THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 10, pp. 7193–7203, March 8, 2013

The Factor VII-activating Protease (FSAP) Enhances the Activity of Bone Morphogenetic Protein-2 (BMP-2)*[S]

Received for publication, November 5, 2012, and in revised form, January 14, 2013 Published, JBC Papers in Press, January 22, 2013, DOI 10.1074/jbc.M112.433029

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Background: Polymorphisms in the gene encoding for Factor VII-activating protease (FSAP) are a risk factor for atherosclerosis and vascular calcification.
Results: FSAP mediates proteolytic cleavage and activation of bone morphogenetic protein-2 (BMP-2).
Conclusion: FSAP regulates BMP-2-dependent proliferation and osteogenic differentiation of several cell types.
Significance: Activation of BMP-2 by FSAP provides a novel mechanistic insight into the actions of FSAP in remodeling-associated diseases.

Factor VII-activating protease (FSAP) is a circulating protease involved in the pathogenesis of atherosclerosis, calcification, and fibrotic processes. To understand how FSAP controls the balance of local growth factors, we have investigated its effect on the regulation of bone morphogenetic proteins (BMPs). BMP-2 is produced as a large pro-form and secreted as a mature heparin-binding growth factor after intracellular processing by pro-protein convertases (PCs). In this study, we discovered that FSAP enhances the biological activity of mature BMP-2 as well as its pro-form, as shown by osteogenic differentiation of C2C12 myoblasts. These findings were complemented by knockdown of FSAP in hepatocytes, which revealed BMP-2 processing by endogenous FSAP. N-terminal sequencing indicated that pro-BMP-2 was cleaved by FSAP at the canonical PC cleavage site, giving rise to mature BMP-2 ($Arg^{282} \downarrow Gln^{283}$), as well as in the N-terminal heparin binding region of mature BMP-2, generating a truncated mature BMP-2 peptide ($Arg^{289} \downarrow Lys^{290}$). Similarly, mature BMP-2 was also cleaved to a truncated peptide within its N-terminal region ($Arg^{289} \downarrow Lys^{290}$). Plasmin exhibited a similar activity, but it was weaker compared with FSAP. Thrombin, Factor VIIa, Factor Xa, and activated protein C were not effective. These results were further supported by the observation that the mutation of the heparin binding region of BMP-2 inhibited the processing by FSAP but not by PC. Thus, the proteolysis and activation of pro-BMP-2 and mature BMP-2 by FSAP can regulate cell differentiation and calcification in vasculature and may explain why polymorphisms in the gene encoding for FSAP are related to vascular diseases.

FSAP[2] is a circulating plasma serine protease with high homology to plasmin. A single-nucleotide polymorphism (SNP) in the FSAP gene (*habp2*) leads to an amino acid substitution at position 534 (G534E; also referred to as Marburg I). Approximately 5% of the Western European population are carriers of this SNP (1). This polymorphism results in a 50–80% decrease in the proteolytic activity of FSAP (2). Carriers of this SNP are more prone to develop carotid stenosis (3), atherosclerosis, and vascular calcification (4, 5). Although these human genetic epidemiological studies suggest a vital role for FSAP in tissue remodeling processes, the precise mechanisms remain to be elucidated. Recent investigations using $FSAP^{-/-}$ mice confirm the contention that FSAP regulates remodeling processes *in vivo* (6).

Growth factor activity is regulated by a variety of proteolytic mechanisms in the intracellular as well as the extracellular space (7). We have previously reported that FSAP can bind to and cleave growth factors containing heparin binding domains like platelet-derived growth factor-BB (PDGF-BB) (8). The inactivation of PDGF-BB by wild type (WT) FSAP was stronger than the Marburg I FSAP variant and contributed to less neointima formation in a mouse model, which emphasizes the importance of the enzymatic activity of FSAP *in vivo* (2). Furthermore, two more growth factors, basic fibroblast growth factor (bFGF, FGF-2) and vascular endothelial growth factor (VEGF) were inhibited by FSAP with respect to endothelial cell activation and angiogenesis (9, 10).

The cystine knot superfamily contains subfamilies of growth factors that include PDGF, VEGF, and transforming growth factor-β (TGF-β)-like growth factors. TGF-β1 to TGF-β4 are well known for their tumor suppressor function (11) and stimulation of extracellular matrix accumulation, which is associated with the progression of fibrosis (12). These TGF-β-like growth factors also contain further subdivisions, such as the bone morphogenetic proteins (BMPs) and the growth and differentiation factors (13, 14). BMPs regulate growth, differentiation, and development in the embryo as well as during tissue remodeling processes in the adult organism. In the vascular system, they play major roles in the regulation of intimal calci-

* This work was supported in part by Deutsche Forschungsgemeinschaft Grant Ka 1468 and grants from the Excellence Cluster in Cardiopulmonary Sciences (ECCPS) and the Behring Röntgen Stiftung (BRS) (to S. M. K.).
[S] This article contains supplemental Tables 1–3 and Figs. 1–6.
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[2] The abbreviations used are: FSAP, factor VII-activating protease; BMP, bone morphogenetic protein; PC, pro-protein convertase; FVIIa and FXa, Factor VIIa and Xa, respectively; ALP, alkaline phosphatase; VSMC, vascular smooth muscle cell.

fication in atherosclerotic plaques (15). BMPs bind to homomeric and heteromeric complexes of BMP receptor type I and II, which are localized on the cell surface (16, 17). Ligand-bound receptor signaling proceeds by Smad1/5/8 phosphorylation, afterwards phosphorylated Smads translocate into nucleus and alters the transcriptional program in cells (18).

BMPs are synthesized as large pro-forms, which dimerize and undergo proteolytic cleavage in the Golgi apparatus by proprotein convertases (PCs) to liberate the mature form expressing full biological activity (19). In a generalized model of BMP activation, largely based on studies with BMP-4, which has high identity to BMP-2, it is clear that there are two major PC cleavage sites, S1 and S2, with the conserved amino acid motif R*XX*R, which separates the pro-domain from the mature protein. Cleavage at the S1 site produces the mature growth factor, but cleavage at the S2 site, more upstream in the pro-domain, facilitates the release of the pro-peptide (20). Between the S1 and S2 sites, a putative S3 cleavage site has been proposed (19). In the intracellular compartment, the pro-domain regulates the correct folding, dimerization, cleavage, and secretion of the mature BMP (21). Limited proteolysis in combination with a biophysical characterization of a protease-resistant subdomain in the pro-region of pro-BMP-2 revealed a core fragment that might mediate correct folding and dimerization of the growth factor (22). The pro-domain of BMPs can also regulate various aspects of the activity of the mature protein, but this aspect remains largely unexplored (23).

Because FSAP inhibits the activity of two major subfamilies of the cystine knot superfamily of growth factors, PDGF-BB and VEGF, we hypothesized that other subfamilies, such as TGF-β, might also be influenced by FSAP. Surprisingly, we found that TGF-β1 activity was not influenced by FSAP but that BMP-2 activity was increased. This indicates that FSAP activation in the context of tissue injury would regulate growth factors of the PDGF and BMP family in opposing ways and channel their growth- and differentiation-regulating properties in remodeling processes.

EXPERIMENTAL PROCEDURES

Materials—The preparation of single-chain FSAP zymogen and Phe-Pro-Arg-chloromethylketone-inactivated FSAP from human plasma has been described before (24). Plasminogen was isolated from human plasma and was activated with urokinase as previously described by our group (25). Recombinant pro-BMP-2 and BMP-2 were produced in *Escherichia coli* as described before (26). Aprotinin was purchased from CSL Behring (Marburg, Germany), Factor VIIa (FVIIa) was from Novo Nordisk (Mainz, Germany), activated protein C was obtained from Lilly, and Factor Xa (FXa) was from Novagen, Merck.

Cell Culture—The mouse myoblast cell line C2C12 was cultivated in RPMI1640 (Invitrogen) with 10% (v/v) fetal calf serum (FCS) (Fisher), 10 units/ml penicillin, and 10 μg/ml streptomycin (Invitrogen) on cell culture-treated plastic (Nunc, Wiesbaden, Germany). Human vascular smooth muscle cells (VSMCs) were isolated as described earlier (27) and were cultivated in basal endothelial growth medium (Promocell, Heidelberg, Germany) with additives as above. Mouse hepatocyte cell line AML12 was cultivated in DMEM (Invitrogen) with additives as above and complemented with 2 mM glutamine, 1% (v/v) sodium pyruvate (all from Invitrogen). All cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

BMP-2 Bioactivity Assay Based on Alkaline Phosphatase Induction in C2C12 Cells—BMP-2 bioactivity was measured by determining the induction of the osteoblastic marker alkaline phosphatase in C2C12 mice myoblasts (28). In brief, C2C12 cells were cultivated for 24 h and stimulated with growth factors in serum-free medium. BMP-2 or pro-BMP-2 was preincubated with FSAP or control buffer (0.2 M arginine, 0.2 M lysine, 5 mM citrate, pH 4.5), and the reaction was stopped with aprotinin (10 μg/ml) before the mixture was added to the cells. After 3 days, cells were washed with PBS and lysed with lysis buffer (100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1% Nonidet P-40, pH 9.6). Lysates were centrifuged and transferred into a new 96-well plate, and a substrate solution (9 mM *p*-nitrophenyl phosphate, Sigma-Aldrich) was added, followed by the measurement of absorption at 405 nm using a plate reader (Biotek, Winooski, VT). Values of untreated controls were set to 1, and treatments were calculated as -fold increase of alkaline phosphatase (ALP) induction compared with controls.

Smad1/5/8 Phosphorylation—C2C12 cells (5×10^4 cells/well) were cultivated in normal medium and thereafter switched to serum-free medium for 2 h. Cells were stimulated with agonists for 30 min, washed with PBS, and lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS) containing 1 mM orthovanadate and PMSF. Western blotting was performed with anti-phospho-Smad1/5/8 antibody. In parallel, total Smad protein was analyzed using anti-Smad1 (both from Cell Signaling Technology (Frankfurt, Germany); anti-Smad1, catalog no. 9743; anti-phospho-Smad1/5 (Ser0463/465), catalog no. 9516).

Western Blot Analysis—SDS-PAGE (15%) was performed, and proteins were transferred to PVDF membrane (GE Healthcare). For Western blot analysis, Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) was used. Polyclonal rabbit antibody against BMP-2 was obtained from Dianova (Hamburg, Germany) (anti-BMP-2, catalog no. CYT-26591).

Edman Sequencing—For determining the cleavage sites, the reducing SDS-polyacrylamide gels were run and blotted onto PVDF membranes. Protein was stained with Coomassie Blue, and subsequently protein bands were cut out for sequence determination. The N-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 (Applied Biosystems, Darmstadt, Germany) in the Proteomics Facility, Justus Liebig University, by Dr. Guenther Lochnit. Five cycles of Edman degradation were performed, and the detected amino acids were aligned with the pro-BMP-2 sequence.

Proliferation Assay—DNA synthesis of C2C12 cells was measured by using the Cell Proliferation ELISA BrdU (calorimetric) kit (Roche Applied Science) using the manufacturer's protocol.

Real-time PCR Analysis—Total RNA was extracted using the Total RNA Miniprep kit (Sigma-Aldrich). Reverse transcription was performed using the High Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR was performed using the SensiMix SYBR kit (Bioline GmbH, Luckenwalde, Germany), and fluorescence of amplified DNA was detected by the Step One Plus real-time PCR system (Applied Biosystems). The temperature program of DNA amplification was the following: cDNA denaturation at 95 °C for 15 s, primer hybridization at 60 °C for 30 s, and elongation at 72 °C for 30 s. The amplification plot was monitored over 40 cycles, and continuous fluorescence measurement indicated mRNA expression of analyzed genes. Fluorescent threshold cycles (*Ct*) were set and normalized against *Ct* of housekeeping gene GAPDH (ΔCt). Afterward, expression of the target gene was controlled with the reference probe, and target *Ct* was calculated as $2^{-\Delta\Delta Ct}$ (29). Primer sequences are provided in supplemental Table 3).

siRNA Knockdown of Endogenous FSAP in Mouse Hepatocyte Cell Line AML12—AML12 cells were transfected with siRNA against FSAP (Flexitube siRNA; Mm_Habp2-2-1, catalog no. SI00238105, Qiagen (Hilden, Germany)) as well as control siRNA (control siRNA, catalog no. 1022076) using Hiperfect (Qiagen). After a 24 h incubation, cells were analyzed for FSAP expression at the protein and mRNA level to confirm knockdown. Rabbit polyclonal anti-FSAP antibody was kindly provided by M. Etscheid (Paul Ehrlich Institute, Langen, Germany). Afterward, cells were incubated with BMP-2 or pro-BMP-2, and their processing was analyzed by Western blotting and mRNA expression of target genes.

Construction of Mutant BMP-2 and Recombinant Expression in HEK293 Cells—The construct pET11a_pro-BMP-2 (26) was used for cloning human pro-BMP-2 cDNA into pSecTag2A (Clontech) expression vector. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (primer sequences are listed in supplemental Tables 1 and 2). Details of the amino acid mutations are shown under "Results." DNA sequencing (Seqlab GmbH, Göttingen, Germany) was performed to check for the correct sequence of the resulting clones. Human embryonic kidney (HEK293) cells were transfected with empty vector, WT cDNA, and the various mutants using Lipofectamine2000 (Invitrogen). Cells were then switched to serum-free medium containing insulin/transferrin/selenium supplement (Invitrogen), and conditioned medium was collected after 24 h. The filtered (0.22 μm) conditioned medium was quantified for BMP-2 by Western blotting using *E. coli*-derived BMP-2 as a standard (26).

Statistical Analysis of the Data—Statistical significance was tested using analysis of variance, and the individual comparisons were made using Bonferroni's test using GraphPad Prism (version 5.02) (GraphPad Software, Inc., La Jolla, CA). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

FSAP Does Not Influence the Activity of TGF-β1 or Latent TGF-β1—In view of the fact that FSAP decreases the activity of two members of the cystine knot family, PDGF and VEGF, we tested whether FSAP influenced the activity of TGF-β1, the prototypic member of the TGF-β family. Incubation of FSAP with mature TGF-β1 did not influence TGF-β activity toward inhibition of mink lung epithelial cell proliferation (supplemental Fig. 1). Besides mature TGF-β1, FSAP did not activate latent TGF-β1 either, although it is known that other proteases are able to activate this pro-growth factor (30). Although TGF-β1 activity is not influenced by FSAP, we found an unexpected effect on another cystine knot family member, BMP-2.

FSAP Activates BMP-2 and Pro-BMP-2 in Vitro—Because BMP-2 is one of the major factors triggering calcification, we analyzed the influence of FSAP on BMP-2 activity. Therefore, mature BMP-2 was incubated with FSAP, and osteogenic differentiation of C2C12 mice myoblasts was evaluated by determining ALP induction as a differentiation marker. Induction of ALP by BMP-2 was stimulated by the presence of FSAP. This increase in ALP was dependent on the enzymatic activity of FSAP because the serine protease inhibitor aprotinin, abolished this effect (Fig. 1*A*). Further controls with aprotinin plus BMP-2 and FSAP alone or through the use of preinhibited FSAP showed that FSAP mediated this effect directly. Because heparin is known to influence the activity of BMP-2 (31) and FSAP (32), we tested its influence in this system. Heparin alone had no effect on C2C12 cell differentiation but decreased the effect of BMP-2 in the absence and presence of FSAP. The effect of FSAP on BMP-2 signaling was further blocked by treatment with the BMP antagonist noggin (33, 34) (Fig. 1*B*). To analyze the FSAP effect in detail, dose and time course experiments were performed. FSAP increased BMP-2 activity in C2C12 cells in a dose- and time-dependent manner (Fig. 1, *C* and *D*), and this was confirmed by Western blot analysis (Fig. 2*A*). SDS-PAGE revealed cleavage of BMP-2 monomer (13 kDa) to a smaller species, which was found to be truncated BMP-2 that lacked the seven N-terminal amino acids (Fig. 2*B*). The cleavage site was identified at position $Arg^{289} \downarrow Lys^{290}$ in a cluster of basic residues (Fig. 2*C*). Hence, proteolytic cleavage of the N-terminal heparin binding region (35) by FSAP produced a truncated peptide (Fig. 2, *trBMP-2*), which increased the activity of mature BMP-2. Trypsin is known to cleave BMP-2 at multiple sites, leading to its activation, and one of these cleavage sites is identical to that obtained with FSAP (36).

Like all members of the TGF-β-like family, BMP-2 is expressed together with a large pro-domain, which is cleaved by PCs (37–39). We then tested whether pro-BMP-2 can be cleaved and activated by FSAP. Pro-BMP-2 had very low activity, but this was highly increased upon incubation with FSAP (Fig. 3*A*). As with BMP-2, the effect of FSAP on pro-BMP-2 was concentration- and time-dependent, and treatment with aprotinin reduced this activity (Fig. 3). Western blot analysis showed processing of the large pro-BMP-2 precursor (45 kDa, under reducing conditions) to the cleaved 13 kDa reduced monomer. N-terminal sequencing indicated that FSAP cleaved pro-BMP-2 at two locations to generate the naturally occurring sequence of the mature BMP-2 (^{283}QAKHK), corresponding to the regular S1 PC cleavage site, and a sequence downstream corresponding to the cleavage site that was identified in mature BMP-2 (^{290}KRLK) (Fig. 4).

Because FSAP is a circulating serine protease, we compared its specificity and effect with other proteases derived from blood. FSAP-mediated proteolysis and activation of mature

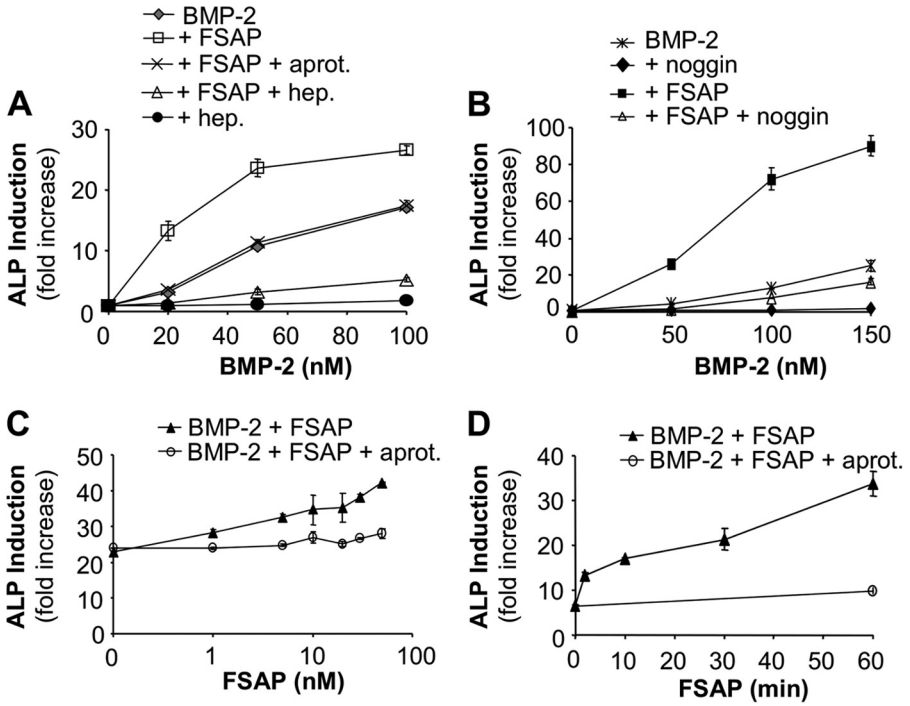

FIGURE 1. **Influence of FSAP on BMP-2 activity.** *A*, FSAP (50 nM) increased BMP-2 (100 nM) activity shown by induction of ALP in C2C12 myoblast cells. Coincubation with heparin (*hep.*; 25 μg/ml) or aprotinin (*aprot.*; 10 μg/ml) reduced BMP-2 activity. *B*, FSAP-mediated BMP-2 activity is reversible by incubation with the BMP inhibitor noggin (50 nM). *C*, dose-dependent enhanced activity of BMP-2 after FSAP preincubation measured by ALP induction in C2C12 cells. In the presence of aprotinin, there was no increase in ALP induction. *D*, time-dependent ALP induction after BMP-2 treatment with FSAP. Means and S.D. values (*error bars*) were calculated from triplicate wells, and similar data were obtained in three independent experiments.

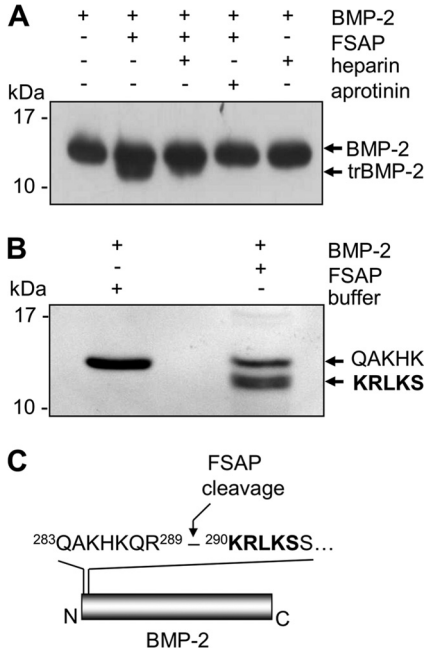

FIGURE 2. **Characterization of FSAP-mediated BMP-2 proteolysis.** *A*, Western blot analysis with preincubated BMP-2 and FSAP (2 h, 37 °C) in the presence or absence of aprotinin or heparin. A low molecular weight band appeared in the presence of enzymatic active FSAP, which was named truncated BMP-2 (*trBMP-2*). *B*, SDS-PAGE with preincubated BMP-2 and FSAP (2 h, 37 °C) for sequencing analysis. FSAP buffer served as control. *Arrows* indicate the identified amino acid sequences of analyzed peptides. *C*, schematic of the amino acid sequence of BMP-2. N-terminal amino acid sequencing revealed a cleaved BMP-2 product ($Arg^{289} \downarrow Lys^{290}$) and the generation of a seven-residue truncated peptide starting with the sequence KRLKS (protein sequence from human pro-BMP-2 was taken from UniProt entry P12643).

BMP-2 (Fig. 5, *A* and *B*) and pro-BMP-2 (Fig. 5, *C* and *D*) was stronger than that with plasmin, whereas other proteases, such as thrombin, FXa, FVIIa, and activated protein C, were not effective. Because FSAP and plasmin show high sequence homology, a detailed comparison between both proteases was performed. Here, FSAP was more effective in activating BMP-2 and pro-BMP-2 than plasmin (supplemental Fig. 2). Analysis of plasmin cleavage sites showed that mature BMP-2 and pro-BMP-2 were cleaved only at one site, corresponding to ^{291}RLKSS, which is one amino acid downstream of the FSAP cleavage site compared with mature BMP-2 (supplemental Fig. 3). Hence, there were some differences between FSAP and plasmin with respect to efficacy and specificity of cleavage sites.

FSAP Modulates BMP-2-mediated Activation of Smad1/5/8 Phosphorylation and Decreases Cell Proliferation—To further characterize the biological activity of processed BMP-2 and pro-BMP-2, the signaling pathway of BMP-2 was analyzed with a focus on the Smad1/5/8 phosphorylation in C2C12 cells. Western blotting revealed that the FSAP enhanced BMP-2/pro-BMP-2-mediated phosphorylation of Smad1/5/8. Smad1 served as an internal loading control for quantification (supplemental Fig. 4). Stimulation of myoblasts by BMP-2 led to their differentiation into osteoblasts with a concomitant inhibition of cell growth. In C2C12 cells, the growth-inhibitory effect of BMP-2/pro-BMP-2 was enhanced by FSAP (supplemental Fig. 5). Similarly in vascular smooth muscle cells (VSMCs), after activation with FSAP and pro-BMP-2, we observed differences in the expression of Id1 and Smad6 (supplemental Fig. 6). Hence, the proteolytic cleavage of BMP-2/pro-BMP-2 by FSAP increased their cellular effects on different cell lines.

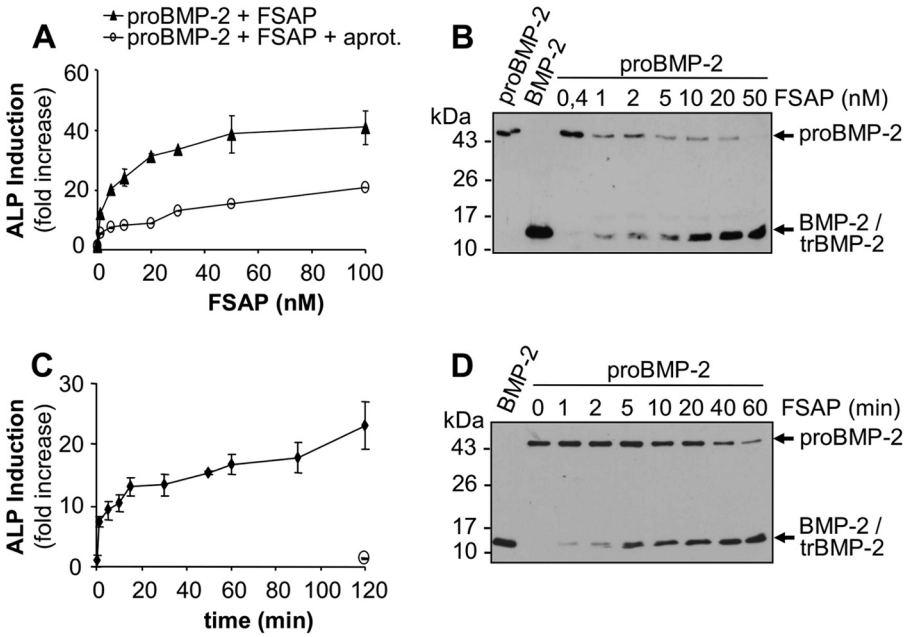

FIGURE 3. **Latent pro-BMP-2 can be activated by FSAP.** *A*, dose-dependent ALP induction in C2C12 cells after pro-BMP-2 (100 nM) and FSAP (0–100 nM) treatment. Enzymatically inhibited FSAP by cotreatment with aprotinin (*aprot.*) served as a negative control. *B*, Western blot analysis of dose-dependent cleavage of pro-BMP-2 (45 kDa) by FSAP into the mature form (12 kDa) indicated by *arrows*. *C*, time-dependent induction of ALP by FSAP (50 nM)-pretreated pro-BMP-2 (100 nM) in C2C12 myoblasts. Enzymatically inhibited FSAP by treatment with aprotinin served as a negative control. *D*, Western blot analysis shows time-dependent cleavage of pro-BMP-2 by FSAP. Graphs represent means and S.D. (*error bars*) from one experiment, which could be reproduced in three independent replicates.

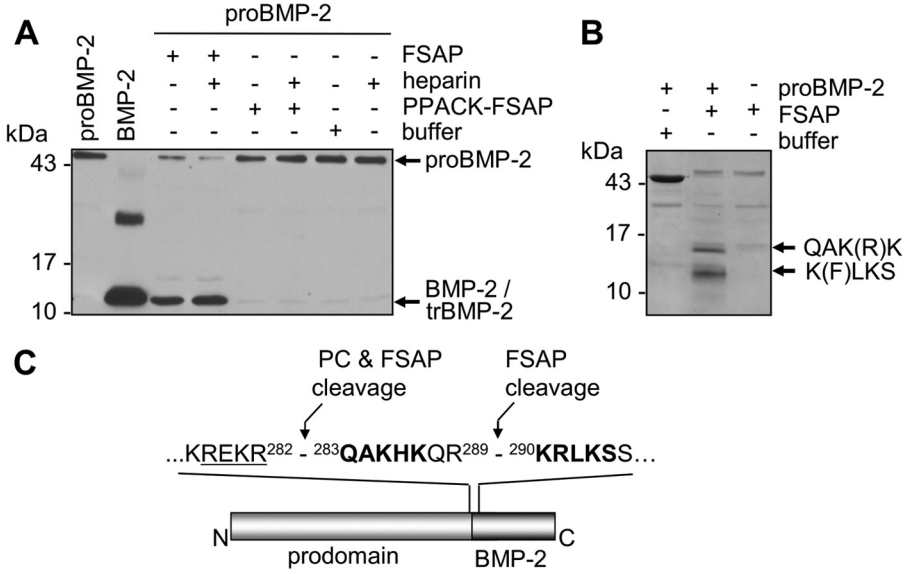

FIGURE 4. **Characterization of FSAP-mediated pro-BMP-2 proteolysis.** *A*, Western blot analysis of pro-BMP-2 cleavage by active FSAP or enzymatically (Phe-Pro-Arg-chloromethylketone) inhibited FSAP (*PPACK-FSAP*). The band pattern revealed cleavage of pro-BMP-2 (45 kDa) to mature BMP-2 (12 kDa) only after incubation with active FSAP (2 h, 37 °C). *B*, SDS-PAGE with preincubated pro-BMP-2 and FSAP (2 h, 37 °C) for sequencing analysis. For controlling nonspecific cleavage, FSAP buffer was used as a negative control. Two generated peptides were analyzed by N-terminal sequencing. *C*, scheme of FSAP-mediated pro-BMP-2 proteolysis. The first cleavage site was identified as the natural PC cleavage site (*underlined*), resulting in liberation of the inactive pro-domain ($Arg^{282} \downarrow Gln^{283}$), and second cleavage ($Arg^{289} \downarrow Lys^{290}$) was identical to mature BMP-2 proteolysis by FSAP.

Role of Endogenous FSAP in BMP-2 Processing in Hepatocytes—We then investigated whether endogenous FSAP could also contribute to the cleavage and activation of BMP-2. Because hepatocytes are the main source of FSAP expression, we tested if hepatic FSAP could cleave and activate BMP-2 and pro-BMP-2. The mouse AML12 hepatocyte cell line was treated with siRNA against the FSAP gene (*habp2*) to knock down endogenous FSAP expression. These, as well as control siRNA-treated cells, were incubated with BMP-2 and pro-BMP-2, and growth factor processing was followed by Western blotting. siRNA treatment significantly reduced FSAP expression at the protein and mRNA level, and this was also coincident with reduced processing of pro-BMP-2 (Fig. 6, *A* and *B*). To further elucidate the activity of processed BMP-2, we analyzed the expression of the BMP-2 target gene hepcidin. Control siRNA-transfected cells led to the induction of hepcidin

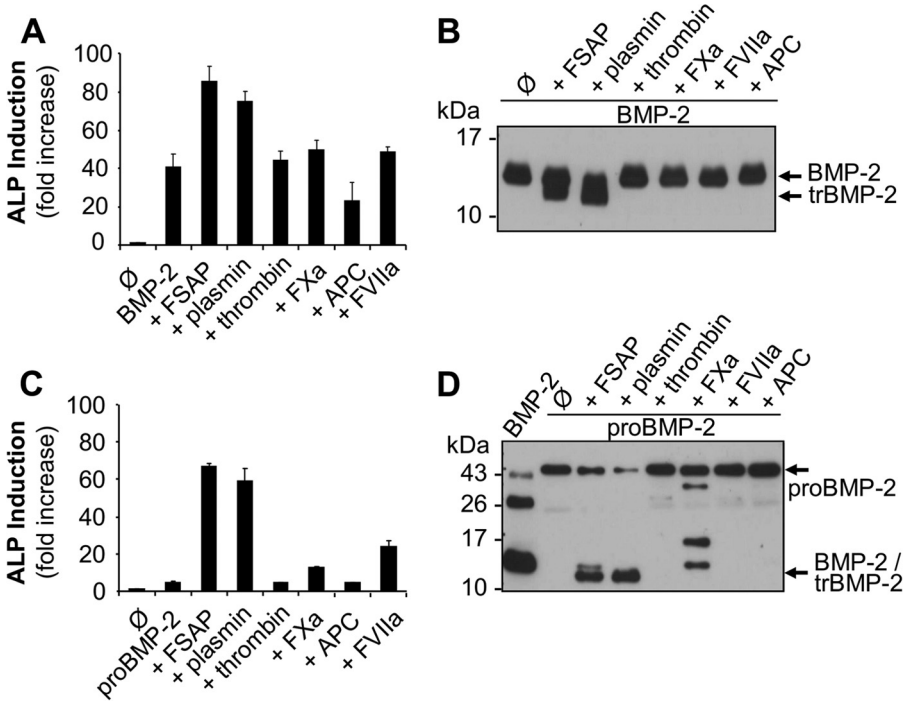

FIGURE 5. **Comparison of different extracellular proteases upon BMP-2 and pro-BMP-2 activation.** *A*, ALP induction of BMP-2 (100 nM) after treatment with different proteases (FSAP, plasmin, thrombin, FVIIa, activated protein C (*APC*), and FXa, each 50 nM) in C2C12 cells. *B*, Western blot analysis of proteases-treated BMP-2 (30 min, 37 °C). The *arrows* indicate cleaved peptides. *trBMP-2*, truncated BMP-2. *C*, pro-BMP-2 (100 nM) activity after incubation with various proteases (50 nM each) in C2C12 cells and induction of ALP. *D*, comparison of pro-BMP-2 cleavage by different extracellular proteases. Western blotting shows cleaved precursor protein bands, which are indicated by *arrows*. The presented data show means and S.D. (*error bars*), which are representative from three independently performed experiments.

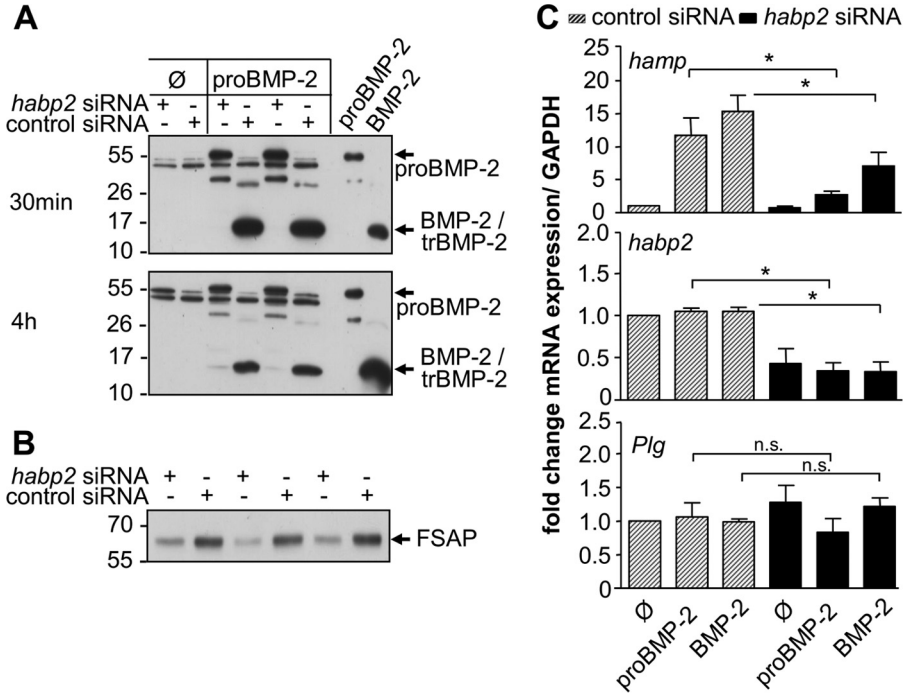

FIGURE 6. **Processing of BMP-2 by endogenous FSAP in mouse hepatocytes.** *A*, AML12 hepatocytes were analyzed for pro-BMP-2 cleavage by Western blotting after siRNA-induced knockdown of the FSAP-encoding gene (*habp2*) compared with control siRNA. Western blot analysis revealed less pro-BMP-2 cleavage (30 min (*top*) and 4 h (*bottom*)) in the presence of FSAP siRNA knockdown. The band pattern was compared with that of recombinant pro-BMP-2 and BMP-2 proteins. *B*, Western blot showing FSAP protein in siRNA-transfected hepatocytes (supernatants), which served as a control for knockdown efficiency. *C*, Real-time PCR analysis of the mRNA expression of the BMP-2 target gene hepcidin in hepatocytes. siRNA-transfected hepatocytes were stimulated with pro-BMP-2 or BMP-2, and expression of hepcidin gene product (*hamp*) was analyzed. Fold change in mRNA expression of hepcidin was relative to GAPDH mRNA. *Middle*, FSAP mRNA was significantly down-regulated by *habp2* siRNA. *Bottom*, plasminogen (*Plg*) mRNA expression was not affected by FSAP siRNA transfection. Graphs are mean values ± S.D. (*error bars*) from four independent experiments. Statistical analysis was carried out by analysis of variance (*, $p < 0.05$ (significant); *n.s.*, not significant).

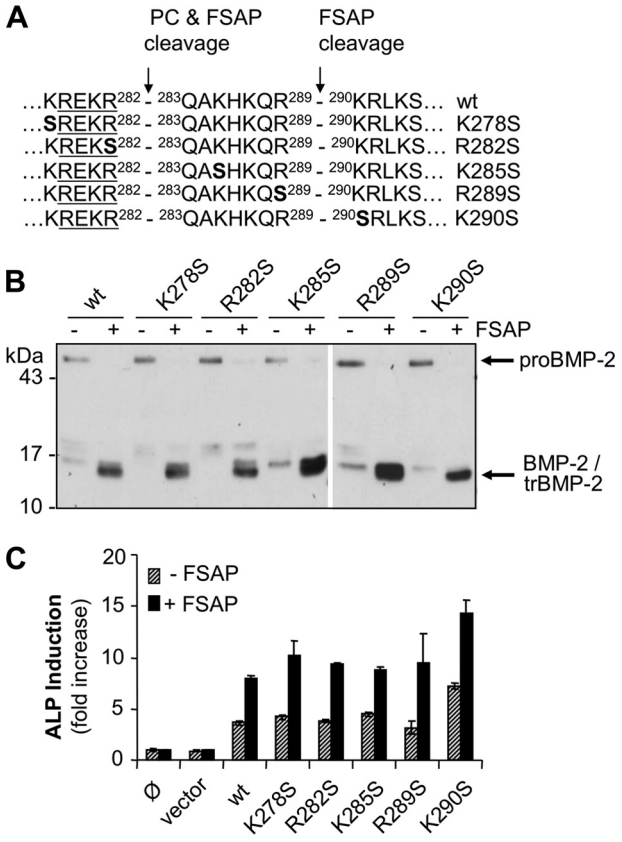

FIGURE 7. **Characterization of different recombinant pro-BMP-2 mutants and FSAP-mediated growth factor activation.** *A*, amino acid sequence of wild type (*wt*) and five different single mutants of human pro-BMP-2. Mutated amino acids are *highlighted*, the PC cleavage site is *underlined*, and putative FSAP/PC cleavage sites are marked by *arrows*. Names of cloned constructs are shown on the *right*, indicating the position of amino acid exchange. *B*, expression of different single mutants of human pro-BMP-2 in HEK293 cells as well as WT pro-BMP-2 and Western blot analysis of conditioned medium after incubation with FSAP. *C*, ALP induction of C2C12 cells after stimulation with conditioned medium from single mutants with and without incubation with FSAP. *Error bars*, S.D.

mRNA after growth factor stimulation, whereas hepcidin expression was low in FSAP siRNA-treated cells (Fig. 6*C*). As a control, endogenous plasminogen mRNA expression was unchanged in hepatocytes, but this was not sufficient to activate pro-BMP-2. These findings suggest that endogenous FSAP from liver cells can activate BMP-2 and pro-BMP-2.

Effect of Mutating the Putative FSAP Cleavage Sites in Pro-BMP-2—We have further characterized the cleavage sites of pro-BMP-2 by FSAP through mutating the two hot spots that were identified above. These cleavage sites fall into two clusters of basic amino acids, where $Arg^{282} \downarrow Gln^{283}$ is located at the canonical PC cleavage site, and the second cleavage site, $Arg^{289} \downarrow Lys^{290}$, is located in the heparin binding region of mature BMP-2. Here, we replaced selected single arginine/lysine residues with a serine using site-directed mutagenesis and transfected HEK293 cells for protein expression (Fig. 7*A*). Western blot analysis of conditioned medium with a BMP-2 antibody showed expression of WT and mutants of pro-BMP-2 (Fig. 7*B*). Surprisingly, the activity of each of these single mutants was increased in the presence of FSAP (Fig. 7*C*). Thus, all mutants were cleaved similarly as the WT pro-BMP-2, indicating that endogenous as well as FSAP-mediated processing is tolerant to single amino acid changes in these two basic clusters.

We then performed triple mutations of three basic residues to serines at sites of both the predicted cleavage hot spots (Fig. 8*A*). The triple mutation of the canonical PC cleavage site (SSS^{282}) resulted in very low secretion of mutant pro-BMP-2 (Fig. 8*B*). This low secretion rate indicates the importance of PC-mediated intracellularly processing as described before (40). However, this mutant was cleaved by FSAP, as indicated by Western blot analysis of FSAP-treated conditioned medium (Fig. 8*B*). The triple mutant SSS^{289} of the heparin binding region in mature BMP-2 was secreted normally, because this site is independent of the PC site, but it could not be cleaved by FSAP (Fig. 8*B*). Analyzing the biological activity of this (SSS^{289}) revealed no significant ALP increase in the presence of applied FSAP (Fig. 8*C*). Hence, mutations of the identified cleavage sites showed cleavage as well as activity patterns as would be expected for the classical PC-mediated cleavage and FSAP-mediated cleavage.

DISCUSSION

TGF-β-related growth factors are locally expressed in the vasculature and regulate fibrotic and degenerative diseases. Our investigations into the regulation of BMP-2 activity by circulating proteases from the coagulation and fibrinolysis system show a novel mechanism of BMP-2 activation by FSAP. This fits well with our previous observations that FSAP inhibits the activity of PDGF-BB, which is a proliferative factor for mesenchymal cells (2). Thus, FSAP inhibits a proliferative factor, PDGF-BB, and activates a differentiating/growth-inhibiting factor, BMP-2, which can potentially alter the phenotype of mesenchymal cells in the vasculature. This may explain the association of polymorphism in the FSAP gene with carotid stenosis, atherosclerosis, and calcification in the vasculature (3–5, 41).

Thus, we propose a model in which tissue injury releases histones and nucleosomes, which leads to local activation of FSAP (42, 43). Thereafter, activated FSAP mediates BMP-2 cleavage and generation of truncated BMP-2, which is characterized by enhanced activity. The N-terminal region of mature BMP-2 contains a 17-amino acid-long heparin binding domain, which shows high affinity to proteoglycans of the extracellular matrix (35). Matrix retention sequences can be found in different growth factors at N or C termini, BMP-2 has a heparin binding site at its N terminus (35), and members of the PDGFs have them on their C terminus (44). Although this is not the active site where receptor binding and activation is mediated, it plays an important role in regulating BMP activity in an environment-dependent context (31). Previous studies have shown that trypsin also cleaves mature BMP-2 within the heparin binding domain at three different sites ($Gln^{289} \downarrow Arg^{290}$, $Arg^{290} \downarrow Lys^{291}$, and $Lys^{291} \downarrow Arg^{292}$ (26). This so-called digit-removed BMP-2 had stronger activity in a limb bud cell assay compared with full-length BMP-2 (35). Furthermore, digit-removed BMP-2 clearly had positive effects on bone formation after implantation in rats (26). Here, we show that FSAP and plasmin cleavage can achieve similar enhanced bioactivity but in a more patho-physiologically relevant manner. The concen-

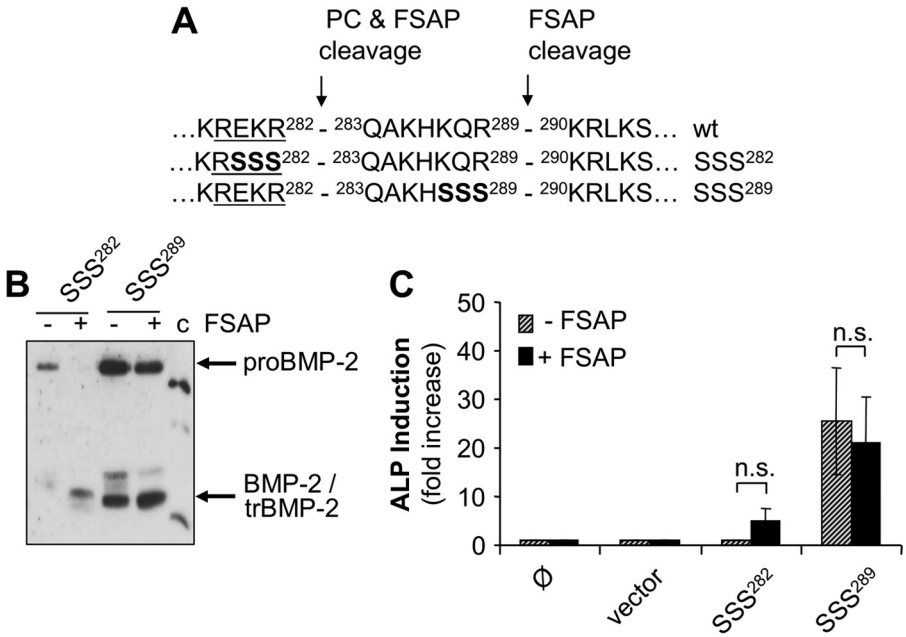

FIGURE 8. **Triple mutation of pro-BMP-2 in the heparin binding domain prevents activation by FSAP.** *A*, mutation sites of generated pro-BMP-2 triple mutants. *B*, Western blot analysis of supernatants of transfected HEK293 cells. SSS^{282} was expressed in very low amounts compared with SSS^{289}. The protein product of triple mutant SSS^{282} showed proteolysis after FSAP treatment, whereas the mutant SSS^{289} was protected. For comparison of immunoreactivity of BMP-2 antibody and molecular weight bands, *E. coli*-produced BMP-2 and pro-BMP-2 (control (*c*)) were included. *C*, ALP induction by triple mutant strains in C2C12 cells. No significant activation was observed after incubating SSS^{289} mutant with FSAP. Data were obtained from three independent experiments with statistical analysis using analysis of variance ($p < 0.05$ (significant); *n.s.*, not significant).

tration of FSAP zymogen in the circulation is 180 nM, and 18–40% is activated under proinflammatory conditions and subsequently forms complexes with inhibitors (42).

PCs are Ca^{2+}-dependent serine endoproteases and can activate different growth factors like IGF-1, TGF-β, or VEGF-C by cleavage of the consensus sequence R*X*(R/K)R (45). For a long time it was assumed that processing of pro-BMPs would take place in the Golgi and that only the processed forms without the pro-domains would be secreted. Association of the pro-domain with the mature growth factor can bind to the extracellular domain of BMPRIA (46). Furthermore, it is known from the literature that, pro-BMP-2 shows no binding characteristics to the extracellular domain of BMPRII (46). It is still an open question whether all of the BMP-2 is secreted in its cleaved mature form or if some is secreted as a pro-form. Our observations in HEK293 cells show that some BMP-2 is secreted as a pro-form, and this confirms earlier observations showing the same phenomenon (47). Apart from the cell culture-based studies, so far, secreted pro-BMP-2 was detected in patients with rheumatic arthritis, where the pro-form has been measured in the synovial liquid (48). Speculations about a putative role of the BMP-2 pro-domain in the extracellular space could be made by the observations that some members of the cystine knot family, such as TGF-β1 (49, 50), PDGF-C (51), and PDGF-D (51), are secreted as *bona fide* pro-forms and then processed in the extracellular environment.

Mutation of single amino acids in the classical S1 cleavage site did not alter processing and secretion via endogenous PCs. This can be explained by the fact that alternative PC cleavage sites, such as S2 and S3, could be used in these circumstances (20, 40, 52), but future studies are required to resolve this issue. All of these single mutants were also cleaved by FSAP. Triple mutation of the canonical PC cleavage site strongly reduced protein secretion, indicating that in these circumstances, alternative cleavage sites are not used. Nonetheless, this mutant was cleaved by FSAP, presumably at the downstream site in the mature BMP-2. The triple mutation SSS^{289} in the mature protein did not influence cleavage by PCs at the S1 site but abolished the cleavage with FSAP, confirming that the KQR^{289} motif in mature BMP-2 is required for FSAP-mediated cleavage. It is interesting to note that this mutation also diminishes the cleavage by FSAP at the classical S1 cleavage site for reasons that are not clear.

One of the unexpected observations we made is that although BMP-2 can be activated by FSAP, this is not true for TGF-β1, despite the high similarities between them. One difference could be the presumed cleavage of the pro-form of BMP-2 in the Golgi, which leads to dissociation of the pro-domain from the mature factor, whereas in the case of TGF-β1, the cleaved pro-domain, called latency-associated peptide, remains associated with the mature growth factor, which is released as a complex (53). Furthermore, another gene product called latent TGF-β-binding protein associates with this complex and also regulates processing and cellular association. Factors such as low pH and proteases in the extracellular milieu then separate these from the active TGF-β (54). Although the plasminogen activation system has been shown to be involved in TGF-β activation (53), here we found that FSAP, despite similarities to plasminogen, did not show the same effect. Our preliminary findings show that GDF-5 (growth and differentiation factor 5)/BMP-14 and BMP-7 are also activated by FSAP (data not shown), indicating that other members of the TGF-β family that are similar to BMP-2 in their sequence and secretion mechanisms are likely targets of FSAP.

It is interesting to note that of all the hemostasis factors tested, we found that only plasmin and FSAP cleaved BMP-2. The serine protease domain of FSAP shows the highest homology to plasmin, which is reflected here in their similar proteolytic actions. There were some differences in their efficacy and preference for cleavage sites. FSAP and plasmin are both likely to be activated in inflammatory remodeling situations but via completely different mechanisms. FSAP is converted into the active form by interaction with nuclear material released by necrotic/apoptotic cells, such as histones and nucleosome (42, 43), whereas plasminogen is activated by urokinase and tissue plasminogen activators as well as plasmin. The link between FSAP and plasmin is reinforced by the fact that FSAP is a potent activator of pro-urokinase (55), which could be a positive regulatory loop by enhancing plasmin activation. We further found evidence that endogenous FSAP expressed by hepatocytes contributes to growth factor cleavage and activation that was identified by transcriptional activation of a hepatic BMP-2 target gene. Hence, using another test system, independently of adding exogenous FSAP, gave the same results, indicating the universality of the observations. Similarly, in VSMCs, the induction of Id1 and Smad6 mRNA by pro-BMP-2 was increased by FSAP, again confirming the same observations in another cell type.

Some of the proteins, which are responsible for development and progression of vascular calcification, belong to the TGF-β family (56). The atherosclerotic calcification process includes changes in gene expression of VSMCs (57, 58), pericytes (59), and adventitial myofibroblast (60). In VSMCs, these changes are mainly regulated by BMP-2, in a process similar to bone formation. Furthermore, BMP-2 was detected in calcified human VSMCs by mRNA level (61) and by immunohistochemistry (62). In mouse models, overexpression of BMP-2 led to calcification of plaques in $ApoE^{-/-}$ mice (63), whereas inhibition of BMP-2 was protective (64). There is also evidence for the involvement of FSAP in atherosclerosis and vascular calcification processes. This evidence is based on genetic studies in human populations that link polymorphism in the FSAP gene to stroke (65), atherosclerosis, and vascular calcification (4, 5). This link between the two observations will need to be explored in further detail in future studies. A further implication of our work is related to the therapeutic use of BMP-2 and pro-BMP-2. Both of these proteins are used to speed up repair of bone fractures (66), and their *in vivo* activation by our described mechanisms may play an important role in regulating their efficacy at the site of injury.

Acknowledgments—We thank Thomas Schmidt Woell for excellent technical assistance, Lars Muhl for sharing the data on TGF-β, and Guenther Lochnit for the N-terminal sequencing (all from Justus Liebig University).

REFERENCES

1. Römisch, J. (2002) Factor VII activating protease (FSAP). A novel protease in hemostasis. *Biol. Chem.* **383,** 1119–1124
2. Sedding, D., Daniel, J. M., Muhl, L., Hersemeyer, K., Brunsch, H., Kemkes-Matthes, B., Braun-Dullaeus, R. C., Tillmanns, H., Weimer, T., Preissner, K. T., and Kanse, S. M. (2006) 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.* **203,** 2801–2807
3. Willeit, J., Kiechl, S., Weimer, T., Mair, A., Santer, P., Wiedermann, C. J., and Roemisch, J. (2003) Marburg I polymorphism of factor VII-activating protease. A prominent risk predictor of carotid stenosis. *Circulation* **107,** 667–670
4. Gardener, H., Beecham, A., Cabral, D., Yanuck, D., Slifer, S., Wang, L., Blanton, S. H., Sacco, R. L., Juo, S. H., and Rundek, T. (2011) Carotid plaque and candidate genes related to inflammation and endothelial function in Hispanics from northern Manhattan. *Stroke* **42,** 889–896
5. Zakai, N. A., Lange, L., Longstreth, W. T., Jr., O'Meara, E. S., Kelley, J. L., Fornage, M., Nikerson, D., Cushman, M., and Reiner, A. P. (2011) Association of coagulation-related and inflammation-related genes and factor VIIc levels with stroke. The Cardiovascular Health Study. *J. Thromb. Haemost.* **9,** 267–274
6. Borkham-Kamphorst, E., Zimmermann, H. W., Gassler, N., Bissels, U., Bosio, A., Tacke, F., Weiskirchen, R., and Kanse, S. M. (2013) Factor VII activating protease (FSAP) exerts anti-inflammatory and anti-fibrotic effects in liver fibrosis in mice and men. *J. Hepatol.* **58,** 104–111
7. Posner, B. I., and Laporte, S. A. (2010) Cellular signalling. Peptide hormones and growth factors. *Prog. Brain Res.* **181,** 1–16
8. Kannemeier, C., Al-Fakhri, N., Preissner, K. T., and Kanse, S. M. (2004) Factor VII activating protease (FSAP) inhibits growth factor-mediated cell proliferation and migration of vascular smooth muscle cells. *FASEB J.* **18,** 728–730
9. Etscheid, M., Beer, N., Kress, J. A., Seitz, R., and Dodt, J. (2004) Inhibition of bFGF/EGF-dependent endothelial cell proliferation by the hyaluronan-binding protease from human plasma. *Eur. J. Cell Biol.* **82,** 597–604
10. Jeon, J. W., Song, H. S., Moon, E. J., Park, S. Y., Son, M. J., Jung, S. Y., Kim, J. T., Nam, D. H., Choi-Miura, N. H., Kim, K. W., and Kim, Y. J. (2006) Anti-angiogenic action of plasma hyaluronan binding protein in human umbilical vein endothelial cells. *Int. J. Oncol.* **29,** 209–215
11. Massagué, J. (2012) TGFβ signalling in context. *Nat. Rev. Mol. Cell Biol.* **13,** 616–630
12. Miyazono, K. (2009) Transforming growth factor-β signaling in epithelial-mesenchymal transition and progression of cancer. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **85,** 314–323
13. Rider, C. C., and Mulloy, B. (2010) Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists. *Biochem. J.* **429,** 1–12
14. Vitt, U. A., Hsu, S. Y., and Hsueh, A. J. (2001) Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. *Mol. Endocrinol.* **15,** 681–694
15. Lowery, J. W., and de Caestecker, M. P. (2010) BMP signaling in vascular development and disease. *Cytokine Growth Factor Rev.* **21,** 287–298
16. Ehrlich, M., Horbelt, D., Marom, B., Knaus, P., and Henis, Y. I. (2011) Homomeric and heteromeric complexes among TGF-β and BMP receptors and their roles in signaling. *Cell. Signal.* **23,** 1424–1432
17. Mueller, T. D., and Nickel, J. (2012) Promiscuity and specificity in BMP receptor activation. *FEBS Lett.* **586,** 1846–1859
18. Cai, J., Pardali, E., Sánchez-Duffhues, G., and ten Dijke, P. (2012) BMP signaling in vascular diseases. *FEBS Lett.* **586,** 1993–2002
19. Zhou, A. J., Zhu, Z., Clokie, C. M., and Peel, S. A. (2012) Mutation of a cleavage site adjacent to the mature domain leads to increase in secreted mature BMP-2 with reduced activity. *Growth Factors* **30,** 267–275
20. Degnin, C., Jean, F., Thomas, G., and Christian, J. L. (2004) Cleavages within the prodomain direct intracellular trafficking and degradation of mature bone morphogenetic protein-4. *Mol. Biol. Cell* **15,** 5012–5020
21. Harrison, C. A., Al-Musawi, S. L., and Walton, K. L. (2011) Prodomains regulate the synthesis, extracellular localisation and activity of TGF-β superfamily ligands. *Growth Factors* **29,** 174–186
22. Kuhfahl, S., Hauburger, A., Thieme, T., Groppe, J., Ihling, C., Tomic, S., Schutkowski, M., Sinz, A., and Schwarz, E. (2011) Identification of a core domain within the proregion of bone morphogenetic proteins that interacts with the dimeric, mature domain. *Biochem. Biophys. Res. Commun.* **408,** 300–305

23. Sengle, G., Ono, R. N., Sasaki, T., and Sakai, L. Y. (2011) Prodomains of transforming growth factor β (TGFβ) superfamily members specify different functions. Extracellular matrix interactions and growth factor bioavailability. *J. Biol. Chem.* **286,** 5087–5099
24. Kannemeier, C., Feussner, A., Stöhr, H. A., Weisse, J., Preissner, K. T., and Römisch, J. (2001) Factor VII and single-chain plasminogen activator-activating protease. Activation and autoactivation of the proenzyme. *Eur. J. Biochem.* **268,** 3789–3796
25. Kanse, S. M., Chavakis, T., Al-Fakhri, N., Hersemeyer, K., Monard, D., and Preissner, K. T. (2004) Reciprocal regulation of urokinase receptor (CD87)-mediated cell adhesion by plasminogen activator inhibitor-1 and protease nexin-1. *J. Cell Sci.* **117,** 477–485
26. Hillger, F., Herr, G., Rudolph, R., and Schwarz, E. (2005) Biophysical comparison of BMP-2, ProBMP-2, and the free pro-peptide reveals stabilization of the pro-peptide by the mature growth factor. *J. Biol. Chem.* **280,** 14974–14980
27. Kanse, S. M., Benzakour, O., Kanthou, C., Kost, C., Lijnen, H. R., and Preissner, K. T. (1997) Induction of vascular SMC proliferation by urokinase indicates a novel mechanism of action in vasoproliferative disorders. *Arterioscler. Thromb. Vasc. Biol.* **17,** 2848–2854
28. Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J. Cell Biol.* **127,** 1755–1766
29. Wong, M. L., and Medrano, J. F. (2005) Real-time PCR for mRNA quantitation. *BioTechniques* **39,** 75–85
30. Jenkins, G. (2008) The role of proteases in transforming growth factor-beta activation. *Int. J. Biochem. Cell Biol.* **40,** 1068–1078
31. Kanzaki, S., Ariyoshi, W., Takahashi, T., Okinaga, T., Kaneuji, T., Mitsugi, S., Nakashima, K., Tsujisawa, T., and Nishihara, T. (2011) Dual effects of heparin on BMP-2-induced osteogenic activity in MC3T3-E1 cells. *Pharmacol. Rep.* **63,** 1222–1230
32. Muhl, L., Galuska, S. P., Oörni, K., Hernández-Ruiz, L., Andrei-Selmer, L. C., Geyer, R., Preissner, K. T., Ruiz, F. A., Kovanen, P. T., and Kanse, S. M. (2009) High negative charge-to-size ratio in polyphosphates and heparin regulates factor VII-activating protease. *FEBS J.* **276,** 4828–4839
33. Brunet, L. J., McMahon, J. A., McMahon, A. P., and Harland, R. M. (1998) Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* **280,** 1455–1457
34. Takayama, K., Suzuki, A., Manaka, T., Taguchi, S., Hashimoto, Y., Imai, Y., Wakitani, S., and Takaoka, K. (2009) RNA interference for noggin enhances the biological activity of bone morphogenetic proteins *in vivo* and *in vitro*. *J. Bone Miner. Metab.* **27,** 402–411
35. Ruppert, R., Hoffmann, E., and Sebald, W. (1996) Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *Eur. J. Biochem.* **237,** 295–302
36. Koenig, B. B., Cook, J. S., Wolsing, D. H., Ting, J., Tiesman, J. P., Correa, P. E., Olson, C. A., Pecquet, A. L., Ventura, F., and Grant, R. A. (1994) Characterization and cloning of a receptor for BMP-2 and BMP-4 from NIH 3T3 cells. *Mol. Cell Biol.* **14,** 5961–5974
37. Constam, D. B., and Robertson, E. J. (1999) Regulation of bone morphogenetic protein activity by pro domains and proprotein convertases. *J. Cell Biol.* **144,** 139–149
38. Meleady, P., Henry, M., Gammell, P., Doolan, P., Sinacore, M., Melville, M., Francullo, L., Leonard, M., Charlebois, T., and Clynes, M. (2008) Proteomic profiling of CHO cells with enhanced rhBMP-2 productivity following co-expression of PACEsol. *Proteomics* **8,** 2611–2624
39. Sopory, S., Nelsen, S. M., Degnin, C., Wong, C., and Christian, J. L. (2006) Regulation of bone morphogenetic protein-4 activity by sequence elements within the prodomain. *J. Biol. Chem.* **281,** 34021–34031
40. Sopory, S., Kwon, S., Wehrli, M., and Christian, J. L. (2010) Regulation of Dpp activity by tissue-specific cleavage of an upstream site within the prodomain. *Dev. Biol.* **346,** 102–112
41. Kanse, S. M., and Etscheid, M. (2011) Factor VII activating protease. Single nucleotide polymorphisms light the way. *Hamostaseologie* **31,** 174–176, 177–178
42. Yamamichi, S., Fujiwara, Y., Kikuchi, T., Nishitani, M., Matsushita, Y., and Hasumi, K. (2011) Extracellular histone induces plasma hyaluronan-binding protein (factor VII activating protease) activation *in vivo*. *Biochem. Biophys. Res. Commun.* **409,** 483–488
43. Stephan, F., Hazelzet, J. A., Bulder, I., Boermeester, M. A., van Till, J. O., van der Poll, T., Wuillemin, W. A., Aarden, L. A., and Zeerleder, S. (2011) Activation of factor VII-activating protease in human inflammation. A sensor for cell death. *Crit. Care* **15,** R110
44. Fredriksson, L., Li, H., and Eriksson, U. (2004) The PDGF family. Four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev.* **15,** 197–204
45. Bassi, D. E., Fu, J., Lopez de Cicco, R., and Klein-Szanto, A. J. (2005) Proprotein convertases. "Master switches" in the regulation of tumor growth and progression. *Mol. Carcinog.* **44,** 151–161
46. Hauburger, A., von Einem, S., Schwaerzer, G. K., Buttstedt, A., Zebisch, M., Schräml, M., Hortschansky, P., Knaus, P., and Schwarz, E. (2009) The pro-form of BMP-2 interferes with BMP-2 signalling by competing with BMP-2 for IA receptor binding. *FEBS J.* **276,** 6386–6398
47. Israel, D. I., Nove, J., Kerns, K. M., Moutsatsos, I. K., and Kaufman, R. J. (1992) Expression and characterization of bone morphogenetic protein-2 in Chinese hamster ovary cells. *Growth Factors* **7,** 139–150
48. Lories, R. J., Derese, I., Ceuppens, J. L., and Luyten, F. P. (2003) Bone morphogenetic proteins 2 and 6, expressed in arthritic synovium, are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis. *Arthritis Rheum.* **48,** 2807–2818
49. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T. A. (2011) Latent TGF-β structure and activation. *Nature* **474,** 343–349
50. Anderson, S. B., Goldberg, A. L., and Whitman, M. (2008) Identification of a novel pool of extracellular pro-myostatin in skeletal muscle. *J. Biol. Chem.* **283,** 7027–7035
51. Hurst, N. J., Jr., Najy, A. J., Ustach, C. V., Movilla, L., and Kim, H. R. (2012) Platelet-derived growth factor-C (PDGF-C) activation by serine proteases: implications for breast cancer progression. *Biochem. J.* **441,** 909–918
52. Akiyama, T., Marqués, G., and Wharton, K. A. (2012) A large bioactive BMP ligand with distinct signaling properties is produced by alternative proconvertase processing. *Sci. Signal.* **5,** ra28
53. Hyytiäinen, M., Penttinen, C., and Keski-Oja, J. (2004) Latent TGF-β binding proteins. Extracellular matrix association and roles in TGF-β activation. *Crit. Rev. Clin. Lab. Sci.* **41,** 233–264
54. Chu, T. M., and Kawinski, E. (1998) Plasmin, substilisin-like endoproteases, tissue plasminogen activator, and urokinase plasminogen activator are involved in activation of latent TGF-β1 in human seminal plasma. *Biochem. Biophys. Res. Commun.* **253,** 128–134
55. Roemisch, J., Feussner, A., Nerlich, C., Stoehr, H. A., and Weimer, T. (2002) The frequent Marburg I polymorphism impairs the pro-urokinase activating potency of the factor VII activating protease (FSAP). *Blood Coagul. Fibrinolysis* **13,** 433–441
56. Ishisaki, A., and Matsuno, H. (2006) Novel ideas of gene therapy for atherosclerosis. Modulation of cellular signal transduction of TGF-β family. *Curr. Pharm. Des.* **12,** 877–886
57. Proudfoot, D., Skepper, J. N., Shanahan, C. M., and Weissberg, P. L. (1998) Calcification of human vascular cells *in vitro* is correlated with high levels of matrix Gla protein and low levels of osteopontin expression. *Arterioscler. Thromb. Vasc. Biol.* **18,** 379–388
58. Boström, K., Watson, K. E., Horn, S., Wortham, C., Herman, I. M., and Demer, L. L. (1993) Bone morphogenetic protein expression in human atherosclerotic lesions. *J. Clin. Invest.* **91,** 1800–1809
59. Collett, G. D., and Canfield, A. E. (2005) Angiogenesis and pericytes in the initiation of ectopic calcification. *Circ. Res.* **96,** 930–938
60. Cheng, S. L., Shao, J. S., Charlton-Kachigian, N., Loewy, A. P., and Towler, D. A. (2003) MSX2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors. *J. Biol. Chem.* **278,** 45969–45977
61. Shanahan, C. M., Cary, N. R., Metcalfe, J. C., and Weissberg, P. L. (1994) High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J. Clin. Invest.* **93,** 2393–2402
62. Dhore, C. R., Cleutjens, J. P., Lutgens, E., Cleutjens, K. B., Geusens, P. P., Kitslaar, P. J., Tordoir, J. H., Spronk, H. M., Vermeer, C., and Daemen, M. J.

(2001) Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* **21,** 1998–2003

63. Nakagawa, Y., Ikeda, K., Akakabe, Y., Koide, M., Uraoka, M., Yutaka, K. T., Kurimoto-Nakano, R., Takahashi, T., Matoba, S., Yamada, H., Okigaki, M., and Matsubara, H. (2010) Paracrine osteogenic signals via bone morphogenetic protein-2 accelerate the atherosclerotic intimal calcification *in vivo*. *Arterioscler. Thromb. Vasc. Biol.* **30,** 1908–1915
64. Derwall, M., Malhotra, R., Lai, C. S., Beppu, Y., Aikawa, E., Seehra, J. S., Zapol, W. M., Bloch, K. D., and Yu, P. B. (2012) Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **32,** 613–622
65. Trompet, S., Pons, D., Kanse, S. M., de Craen, A. J., Ikram, M. A., Verschuren, J. J., Zwinderman, A. H., Doevendans, P. A., Tio, R. A., de Winter, R. J., Slagboom, P. E., Westendorp, R. G., and Jukema, J. W. (2011) Factor VII activating protease polymorphism (G534E) is associated with increased risk for stroke and mortality. *Stroke Res. Treat.* **2011,** 424759
66. Lissenberg-Thunnissen, S. N., de Gorter, D. J., Sier, C. F., and Schipper, I. B. (2011) Use and efficacy of bone morphogenetic proteins in fracture healing. *Int. Orthop.* **35,** 1271–1280