TF/FVIIa Transactivate PDGFRβ to Regulate PDGF-BB–Induced Chemotaxis in Different Cell Types: Involvement of Src And PLC

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Involvement of Src And PLC

Agneta Siegbahn, Matilda Johnell, Anna Nordin, Mikael Åberg, Teet Velling

Background—We have previously reported the potentiation of PDGF-BB–induced chemotaxis of fibroblasts, vascular smooth muscle cells, and endothelial cells by FVIIa. Here we studied the role of TF/FVIIa and the induced signaling pathways in regulation of chemotaxis of human monocytes, fibroblasts, and porcine aorta endothelial cells.

Methods and Results—Human monocytes were obtained by using Ficoll-Paque gradient and the MACS system (for highly purified population), fibroblasts and PAE cells have been characterized previously. Inhibitors of selected signaling intermediates were used, and the effect of TF/FVIIa on the migratory response of all cells to chemotactic agents was analyzed. The induced signaling was studied by immunoprecipitation and Western blotting. TF/FVIIa complex selectively enhanced PDGF-BB–induced chemotaxis in a Src-family, PLC, and PAR-2–dependent manner. Using PAE cells we identified c-Src and c-Yes as the Src-family members activated by TF/FVIIa. We report for the first time the PAR-2 and Src family-dependent transactivation of PDGFRβ by TF/FVIIa involving phosphorylation of a subset of PDGFRβ tyrosines.

Conclusions—The described transactivation is a likely mechanism of TF/FVIIa-mediated regulation of PDGF-BB–induced chemotaxis. Similar behavior of 3 principally different cell types in our experimental setup may reflect a general function of TF in regulation of cell migration. (Arterioscler Thromb Vasc Biol. 2008;28:135-141.)

Key Words: TF/FVIIa ■ PDGFRβ ■ transactivation ■ cell signaling ■ chemotaxis

On binding of the coagulation factor VII (FVII) to its receptor tissue factor (TF), the proteolytically active TF/FVIIa complex forms. Besides initiating the coagulation process in vivo,1 this complex is involved in various nonhemostatic functions such as wound healing, local inflammation, atherosclerosis, angiogenesis, metastasis and tumor invasion,2–5 and regulation of gene expression.6–8 Directed cell migration, regulated by complex signaling reactions, is a critical component of these processes.9,10 The importance of TF cytoplasmic domain, and the role of serine residues in the TF cytodomain for cell migration have been recently established11,12 and phosphorylation of several signaling intermediates on the TF/FVIIa interaction has been reported.11,13–16 Nevertheless, the precise TF-dependent mechanism regulating cell migration, and the utilized signaling pathways have not been exhaustively explored.

A G protein–coupled receptor (GPCR), protease activated receptor-2 (PAR-2), activated on different cell types by the TF/FVIIa complex,6 is believed to mediate the nonhemostatic functions of TF/FVIIa, including transmission of cellular signals,6,5 although there is evidence for the PAR-2-independent reactions17,18 (and our unpublished results). Recently, transactivation of epidermal growth factor receptor (EGFR) by GPCR was reported,19 and the precise mechanism was characterized.20,21 Transactivation of the platelet-derived growth factor receptor β (PDGFRβ) by different GPCR has also been described,22,23 but the used mechanism is unknown.

We have previously shown that the TF/FVIIa complex positively regulates the platelet derived growth factor-BB (PDGF-BB)–induced chemotaxis.3,12 The aim of this study was to investigate the molecular mechanism by which TF/FVIIa enhances cell migration toward the PDGF-BB. We used cell lines with constitutive TF expression (porcine aorta endothelial cell line transfected with human TF and PDGFRβ, and human foreskin fibroblasts 1064Sk), and freshly isolated lipopolysaccharide (LPS)-stimulated human monocytes.3,12,24

We show that TF/FVIIa engages in a cross-talk with PDGFRβ by selectively potentiating the response of the cells to PDGF-BB different from 5 other chemotactic agents. This potentiation is regulated by PAR-2, c-Src and c-Yes kinases, and PLC isoform(s) other than PLCγ1 and PLCβ3, but not by MAP kinases. Most importantly we report, for the first time, the Src kinase–dependent transactivation of PDGFRβ by TF/FVIIa, and suggest this as the mechanism of the observed potentiation of PDGF-BB–induced cell migration by TF/FVIIa.
Methods

Cell Lines
PAE cells transfected with human PDGF β-receptors and TF (PAE/PDGFRβ-TF cells), or only PDGF β-receptors, and human foreskin fibroblasts 1064Sk and 1523 expressing endogenous TF, have been characterized previously.3,12,25

Isolation of Monocytes
The monocyte-containing fraction was isolated from whole blood of healthy donors by centrifugation in a Ficoll-Paque gradient. The cells were stimulated with 10 ng/mL bacterial LPS for 3 hours, washed twice with PBS to remove the platelets and lymphocytes, and the adherent mononuclear cells were recovered. A highly purified monocyte population was obtained using the MACS system (Miltenyi Biotech Inc).

Reagents
Recombinant human FVIIa and cataphylactically inactive FVIIa (FRR-FVIIa) were from Novo Nordisk A/S, recombinant human PDGF-BB from Amgen Inc, PAR-2 agonist peptide SLIGKV-NH2, LDS, f-MLP, Zymosan A and pertussis toxin (PTX) from Sigma-Aldrich, and the polyclonal neutralizing antibody to PAR-2 was a gift from Dr Wolfram Ruf (La Jolla, Calif). c-Reactive protein (CRP) was from Chemicon, MCP-1, and interleukin (IL)-8 from R&D systems, and Ficoll-Paque from Amersham Biosciences. The used inhibitors were: PD90859 (MEK) (Cell Signaling); SU6656 (Src family), U73122 (PLC), and the inactive compound U73343 (Calbiochem). The following antibodies were purchased: phospho-Src family (pY416), phospho-PLCγ1 (pY783), phospho-PLCβ3 (p557), PLCγ1 and PLCβ3 pAb, and HRP-linked anti-rabbit and anti-mouse IgG (Cell Signaling); c-Src (H-12) and c-Fyn (15) mAb, and c-Yes (3) and PDGFR pAb, and HRP-anti-goat (Santa Cruz); pY579 (GeneTex); rabbit monoclonal pY740 (32A9), pY771 (sc-17173), pY857 (sc-12907) and pY716 (sc-16569) (Santa Cruz); anti-phosphotyrosine (PY20-HRPO) and anti-FAK (Transduction Laboratories). The PDGFRβ site-specific phosphoantibodies were: pY740 (sc-17173), pY857 (sc-12907) and pY716 (sc-16569) (Santa Cruz); pY579 (GeneTex); rabbit monoclonal pY740 (32A9), pY771 (76D6), pY1021 (6F10) and pY751 (88H8) (Cell Signaling); and pY579 and pY1009 (LabFrontier). The Human PDGF-AB and PDGFB-B ELISA kits were from R&D Systems.

Complement Activation
Zymosan-activated serum (ZAS) with its predominant component, complement factor C5a, was obtained as described previously,26 aliquoted and stored at −70°C.

Chemotaxis Assay
The effect of FVIIa, FFR-FVIIa, or SLIGKV, and the selected pharmacological inhibitors, on chemotactic migration of the LPS-stimulated (10 ng/mL) monocytes, and fibroblasts and endothelial cells, was analyzed in a modified Boyden chamber assay applying a leading front technique essentially as described previously.12,27,28 For each set of experiments, the migration of cells toward the assay medium without chemotactic agents served as a control and was set as 100%. The results are expressed as the mean of at least 3 observations in different parts of the filter, analyzed in duplicates, and presented as % of control.

Immunoprecipitation and Western Blotting
Subconfluent PAE/PDGFRβ-TF cells were serum-starved for 24 hours and seeded to type I collagen (Vitrogen) before analysis of Src family kinases. A subset of cells was preincubated with SU6656, and FVIIa, SLIGKV, or PDGF-BB (a positive control) were added as agonists for the indicated times. Immunoprecipitation with c-Src, c-Yes, c-Fyn, or PDGFRβ antibodies and immunoblotting were carried out as described.29 The membranes were exposed to autoradiography films, bands from the representative experiments were scanned with the CanonScan 4200F scanner, and the relative extent of the protein phosphorylation was quantified densitometrically using the Image J software.

Statistical Analyses
Statistical analysis of the data were performed using Student unpaired t test in Statistica for Windows and Graph Pad Prism 5.0 (GraphPad software, Inc). The results are expressed as mean±SEM, and probability values (2-tailed) <0.05 are considered statistically significant.

Results
TF/FVIIa Enhances the Migration of Human Monocytes, Fibroblasts, and PAE/PDGFRβ-TF Cells to PDGF-BB, but not to Five Other Potent Chemoattractants, in a PAR-2, Src-Family, and PLC-Dependent Manner
The cell surface expression of TF was comparable in LPS-stimulated monocytes, fibroblasts, and PAE/PDGFRβ-TF cells agreeing with our previously published data.3,12,30 Concentration gradients of MCP-1, f-MLP, CRP, complement factor ZAS/C5a, and IL-8 all induced migration of human monocytes without any significant effect of the added FVIIa or FFR-FVIIa (Figure 1A through 1E). However, migration of all 3 cell types toward a concentration gradient of PDGF-BB was strongly enhanced by both 10 nmol/L and 100 nmol/L FVIIa or 50 pmol/L SLIGKV, and it was abolished by blocking the function of PAR-2 (Figures 1F and 2B). To investigate the role of Src-family kinases in the effect of FVIIa, the cells were treated with 2 pmol/L of the Src family kinase inhibitor SU6656. SU6656 abolished the hyperchemotactic response induced by FVIIa as well as by SLIGKV in all cell types tested (Figures 1F and 2B). Incubation of the cells with 0.5 µg/mL PTX, which interferes with Goα and Gai-type G protein α-subunit–mediated signals, abolished chemotaxis toward 10 nmol/L f-MLP but did not reverse the effect of FVIIa on PDGF-BB–induced migration (Figure 2B). Similarly, the MEK-inhibitor PD90859 had no effect on any of the cell types in the same assay (Figure 2B). We have previously shown that the TF/FVIIa complex formation leads to the activation of PLC.3 The widely used PLC inhibitor U73122 eliminated the enhanced chemotactic response (Figure 2B). To exclude that the inhibitors affect the basal cell migration the cells pretreated with the increasing concentrations of SU6656 and U73122, and the same concentrations of pertussis toxin and PD90859 as used in Figure 2, were subjected to migration assays. No change in cell migration was noted (see supplemental Figures 1 and II, available online at http://atvb.ahajournals.org), and our results thus confirm the selective inhibition of the effect of FVIIa by the used inhibitors.

PLCγ1, which was recently suggested to regulate the early migratory response mediated by adhesion and growth factor receptors,31 was not activated by TF/FVIIa or by the SLIGKV peptide in PAE/PDGFRβ-TF cells. Likewise, the phosphorylation of PLCβ3 active site Ser 537 was not affected by TF/FVIIa (data not shown) leaving the involved U73122-sensitive PLC isoform to be identified.

c-Src and c-Yes, but not c-Fyn, Are Activated by TF/FVIIa
Analysis of Src family kinases in monocytes was hampered by a strong activation of these enzymes during the purification process, and therefore the PAE/PDGFRβ-TF cells were
chosen for this purpose. Prominent phosphorylation of the active site Y416 of Src-family kinases was detected on the addition of FVIIa or SLIGKV to the cells adhering to type I collagen, and it was reduced to the background level by SU6656 (Figure 3A; collagen was used as an adhesive substrate to ensure comparable conditions to the migration assay). To discriminate between c-Src, c-Yes, and c-Fyn kinases, the common Src-family members in the 3 cell types used, these enzymes were immunoprecipitated from the PAE/PDGFR/H9252,TF cells treated as above, and analyzed in Western blotting with the pY416 Src-family antibody. Figure 3B depicts the result of this experiment whereby c-Src and c-Yes, but not c-Fyn, are activated on the formation of the TF/FVIIa complex. Activation of these enzymes by integrin type collagen receptors was not detected under these conditions (Figure 3B, “Col”).

TF/FVIIa Complex Transactivates PDGFRβ
To test the possibility that the potentiation of chemotaxis toward PDGF-BB by TF/FVIIa is attributable to a direct effect of TF/FVIIa on PDGFRβ, the PAE/PDGFRβ,TF cells were serum starved overnight and incubated with the suggested physiological concentration of FVIIa (10 nM). PDGFRβ was immunoprecipitated from cell lysates followed by Western blotting with the pan-phosphotyrosine antibody. In repeated experiments both a time and concentration-dependent increase in PDGFRβ phosphorylation was detected (P ranging from 0.003 to 0.04, and from 0.032 to 0.036; Figure 4A and 4B, respectively) indicating that the engagement of TF by FVIIa triggers activation of PDGFRβ. The phosphorylation of PDGFRβ induced by low concentrations of PDGF-BB (0.1 ng/mL) was enhanced by 10 nmol/L FVIIa (data not shown) thus correlating with the results of cell migration assays performed under similar conditions (Figure 1F). Reduction of this phosphorylation by a Src family inhibitor SU6656 suggested a role of the TF/FVIIa-activated Src kinase(s) in this process (Figure 4B). To exclude the possibility that the phosphorylation of PDGFRβ was induced by a nonspecific binding of FVIIa to the cell surface protein other than TF, the PAE cells transfected with PDGFRβ (but not with TF), were incubated with 100 nmol/L of FVIIa, but no change in PDGFRβ phosphorylation was detected (data not shown). Increasing concentrations of SLIGKV led to phosphorylation of PDGFRβ that was significantly (P=0.012) reduced by SU6656 (Figure 4C) supporting the possibility that at least one of the ways to activate PDGFRβ by TF/FVIIa may be mediated by PAR-2. Thrombin binding to PAR-1 has been shown to induce secretion of PDGF and activation PDGFR. To test whether similar mechanism was used by FVIIa/PAR-2 we analyzed PDGF-AB and PDGF-BB in conditioned media of the FVIIa-treated cells. As no secreted PDGF was detected this mech-
anism of PDGFRβ activation could be excluded (data not shown). These results show for the first time that the TF/FVIIa complex transactivates PDGFRβ.

**TF/FVIIa Induces Phosphorylation of a Subset of PDGFRβ Cytoplasmic Tyrosines**

To get an insight into the signaling pathways potentially activated by TF/FVIIa through PDGFRβ transactivation we analyzed the phosphorylation of individual PDGFRβ tyrosines, which in their phosphorylated state are known to regulate major cellular functions by binding the SH-2 domain-containing enzymes and adapter proteins. Of the 8 PDGFRβ autophosphorylation sites analyzed only 4 (tyrosines 579, 771, 1009, and 1021) were phosphorylated on the TF/FVIIa complex formation (Figure 5 and Table). Four other sites, Y716, 740, 751, and 857, were phosphorylated on PDGF-BB stimulation but no significant increase of the signal in FVIIa-stimulated cells was detected (Figure 5).

**Discussion**

We have previously shown that TF/FVIIa complex potentiates PDGF-BB–induced chemotaxis in fibroblasts and vascular smooth muscle cells and endothelial cells. In this study, using the freshly isolated LPS-stimulated human monocytes together with fibroblasts and endothelial cells, we studied the molecular mechanism by which TF/FVIIa influences PDGF-BB–induced cell migration. Whereas monocyte migration induced by 5 well-characterized chemotactic factors was unaffected by TF/FVIIa, PDGF-BB–induced migration of all 3 cell types was strongly enhanced by TF/FVIIa indicating a cross-talk between TF/FVIIa and PDGFRβ signaling. Here we show that TF, after ligation of FVIIa, induces the PAR-2 and Src family-dependent phosphorylation of PDGFRβ. We thus report, for the first time, the phenomenon of transactivation of PDGFRβ by TF/FVIIa and suggest a role for this event in regulation of cell migration.

Incubation of cells with FVIIa-induced PDGFRβ tyrosine phosphorylation in time and concentration-dependent manner (Figure 4). Involvement of PAR-2 and the TF/FVIIa-activated Src kinases in this reaction was supported by a similar induction of pY PDGFRβ by SLIGKV and the reduction of the effect of both FVIIa and SLIGKV by a Src family inhibitor SU6656. Closer mapping of specific tyrosines in PDGFRβ with the site-specific phosphoantibodies revealed a distinct TF/FVIIa-induced pattern of phosphorylation compared with that induced by PDGF-BB (Figure 5). Phosphorylation of the Src binding site (Y579) supports the role of Src kinases in this event in regulation of cell migration.

**Figure 2.** FVIIa or PAR-2 agonist-induced potentiation of the migratory response of the indicated cells to 0.1 ng/mL PDGF-BB (A) is eliminated by SU6656, U73122, or anti-PAR-2 antibody but is not affected by PD98059 or pertussis toxin (PTX). Data are presented as mean±SEM of 3 separate experiments. **P<0.01; ***P<0.001; NS, non-significant.

**Figure 3.** Phosphorylation of the Src-family active-site Y416 in total cell lysates (A) and c-Src, c-Yes, and c-Fyn immunoprecipitates (B) of the FVIIa and PAR-2 agonist-stimulated PAE/PDGFRβ, TF cells. Shown are the results of 1 representative experiment out of 3. *P<0.05; **P<0.001.
Table 1 indicate that the TF/FVIIa-induced PDGFRβ transactivation is likely to amplify some of the TF/FVIIa-induced signals in parallel with those triggered by TF/FVIIa via separate mechanisms.

Transactivation of receptor tyrosine kinases (RTK) by activated GPCR has been recognized as one of the means to mediate a variety of cellular responses and references therein). E.g. PAR-1–induced signals have been shown to trigger activation of different growth factor receptors in this manner, and the involvement of EGFR transactivation trigger activation of different growth factor receptors in this manner, and the involvement of EGFR transactivation activation is likely to amplify some of the TF/FVIIa-induced signals in parallel with those triggered by TF/FVIIa via separate mechanisms.

In Figure 2 we have used the published optimal concentrations of the inhibitors; e.g. in the case of SU6656 the selectivity for Src (at the 2 μmol/L concentration) relative to a number of other kinases was shown to be over 6 times higher. Under these conditions the used inhibitors that were found not to influence the basal chemotaxis induced by PDGF-BB (supplemental Figures I and II) strongly inhibited the TF/FVIIa-induced enhanced cell migration (Figure 2B). This finding is supported by a previous report on stimulation of Src-kinases by TF and suggests a similar functional role for these enzymes in all 3 cell types used. Our results further show that the c-Src and c-Yes kinases are selectively activated by TF/FVIIa and participate in transactivation of PDGF-BB. Although our data support the dependence of TF/FVIIa-induced transactivation of PDGFRβ, and hyperchemotaxis, on the activation of PAR-2 (see Figures 1F, 2A, and 4C), parallel PAR-2–independent pathway(s) may exist that are regulated by TF cytoplasmic domain or the lateral interactions with other cellular receptors (and our unpublished results).

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Figure 4. The TF/FVIIa-induced transactivation of PDGFRβ in PAE/PDGFRβ, TF cells is time (A), concentration (B), and PAR2 (C)-dependent, and is sensitive to Src-inhibitor SU6656 (B and C). The experiments were repeated 3 to 5 times. *P<0.05; **P<0.01; ***P<0.001.

Figure 5. TF/FVIIa induces a rapid phosphorylation of a subset of PDGFRβ tyrosines in serum-starved PAE/PDGFRβ, TF cells. The 4-minute point is missing on the pY1009 blot. One of at least 3 representative experiments is shown. *P<0.05; **P<0.01.
PDGFβ. Other specific functions for these Src family enzymes in a given context remain to be identified.

Recently, a role for c-Src–mediated activating phosphorylation of PLCγ1 on Y783 for cell migration was demonstrated.31 We have shown that TF/FVIIa complex triggered PLC activity,3 and U73122 eliminated the enhanced cell migration in our assays (Figure 2). However, neither PLCγ1 nor PLCβ3 that both are targets of U73122, were activated by TF/FVIIa (data not shown). c-Src and c-Yes are therefore likely to act via other U73122-sensitive PLC isoform(s), or function in parallel with a putative Src-family–independent PLC subtype. Pertussis toxin, which perturbs the signaling through Goq-type nucleotide-binding proteins32 that are engaged in PAR-2–activated pathways,43 had no effect on cell migration (Figure 2B). Our results rather implicate the pertussis toxin-insensitive Goq subfamily members, which also connect to PAR-2, in TF/FVIIa signaling.

TF/FVIIa signaling in monocyte-derived macrophages has been described earlier,44,45 but our study is so far the first one to use freshly isolated human monocytes in this context. A recent paper by Muth et al45 reported a consistent upregulation of IL-8 gene in response to FVIIa in activated monocyte-derived macrophages, and migration of breast cancer cells with constitutive TF-expression toward FVIIa was entirely dependent on TF/FVIIa-induced IL-8 production.7 In our experimental system a weak effect of IL-8 on chemotaxis of the short-term cultured human monocytes was not altered by addition of FVIIa, which rules out the possibility that the FVIIa-dependent potentiation of chemotaxis is regulated by IL-8 via an autocrine loop.

Our findings with 3 principally different cell types indicate that the TF/FVIIa-induced elevated migratory response to PDGF-BB could be of broader functional importance. However, in a different cellular context FVIIa does not have an additive effect on PDGF-BB–stimulated events that involve cell migration.4 Nevertheless, a synergy between TF and PDGFβ has been suggested in support of our results.46

In conclusion, we report the functional cooperation between TF/FVIIa and PDGF β-receptor in chemotaxis of 3 principally different cell types. This cross talk involves PAR-2, Src-family kinases c-Src and c-Yes, and isoform(s) of phospholipase C other than γ1 or β3. We show that TF/FVIIa induces the phosphorylation of a defined subset of PDGFβ cytoplasmic tyrosines and thus report, for the first time, the phenomenon of transactivation of PDGFβ by TF/FVIIa that is likely to play a role in the regulation of PDGF-BB–induced cell migration. Characterization of the signaling intermediates involved in this process, the activated signaling pathways, and their role in TF/FVIIa-dependent biological functions are a subject of ongoing studies.

Acknowledgments
The authors thank Dr Wolfram Ruf for the gift of the anti–PAR-2 antibody and acknowledge Birgitta Fahlström for skillful technical assistance.

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Disclosures
None.

References

Table. TF/FVIIa-Induced PDGFR-β Phosphorylations in PAE/PDGFRβ, TF Cells

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<td>Y1021</td>
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<td>PLCγ</td>
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Figure I
Figure II
Legends to the supplemental figures

Supplemental Figure I. Human fibroblasts were treated with SU6656 and U73122, and subjected to the chemotaxis assay in the concentration gradient of PDGF-BB. At the inhibitor concentrations used in the study the basal PDGF-BB-induced cell migration was not affected. n=4

Supplemental Figure II. The indicated cells were pre-incubated with increasing concentrations of SU6656 (A, n=4), U73122 (B, monocytes, n=6; PAE cells, n=2; fibroblasts, n=2) and its inactive control compound U73343 (C, n=2), and with 50 mM of PD98059 (D, n=6) and 500 ng/ml pertussis toxin (PTx, E, n=6), and analysed in a migration assay in the presence of 0,1 ng/ml PDGF-BB. Note that the basal cell migration was not affected by the concentrations of the inhibitors used in our study thereby also excluding the aspect of potential cytotoxicity.
Detailed Figure Legends

Figure 1. Effects of FVIIa and FFR-FVIIa on monocyte chemotactic protein-1 (MCP-1), zymosane-activated serum (ZAS), f-MLP, C-reactive protein (CRP), interleukin-8 (IL-8) and platelet-derived growth factor-BB (PDGF-BB)-induced chemotaxis of human TF-expressing monocytes. (A-E) Monocytes, untreated or pre-incubated with FVIIa or with FFR-FVIIa, were exposed to assay media without additives, or containing different concentrations of the indicated chemoattractants, and analyzed in duplicates in a modified Boyden chamber assay. (F) Results of the analysis of the effect of FVIIa and SLIGKV, alone or in combination with the Src-family kinase inhibitor SU6656, on the PDGF-BB-induced chemotaxis are shown. Data are presented as mean±SEM of three (A-E) or five (F) separate experiments. * = p<0,05; ** = p<0,01; *** = p<0,001

Figure 2. The role of selected signaling pathways in the TF/FVIIa-potentiated migratory response to PDGF-BB. (A) The indicated cell types were allowed to migrate towards 0,1 ng/mL PDGF-BB and migration was analyzed as in Figure 1 in the absence or presence of FVIIa and PAR-2 agonist SLIGKV. (B) The same cells as in (A) were stimulated with FVIIa or SLIGKV in the presence of pharmacological inhibitors (SU6656, U73122, PD98059 or pertussis toxin (PTX)), or the anti-PAR-2 antibody
The rates of induction (A) and the inhibition (B) of cell migration were calculated relative to the unstimulated cells in assay medium, or to the stimulated cells, respectively. Data are presented as mean±SEM of three separate experiments. ** = \( p < 0.01 \); *** = \( p < 0.001 \), NS – non-significant.

Figure 3. FVIIa and PAR-2 agonist activate Src family kinases in PAE/PDGFRβ,TF cells. (A) The serum-starved cells were allowed to adhere to type I collagen (Col) for 10 minutes in the presence of 100 nM FVIIa, 50 µM SLIGKV, and 2 µM SU6656 as indicated, lysed in reducing SDS sample buffer and subjected to Western blotting with pY416 Src family antibody. The FAK antibody was used as a loading control. (B) The cells treated as in (A) and a control of non-adherent cells (BSA), were processed for immunoprecipitation with the indicated antibodies followed by western blotting with the pY416 Src family antibody. Membranes were re-probed with the c-Src, c-Yes and c-Fyn antibodies detecting whole proteins. Shown are the results of one representative experiment out of three. * = \( p < 0.05 \); *** = \( p < 0.001 \)

Figure 4. TF/FVIIa induces the transactivation of PDGFRβ in PAE/PDGFRβ,TF cells. (A) The serum-starved cells were stimulated with FVIIa for the indicated times and the tyrosine-phosphorylated PDGFRβ was detected by immunoprecipitation and Western blotting (2 min, \( p = 0.017 \); 5 min, \( p = 0.010 \); 10 min, \( p = 0.030 \); 20 min, \( p = 0.003 \); 30 min, \( p = \)
0.040; PDGF-BB, \( p < 0.0001 \)). (B) The serum starved cells were incubated with increasing concentrations of FVIIa during 10 minutes and tyrosine phosphorylation of PDGFR\( \beta \) was detected as in A (10 nM, \( p = 0.036 \); 100 nM, \( p = 0.032 \), 100 nM + SU6656 not significant, the analyses were performed compared to unstimulated control). (C) Cells were treated identically to B except that increasing nanomolar concentrations of the SLIGKV peptide were added instead of FVIIa. 2 \( \mu \)M of SU6656 was added where indicated and PDGF-BB was used as a positive control (10 nM, \( p = 0.053 \); 20 nM, \( p < 0.0001 \); 50 nM, \( p = 0.006 \); 50 nM + SU6656, \( p = 0.005 \), analyses were performed compared to unstimulated control, 50 nM vs 50 nM + SU6656, \( p = 0.012 \)). All experiments were repeated for three to five times, the representative films were scanned and the bands were quantified densitometrically as described in Methods. * = \( p < 0.05 \); ** = \( p < 0.01 \); *** = \( p < 0.001 \).

Figure 5. TF/FVIIa induces a rapid phosphorylation of a subset of tyrosines in PDGFR\( \beta \). Serum-starved PAE/PDGFR\( \beta \),TF cells were incubated with FVIIa or PDGF-BB for the indicated times and the lysates were analyzed by Western blotting with the site-specific anti-PDGFR\( \beta \) phosphotyrosine antibodies. The 4-minutes point is missing on the pY1009 blot. The diagram depicts the results of a densitometric analysis where the strongest signal obtained within the given time scale was related to the signal from unstimulated cells for each phosphorylation site. One out of at
least three representative experiments is shown. * = \( p < 0.05 \); ** = \( p < 0.01 \)