DNA.

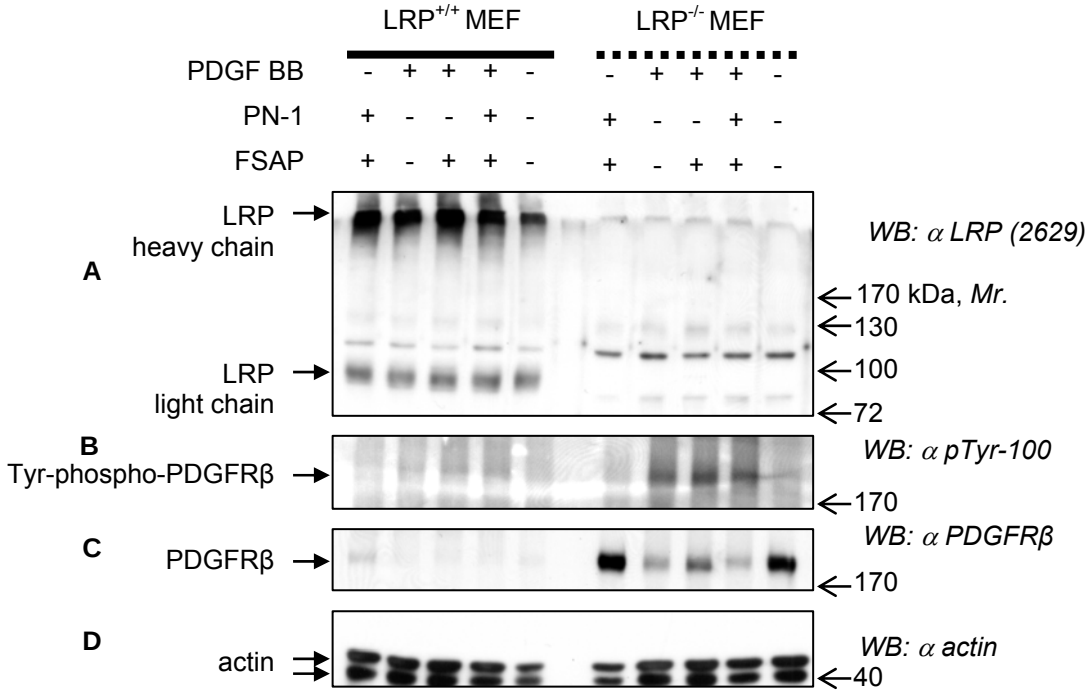
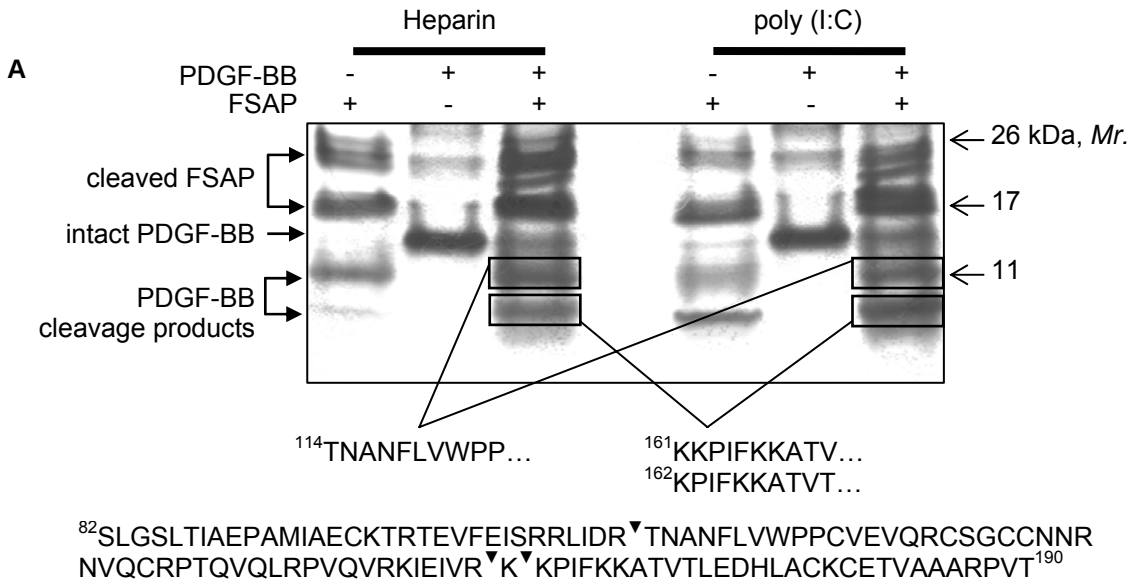


Figure 17: *LRP-dependent PDGFRβ distribution and activation.* 1 µg/ml FSAP and 2 µg/ml PN-1 were preincubated for 30 min. 20 ng/ml PDGF BB was then preincubated with either buffer, FSAP or FSAP-PN-1 for 60 min and the mixtures were used for stimulating cells for 10 min. MEF lysates were processed for Western Blot analysis. Heavy chain and light chain of LRP is expressed in $LRP^{+/+}$ MEF but not in $LRP^{-/-}$ MEF (A). Since LRP internalizes PDGFRβ, more PDGFRβ is present in $LRP^{-/-}$ than $LRP^{+/+}$ cell (C). Stimulation of cells with PDGF-BB leads to tyrosine phosphorylation of PDGFRβ (B) and its internalization and degradation (C). FSAP or FSAP-PN-1 complex do not influence this process. Actin blot shows equal loading of cell extracts in all lanes (D).

6.3 FSAP cleaves PDGF-BB at distinct sites

6.3.1 A cluster of basic residues is the target motif for FSAP-dependent cleavage of PDGF-BB

FSAP inhibits PDGF-BB-dependent signaling by a proteolytic mechanism. To specify this process the generated PDGF-BB fragments were analyzed for their amino-acid sequences. Thereby the precise cleavage sites of FSAP were identified. Fragments of cleaved PDGF-BB (Figure 18A) were resolved by SDS-PAGE and blotted onto PVDF membranes and analyzed by amino-terminal sequencing (Figure 18A).This led to the determination of preferential proteolytic sites after amino-acid R_{113}, R_{160} and K_{161}. R_{113} is a known cleavage site in PDGF-BB and natural fragments starting at this point are found in platelets but the responsible protease was unknown[149]. The latter two cleavage sites are in loop III of PDGF-BB, important for receptor binding and activation[150] (Figure 18B).



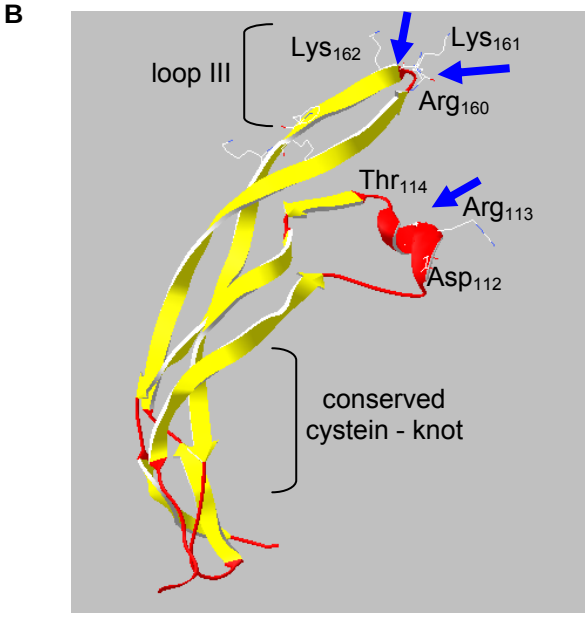


Figure 18: *Determination of FSAP cleavage-site in the PDGF-BB molecule.* (A) PDGF-BB (20 µg/ml) was incubated with FSAP (200 µg/ml) in the presence of heparin or poly (I:C) (10 µg/ml) as indicated for 60 min at 37°C. SDS-PAGE was performed under reducing conditions and the protein fragments were detected by silver-staining or were blotted onto a PVDF membrane and stained with Coomassie blue (Data not shown). Bands were analysed by amino-terminal sequencing. Molecular mass standards are indicated in kDa. The results of the N-terminal sequencing are shown and the complete sequence of PDGF-B chain is marked with the FSAP-cleavage sites. (B) Swiss-model generated structure of PDGF-B (1pdgA). Blue arrows highlight FSAP cleavage sites. Location of loop III region, important for receptor binding, and of the conserved cystein – knot structures are given.

6.3.2 Nucleic acids are cofactors for FSAP-mediated cleavage of PDGF-BB

It has been previously shown that FSAP cleaves PDGF-BB in a dose- and time-dependent manner in the presence of the cofactor heparin. Here the relation of nucleic acids with respect to FSAP-dependent PDGF-BB cleavage could be evaluated. The fragmentation of PDGF-BB was only discernable under reducing conditions, and not under non-reducing conditions (Figure 18). This indicates that after limited proteolysis the PDGF fragments were held together through disulphide bridges and that the

antibody binding site was intact[141]. ^{125}I-PDGF-BB cleavage was determined in the presence of natural RNA, DNA as well as synthetic poly (I:C) or synthetic poly (dI:dC). Natural or artificial RNA was a more effective cofactor for the cleavage of PDGF-BB than DNA (Figure 19A, B). This effect was dependent on the concentration of RNA (Figure 19A, B) and could be inhibited by protease inhibitors (Figure 19C). These results suggest that RNA is an effective cofactor for the limited proteolysis of PDGF-BB by FSAP, in line with its propensity to increase auto-activation of FSAP [66].

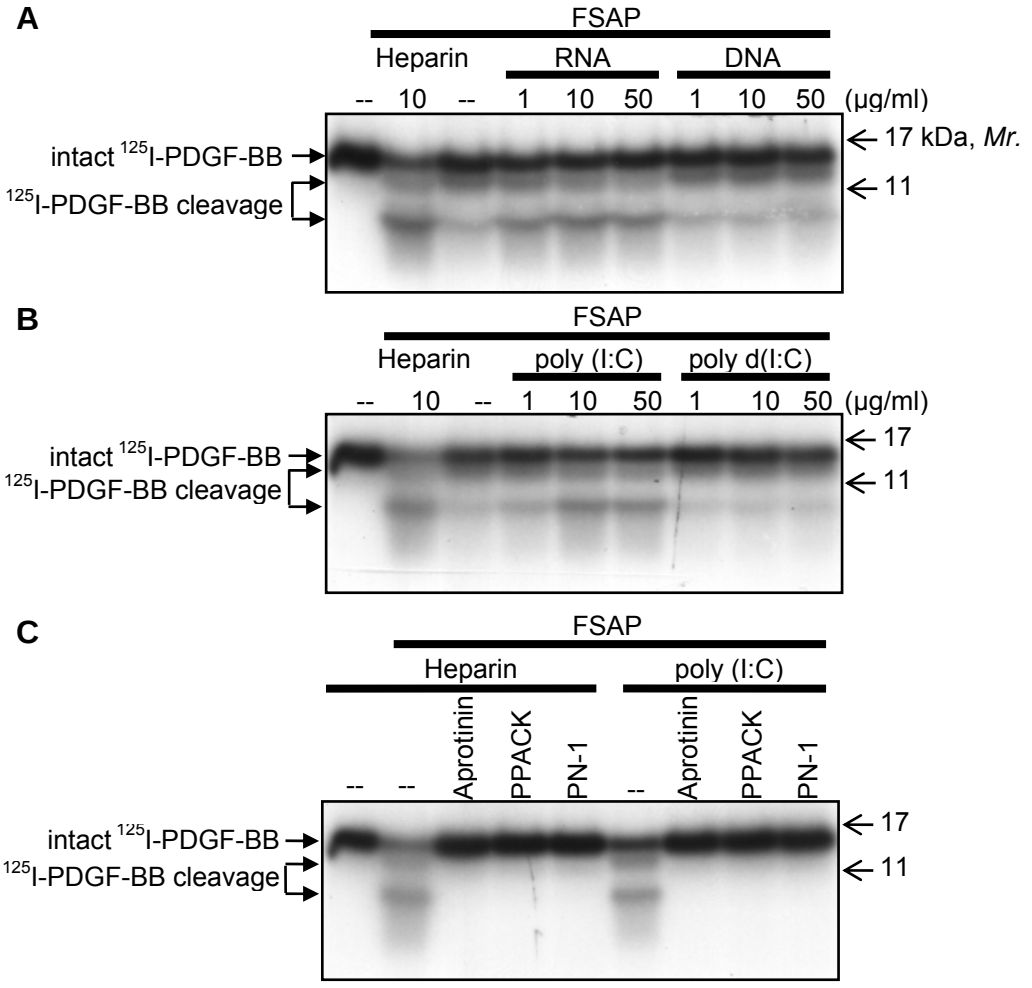


Figure 19: *Cleavage of PDGF-BB by FSAP is enhanced by nucleic acids.* (A) ^{125}I-PDGF-BB was incubated with FSAP (1 µg/ml) in the presence of heparin (10 µg/ml), natural RNA or natural DNA for 60 min at 37°C. (B) Same as above except that poly (I:C) or poly (dI:dC) were used as cofactors. (C) Similarly, FSAP was compared to inactivated PPACK-FSAP, or FSAP in the presence of the protease inhibitor aprotinin (2 µg/ml) or protease-nexin-1 (8 µg/ml) in the presence of poly (I:C) or heparin (each 10 µg/ml). After SDS-PAGE under reducing conditions autoradiography was performed.

6.4 Structure-function analysis using recombinant FSAP-mutants

6.4.1 Expression of recombinant FSAP-isoforms in HEK-293 cells

Different FSAP-variations were produced to investigate mechanisms of FSAP activation and inhibition in more detail. The evaluation of recombinant FSAP expression will be a basis for a large-scale production later on. Recombinant WT-FSAP, MI- and MII-FSAP was generated to proof the functionality of the recombinant versions. Furthermore, two isoforms that were suggested to be completely inactive were produced, the cleavage-site mutant $R_{313}G$ and the active-site mutant $H_{363}F$. To investigate the binding of polyanions to FSAP, a FSAP-variation that lacks the EGF-3 domain (ΔEGF-3-FSAP), which is the putative polyanion binding-site, was generated.

The highest number of stably transfected clones were obtained with vector control, $R_{313}G$ cleavage site mutant, $H_{362}F$ active site mutant and MI SNP, whereas the number of clones obtained with WT, ΔEGF-3 and MII was very low (Figure 20A). Amount of FSAP in the conditioned media was the highest in $R_{313}G$, $H_{362}F$ and MI, whereas WT, ΔEGF-3 and MII contained very low levels of FSAP protein, empty vector exhibited no expression of FSAP (Figure 20B). To equalize and standardize these differences in FSAP concentration for further experiments, the amounts were calibrated using ELISA and Western blotting (Figure 20C). These results show that 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 why fewer clones with WT-FSAP compared to the inactive mutants were obtained, FSAP immunocytochemistry on cells transfected with WT-, MI- and ΔEGF-3-FSAP plasmids were performed. WT-FSAP appeared as a dense irregular structure within the cytoplasm, whereas MI- and $R_{313}G$-FSAP (Figure 21A) exhibited a more granular and dispersed localization. Initially, the number of cells

transfected with WT-FSAP was similar to those transfected with FSAP variants, exhibiting lower or no enzymatic activity. Hence, it was reasoned that cells expressing WT-FSAP had a selective disadvantage or died, leading to fewer viable cells. To test this point more systematically an inducible expression system was used. Without induction, the expression of FSAP and staining for the apoptosis marker, cleaved PARP [138] was negligible. With the induction of FSAP expression there was a concomitant increase in cleaved PARP staining. Addition of tetracycline to cells transfected with the control vector did not show any alterations in FSAP or cleaved PARP staining (Figure 21B). As a control, the expression of a related protease, uPA, was efficiently expressed and did not induce apoptosis.

6.4.2 Interaction of FSAP-isoforms with heparin

Polyanion binding to FSAP is important for a subsequent activation of the zymogen. Using the recombinant FSAP-variations it was possible to study this relationship in more detail. Previous studies have alluded to the necessity of FSAP EGF-3 domain in heparin to binding [151]. The ΔEGF-3 variant, lacking the EGF-3 domain, exhibits a clearly diminished ability to bind heparin (Figure 22A). The recombinant proteins WT-, MI-, and R_{313}G-FSAP (Figure 22A) as well as MII- and H_{362}F-FSAP (data not shown) bound to a similar extent to immobilized heparin. As a control the binding of WT-, ΔEGF-3-, MI- and R_{313}G-FSAP to immobilized pAb against FSAP was similar (Figure 22B). Using recombinant protein lacking the EGF-3 domain confirmed its importance for binding to heparin. Heparin binding leads to an auto-activation of latent FSAP [62]. WT- and ΔEGF-3-FSAP exhibited a similar amidolytic activity without the addition of heparin whereas MI- and R_{313}G-FSAP exhibited no activity (Figure 23A, black symbols). After addition of heparin, the

activity of WT- and MI-FSAP was increased 1.5-fold, whereas the activity of ΔEGF-3-FSAP remained unchanged (Figure 23A, white symbols).

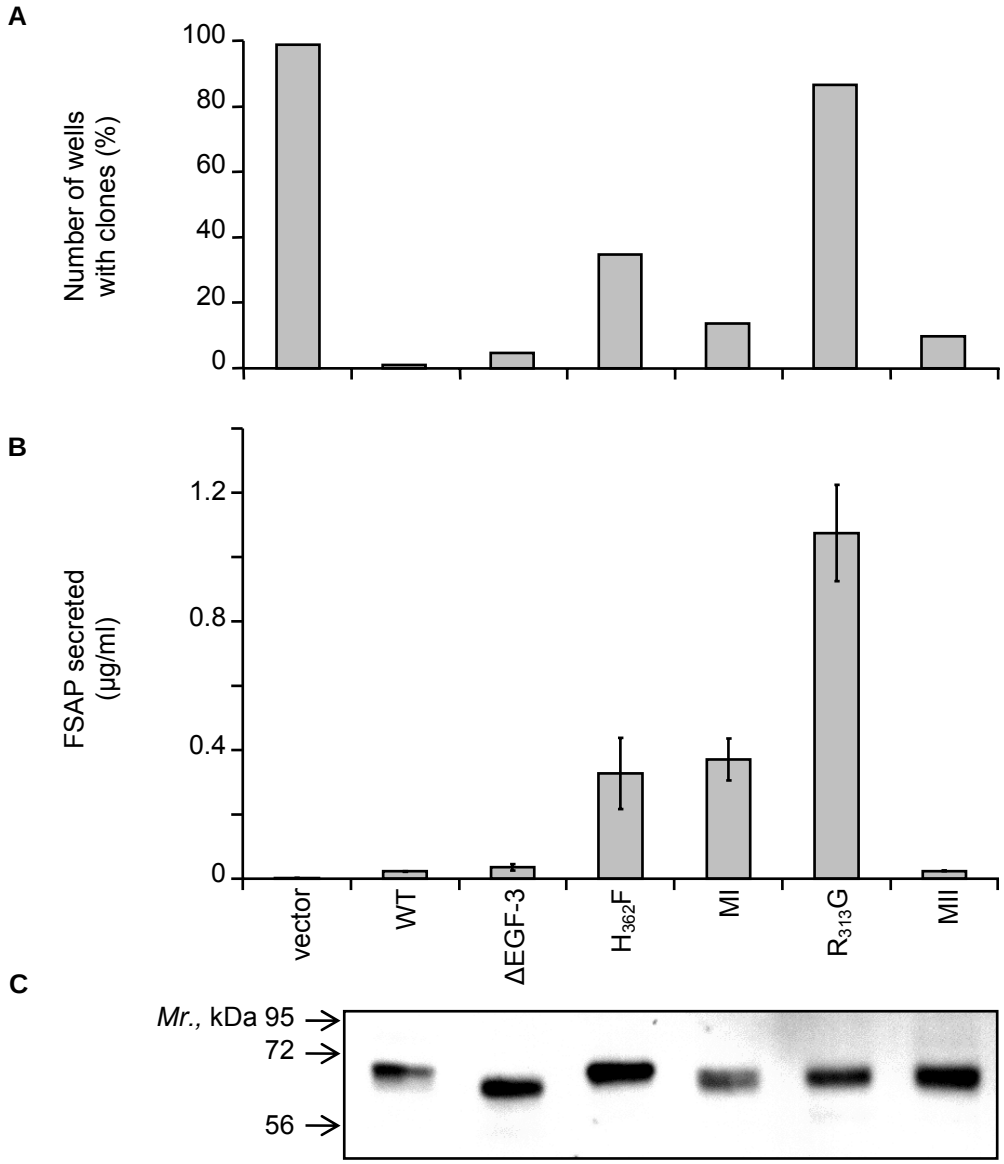


Figure 20: *Expression of FSAP isoforms in HEK-293 cells.* (A) After transfection of cells and transfer into selection medium, the percentage of wells with viable clones was determined. (B) The amount of FSAP in conditioned medium, was determined by ELISA (mean ± SD, n = 3). (C) Supernatants of different FSAP variants, standardized by ELISA, were analyzed by Western blotting performed with a mixture of mAb 677 and mAb 1189.

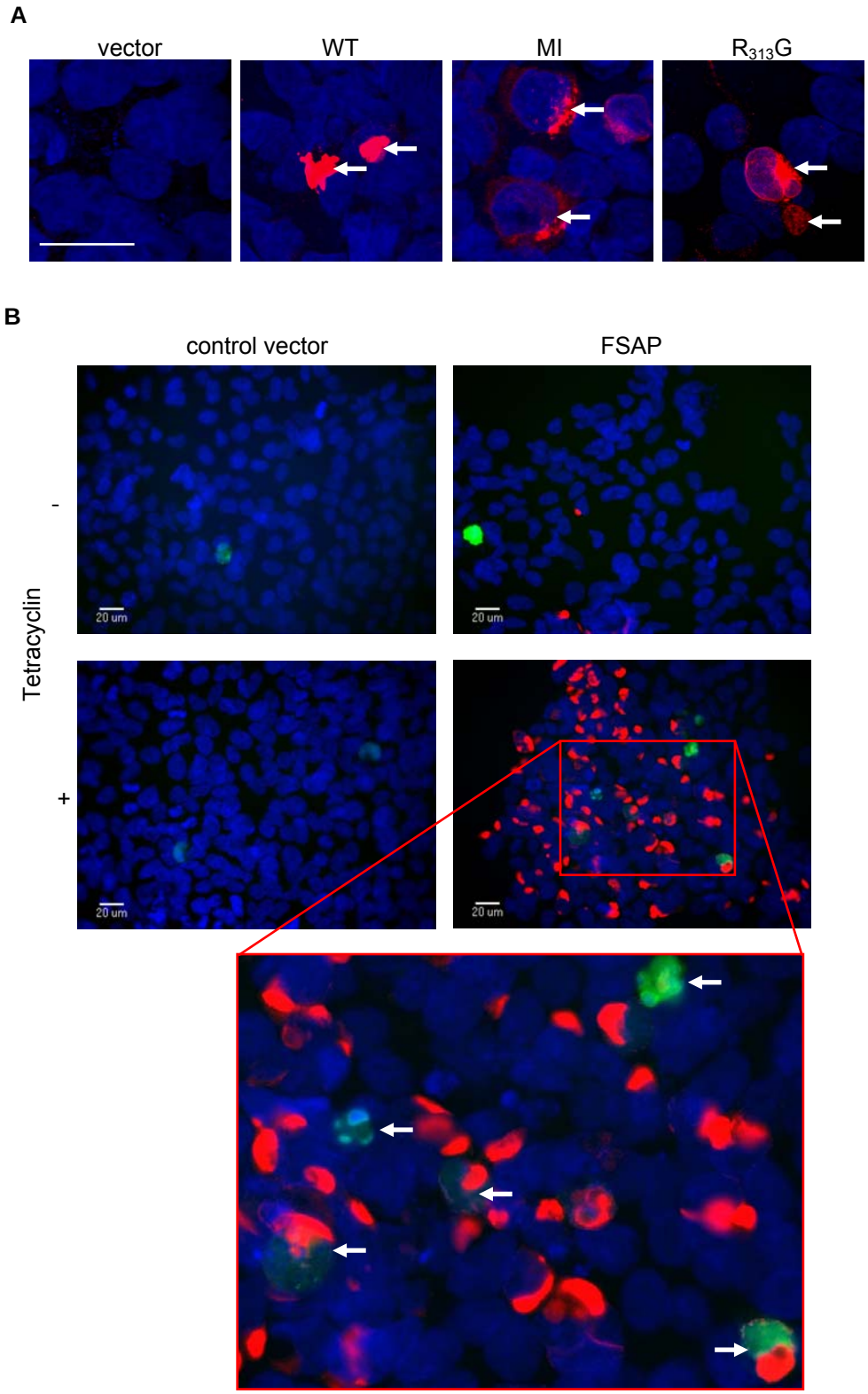


Figure 21: *Cellular effects of over-expressed FSAP-variants.* (A) Confocal microscopy of transiently transfected HEK-293 cells with empty vector, WT-, MI- and $R_{313}G$-FSAP. FSAP was stained using mAb 1189 (red) and nuclei with DAPI (blue). White arrows highlight staining for FSAP. (B) Transiently 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 indicate staining for cleaved PARP. Calibration bars = 20 µm.

PAI-1 is known to inhibit FSAP efficiently in the presence a cofactor [152]. PAI-1-dependent inhibition of WT-, but not ΔEGF-3-FSAP was increased by heparin (Figure 23B). Hence, the EGF-3 domain is not only important for heparin binding but contributes largely to the heparin mediated activation as well as the inhibition of FSAP by PAI-1.

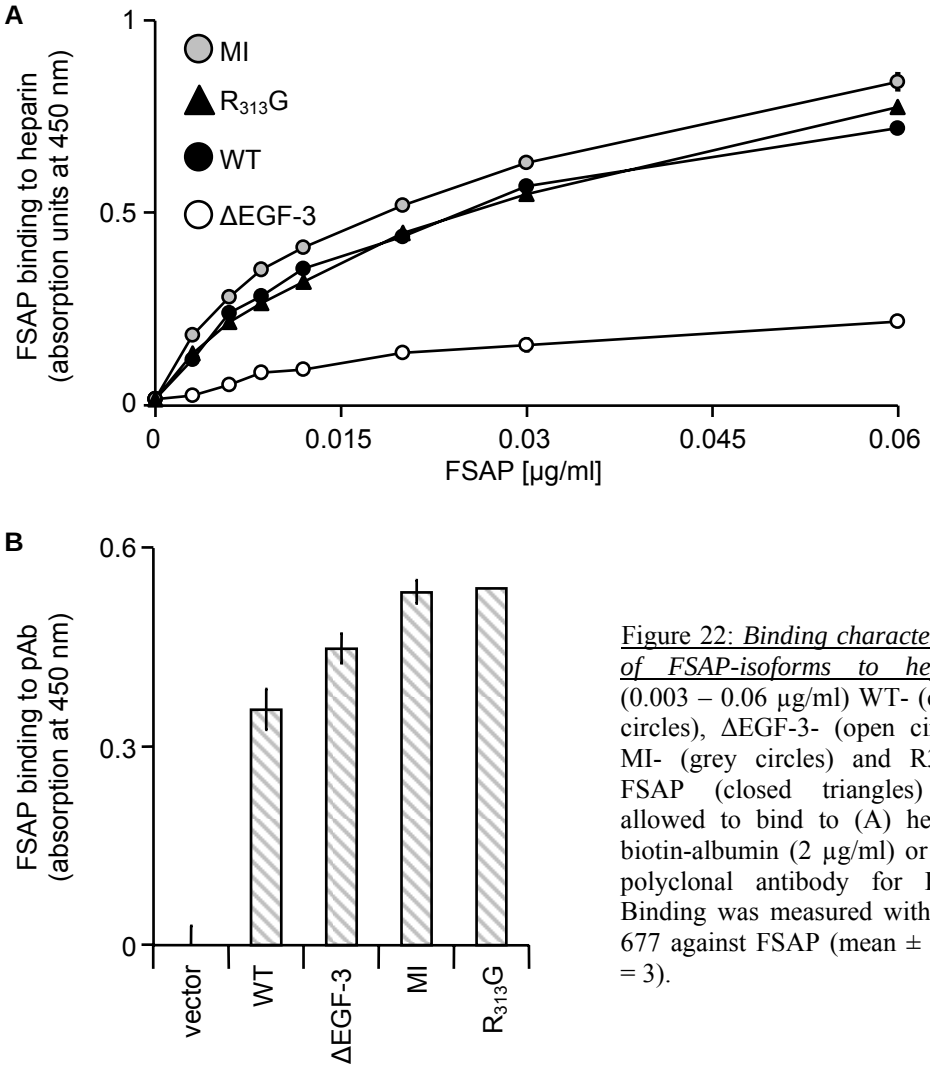


Figure 22: *Binding characteristics of FSAP-isoforms to heparin.* (0.003 – 0.06 µg/ml) WT- (closed circles), ΔEGF-3- (open circles), MI- (grey circles) and R313G-FSAP (closed triangles) was allowed to bind to (A) heparin-biotin-albumin (2 µg/ml) or (B) a polyclonal antibody for FSAP. Binding was measured with mAb 677 against FSAP (mean ± SD, n = 3).

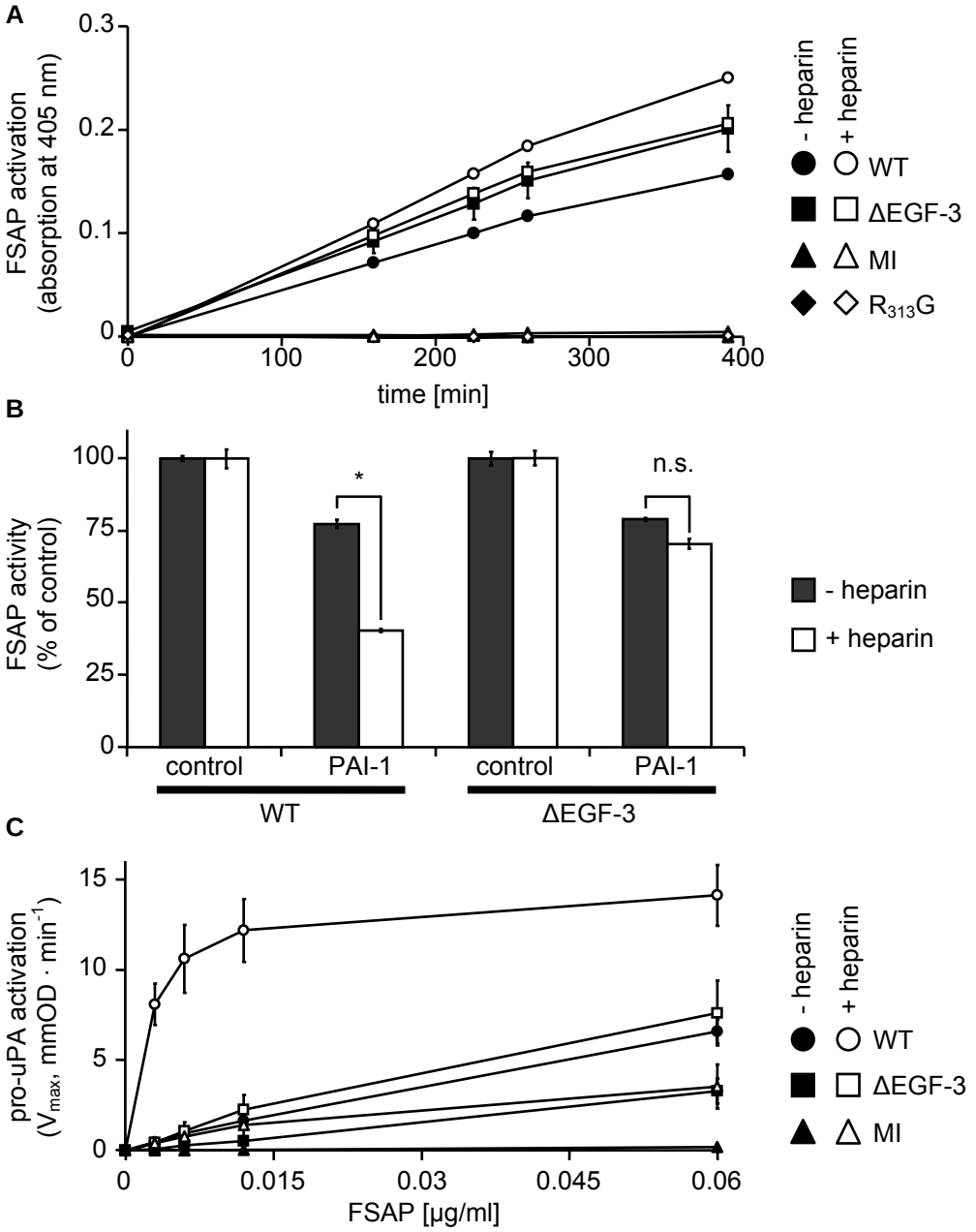


Figure 23: *Characterization of the enzymatic activity of different FSAP-isoforms.* (A) WT- (circles), ΔEGF-3- (squares), MI- (triangles) and $R_{313}G$-FSAP (diamonds) (0.06 µg/ml) was captured with mAb 677. FSAP activity without (closed symbols) or with (open symbols) heparin (1 µg/ml) was measured by the conversion of S-2288, mean ± SEM (n=3). (B) FSAP inhibition by PAI-1 (0.01 µg/ml), without (closed column) and with (open column) heparin (1 µg/ml), mean ± SEM (n = 3), * indicates $p < 0.005$, using unpaired two tailed "t" test. (C) FSAP isoforms (0.003 – 0.06 µg/ml) were captured as described above and pro-uPA (1 µg/ml) activation without (closed symbols) or with (open symbols) heparin (1µg/ml) was measured by the conversion of S-2444 (mean ± SEM, n = 4 – 6).

6.4.3 Pro-uPA activation by FSAP-isoforms

Pro-uPA is efficiently activated by FSAP [71]. Therefore, its activation by different FSAP-isoforms was tested. WT-FSAP activated pro-uPA more effectively than ΔEGF-3-FSAP and MI-FSAP (Figure 23C, black symbols). In the presence of heparin the activity of WT-FSAP increased 10-fold, at 0.006 µg/ml, but that of ΔEGF-

3-FSAP by 2-fold only (Figure 23C, white symbols). MII-FSAP exhibited a comparable activity to WT-FSAP, whereas R_{313}G- and H_{362}F-FSAP showed no activity towards pro-uPA as would be predicted (Figure 23C). The same pattern could be observed with respect to pro-uPA cleavage by FSAP-isoforms, using Western blotting. FSAP-isoform-dependent uPA-β-chain generation was inhibited by PN-1 or PAI-1 (Figure 24)

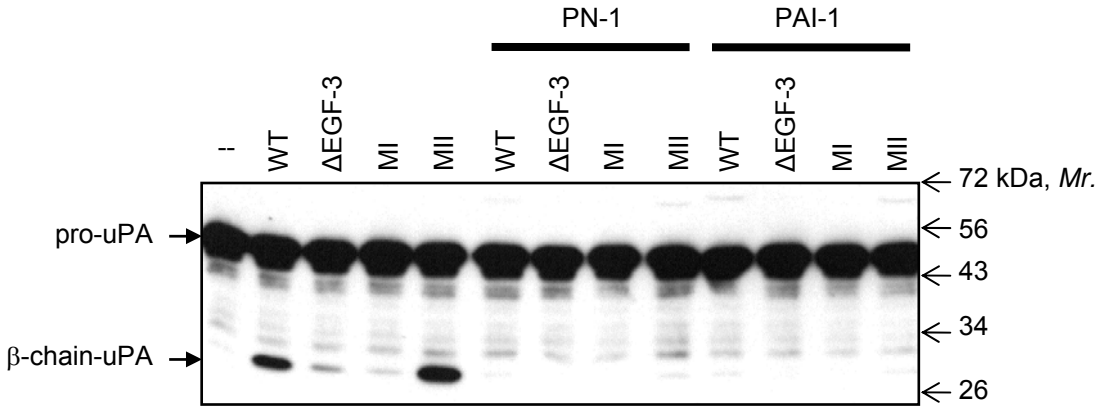


Figure 24: *Pro-uPA cleavage by FSAP-isoforms.* Western blot analysis of pro-uPA (100 ng/lane) incubated with different FSAP-isoform conditioned media with heparin (10 µg/ml) alone or with PN-1 or PAI-1 (both 25 ng/lane). SDS-Page was performed under reducing conditions and uPA bands were detected using a polyclonal anti uPA antibody. Arrows indicate pro-uPA and after cleavage appearing uPA-β-chain.

6.4.4 Binding to and cleavage of PDGF-BB by FSAP-isoforms

Besides pro-uPA, PDGF-BB is another important substrate of FSAP vascular tissues [141]. PDGF-BB is cleaved by FSAP at distinct sites [142]. Polyanions, such as heparin or RNA are important cofactors for this mechanism (Figure 19). Using recombinant FSAP-variations the importance of FSAP EGF-3 domain for FSAP-dependent PDGF-BB inhibition was analyzed. The reduced ability of MI-FSAP to inhibit PDGF-BB signaling [141] was additionally analyzed with the recombinant mutants. Without heparin the binding of WT-, ΔEGF-3- and R_{313}G-FSAP to PDGF-BB was low. This binding was increased with heparin in case of WT-and R_{313}G-FSAP, but not for ΔEGF-3-FSAP indicating again the importance of the EGF-3 domain of FSAP (Figure 25A). Cleavage of ^{125}I-PDGF-BB was detected by WT- and plasma-derived

FSAP, but not by ΔEGF-3- or MI-FSAP either in the absence or presence of heparin (Figure 25B).

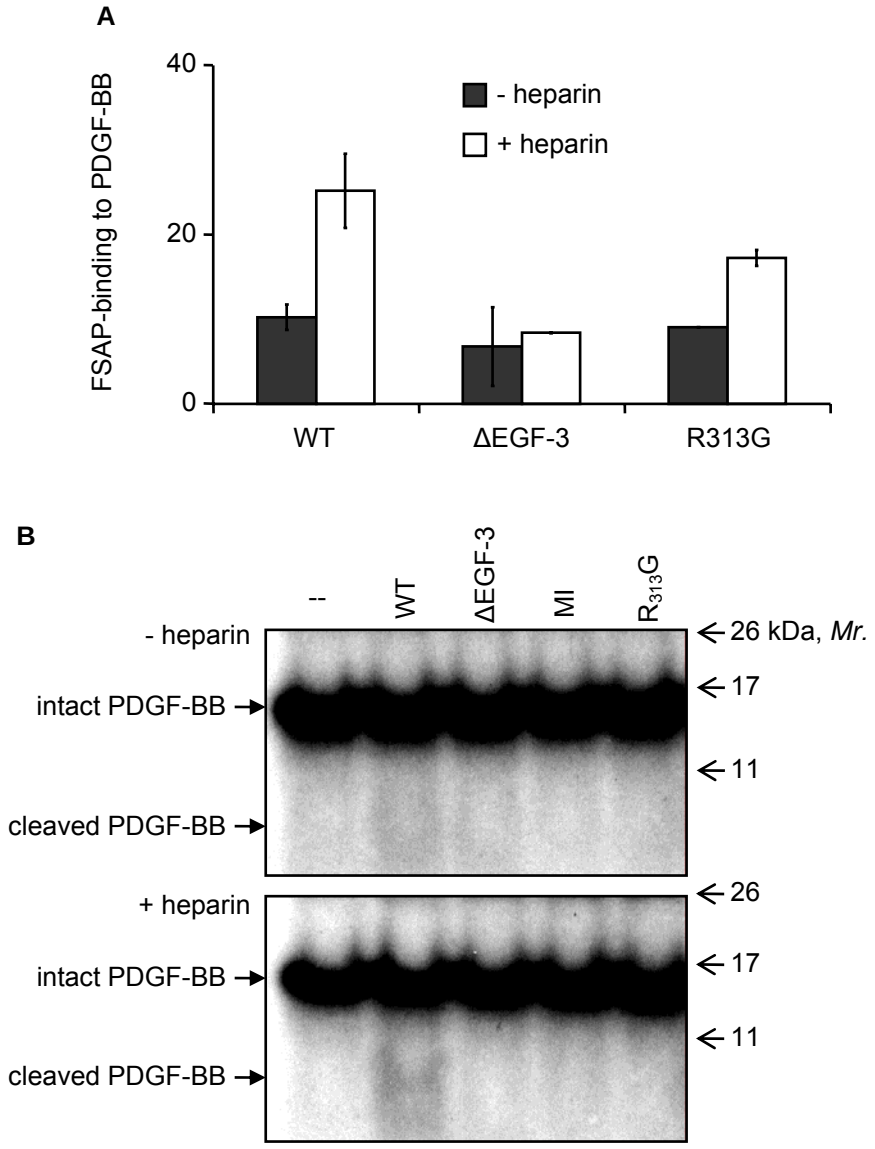


Figure 25: *Binding to and cleavage of PDGF-BB by FSAP-isoforms.* WT-, ΔEGF-3- and $R_{313}G$-FSAP (0.06 µg/ml) were added to immobilized PDGF-BB. Binding of FSAP-isoforms without (closed columns) or with (open column) heparin (10 µg/ml) was determined with a FSAP antibody, mean ± SD (n=3). FSAP binding to PDGF-BB was normalized with respect to FSAP binding to polyclonal anti-FSAP Ab. (B) Autoradiogram of cleaved ^{125}I-PDGF-BB by WT-, ΔEGF-3-, MI-, $R_{313}G$- or from plasma derived FSAP. Arrows indicate intact- and cleaved-^{125}I-PDGF-BB.

7. Discussion

The correlation of the MI-single nucleotide polymorphism ($G_{534}E$) of FSAP with cardiovascular diseases [61, 75, 76] and its reduced proteolytic activity [74], indicate the functional importance of FSAP. Smooth muscle cells contribute to the development of atherosclerotic lesions [51, 115], and are major effectors in restenotic processes, post balloon-angioplasty and stent implantation [140]. Since FSAP is able to inhibit VSMC proliferation *in vitro* [70] and *in vivo* [141], FSAP is of crucial importance to the development of vascular diseases. Additionally, FSAP protein and mRNA were found in atherosclerotic plaques [114], demonstrating FSAP's importance for the development of atherosclerotic lesions. The evaluation of the regulation of FSAP activity will provide new insights for novel therapeutic approaches in the treatment of cardiovascular and related diseases.

7.1 Activation of FSAP

It is not known which events lead to the activation of FSAP in the vasculature. This prompted us to investigate different forms of heparin and other polyanions present in the vascular system with respect to FSAP activation. When assessing the different heparin isoforms (unfractionated heparin, LMW heparin, *N*-acetyl heparin, de-*N*-sulfate heparin and *N*-acetyl-de-*O*-sulfate heparin) with respect to their ability to bind to and activate FSAP, strong effects only with unfractionated heparin were observed. The interaction of heparin with FSAP was found to be dependent on its molecular size and even more on its negative charge density. LMW heparin, which exhibits reduced size but comparable negative charge density, showed only weak effects. However, heparin variations exhibiting the same size and reduced negative charge density (*N*-acetyl heparin, de-*N*-sulfate heparin and *N*-acetyl-de-*O*-sulfate heparin), showed no effect at

all, except for heparan sulfate, which activated FSAP at high concentrations. These observations demonstrate that FSAP activation by heparin-like molecules can only occur at sites of glycosaminoglycans that exhibit a high rate of sulfation. Hence, the known release of heparin by mast cells within atherosclerotic lesions [153] may represent a functional situation in which FSAP activation occurs. This represents a process limited to a specified location and time. After their stimulation, mast cells secrete a variety of factors, including heparin, and thereby contribute to the development of atherosclerotic lesions. To test the native material with respect to binding to and activation of FSAP mast cell-derived heparin has been analyzed. Native mast cell-derived heparin exhibited the same features as the synthetic heparin. Thus, the relation of heparin within atherosclerotic plaques to a potential FSAP activation could be confirmed using native material.

Another highly charged negative molecule is polyphosphate, which has been recently reported to activate the contact factor pathway, FV, TFPI [15], and to modulate fibrin clot structure [104]. Synthetic polyphosphate of different average molecular sizes and phosphatase-treated polyphosphate have been used to activate FSAP. Polyphosphate, in a similar way to heparin, binds to and activates FSAP in a size-dependent manner. Polyphosphate is stored in the platelete's dense granules [102] and released upon platelet activation. The localization of polyphosphate to the events of hemostasis and fibrinolysis raises considerable indirect evidence for a physiological contribution of polyphosphate to the activation process of FSAP. In order to test this conjecture, isolated native platelet-derived polyphosphate was used with respect to FSAP binding and activation. Platelet-derived polyphosphate exhibited anomalous properties. While the binding of platelet-derived polyphosphate to FSAP was as expected, activation studies reported only a very weak ability of native polyphosphate to activate FSAP. This anomaly may have occurred because the synthetic

polyphosphate exhibits a relatively heterogeneous chain-size distribution, with up to 200 phosphate residues, whereas the native platelet-derived polyphosphate is restricted to chain-lengths of 70 to 75 units [102, 154]. Since our investigation is one of the first in comparing native polyphosphate with its synthetic analog, no other reports are available at this time.

Platelets contribute to the development of atherosclerotic plaques, because activated platelets secrete various inflammatory cytokines [37]. After the establishment of the atherosclerotic plaque, platelets are important for the hemostatic response after plaque rupture. Polyphosphate released from activated platelets, either during blood coagulation or during the development of atherosclerotic lesions, represents a putative activator for FSAP within these distinct events. Polyphosphate released by platelets exhibit a strong potential to interact with FSAP and locate possible activation of the latent zymogen in pathophysiological situations. These findings are in agreement with the observation that the FSAP-antigen is located in atherosclerotic plaques. Further studies are needed to explain why native polyphosphate has different properties compared to synthetic polyphosphate and how this relates to FSAP activation in physiological situations.

Since FSAP is able to inhibit PDGF-BB-dependent cell activation and VSMC are important in the development of atherosclerotic plaques, the interest as to whether heparin or polyphosphates enable PDGF-BB inhibition by FSAP was high. Both polyanions demonstrate a strong cofactor function with respect to FSAP-dependent PDGF-BB inhibition. This mechanism represents another possible function for FSAP activation within atherosclerotic plaques, regulating the activation of VSMC.

Apart from heparin, it has been found that nucleic acids, especially RNA, can serve as a cofactor for the auto-catalytic activation of FSAP [66]. After more detailed studies, it could be confirmed that RNA or its synthetic analog poly (I:C), but not DNA

or poly (dI:dC), are efficient cofactors for FSAP-dependent inhibition of PDGF-BB-mediated VSMC activation. These findings and the fact that FSAP protects RNA from enzymatic breakdown may provide an additional possible activation mechanism at sites of cell destruction that occur in areas of atherosclerotic lesions [155] or disruption of the endothelial cell layer where blood coagulation is initiated. Similar to heparin and polyphosphate, RNA released from cells will locally activate FSAP that in turn can regulate PDGF-BB-dependent cell stimulation.

7.2 Inhibition of FSAP

While FSAP is efficiently inhibited by SERPINs, the effect of the different polyanions in respect to their contribution to FSAP regulation by inhibitors was investigated. AT is strongly dependent on the presence of heparin or heparan sulfate to interact with its targets FIIa and FXa [3]. Only unfractionated heparin exhibited a cofactor function for the AT-dependent inhibition of FSAP. This indicates a potential regulation of FSAP activity via AT at sites where mast cells have secreted unfractionated heparin or to circumstances where unfractionated heparin was administered exogenously. PAI-1 was reported to require cofactors for the efficient inhibition of its target proteases [139]. In this regard, RNA was recently demonstrated to serve as a cofactor for PAI-1-dependent inhibition of FSAP, whereas heparin was not [73]. Here it could be shown that unfractionated heparin as well as polyphosphate serve as efficient cofactors for the inhibition of FSAP by PAI-1. Heparan sulfate, due to its reduced negative charge density, is a cofactor for PAI-1 only at high concentrations. These findings would indicate a minor role for heparan sulfate with respect to FSAP-regulation *in vivo*. Similar to the potential to activate FSAP, the inhibition of FSAP by AT or PAI-1 is limited to sites where a cofactor is present. AT-dependent inhibition of FSAP appears

to be of less importance, since approximately 5-fold more AT is required to achieve the same inhibition efficiency as with PAI-1. However, AT is constitutively present at high concentrations in the circulation, which would still suggest a contribution to the regulation of FSAP activity. PAI-1, in addition to its constitutive expression, is released by activated platelets [21]. Therefore, similar to polyphosphate, it is present during the development of atherosclerotic lesions, in the case of plaque rupture and of initiated blood coagulation. Thus, PAI-1 appears to be of importance for the regulation of FSAP activation at sites of platelet activation.

The regulation of FSAP activity by inhibitors present in the vasculature is an important mechanism to abrogate FSAP activity and prevent excessive proteolytic degradation. PN-1 is up-regulated in hypertension [109] and, like PAI-1 [156], during atherosclerosis [146]. Recent studies revealed that macrophages and platelets are the major source of PN-1 within atherosclerotic plaques [108]. Despite studies that have shown that PN-1 activity is regulated by cofactors [110, 134], FSAP is efficiently inhibited by PN-1 independently of any cofactor [152]. The physiological regulation of FSAP via either PAI-1 or PN-1 is evident, since the inhibitors, FSAP and cofactors are detected in close proximity during all stages of atherosclerotic lesion development.

7.3 Internalization of FSAP

LRP is part of the LDL receptor family and has a large number of different ligands, including proteases, protease-protease-inhibitor complexes, lipids and -cellsurface receptors [113, 143, 157, 158]. Bound ligands become internalized and directed to early endosomes for subsequent lysosomal degradation, whereas LRP is recycled back to the cell membrane [112, 159, 160]. In mouse models of atherosclerosis, LRP was shown to be a protective molecule with respect to atherosclerotic lesion development [50-52, 161, 162].

LRP is involved in the regulation of the PDGF-BB-PDGFRβ signaling axis, by internalizing the activated PDGFRβ. Thereby, LRP abrogates the PDGF-BB-dependent cell activation [160, 163]. Apart from the LRP-dependent uptake of lipids and its regulation of PDGF-BB-signaling, LRP also was shown to interact with proteases, especially hemostasis factors and their inhibitors [113]. Therefore, the possibility of an interaction for FSAP or FSAP-inhibitor complexes with the scavenger receptor, LRP was investigated.

It could be demonstrated that FSAP in complex with either PN-1 or PAI-1 specifically binds to LRP. This could be shown with, either isolated LRP protein or with LRP present on cell-surfaces. FSAP-Inhibitor complex binding to LRP was efficiently inhibited by the addition of the LRP antagonist RAP [164, 165], which confirmed a specific binding. This conclusion could be further strengthened by the observation that FSAP-inhibitor complexes were not internalized in LRP-deficient fibroblasts.

Since LRP interacts directly with the PDGF-BB-PDGFRβ signaling cascade, by internalizing the activated receptor and also by binding directly to PDGF-BB, thereby undergoing intracellular phosphorylation [166] which generates an Shc-docking site and subsequently propagates signal transduction [166, 167]. An alteration of LRP-function following its interaction with FSAP-inhibitor complexes was predicted. The occurrence of FSAP-inhibitor complexes that bind to LRP might thereby block LRP for other binding partners. This would mean that the inhibition of FSAP by PN-1 or PAI-1 would not only preserve PDGF-BB signaling by inhibiting FSAP-dependent cleavage, but also due to the binding of FSAP-inhibitor complexes to LRP the internalization of activated PDGFRβ would be slowed down. Therefore, PDGF-BB-signaling would be prolonged.
In these studies, an intracellular co-localization of FSAP with PDGFRβ was observed when PDGF-BB signaling occurred. If PDGF-BB was cleaved by FSAP, no intracellular staining of FSAP or PDGFRβ was observed. The distribution of PDGFRβ

throughout the cell-surface is diffuse in unstimulated cells. Therefore, no membrane-localization for quiescent PDGFRβ is detectable [148].

The presence of FSAP-PN-1 complexes exhibited no effect on PDGF-BB-PDGFRβ signaling, indicating that the LRP-internalization system is a high capacity system, which is not easily saturated. Another explanation could be that the binding of FSAP-PN-1 complexes to LRP occurs at another epitope than the one for PDGF-BB or PDGFRβ within the LRP molecule. It might also be possible that the binding happens in different cellular compartments. Therefore, there appears to be no effect of FSAP complexed with SERPINs with respect to an alteration of the PDGF-BB signaling cascade [152].

7.4 FSAP-dependent inhibition of PDGF-BB

The inhibitory effect of FSAP on PDGF-BB is strongly dependent on proteolytic processing. Therefore, the interest in the existence and location of putative FSAP cleavage-sites within the PDGF-BB molecule was high.

The family of platelet-derived growth factors has been studied extensively, and the molecular structure of PDGF-BB has been revealed by crystallography [168]. The important region in PDGF-BB and PDGF-AA for their binding to and activation of their receptors has been reported as a cluster of cationic amino-acid residues Arg_{159}-Lys_{160}-Lys_{161} [169], located in the so-called loop III region [150].

Usually the regulation of PDGF-BB activity happens via its secretion or on its release from cell membranes or the extracellular matrix [170]. Another mechanism that controls the signaling of PDGF-BB is the internalization of its activated receptor, PDGFRβ. LRP and α_2-MG receptor represent important candidates for this regulation of PDGF-BB signaling [51, 171]. PDGF-BB signaling is also regulated via other signaling

molecules, such as the hyaluronan-dependent activation of its receptor CD44. This process inhibits PDGF-BB-mediated cell activation by involvement of an intracellular tyrosine phosphatase [172]. Another mechanism dependent on the activation of PAR-1 by FIIa inhibits PDGF-BB-induced migration of fibroblasts [173]. While this mechanism is controlled by the proteolytic activity of FIIa, it is not a direct function towards the PDGF-BB or PDGFRβ molecules. For periodontal cells, a regulation mechanism that directly affects the PDGFRβ molecule has been recently described. PDGFRβ is proteolytically cleaved by elastase, which leads to the inhibition of PDGF-BB-mediated cell activation [174]. Proteolytically modified PDGF-BB was found in platelets, but this modification exhibits no altered mitogenic activity [149]. Studies on recombinant PDGF-BB revealed a proteolytic cleavage site of PDGF-BB at position R_{113}, whilst proteinase-resistant mutations exhibited increased secretion levels and higher mitogenic activity than the cleavage-susceptible variants [175, 176]. The proteases responsible for this processing at R_{113} have not yet been identified.

Our data, have shown for the first time, a direct effect of a protease on the PDGF-BB molecule which results in the complete inhibition of PDGF-BB-dependent signal transduction. The determination of FSAP cleavage sites in PDGF-BB by N-terminal sequencing revealed two distinct sites. The first cleavage site is located at $R^{\blacktriangledown}T_{114}$, which is a reported natural cleavage site in PDGF-BB. PDGF-BB which has been processed in this way represents 30 % of that found in platelets [149]. The second cleavage site of FSAP in PDGF-BB is located directly in the loop III region: $Arg^{\blacktriangledown}Lys^{\blacktriangledown}Lys_{161}$ (RKK-motif). PDGF-BB-cleavage by FSAP in this region which is vital for the activation of its downstream receptor PDGFRβ, explains the inhibitory action of FSAP towards PDGF-BB-dependent cell activation. However, PDGF-AA does not seem to be cleaved by FSAP, although it exhibits the same amino-acid

sequence as PDGF-BB in the loop III region, so not only the primary sequence but the neighboring sequences as well as the overall accessibility are important.

Since MI-FSAP is correlated with vascular diseases in its attribute of reduced proteolytic activity, it could be shown that MI-FSAP, in contrast to WT-FSAP, was not able to inhibit neointima formation in a mouse model [141]. Neointima formation or restenosis occur following stent implantation or balloon angioplasty [140], therefore, represent late complications for patients with a myocardial infarction. The narrowing of the lumen is, at least in part, dependent on PDGF-BB-mediated activation of VSMC. With this finding, the contribution of FSAP in atherosclerotic development and also to restenosis, a mechanism characterized by extensive VSMC proliferation has been elucidated. The inhibition of neointima formation is dependent on FSAP's proteolytic activity. This together with the fact that FSAP specifically cleaves PDGF-BB raises the possibility that FSAP may regulate other diseases also characterized by excessive cell stimulation dependent on PDGF-BB signaling.

Fibrotic diseases, such as liver or lung fibrosis, are at least partially driven by PDGF-BB signaling [126, 177, 178], hence a beneficial effect of exogenously applied FSAP is most likely. Newly generated blood vessels have to be stabilized by pericytes and later on by smooth muscle cells. Both these mechanisms are dependent on PDGF-BB signaling [126, 179, 180]. The investigation as to whether exogenously applied FSAP will interfere with angiogenic stimuli, e.g., in cancer-driven angiogenesis, will reveal potentially new functions or therapeutic medication possibilities for FSAP. In general, all diseases which are accompanied by extensive proliferation of cells that become activated by PDGF-BB are potential target treatments for FSAP.

7.5 Expression of recombinant FSAP

Recombinant FSAP variants were produced to gain more insight into the regulation and function of FSAP. Our first observation was that enzymatically inactive FSAP-mutants (active-site mutant, cleavage-site mutant, MI-FSAP) generated a high number of stably transfected clones. Whereas the selection of enzymatically active WT, MII- and ΔEGF-3 FSAP transfected cells generated very low numbers of viable clones. These results are in agreement with the measurement of secreted FSAP by the different mutants, which was high for inactive and low for active FSAP mutants.

Furthermore, the intracellular appearance of active FSAP-mutants compared to inactive variants differed. WT-FSAP was present in a dense-staining pattern, suggesting intracellular accumulation, whereas inactive mutants appeared in a regularly dispersed, granular pattern. Cells that expressed WT-FSAP showed signs of apoptosis, suggesting that intracellular accumulation and toxicity for host cells are dependent on FSAP enzymatic activity. A possible explanation for these cytotoxic effects might be due to the interaction of FSAP with nucleic acids [66], as in the recently described function for FSAP of nucleosome proteolysis in apoptotic cells [181]. Over-expression of other serine proteases, such as uPA, did not show any of these effects (unpublished observations). The restriction of this pro-apoptotic effect to enzymatically active FSAP suggests that FSAP cleaves or activates defined proteins within cells. If these affected proteins are of crucial importance in cellular mechanisms, such as gene-expression, cell-cycle regulation, metabolism or apoptotic regulation, the cell-death ensues. Usually FSAP is almost exclusively expressed in hepatocytes, which potentially have a specialized mechanism for the expression and secretion of proteases. Therefore, hepatocytes should be tested for the expression of recombinant FSAP and analyzed with respect to apoptotic events dependent on the enzymatic activity of FSAP.

Recombinant WT-FSAP was similar to plasma-derived, isolated FSAP. The enzymatic activity of recombinant MI-FSAP was, in line with results obtained from MI-FSAP purified from human plasma [74, 141], very low. MII-FSAP, which also exhibits an amino-acid exchange located in the serine protease domain, showed no change in enzymatic properties when compared to WT-FSAP. The two point-mutation variants, cleavage-site ($R_{313}G$) and active-site ($H_{362}F$) mutant were as expected, completely inactive. In order to perform structure-studies it is a prerequisite to produce large amounts of recombinant FSAP. Here the foundation for such production has been laid.

Studies on proteolytic FSAP fragments [151], showed that the EGF-3 domain is important for the binding of cofactors to FSAP. When using the binding competition assay and from older studies, one could conclude that heparin, RNA and polyphosphate use the same binding region in the FSAP molecule, namely the EGF-3 domain. The FSAP-isoform that lacks the EGF-3 domain is not activated by polyanions to the same efficiency as WT-FSAP. A strong effect of heparin and polyphosphate in the context of PAI-1-dependent inhibition of FSAP was observed. As expected, this effect was abolished when the inhibition of ΔEGF-3-FSAP by PAI-1 was measured. Hence, a crucial role for the EGF-3 domain with respect to regulation of FSAP activity has been confirmed.

7.6 Conclusion

With the herein presented data new findings that shed light on the complex regulatory mechanism of FSAP activation, inhibition and turnover are provided.

The ability of FSAP to inhibit PDGF-BB-dependent cell activation is the first mechanism of its kind to be described. Therefore, it is concluded that FSAP is important for the regulation of PDGF-BB activity in the vasculature. Since PDGF-BB

signaling has been reported in many diseases, a correlation with the MI-SNP of FSAP in respect to the occurrence of diseases or the type of their progression should be investigated. Thus, FSAP represents a possible new protein with a high potential that can be used for therapeutic applications to treat atherosclerosis, fibrosis, cancer and other related diseases (Figure 26).

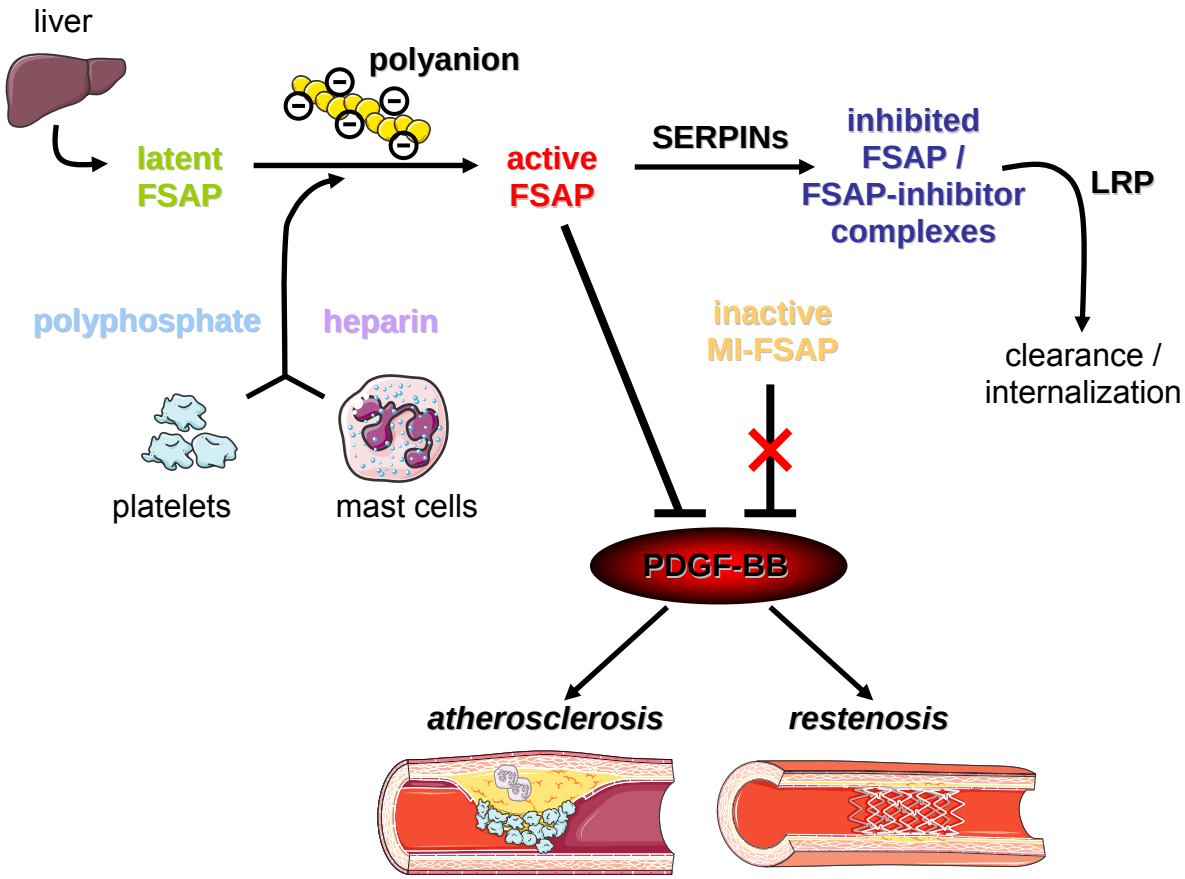


Figure 26: *Summarizing scheme.* Latent FSAP is secreted by the liver and undergoes auto-catalytic activation, which is amplified by negatively charged polyanions, such as, polyphosphate or heparin. The active FSAP is efficiently inhibited by serine protease inhibitors (SERPINs), such as PAI-1 and PN-1 and thereafter, the complexes can bind to LRP and undergo internalization. FSAP is a potent inhibitor of PDGF-BB-mediated cell activation, whereas the MI-FSAP SNP exhibits reduced proteolytic activity and therefore, is not able to inhibit PDGF-BB signaling. Hence, FSAP contributes to the development of vascular diseases, such as atherosclerosis and restenosis through its regulation of PDGF-BB signaling.

8. Summary

The functions of factor VII activating protease (FSAP) in the vascular system have been investigated. Marburg I (MI)-FSAP-polymorphism correlates with various vascular diseases and is characterized by a reduced proteolytic activity. Hence, the activity of FSAP appears to be of pivotal importance in vascular homeostasis. Further evidence for the contribution of FSAP to vascular pathophysiology was provided by the fact that FSAP-antigen and mRNA have been found within atherosclerotic plaques. FSAP circulates as an inactive zymogen in plasma and is activated by negatively charged polyanions. However, the precise mechanisms of FSAP-activation have not yet been elucidated. FSAP is known to inhibit platelet-derived growth factor (PDGF)-BB-mediated cell activation, whilst PDGF-BB is an important factor in the development of atherosclerosis and contributes to other fibrotic and cancer-related diseases.

FSAP has been shown to be activated by heparin and RNA. Herein it has been demonstrated that polyphosphate, released by activated platelets, is a newly detected cofactor for FSAP. The high negative charge-to-size ratio and overall molecular size of polyanions qualify them as cofactors for FSAP. Furthermore, by using recombinant ΔEGF-3-FSAP, the importance of FSAP's EGF-3 domain in its interaction with polyanions could be confirmed. Heparin is released by mast cells, polyphosphate by platelets and RNA might be released from dying cells. Therefore, these cofactors can be present at sites of atherosclerosis and regulate FSAP function in these circumstances.

FSAP is inhibited by serine protease inhibitors, such as protease nexin (PN)-1, plasminogen activator inhibitor (PAI)-1 and antithrombin III. If FSAP was complexed with either PN-1 or PAI-1 its internalization in a LDL receptor-related protein (LRP)-dependent mechanism by smooth muscle cells and fibroblasts could be shown. Hence, LRP accounts for the clearance of FSAP-inhibitor complexes. The functionality of LRP

is crucial in the regulation of PDGF-BB signaling. However, the interaction of FSAP-inhibitor complexes with LRP had no impact on the LRP-mediated regulation of PDGF-BB signal-transduction.

The inhibitory effect of FSAP on PDGF-BB-dependent cell activation occurs through specific cleavage of the PDGF-BB molecule. The cleavage-sites of FSAP in the PDGF-BB molecule have been identified. This revealed that FSAP-dependent cleavage had destroyed the receptor binding motif of PDGF-BB. Hence, the reduced proteolytic activity of MI-FSAP is the determinant for its correlation with vascular diseases, which has been demonstrated by its inability to inhibit PDGF-BB-dependent smooth muscle cell activation.

With the herein presented results, new insights into the complex mechanisms that are involved in FSAP-regulation have been created. The improved description of these mechanisms may lead to novel therapeutic approaches in the treatment of fibro-proliferative, cancer-related and atherosclerotic diseases.

9. Exposé

Die Aufgaben der Faktor VII aktivierenden Protease (FSAP) im vaskulären System wurden untersucht. Der Marburg I (MI)-FSAP Polymorphismus zeigt eine Verbindung zu verschiedenen vaskulären Erkrankungen auf, und zeichnet sich durch eine verringerte enzymatische Aktivität aus. Daraus wird ersichtlich, dass die Aktivität der FSAP von entscheidender Bedeutung für die vaskuläre Homeostase ist. Weitere Hinweise auf eine Verbindung zur vaskulären Pathophysiologie wurden durch die Tatsache, dass Protein und mRNA der FSAP in arteriosklerotischen Plaques gefunden wurden, geliefert. Die FSAP zirkuliert als inaktives Zymogen im Plasma und wird von negativ geladenen Polyanionen aktiviert. Die genauen Mechanismen zur Aktivierung der FSAP sind jedoch noch nicht aufgeklärt. Es ist bekannt, dass die FSAP die platelet-derived growth factor (PDGF)-BB abhängige Zellaktivierung inhibiert, während PDGF-BB ein wichtiger Faktor für die Entstehung von Arteriosklerose, und beteiligt an fibrotischen und krebs-assoziierten Erkrankungen, ist.

Es wurde gezeigt, dass die FSAP von Heparin und RNA aktiviert wird. Hier wird nun demonstriert, dass Polyphosphat, welches von aktivierten Thrombozyten freigesetzt wird, ein neu entdeckter Kofaktor der FSAP ist. Ein hohes Verhältnis von negativer Ladung zur Größe und die allgemeine molekulare Größe qualifizieren Polyanionen zu Kofaktoren der FSAP. Weiterführend konnte, durch die Verwendung von rekombinantem ΔEGF-3-FSAP, die Bedeutung der FSAP-EGF-3 Domäne, für ihre Interaktion mit Polyanionen, bestätigt werden. Heparin wird von Mastzellen freigesetzt, Polyphosphate von Thrombozyten und RNA könnte von sterbenden Zellen freigesetzt werden. Demnach können diese Kofaktoren in Bereichen von Arteriosklerose vorhanden sein und dort die Funktionen der FSAP regulieren.

Die FSAP wird durch Serinprotease Inhibitoren wie Protease Nexin (PN)-1, Plasminogen Aktivator Inhibitor (PAI)-1 und Antithrombin III inhibiert. Wenn die FSAP in Komplexen mit PN-1 oder PAI-1 vorlag, konnte ihre Internalisierung durch einen LRP abhängigen Mechanismus, in glatten Muskelzellen und Fibroblasten, aufzeigen werden. Demzufolge ist LDL Receptor-releated protein (LRP) für die Beseitigung der FSAP-Inhibitor Komplexe verantwortlich. Die Funktionalität des LRP ist wichtig für die Regulation des PDGF-BB Signalweges. Die Interaktion der FSAP-Inhibitor Komplexe mit LRP hatte jedoch keinen Einfluss auf die LRP abhängige Regulation der PDGF-BB Signalübertragung.

Der inhibitorische Effekt der FSAP auf die PDGF-BB abhängige Zellaktivierung wird durch eine spezifische Spaltung des PDGF-BB Moleküls erzielt. Die Spaltstellen der FSAP im PDGF-BB Molekül wurden identifiziert. Damit wurde aufgezeigt, dass durch die FSAP abhängige Spaltung das rezeptorbindende Motiv des PDGF-BB zerstört wird. Demzufolge ist die verringerte proteolytische Aktivität der MI-FSAP der bestimmende Faktor für ihre Verbindung zu vaskulären Krankheiten, welches durch ihre Unfähigkeit zur Inhibition der PDGF-BB abhängigen Aktivierung glatter Muskelzellen aufgezeigt wurde.

Mit den in dieser Arbeit präsentieren Resultaten wurden neue Einsichten in die komplexen Mechanismen, welche an der Regulation der FSAP beteiligt sind, geschaffen. Ein besseres Verständnis dieser Mechanismen könnte zu neuen therapeutischen Ansätzen zur Behandlung von fibro-proliferativen, krebs-assoziierten und arteriosklerotischen Krankheiten führen.

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11. Supplement

11.1 Danksagung

Mein erster Dank gilt der gesamten Arbeitsgruppe Preissner, die mich ich den letzten Jahren aufgenommen und zum gelingen meiner Arbeiten maßgeblich beigetragen hat. Besonderen Dank möchte ich Professor Klaus T. Preissner aussprechen, für die Bereitstellung der Gerätschaften und finanzielle Realisierung meiner Arbeiten sowie sein stetiges Interesse an meiner Arbeit, die kritischen Diskussionen und seine Ideen zur Erforschung der FSAP.

Meinen größten Dank möchte ich PD Dr. Sandip M. Kanse entgegenbringen. Für Alles was er mir in den vergangenen Jahren ermöglicht hat, für die vielen Stunden des Ideenaustausches, für seinen uneingeschränkten Enthusiasmus was meine Forschung betrifft und die ausgezeichnete Unterstützung während meiner Zeit im Biochemischen Institut.

Thomas Schmidt-Wöll, Susanne Tannert-Otto, Dr. Karin Hersemeyer und Bärbel Fühler möchte ich für ihre Geduld und Hingebung danken, die sie mir zu teil werden ließen um mir die methodischen Geschicke im Labor zu erklären.

Dr. Sigrid Schmitt und Hans Günter Welker möchte ich für ihre Unterstützung beim Sequenzieren danken.

Professor Dr. Alfred Pingoud möchte für die Ermöglichung meiner Promotion danken, für seine Bereitschaft meine Arbeit von Seiten des Fachbereichs 08 zu betreuen.

Professor Dr. Adriaan Dorresteijn möchte ich danken, für sein Interesse an dieser Arbeit und seine Bereitschaft diese aus der Sichtweise der Entwicklungsbiologie zu prüfen.

PD Dr. Thomas Noll, Anna Reis und Annika Krautwurst möchte ich für die Aufnahme und die schöne Zeit im Graduiertenkolleg 534 „Biologische Grundlager der

vaskulären Medizin“ danken. Diese Arbeit wurde durch den Graduiertenkolleg 534 finanziert.

PD Dr. Roger Dennis und Anja Müller möchte ich Danken, für die vielen Stunden die sie, für die Korrektur der englischen Sprache, für mich geopfert haben.

Christiane Hild gebührt mein innigster Dank für die seelisch-moralische Unterstützung während der letzten Wochen. Vielen Dank!

Diese Arbeit ist meinen Eltern, Gertrud und Reinhold Muhl, gewidmet, und dies aus gutem Grund. Ich möchte euch von ganzem Herzen danken, für eure Unterstützung seit nunmehr 28 Jahren. Ohne euch währe dies alles nicht möglich gewesen. Vielen Dank für den Rückhalt den ihr mir biete, für das Interesse an meinem Tun, für all die Annehmlichkeiten die ich ohne euch niemals hätte erleben können, für den Mut den ihr mir gebt und die Ermöglichung meiner Zukunft. VIELEN DANK!

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

11.2 Erklärung

Ich erkläre:
Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Gießen, den 03. August 2009 _____

Lars Muhl