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Inhibition of PDGF-BB by factor VII activating protease is neutralized by protease nexin-1 and the FSAP-inhibitor complexes are internalized via LRP.

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Abstract

Factor VII activating protease (FSAP) can inhibit neointima formation and vascular smooth muscle (VSMC) proliferation by cleavage of platelet derived growth factor-BB (PDGF-BB). Negatively charged polyanions lead to autoactivation of the FSAP but no information is **available concerning the potential regulation of FSAP activity and its metabolism** in the vessel wall. We now demonstrate that the enzymatic activity of FSAP can be inhibited by the serine protease inhibitor (SERPIN), protease nexin-1 (PN-1), that **is** found in the vasculature. This leads to the loss of the inhibitory effect of FSAP on PDGF-BB-mediated DNA synthesis and MAPK phosphorylation in VSMC. The FSAP-PN-1 complexes bind to the low density lipoprotein receptor related protein (LRP) and **are** subsequently internalized. This binding is inhibited by receptor associated protein (RAP) an antagonist of LRP **as well as heparin**. While PDGF β R is internalized by an LRP-dependent mechanism after stimulation of cells by PDGF-BB, the FSAP-PN-1 complexes neither influenced PDGF-BB-mediated phosphorylation of PDGF β R nor its internalization via LRP. Hence, PN-1 inhibits the **enzymatic activity of FSAP and neutralizes its effect on PDGF-BB-mediated VSMC proliferation**. The FSAP-inhibitor complexes are internalized via LRP without influencing the PDGF-BB signal transduction pathway.

Introduction

The factor seven activating protease (FSAP) is a serine protease containing three EGF domains a kringle domain and a serine protease domain [1]. We have previously shown that FSAP is a potent inhibitor of platelet derived growth factor-BB (PDGF-BB)-dependent proliferation and migration of vascular smooth muscle cells (VSMC) [2]. These are important processes in the development of atherosclerotic disease and restenosis after angioplasty or stenting. Furthermore, the level of active FSAP in the vessel wall is a key determinant of neointima formation in a mouse model of injury-induced stenosis [3].

FSAP is auto-activated in the presence of negatively charged polyanions such as heparin, hyaluronic acid or nucleic acid [4-7]. The Marburg I single nucleotide polymorphism leads to a single amino acid exchange (G534E) in the protease domain of the molecule and exhibits diminished proteolytic activity compared to wild type FSAP [8]. The inhibitory effect of **Marburg I** FSAP on PDGF-BB dependent cell proliferation and neointima formation was diminished compared to **wild type** FSAP [3]. This indicates that the proteolytic activity of FSAP is a key determinant of the VSMC activation status **in vivo**. The enzymatic activity of FSAP is known to be inhibited by plasma serine protease inhibitors (SERPINS) such as C1-inhibitor, α 2-antiplasmin, antithrombin / heparin or aprotinin [9]. The SERPIN, protease nexin-1 (PN-1) is produced mainly by vascular smooth muscle cells (VSMC) [10], pericytes [11] and fibroblasts [12] and it is not known if **it influences** FSAP activity.

The low density lipoprotein receptor-related protein (LRP) is a member of a large receptor family responsible for **the** endocytosis of a variety of ligands [13]. LRP is responsible for the internalization of proteases, protease-inhibitor complexes and lipoproteins [14]. The ligands, once bound to LRP, are internalized and directed to endosomes and undergo degradation whereas LRP is recycled to the cell membrane [15]. Urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) bind to LRP alone as well as in complex with SERPINS [13]. It is not known if FSAP can interact with LRP. Recently it has been reported that LRP **is a signal transduction receptor that** is involved in the regulation of the PDGF-BB- PDGF β receptor (PDGF β R) signaling pathways [14].

In this study we demonstrate that the SERPIN PN-1, found in the vasculature, can inhibit FSAP. FSAP-PN-1 complexes, but not FSAP alone, interact with the scavenger receptor LRP and are **subsequently** internalized. PN-1 inhibits FSAP-mediated cleavage and inhibition of PDGF-BB but, FSAP-PN-1 complexes do not influence the PDGF-BB-PDGF β R internalization **by LRP** or signal transduction processes.

Material and Methods

Cell culture: Wild type (WT) and LRP^{-/-} mouse embryo fibroblasts (MEF) were cultivated in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) with 10% 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). Mouse vascular smooth muscle cells (VSMC) were cultured in Iscov's modified medium (Invitrogen) with the same supplements as above. Cells were growth arrested in serum-free medium for 18 h prior to experiments.

Immunocytochemistry: Cells in 8-well chamber slides were incubated with the test substances for the indicated times and washed and fixed with 3.7% (wt/vol) paraformaldehyde, permeabilised with 0.2% (wt/vol) Triton X-100 (Sigma, Munich, Germany) and blocked with 3% (wt/vol) BSA (Sigma). After incubation with the primary antibody against the FSAP (mAb 1189 or mAb 677) (ZLB Behring, Marburg, Germany), LRP (Rabbit polyclonal #2629 generously provided by Dr. Dudley Strickland, American Red Cross, Rockville, Maryland), secondary antibodies labeled with **fluorescence 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 and the images were prepared with the Metamorph software (Visitron, Puchheim, Germany).

DNA-synthesis assays: VSMC were stimulated in **0.2% FCS containing** medium for 24 h with the test substances. For the last 4 h BrdU was added and the cells were processed with a BrdU detection kit (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer.

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. PhosphoMAPK antibody (ERK-p42/44) was from Cell Signaling Technology (Danvers, Massachusetts) and the total MAPK antibody from Upstate (Charlottesville, Virginia)

Western blot analysis: After SDS-polyacrylamid gel proteins were transferred to **polyvinylidene fluoride (PVDF)** -membranes. Western blotting was performed using

enhanced chemiluminescence (ECL) plus reagent (GE Healthcare, Uppsala, Sweden) as described by the manufacturer. **For Western blot analysis of FSAP a mixture of antibodies directed against the N-terminal end (mAb 1189) and against the C-terminal end (mAb 677) was used as described before [2] in order to detect degraded forms of FSAP, if present.**

FSAP activity assay: FSAP was isolated from human plasma as described before [5]. To determine activation and enzymatic activity of FSAP, the hydrolysis of the chromogenic substrate **H-D-Isoleucyl-L-prolyl-L-arginine-p-nitroaniline dihydrochloride** (S-2288) (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.). **Recombinant PN-1 was produced in Sf9 insect cells and purified in Blue Agarose and Sephacryl S-200 as described before [16]. Concentration of active PN-1 was determined by inhibition of thrombin and determination of the residual thrombin activity as described [16].** PN-1 and aprotinin were preincubated with FSAP for 30 min prior to the enzymatic activity assay. The assays were performed with a final FSAP-concentration of 1 µg/ml in buffer containing 10 µg/ml heparin.

FSAP binding to LRP: LRP was isolated from human placenta as described before [17]. LRP and the respective control buffer containing 2 µg/ml BSA were immobilized, in Tris (50 mM, pH 7.4) containing NaCl (150 mM) in a Maxisorp plate (Nunc, Wiesbaden, Germany). Thereafter the plate was blocked with 3% (wt/vol) 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) (ZLB Behring) followed by a horse reddish peroxidase (HRP) conjugated secondary antibody (Dako, Glosstrup, 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.

Expression and purification of receptor associated protein (RAP): The constructed plasmid with His-tag, ampicillin (Amp)-resistance and human RAP cDNA was a kind gift from Dr. Willnow (MDC, Berlin, Germany). Extracts were purified over a ProBondTM column (Promega, Mannheim, Germany) and fractions containing the protein were dialyzed against TBS pH 8.0 containing 5 mM reduced glutathione (GSH) and 1mM oxidized glutathione (GSSG) for refolding and finally dialyzed against TBS pH 8.0.

Results

Inhibition of FSAP by PN-1 and the binding of the FSAP-PN-1 complex to LRP: PN-1 is a SERPIN produced locally in the vessel wall **and it** neutralized the enzymatic activity of FSAP as measured by the direct chromogenic substrate S-2288 (Fig. 1A). **The presence of heparin did not influence the FSAP inhibition characteristics (Fig. 1A).** The formation of complexes between FSAP and PN-1 could be observed in a dose dependent manner with Western blotting (Fig. 1B). Several protease protease-inhibitor complexes like uPA and tPA in complex with PN-1 or plasminogen activator inhibitor (PAI)-1 are known to bind to LRP hence the binding of FSAP-inhibitor complexes to the immobilized LRP was investigated. FSAP-PN-1 complex showed a strong specific binding whereas FSAP by itself, or in complex with aprotinin (**data not shown**), exhibited no binding at all to LRP (Fig. 2A). **The binding of FSAP-PN-1 complex was inhibited by heparin (Fig. 2A).** Concentration-dependent analysis confirmed that FSAP-PN-1 complex binding **showed saturation** (Fig. 2B). The binding of FSAP-PN-1 complex could be inhibited with receptor associated protein (RAP) (Fig. 2C), a 39 kDa LRP antagonist, known to inhibit ligand binding to LRP [18]. Maximal inhibition ($\approx 70\%$) was observed with 10 $\mu\text{g/ml}$ RAP.

Binding of FSAP-PN-1 complex to LRP on cells: The binding of FSAP and FSAP-PN-1 complexes to cells was analyzed with immunofluorescence microscopy. Application of FSAP alone to VSMC did not lead to an accumulation of cellular FSAP (Fig. 3b), whereas FSAP-PN-1 complexes were internalized by VSMC (Fig. 3d). Co-staining of FSAP (Fig. 3d; green) and LRP (Fig. 3d; red) on VSMC shows a co-localization of the ligand and the receptor in the intracellular compartment (Fig. 3d). 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 (Fig. 3h). Time course analysis showed that FSAP-PN-1 complexes were internalized within minutes (Online supplemental data 1) and that heparin inhibited this internalization (Online supplemental data 2). FSAP-PN-1 complex but not FSAP alone was internalized by wild type-mouse embryo fibroblasts (MEF) but not by LRP^{-/-} MEF (Online supplemental data 3). FSAP-PN-1 complex binds to LRP, **undergoes** internalized via the scavenger receptor and is directed to lysosomes.

Effect of FSAP-PN-1 complex on PDGF-BB-induced internalization of PDGF β R: Since FSAP is a strong inhibitor of the PDGF-BB signaling pathway and LRP is also involved in modulating the same pathway we investigated the interactions between FSAP, LRP and

PDGF β R. In unstimulated cells PDGF β R is diffusely distributed over the whole cell membrane and upon stimulation with PDGF-BB internalization of PDGF β R into lysosomes is observed (Fig. 3i; red) as has been described before [19, 20]. This PDGF-BB-induced internalization of PDGF β R was not influenced by the presence of FSAP-PN-1 complex (Fig. 3l; red). Hence, FSAP-PN-1 complex is internalized via LRP but this does not influence the internalization of PDGF-BB-PDGF β R by LRP even though there was co-localization of FSAP-PN-1 complex (Fig. 3m; green) with internalized PDGF β R (Fig. 3m; red). **No PDGF β R staining was observed in the presence of PDGF-BB and FSAP due to cleavage and inactivation of PDGF-BB so that the diffuse distribution of PDGF β R remained unchanged (Fig. 3j).** Although FSAP-PN-1 complexes as well as the PDGF-BB-PDGF β R complexes bind to LRP, their internalization was completely independent of each other.

Effect of FSAP-PN-1 complex on PDGF BB-dependent cell activation: Finally, we have investigated if FSAP-PN-1 complex can influence PDGF-BB-mediated signal transduction. Preincubation of PDGF-BB with FSAP diminished 42/44-MAPK phosphorylation in VSMC (Fig. 4a) because of cleavage and inactivation of PDGF-BB (Online supplemental data 4). Preincubation with FSAP-PN-1 did not alter PDGF-BB-mediated phosphorylation of 42/44 MAPK in VSMC (Fig. 4A). Inhibition of the proteolytic activity of FSAP by PN-1 led to neutralization of its ability to inhibit PDGF-BB-mediated DNA synthesis (Fig. 4B). 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 PDGF β R was present in LRP^{-/-} MEF than in wild type MEF and higher levels of tyrosine phosphorylation were observed in LRP^{-/-} MEF than in WT MEF after PDGF-BB stimulation (Online supplemental data 5). 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 PDGF β R (Online supplemental data 4).

Discussion

Inhibition of PDGF-BB-mediated VSMC proliferation by FSAP was observed *in vitro* [2] and *in vivo* in a mouse model of neointima formation [3]. The naturally occurring Marburg I form of FSAP has diminished proteolytic activity and is a weaker inhibitor of VSMC proliferation [3]. This could explain why this form of FSAP is a strong risk factor for cardiovascular diseases in general [21, 22]. Hence, the proteolytic activity of FSAP is a key

determinant in this scheme of atherothrombosis. PN-1 is a prominent SERPIN in the vessel wall produced by vascular smooth muscle cells and it is capable of inhibiting the enzymatic activity of FSAP. There are numerous studies indicating that PN-1 is up-regulated in hypertension [10] and atherosclerosis [23]. Hence, through inhibition of FSAP, PN-1 can regulate the activity of FSAP towards PDGF-BB.

FSAP is homologous to factors from the coagulation and the fibrinolysis system and LRP plays a critical role in the regulation of their activity and metabolism. Through internalization of the active factor IXa [24] and VIIIa [25] LRP also indirectly regulates the activity of the coagulation system. Complexes of plasminogen activator inhibitor-1 (PAI-1) with proteases are internalized by LRP and this process is heparin-dependent [26]. The binding of PN-1-thrombin but not PN-1-uPA complex was also heparin-dependent [27]. With some exceptions such as PN-1 [28] or factor VIII [29] the binding of individual factors to LRP is weak.

FSAP-PN-1 complexes bound to LRP and were internalized by cells as are other protease-inhibitor complexes [30]. Internalization was not observed in LRP^{-/-} MEF cells or in the presence of the specific LRP inhibitor, RAP. Neither FSAP alone nor the FSAP-aprotinin complex binds to LRP indicating that the recognition by LRP is only possible when FSAP is in complex with PN-1. **Hence PN-1 contributes binding elements necessary for the binding of FSAP-PN-1 complex to LRP. The binding of FSAP-PN-1 complex was inhibited by heparin, the likely reason is that PN-1 is a strong heparin binding protein [31] analogous to FSAP. Our preliminary data shows that another SERPIN, plasminogen activator inhibitor-1 (PAI-1) also inhibits FSAP (Wygrecka et al. unpublished results) and the FSAP-PAI-1 complex binds to LRP and is internalized.**

LRP also plays an important role in regulating the signal transduction pathway at many levels. LRP seems to bind to PDGF-BB directly [32], but not bFGF, thereby increasing the local concentration of PDGF-BB. LRP is phosphorylated upon stimulation of cells with PDGF-BB and there is a crosstalk between PDGFβR and LRP. Phosphorylation of LRP generates a Shc docking site propagating the signal [32-34]. In its unphosphorylated state LRP functions as an endocytic receptor and inhibits PDGF-BB-mediated signal transduction. In the absence of LRP, PDGF-BB dependent signal transduction is increased *in vivo* and leads to atherosclerosis [35, 36].

Since FSAP-inhibitor complexes also bind to LRP we have hypothesized that PDGF-BB signal transduction might be altered since this is partially dependent on LRP. The binding of FSAP-PN-1 complex to LRP does not influence the function of LRP with respect to the

PDGF-BB-PDGF β R axis. The possible reasons are that FSAP-PN-1 complexes bind to a different region of LRP than the PDGF-BB-PDGF β R complex and no competition ensues. **Alternatively**, LRP that is involved in internalization of the FSAP-inhibitor complex is localized in a different cellular compartment compared to LRP involved in PDGF-BB / PDGF β R signal transduction. Another reason could be that the LRP system is high a capacity system that is not easily saturated, hence the lack of competition between the different LRP ligands.

We have recently shown that a naturally occurring variant of FSAP, the Marburg I variant, has reduced proteolytic activity and cannot inhibit neointima formation *in vivo* in a mouse injury model. Hence, the inhibition of FSAP activity by a locally produced SERPIN such as PN-1 is a likely regulatory element in this pathway [3]. The FSAP-inhibitor complexes are internalized via LRP without influencing the signal transduction-related functions of LRP with respect to PDGF-BB and PDGF β R. In human atherosclerotic plaques high intracellular FSAP staining has been observed in VSMC and macrophages indicating that this mechanism is likely to function *in vivo* [2].

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Figure legends

Figure 1: Inhibition of FSAP by PN-1 and complex formation. (A) FSAP (1 $\mu\text{g/ml}$) was preincubated with PN-1 in the absence (closed circles) or presence of heparin (10 $\mu\text{g/ml}$) (open circles) in the concentration range 0.5 – 2.0 $\mu\text{g/ml}$ for 30 min. FSAP-activity was measured with the conversion of 0.2 mM specific chromogenic substrate S-2288; mean \pm SD (n=3). (B) FSAP (30 ng/lane) was preincubated with PN-1, in the concentration range 10 – 80 ng/lane in the presence of heparin (10 $\mu\text{g/ml}$). Western blot analysis was performed using two monoclonal antibodies against FSAP. These experiments were repeated 3 times with similar results.

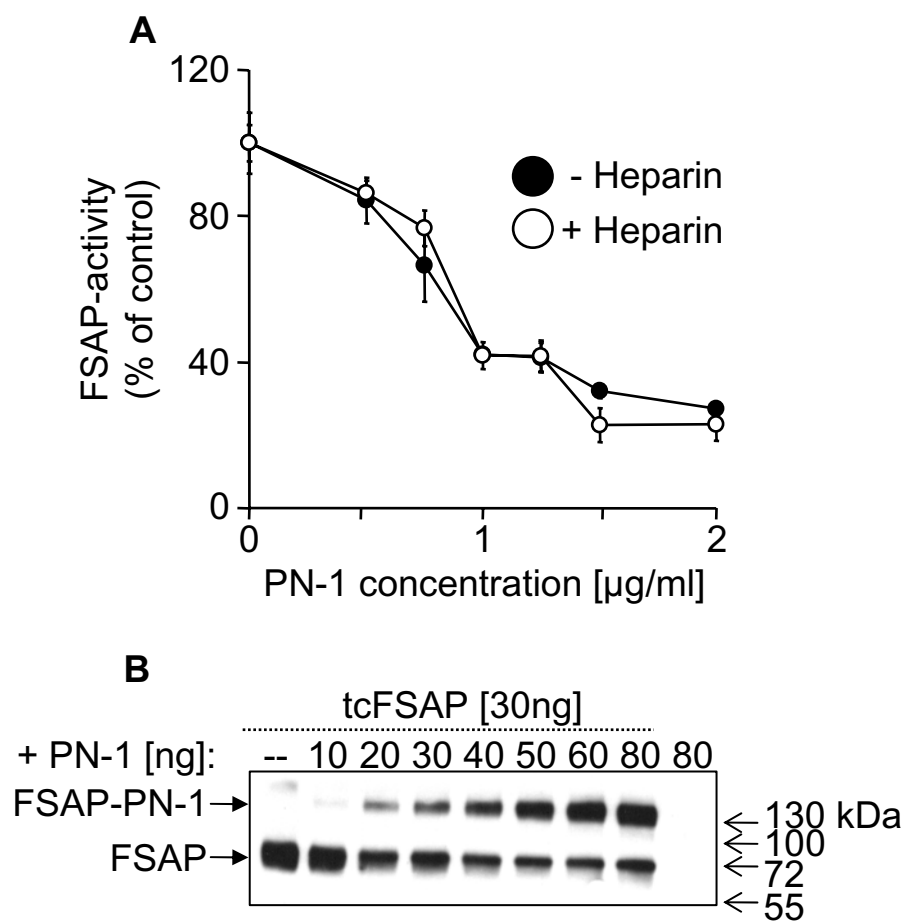
Figure 2: FSAP-PN-1 complex binding to LRP. (A) FSAP (1 $\mu\text{g/ml}$) was preincubated for 30 min with PN-1, (2 $\mu\text{g/ml}$) to allow complex formation and the binding to immobilized LRP (1 $\mu\text{g/ml}$) was measured in absence (dark bars) or presence of heparin (10 $\mu\text{g/ml}$) (open bars) using a monoclonal antibody against FSAP; mean \pm SD (n=3). (B) FSAP (1 $\mu\text{g/ml}$) was preincubated with PN-1 in the concentration range of 0.05 – 2 $\mu\text{g/ml}$ for 30min to allow complex formation and binding of the FSAP-inhibitor complexes to immobilized LRP (1 $\mu\text{g/ml}$) and BSA was measured by specific monoclonal antibody against FSAP, mean \pm SD (n=3). (C) FSAP (1 $\mu\text{g/ml}$) was preincubated with 2 $\mu\text{g/ml}$ PN-1 for 30 min to allow complex formation. Binding of the FSAP-inhibitor complexes to immobilized LRP (1 $\mu\text{g/ml}$), without (dark columns) or with 10 $\mu\text{g/ml}$ RAP (striped columns), and control buffer, containing BSA (2 $\mu\text{g/ml}$) was measured by specific monoclonal antibody against FSAP; mean \pm SD (n=3). These experiments were repeated 3 times with similar results.

Figure 3: Binding of FSAP-PN-1 complex to LRP and the influence of this complex on PDGF β R distribution in VSMC. (Top panel) Buffer control (a, e), FSAP (1 $\mu\text{g/ml}$) alone (b, f) and PN-1 (2 $\mu\text{g/ml}$) alone (c, g) or together (d, h) were preincubated for 30 min to allow complex formation and added to VSMC without (upper panel, a, b, c, d) or with RAP (10 $\mu\text{g/ml}$) (lower panel, e, f, g, h) for 45 min. FSAP was stained with FITC-labeled secondary antibody indicated in green, LRP with Rhodamine red-X-labeled secondary antibody indicated in red and nuclei with DAPI indicated in blue (a-h). Yellow color indicates co-localization of FSAP and LRP and is highlighted by white arrows. (Bottom panel) PDGF-BB (20 ng/ml) was preincubated with buffer (i), FSAP (1 $\mu\text{g/ml}$) (j), PN-1 (2 $\mu\text{g/ml}$) (k) or FSAP-PN-1 complex (l) for 60 min on 37°C. VSMC were incubated with these mixtures for 30 min on 37°C. PDGF β R was stained with Rhodamine red-X-labeled secondary antibody indicated in red and nuclei with DAPI indicated in blue (i-l). White arrows highlight examples of intracellularly accumulated PDGF β R. To examine the co-localization of FSAP and PDGF β R cells were prepared as in (l) and FSAP was stained with FITC-labelled secondary antibody indicated in green, PDGF β R with Rhodamine red-X-labeled secondary antibody indicated in red and nuclei with

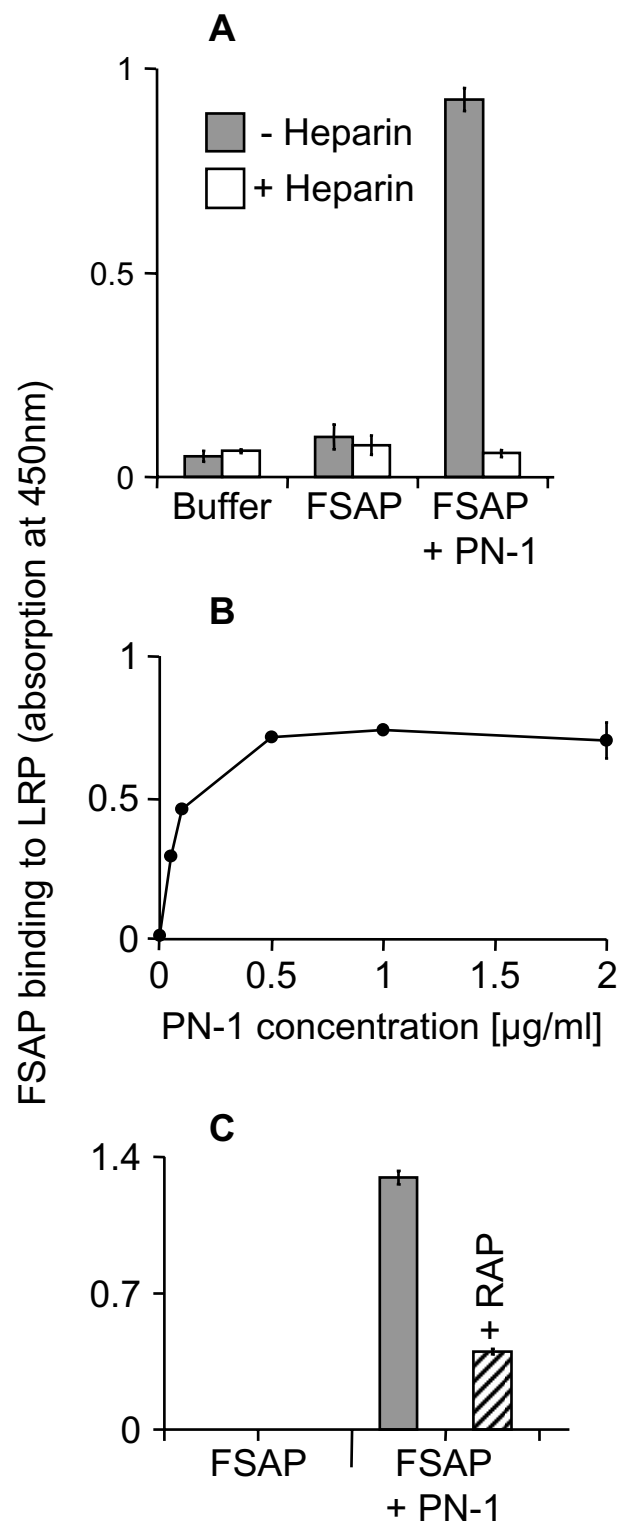
DAPI indicated in blue. Yellow colour and white arrows highlight examples of intracellular co-localization of FSAP-PN-1 complex with PDGF β R (m). Calibration bar = 20 μ m. This staining pattern was observed in 80% of cells on a slide and this experiment was repeated 2 times.

Figure 4: Influence of LRP on the inhibitory effect of FSAP on PDGF-BB-dependent VSMC activation. (A) In serum-free medium containing heparin (10 μ g/ml) VSMC were stimulated with PDGF-BB (20 ng/ml), PDGF-BB preincubated with FSAP (1 μ g/ml) or PDGF-BB preincubated with FSAP-PN-1 complex in the absence or presence of RAP (10 μ g/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 μ g/ml) (open column). DNA-synthesis was measured (mean \pm SD, n=3) using a kit to measure BrdU incorporation into newly synthesized DNA. Similar results were obtained in 3 independent replicates of these experiments.

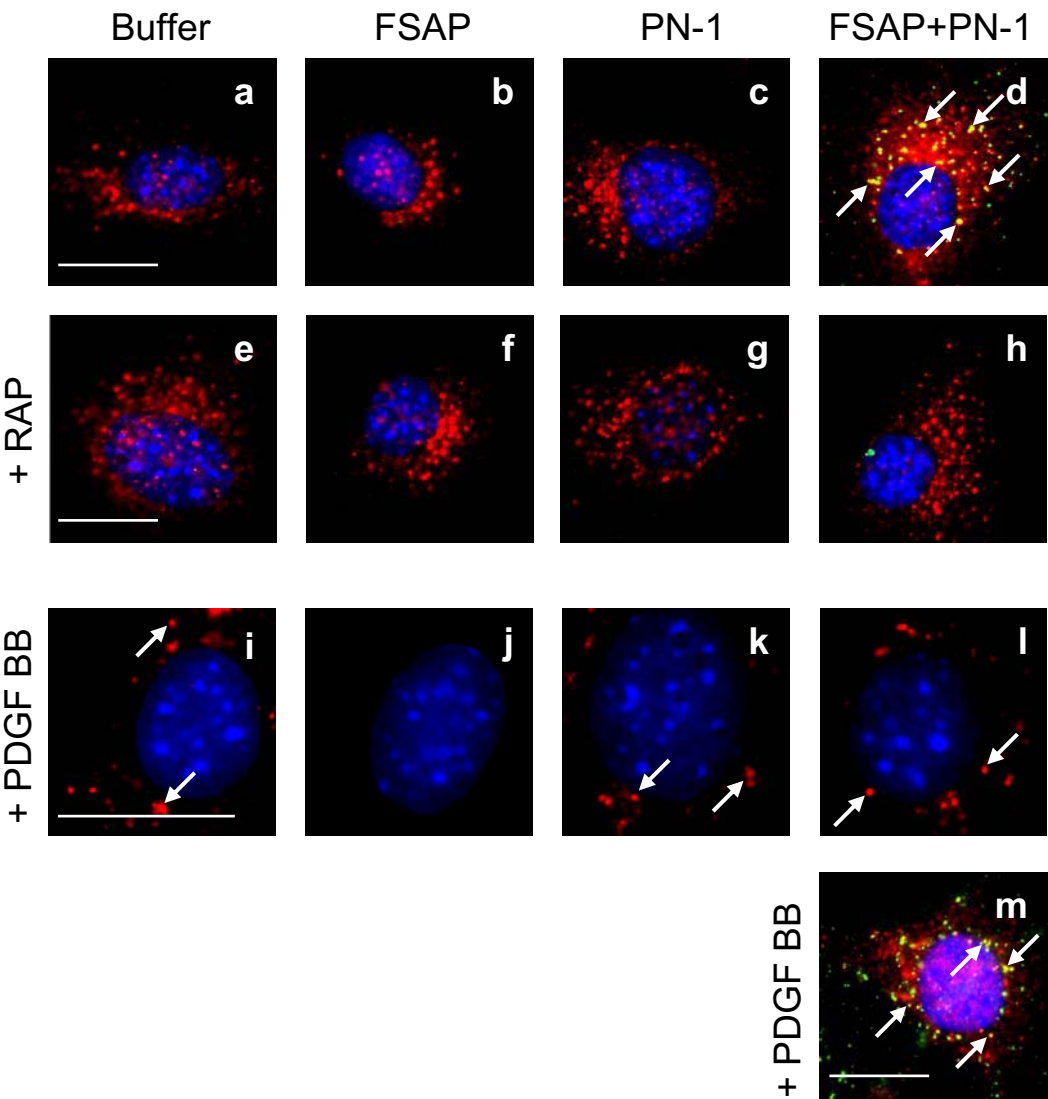
Muhl et al. Figure 1 A-B



Muhl et al. Figure 2 A-C



Muhl et al. Figure 3



Muhl et al. Figure 4 A-B

