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Citation for published version (APA):
Versteeg, H. H. (2003). Factor VIIa-induced signal transduction; possible explanations for tissue factor-associated events

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FVIIa/tissue factor interaction results in a tissue factor cytoplasmic domain-independent activation of protein synthesis, p70 and p90 S6 kinase phosphorylation

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Based on: Journal of Biological Chemistry 2002;277:27065-27072
Abstract

FVIIa binding to tissue factor (TF) and subsequent signal transduction has now been implicated in a variety of pathophysiological processes including cytokine production during sepsis, tumor- and neoangiogenesis, and leukocyte reverse transmigration. The molecular details, however, by which FVIIa/TF affects gene expression and cellular physiology, remain obscure. Here we show that FVIIa induces a transient phosphorylation of p70/p85$^{S6K}$ and p90$^{RSK}$, in BHK cells stably transfected with either full length TF or with a cytoplasmic domain-truncated TF, but not in wild type BHK cells. Phosphorylation of these kinases was also observed in HaCaT cells, expressing endogenous TF. Phosphorylation of p70/p85$^{S6K}$ coincided with protein kinase B and GSK-3$^\beta$ phosphorylation. Activation of p70/p85$^{S6K}$ was sensitive to inhibitors of PI3-kinase and to rapamycin, whereas phosphorylation of p90$^{RSK}$ was sensitive to PD98059. FVIIa stimulation of p70/p85$^{S6K}$ and p90$^{RSK}$ correlated with phosphorylation of the eukaryotic initiation factor eIF-4E, upregulation of protein levels of eEF1$\alpha$, eEF2, and enhanced $^{35}$S-methionine incorporation. These effects were not influenced by inhibitors of thrombin or FXa generation and were strictly dependent on the presence of the extracellular domain of TF, but did not require the intracellular portion of TF. We propose that a TF cytoplasmic domain-independent stimulation of protein synthesis via activation of S6 kinase contributes to FVIIa effects in pathophysiology.
Introduction

Tissue Factor (TF) is the principal initiator of the coagulation cascade in vivo (1,2,3). After binding to the zymogen FVII, activation of the serine proteases FIX, FX and prothrombin takes place, eventually leading to platelet activation and fibrin deposition, inducing the formation of a blood clot. In addition, recent research suggests that TF is implicated in various other physiological events, as is most dramatically demonstrated by the observation that TF knock-out mice die at day 9.5-10.5 of embryogenesis as a consequence of defective neoangiogenesis (4). Also, TF plays an eminent role in tumor angiogenesis, since interfering with the association of FVIIa to TF results in a dramatic reduction in tumor potential (5) as a consequence of reduced vascularization. Finally, TF/FVIIa complex formation is implicated in the inflammatory response, as evident from the upregulation of mRNA of pro-inflammatory cytokines (6), and from the reduction of LD_{100} E. coli-induced mortality of baboons infused with active site-inhibited FVIIa (7). Moreover, TF also regulates leukocyte trafficking since inhibiting TF with an antibody resulted in a significant reduction of mononuclear phagocyte's trans-endothelial migration (8). The molecular details of these effects, however, remain largely unresolved but are generally assumed to be a consequence of FVIIa-induced signal transduction in TF-expressing cells (reviewed in 9,10,11).

A significant research effort has been devoted to FVIIa/TF-induced signal transduction resulting in the identification of FVIIa/TF-dependent calcium transients (12,13), activation of polyA-polymerase (14) and activation of the p42/p44 and the p38 MAP kinase pathway (15-18). The mechanism for the signal transduction across the membrane is still unclear but may involve the sequestration of FVIIa to the membrane by TF and subsequent proteolytic cleavage and activation of a protease activated receptor (PAR) that remains yet to be unequivocally identified (19-21). More recently, we showed that FVIIa induces the activation of the Src family members c-Src, Lyn, and Yes, followed by stimulation of the small GTPases Rac and Cdc42. In turn, Rac mediates p38 mitogen-activated protein (MAP) kinase activation and cytoskeletal reorganization, events which may be related to TF effects in angiogenesis and inflammation (18). Other studies have shown changes in the transcription of various cytokines and extracellular matrix components (6,22). Thus, although important aspects of TF/FVIIa signal transduction have now been uncovered, significant sections of this signal...
transduction pathway remain to be explored, and in particular links to the translational process are largely unknown.

This prompted us to study possible links between TF/VIIa-induced signal transduction and changes in translation. Initiation of protein synthesis is largely regulated by the kinases c-Akt/PKB and the S6 kinases (e.g. 23,24). The latter proteins activate both the ribosome, by phosphorylation of the Ribosomal S6 protein, and eukaryotic initiation factors, that facilitate binding of the mRNA to the ribosome. In this study, we have investigated the activation of several protein synthesis mechanisms, and we show that FVIIa induces phosphorylation of c-Akt/PKB, p70/p85\textsuperscript{S6K} as well as p90\textsuperscript{RSK} in HaCaT cells and BHK cells transfected with full length or cytoplasmic domain-deleted TF, but not in untransfected BHK cells. Furthermore, eukaryotic initiation factor eIF-4E was similarly activated in the same cells. We show that activation of the translational machinery was specific for FVIIa and that this effect could not be inhibited by the specific thrombin inhibitor hirudin or the specific FXa inhibitor Tick Anticoagulant Protein (TAP). From these data we conclude that binding of FVIIa to cellular TF results in protein synthesis, independent of the TF cytoplasmic domain.
Experimental Procedures

Cell culture- Wild type Baby Hamster Kidney (BHK) cells, BHK cells stably transfected with a construct, expressing full length TF (BHK\textsuperscript{TF}) or a cytoplasmic domain truncated mutant (BHK\textsuperscript{TF\_Asy}) (TF 1-247) were cultured, as described before (15,16) in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 µg/ml streptomycin, in a humidified environment at 37°C. HaCaT human keratinocytes were cultured likewise. Prior to experiments, BHK cells were serum starved in DMEM for 16-18 hrs, whereas HaCaT cells were starved for 24 hrs. Half an hour before stimulation, hirudin (25 U/ml), TAP (200 nM), LY294002 (10 µM), or PD98059 (20 µM) were added. The mTOR inhibitor rapamycin (20 ng/ml) was added for 15 minutes.

Materials- Antibodies directed against phospho-PKB\textsuperscript{Ser473}, phospho-p70\textsuperscript{S6K-Thr389}, phospho-GSK3\alpha/\beta\textsuperscript{Ser21}, phospho-p90\textsuperscript{RSK-Ser381}, phospho-eIF-4E\textsuperscript{Ser209} as well as peroxidase-conjugated Goat-anti-Rabbit antibody were purchased from Cell Signaling Technologies (Beverly, MA). All non-phosphospecific antibodies were purchased from Santa Cruz (Santa Cruz, CA), except from the antibody raised against eEF1\alpha (Upstate Biotech; Waltham, MA). The eEF2 antibody was a generous gift from Dr. Angus Nairn (Rockefeller University, New York). \textsuperscript{35}S-methionine (Promix) was obtained from Amersham Biosciences (Piscataway, NJ). Bovine Serum Albumin (BSA), hirudin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and rapamycin were from Sigma (St. Louis, MO). LY294002 as well as PD98059 were from Alexis. Recombinant human FVIIa (NovoSeven\textsuperscript{®}) and active site-inhibited FVIIa (FVIIai or FFR-FVIIa) were from Novo-Nordisk A/S (Bagsvaerd, Denmark). Insulin was from Eli Lilly (Nieuwegein, The Netherlands). TAP was a generous gift from Dr George Vlasuk (Corvas International, Inc., San Diego, California).

Proliferation assay- BHK\textsuperscript{TF} cells were seeded in 24-well plates and serum starved for 16 hours in the presence of 0.1 mg/ml BSA. Subsequently, the cells were stimulated mock, with FVIIa, or with 10% FCS in DMEM. Cell proliferation was determined, using an MTT assay as described by Rubinstein et al (25). Briefly, after mock-stimulation or incubation with FVIIa or FCS, 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was directly added to the media, for 30 min at 37°C. Subsequently, the media were
aspirated and the cells were lysed in isopropanol/0.04 N HCl. The OD550 of this solution was determined, using an ELISA reader.

**Western blotting**- Cells at 80% confluence were serum starved in DMEM for 16 hrs before incubation with the indicated compounds. After incubation, cells grown in 20 cm\(^2\) dishes were kept on ice and washed with ice-cold phosphate buffered saline (PBS). After that, the cells were rapidly harvested by adding 100 μl of heated (95°C) sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue) lysates were collected by scraping. After a 5 min incubation at 95°C, 30 μl of the lysates were loaded onto SDS-PAGE and subsequently transferred to a PVDF membrane. The membranes were blocked with tris-buffered saline (TBS) supplemented with 0.1% tween-20 (wash buffer) and 2% low-fat milk powder, and incubated with primary antibody over night at 4°C, diluted 1:1000 in wash buffer containing 2.5% BSA. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody in wash buffer, containing 2% low-fat milk powder. The bands were visualized, using Lumilight plus\(^\circ\) ECL substrate from Roche and a chemiluminescence detector with a cooled CCD-camera (Genetools) from Syngene. Antibody bands were quantified using Genetools from Syngene.

\(^{35}\text{S-methionine labeling} -\) The cells were grown in 6 well plates until 75% confluence and serum starved for 16 hours. The next day, 20 μCi/well of \(^{35}\text{S}-\text{methionine} was added, and incubated for 5 hours. Cells were stimulated with 100 nM FVIIa for the times indicated, in the presence or absence of inhibitors, or 170 nM (1 μg/ml) insulin. Control cells were left unstimulated. After incubation, the medium was aspirated and the cells were lysed in a 1% SDS solution in H\(_2\)O. Trichloro acetic acid (TCA) was added to a final concentration of 15% and proteins were precipitated for 30’ on ice. The lysates were centrifuged for 5’ at 21,000 x G and the pellets were washed with 15% TCA. The pellets were resuspended in 400 μl 0.1 N NaOH, 3.6 ml scintillation fluid (Ultima Gold; Packard) was added and the number of disintegrations per minute (DPM) was determined using a scintillation counter (Packard).

For immunoprecipitation, cells were grown and labeled as described above. After stimulation, cells were lysed in 600 μl non-denaturing lysisbuffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na\(_2\)VO\(_4\), 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM PMSF) and scraped. The insoluble fraction was collected by centrifugation and the soluble fraction
was precleared with either protein G-sepharose or protein A-sepharose respectively. Subsequently, monoclonal anti-eEF1α or polyclonal anti-eEF2 was added over night or for 2 hours respectively, and the immune complexes were precipitated with either protein G-sepharose or protein A-sepharose for 1.5 hours. After centrifugation, the pellet was washed three times with lysisbuffer and resuspended in 400 µl 0.1 N NaOH. Again, 3.6 ml scintillation fluid was added and the number of disintegrations per minute (DPM) was determined.
Chapter 4

Results

**FVIIa does not induce cell proliferation**— TF plays a pivotal role in vascular development and neoangiogenesis (e.g. 3,4), but the role of TF in such phenomena remains unclear. Therefore we investigated the capability of FVIIa of inducing proliferation in Baby Hamster Kidney cells stably transfected with TF (BHK\(^{TF}\), (15)). Cells were serum starved in DMEM and after 16 hours, proliferation was assessed, using an MTT assay, as described under Experimental Procedures. After mock stimulation, 100 nM of FVIIa, or 10% FCS for 24 hours, proliferation was assessed again Fig. 1 shows that in contrast to stimulation with FCS, exposure to FVIIa did not result in any increase in proliferation within 24 hours. Thus cell proliferation appears not to be a plausible cause for the FVIIa/TF-induced effect on neovascularization and other mechanisms involving protein synthesis without cell division may be involved.

**Figure 1:** FVIIa does not induce cell proliferation in TF expressing cells. BHK\(^{TF}\) cells were serum starved for 16 hours in the presence of 0.1 % BSA. Subsequently, the cells were incubated with DMEM containing 100 nM FVIIa or DMEM containing 10% FCS for 24 hours. The cells were subjected to an MTT assay on \(t=0\) as a control and after 24 hours. Experiments were performed in quadruple, standard deviations are shown.

FVIIa induces c-Akt/PKB, S6 kinases, GSK3 \(\alpha/\beta\), and eIF-4E phosphorylation in BHK cells, stably transfected with TF— Recently we have shown that exposure of A14 fibroblasts to FVIIa leads to activation of the anti-apoptotic kinase PKB (18). To investigate whether stimulation of PKB by FVIIa is restricted to these fibroblasts or may constitute a more general phenomenon in TF-dependent signal transduction, we subjected BHK\(^{TF}\) cells to 100 nM FVIIa for various times ranging from 5 min to 60 min. Lysates were analyzed for
phosphorylation of PKB, S6 kinases, GSK3 and eIF-4E by immunoblotting using phosphospecific antibodies. As can been seen in Fig. 2A, BHK\textsuperscript{TF} cells show a transient and concentration-dependent phosphorylation of PKB in response to FVIIa, whereas media control experiments had no effect. Also, wild type BHK cells did not react to FVIIa (Fig. 2B). PKB and its upstream activator PI3-kinase are frequently associated with signaling events leading to protein synthesis; PKB activation leads to phosphorylation of the downstream

**Figure 2:** FVIIa induces transient phosphorylation of PKB, p70/p85\textsuperscript{S6K}, GSK3, p90\textsuperscript{PSK} and eIF-4E in BHK\textsuperscript{TF}, but not in wild type BHK cells. The cells were serum starved as described under Experimental Procedures and incubated with 100 nM FVIIa for the indicated times. The cells were lysed and analyzed by Western Blot for phosphorylation of the indicated proteins using phosphospecific antibodies. Panel (A) represents phosphorylation states of PKB, p70/p85\textsuperscript{S6K}, GSK3, and p90\textsuperscript{PSK} in BHK\textsuperscript{TF} cells. Panel (B) represents the phosphorylation state of the same proteins in wild type BHK cells. A 10 min incubation of the cells with 170 nM insulin (I), a well-established inducer of protein synthesis, was used as a positive control. To test for equal loading, the Western Blots were reprobed for \(\beta\)-actin (42 kDa). (C,D) Cells were treated as described above and analyzed for eIF-4E phosphorylation. An antibody against total eIF-4E was used to demonstrate equal loading. The Western Blots represent three independent experiments.
targets p70/p85<sub>S6K</sub> via mTOR, and GSK3. The S6 kinases mediate activation of the ribosome whereas phosphorylation and subsequent inactivation of GSK3 is a prerequisite for protein synthesis (26). Therefore we also investigated the effect of FVIIa on these kinases. Fig. 2A shows a transient phosphorylation of both p70/p85<sub>S6K</sub> and GSK3α/β, after stimulation with FVIIa. In addition we investigated the FVIIa-induced phosphorylation of p90<sup>RSK</sup>, which phosphorylates the ribosomal protein S6<sub>i</sub>n<sub>v</sub>itro and, unlike the p70/p85<sub>S6K</sub>, is activated through the MAP kinase pathway (27). Activation of both p90<sup>RSK</sup> was observed in BHK<sup>TF</sup> cells (Fig 2A), but untransfected BHK cells did not display p90<sup>RSK</sup> activation in response to FVIIa (Fig 2B). Apparently, S6 kinase phosphorylation is a major event in FVIIa/TF-induced signal transduction.

Next, we determined the phosphorylation status of the eukaryotic translation factor eIF-4E, which regulates translation initiation of a subset of highly structured growth-regulatory mRNAs. Phosphorylation of both eIF-4E and 4E-BP, the latter leading to its dissociation from eIF-4E, are brought about by the p70/p85<sub>S6K</sub> pathway, specifically mTOR (28). Accordingly, we observed a sustained phosphorylation of eIF-4E in BHK<sup>TF</sup> in response to FVIIa (Fig. 2C), whereas untransfected BHK cells were not responsive in this respect. (Fig. 2D).

**Figure 3:** Insulin-induced phosphorylation of PKB, p70/p85<sub>S6K</sub>, GSK3 and p90<sup>RSK</sup> in BHK<sup>TF</sup> cells. The cells were serum starved as described under Experimental Procedures and incubated with 170 nM insulin for the times indicated. The cells were lysed and analyzed by Western Blot for phosphorylation of the indicated kinases and proteins using phosphospecific antibodies on Western Blot. Equal loading was tested by reprobing the blots with antibodies directed against total protein of interest. The Western Blots represent three independent experiments.
To compare FVIIa-induced phosphorylation events with a more established activator of these proteins, we also treated BHK^T^ cells with 170 nM insulin. Fig. 3 shows that the effect of insulin on phosphorylation of PKB, p70/p85S^6K^, p90RSK and GSK3 is much less transient, suggesting a more profound, or longer effect on protein synthesis.

**FVIIa induces PKB, S6 kinases, p42/44 MAP kinase, GSK3 α/β, and eIF-4E phosphorylation in HaCaT cells-** With the above demonstration of sequential activation of PKB, the S6 kinases, GSK3 and eIF-4E in BHK cells type stably transfected with TF, it was of interest also to study the FVIIa-induced activation of these components in a cell type ubiquitously expressing TF. Hence we challenged HaCaT keratinocytes with 100 nM FVIIa for various times and screened phosphorylation states of these proteins as described before. Fig. 4 shows that HaCaT cells, exposed to FVIIa respond with a much less transient phospho-

![Phosphorylation States](image)

**Figure 4: FVIIa induces transient phosphorylation of PKB, p70/p85S^6K^, GSK3, p90RSK and eIF-4E in HaCaT cells.** The cells were serum starved and incubated with 100 nM FVIIa for the times indicated. The cells were lysed and analyzed by Western Blot for phosphorylation of the indicated kinases and proteins using phosphospecific antibodies on Western Blot. 170 nM insulin (I) was used as a control. To test for equal loading, Western Blots were reprobed with antibodies directed against total protein. The Western Blots represent three independent experiments.
rylation of PKB, the S6 kinases, GSK3 and eIF-4E, compared to BHK<sup>TF</sup> cells. We conclude from this that FVIIa activation of these proteins is not restricted to TF-transfected cells, but also occurs in cells, naturally expressing TF constitutively, at physiological density on the cell membrane.

Figure 5: Concentration-dependent effects of FVIIa on phosphorylation of p42/p44 MAP kinase, PKB and p90<sup>RSK</sup> in BHK<sup>TF</sup> and HaCaT cells. (A) Quiescent BHK<sup>TF</sup> cells were stimulated with the indicated concentrations of FVIIa for 10 min. The cells were lysed and analyzed for phosphorylation of MAP kinase, PKB and RSK by Western Blot, using phospho-specific antibodies. Equal loading was shown using an antibody against total PKB. (B) HaCaT cells were starved and treated with the concentrations of FVIIa indicated. Subsequently, phosphorylation of GSK3, p42/p44 MAP kinase and p90<sup>RSSK</sup> was assayed on Western Blot, equal amounts were demonstrated, using an antibody against total protein. The Western Blots represent three independent experiments.
FVIIa-induced activation of protein synthesis signaling pathways is concentration dependent. To establish the physiological relevance of FVIIa-induced signaling, we subjected both BHK\textsuperscript{TF} and HaCaT cells to various concentrations of FVIIa. From Fig. 5A it is evident that PKB, p42/p44 MAP kinase and its downstream target 90\textsuperscript{RSK} were clearly phosphorylated at concentrations as low as 17 nM in BHK\textsuperscript{TF} cells. In HaCaT cells concentrations, lower than 10 nM, which is the FVII plasma concentration, were sufficient to induce phosphorylation of both p90\textsuperscript{RSK}, being an effector of the MAP kinase pathway, and GSK3\textbeta, activated through the PI3-kinase pathway (Fig. 5B). We conclude that in cells ubiquitously expressing TF, FVIIa acts on signal transduction at physiological concentrations.

Activation of PKB, the S6 kinases, GSK3 and eIF-4E is independent of the cytoplasmic domain. Although the relatively short cytoplasmic tail of TF contains two putative phosphorylation sites and has been shown to bind to the protein ABP-280 in yeast-two-hybrids studies (29), so far no physiological function has been found for this 21-amino acid domain, despite its strong sequence conservation in divergent species. We decided to explore

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\caption{FVIIa induces transient PKB, p70/p85\textsuperscript{S6K}, GSK3, p90\textsuperscript{RSK} and eIF-4E phosphorylation in BHK\textsuperscript{TF} cells. (A) The cells were serum starved as described under Experimental Procedures and incubated with 100 nM FVIIa for the times indicated. The cells were lysed and analyzed by Western Blot for phosphorylation of the indicated kinases and proteins using phosphospecific antibodies on Western Blot. 170 nM insulin (I) was used as a control. To test for equal loading, the Western Blots were reprobed for \textbeta-actin. (B) Cells were treated as described above and analyzed for eIF-4E phosphorylation. An antibody against total eIF-4E was used to demonstrate equal loading. The Western Blots shown represent three independent experiments.}
\end{figure}
the requirement of the TF-cytoplasmic domain for the aforementioned signal transducers. Sørensen et al (16) showed that BHK cells expressing a truncated form of TF (BHK\textsuperscript{TF\textsubscript{Acetylo}}) responded to FVIIa treatment with a prominent phosphorylation of p44/p42 MAPK similar to that observed in BHK cells, transfected with full length TF. As can be seen in Fig. 6, this was also true for PKB activation and subsequent signaling; BHK\textsuperscript{TFAcetylo} cells were equally responsive to FVIIa, as were the BHK\textsuperscript{TF} cells. It thus appears that activation by FVIIa of neither the MAP kinase pathway nor the PI3-kinase pathway requires the cytoplasmic TF domain.

**FVIIa-induced signals are FXa- and thrombin-independent, but are sensitive to inhibitors of the MAP kinase and PI3-kinase pathway.** To study the specificity of FVIIa effects on translation-related signal transduction as well as the contribution of the various pathways to the intracellular signaling generally associated with protein translation, we stimulated BHK\textsuperscript{TF} cells with 100 nM FVIIa in the presence of the thrombin inhibitor hirudin (25U/ml) or the FXa inhibitor TAP (200 nM). As is clear from Fig. 7A, neither hirudin nor TAP abrogated FVIIa-induced activation of PKB, p90\textsuperscript{RSK}, p70/p85\textsuperscript{S6K} and eIF-4E, demonstrating that the FVIIa-induced signals were direct and not dependent on FXa and thrombin generation.

To establish the possible relative contribution of the different pathways involved, cells were preincubated with rapamycin, being the inhibitor to the upstream p70/p85\textsuperscript{S6K} activator mTOR, as well as the PI3-kinase inhibitor LY294002 and the MEK inhibitor PD98059. Whereas PD98059, but not LY294002 or rapamycin, had an effect on p90\textsuperscript{RSK} phosphorylation, all three inhibitors had an effect on p70/p85\textsuperscript{S6K} and eIF-4E phosphorylation in BHK\textsuperscript{TF} cells, suggesting possible roles for both the MAP kinase pathway and the PI3-kinase pathway in FVIIa-induced protein translation. In this experimental set-up, the use of active-site-blocked FVIIa (FVIIai) did not result in increased phosphorylation of the signaling components, demonstrating the requirement for FVIIa proteolytic activity.

The specificity of FVIIa effects on translation-related signal transduction was also determined in HaCaT cells; thrombin- and FXa-inhibitors showed no effect on phosphorylation of p90\textsuperscript{RSK}, GSK3β, eIF-4E, p70/p85\textsuperscript{S6K} and the downstream p70/p85 S6 kinase target S6 (Fig. 7B). PD98059 inhibited the p90\textsuperscript{RSK} pathway and LY294002 inhibited GSK3β. Importantly, whereas in BHK\textsuperscript{TF}, PD98059 reduces FVIIa-mediated eIF-4E phosphorylation, this MEK-
**Figure 7:** FVIIa-induced signals are sensitive to signal transduction inhibitors but not to thrombin or FXa inhibitors. (A) BHK TF cells were serum starved for 16 hrs and pretreated with 25 U/ml hirudin (Hir), 200 nM Tick Anti-coagulant Protein (TAP), 10 μM LY294002 (LY), or 20 μM PD98059 (PD) for 30 min. The mTOR inhibitor rapamycin (20 ng/ml) was added for 15 minutes. Subsequently, the cells were treated with 100 nM FVIIa for 10 min. Cells were also treated with 100 nM active site-inhibited FVIIa (FVIIai) or 170 nM insulin (I). Phosphorylation of PKB, p70/p85 S6K, p90 RSK, and eIF-4E was assayed by Western Blot using phosphospecific antibodies. Equal loading was verified by antibodies against β-actin. (B) HaCaT cells were serum starved for 24 hrs and pretreated with the inhibitors mentioned above and used as described above. Subsequently, the cells were treated with 100 nM FVIIa for 20 min. Cells were also treated with 100 nM active site-inhibited FVIIa (FVIIai) or 170 nM insulin (I). Phosphorylation of GSK3, p90 RSK, eIF-4E, p70/p85 S6K and S6 was assayed by Western Blot using phosphospecific antibodies. Equal loading was verified by antibodies against protein of interest. The Western Blots represent three independent experiments.

Inhibitor did not inhibit eIF-4E phosphorylation in HaCaT, excluding a role for the MAP kinase pathway in eIF-4E phosphorylation in this cell type. Contrasting, MEK activity appears to be essential for both p70/p85 S6K and S6 phosphorylation, suggesting that the MAP kinase pathway is involved in ribosomal activation.

Finally, as was observed in BHK TF cells, FVIIai did not elicit any effects in HaCaT cells.
FVIIa/TF-interaction results in protein synthesis - Since exposure of TF-expressing cells showed a dramatic activation of protein synthesis key regulators, we decided to investigate the potential of FVIIa for initiating protein synthesis. Therefore, BHK TF cells were metabolically labeled with 35S-methionine and stimulated with either insulin or FVIIa for various times. Strikingly, in this cell type FVIIa was as potent as insulin on the induction of protein synthesis (Fig. 8A). However, after 2h., FVIIa-induced protein levels decreased again, whereas insulin-induced protein synthesis appeared to be more persistent, correlating with the less transient phosphorylation of key signal transducers by insulin (see above). Subsequently, we investigated the contribution of possible FXa and thrombin generation to the FVIIa-induced protein synthesis. Fig. 8B shows that the use of hirudin or TAP did not result in any decrease of FVIIa-induced protein synthesis, ruling out a possible role for FXa or thrombin. Thus FVIIa/TF interaction has a strong and specific stimulatory effect on protein synthesis.

Figure 8: FVIIa induces protein synthesis rates, similar to insulin, independent of FXa and thrombin generation. (A) BHK TF cells were metabolically labeled with 35S-methionine and stimulated with 100 nM FVIIa or 170 nM insulin for various times. At the end of the experiment the proteins were precipitated with 15% TCA and protein synthesis was determined by measuring the number of disintegrations per minutes (DPM). (B) Cells were labeled as described under (A) and preincubated with 25 U/ml hirudin (Hir) or 200 nM Tick Anticoagulant Protein (TAP) for 30 min, and subsequently stimulated with FVIIa for 2 hours. In both experiments, background was subtracted. SEMs are depicted.
To identify a possible contribution of the intracellular domain of TF in the FVIIa effects on translation, we decided to compare protein synthesis in BHK\textsuperscript{TF}, BHK\textsuperscript{TF\textasciitilde cyto} and wildtype BHK cells. For this purpose, cells were stimulated for 0, 2 and 4 hours, and Fig. 9 shows that whereas BHK cells exposed to 100 nM of FVIIa did not show any response, BHK\textsuperscript{TF}, stimulated with the same concentration of FVIIa, showed a dramatic induction of protein synthesis after 2 and 4 hours. Similarly, FVIIa elicited a very similar induction in BHK\textsuperscript{TF\textasciitilde cyto} cells, demonstrating that FVIIa TF-mediated translation is independent of the TF cytoplasmic domain.

![Figure 9: FVIIa treatment of cells induces protein synthesis in BHK\textsuperscript{TF} and BHK\textsuperscript{TF\textasciitilde cyto}, but not in wildtype BHK cells. BHK cells, BHK\textsuperscript{TF} and BHK\textsuperscript{TF\textasciitilde cyto} were starved for 16 hrs and labeled with 20 \mu Ci/ml 35S-methionine for 5 hrs. Cells were stimulated with 100 nM FVIIa for the indicated times. Proteins were precipitated with 15% TCA and 35S-methionine incorporation was determined by scintillation counting. To enable comparison between the three cell lines, the net amount of incorporation, measured at 0 hours was set at a value of 1. The experiment was performed in triplicate and the figure is representative for 2 independent experiments, standard deviations are depicted.](image)

FVIIa induces synthesis of eEF1\textalpha and eEF2, regulators of protein synthesis - The p70/p85 S6 kinases often induce rapid synthesis of proteins that are direct regulators of protein synthesis, such as the elongation factors eEF1\textalpha and eEF2 (reviewed in 30). These proteins bind aminoacyl-tRNA and deliver it to the ribosomal aminoacyl site during the
Figure 10: FVIIa induces upregulation of the elongation factors eEF1α and eEF2 in BHK<sup>TF</sup> and HaCaT cells. (A) BHK<sup>TF</sup> cells were serum starved and treated with 100 nM FVIIa, 170 nM insulin or 100 nM FVIIai for various times. Subsequently, the cells were lysed and analyzed for eEF1α expression on Western Blot. The blots represent three independent experiments. (B) BHK<sup>TF</sup> cells were labeled with 20 μCi/ml <sup>35</sup>S-methionine and stimulated with 100 nM FVIIa for various times. eEF2 was immunoprecipitated and the number of DPMs of the pellet was determined using a scintillation counter. (C,D) HaCaT cells were labeled and treated as under B. eEF1α and eEF2 were immunoprecipitated and the number of DPMs of the pellet was determined using a scintillation counter. (E) Radiolabeled HaCaT cells were treated mock, with FVIIa, FVIIai or with insulin. Subsequently, eEF1α production was measured as described above. In all experiments, background levels were subtracted and SEMs are shown. The experiments were performed in triplicate, the graphs represent 2 independent experiments.
elongation phase of protein biosynthesis. Interestingly, these proteins are translated from mRNA containing a 5' polypyrrimidine tract; upon stimulation of the S6 kinase pathway these pre-existing mRNA sequences are preferentially translated within 30 min (31,32), almost parallel with S6 kinase activation. Thus, synthesis of these proteins is a good measure for stimulus-induced activation of translation, independent of enhanced mRNA synthesis.

Fig. 10A shows the FVIIa-induced increase in cellular eEF1α contents; eEF1α levels are upregulated in a similar fashion as those after stimulation with 170 nM insulin. FVIIai, however, does not appear to have an effect on eEF1α. Levels of eEF2 were also upregulated by FVIIa within 30 min, as was demonstrated after 35S-methionine labeling and immunoprecipitation of eEF2 (Fig. 10B). Both proteins were also upregulated in HaCaT cells, as determined by labeling with 35S-methionine and immunoprecipitation of both proteins (Fig. 10C,D). Finally, in HaCaT eEF1α was not upregulated by active site-blocked FVIIa (FVIIa), whereas in the same experiment, FVIIa and insulin induced significant levels of eEF1α (Fig 10E). Thus FVIIa regulates protein synthesis, further supporting the concept that enhanced translation is a principal effector of TF signal transduction.
Discussion

A picture has emerged in recent years, which considerably broadens the present understanding of the TF/FVIIa functionality. Apart from initiating the coagulation cascade, TF/FVIIa has been implicated in a number of other biological functions as a consequence of FVIIa's capability of mediating intracellular signaling in TF expressing cells. It remains, however, elusive exactly how TF/FVIIa signaling links to the presumed effects of TF in (patho)physiology, especially angiogenesis and inflammation. One theory relies on a TF/FVIIa-dependent MAP kinase activation and subsequent proliferation, necessary for neovascularization. Another option might be that FVIIa/TF-signal transduction leads to translation and secretion or membrane expression of factors, such as cytokines or angiogenesis-promoting agents, which in turn are mediating TF/FVIIa's effects in pathophysiology. In general, relevant production of such factors requires substantial stimulation of the translational machinery, and this led us to focus on TF/FVIIa's effect on sections of the signal transduction pathways implicated in regulation of protein synthesis. The present study revealed a TF/FVIIa-dependent activation of p70/85S6K and p90RSK, especially the first kinase implicated in enhanced translation. Further evidence for enhanced translation was suggested by the observation of GSK3 phosphorylation leading to deactivation of this translation inhibitor. Thus, binding of FVIIa to TF stimulates multiple pathways leading to enhanced translational activity. In agreement with this concept, exposure of TF-expressing cells to FVIIa led to 1) a strong phosphorylation of eukaryotic initiation factor, eIF-4E, 2) increased protein synthesis within 20 min of 5' polypyrimidine tract-containing mRNAs and 3) within 15 min to 4 hours an enhanced protein synthesis, as assayed by 35S-methionine incorporation. The effect of FVIIa/TF interaction on translation was comparable to that observed with insulin and hence it seems fair to suggest that enhanced translational activity might be a major effect of FVIIa signaling on cellular physiology.

Expression of the extracellular part of TF was mandatory for the effect of FVIIa on translational regulation. However, as indicated by the results with BHK cells transfected with truncated TF, the cytoplasmic domain was not required for translational regulation. Finally, the effects were not sensitive to the specific thrombin- and FXa-inhibitors hirudin and TAP and thus the cellular effects of FVIIa addition are direct effects of this coagulation factor on TF-expressing target cells, however, they are not transmitted by the TF-receptor itself but
Figure 11: Proposed mechanism for FVIIa:TF-induced protein synthesis in BHK\textsuperscript{TF} and HaCaT cells. The FVIIa:TF complex targets a still unknown transmembrane protein or PAR-2 in BHK\textsuperscript{TF} and HaCaT cells respectively. Subsequently, activation of the PI3 kinase (PI3K) pathway takes place, leading to the indirect activation of S6 kinase (p70/p85), the ribosome (ribosomal protein S6), and initiation factors (among which eIF4E). This will lead to upregulation of the protein synthesis machinery by means of eEF1\textalpha and eEF2 production. Furthermore, GSK3-mediated inhibition of protein synthesis is abrogated. FVIIa:TF interaction also leads to subsequent activation of MAP kinase and p90 RSK. Note that whereas the MAP kinase pathway significantly contributes to eIF4E activation in BHK\textsuperscript{TF} cells, it does not in HaCaT cells.

they are probably mediated via a FVIIa-cleavable alternative receptor, e.g. the often-proposed PARs (e.g. 19).

So far, the role of the cytoplasmic domain in TF has been a matter of debate. Some investigators have found evidence for functional interactions of the intracellular domain with
downstream signaling components, for example in FVIIa-induced production of Reactive Oxygen Species in macrophages (33), or suggested an association of the domain with filamin-A (ABP-280) (29), whereas others have found no function for this domain in FVIIa signaling (16). Furthermore, deletion of the cytoplasmic domain of TF does not result in abnormal embryonic development, in spite of the essential role of TF for normal angiogenesis (34). In our studies, we confirm that the lack of this domain does not abrogate signaling nor protein synthesis. However, it did not escape our attention that cells expressing the cytoplasmic domain-deleted TF showed a prolonged protein synthesis. This might correlate with previous data (17) that showed increased mRNA synthesis in cells expressing the mutant when compared to cells expressing wild type TF. The mechanism behind this phenomenon is currently not known, but it is tempting to suggest that the cytoplasmic tail might, in some way, inhibit protein synthesis.

Further attention should be paid to the activation of the p90RSK pathway, which like p70S6K is often suggested to phosphorylate the ribosomal S6 protein. However, although p90RSK readily phosphorylates S6 upon activation in vitro, reports about in vivo phosphorylation have not been made (for review: 35). Although this makes the contribution of the MAP kinase pathway in FVIIa-induced protein translation somewhat unclear, it may very well be that another MAP kinase-activated protein plays a role in FVIIa-induced translation. Especially, Mnk1, which associates with, and phosphorylates eIF-4E in vivo (36,37), might be a direct keyplayer in FVIIa-induced protein synthesis via the MAP kinase pathway.

Interestingly, the role of the MAP kinase pathway in eIF-4E phosphorylation appears to differ between cell lines; whereas the MEK-inhibitor PD98059 reduced eIF-4E phosphorylation to basal levels in BHK\textsuperscript{TF} cells, pretreatment with PD98059 did not have any effect on eIF-4E phosphorylation in FVIIa-stimulated HaCaT cells, but prevented both FVIIa-induced p70/p85\textsuperscript{S6K} and S6 phosphorylation. Apparently, activity of the MAP kinase pathway is essential for p70/p85\textsuperscript{S6K} phosphorylation in this system.

It is noteworthy that FVIIa exhibits a large degree of cell-type specificity in its intracellular effects. Evidently TF expression is mandatory for FVIIa-induced signaling, however, there appears still to exist a high degree of variability in the response to VIIa between various TF-expressing cell lines. For instance, whereas FVIIa signal transduction provokes activation of the MAP kinase family member JNK in keratinocytes, it does not in A14 fibroblasts (18). Furthermore, whereas A14 fibroblasts, HaCaT keratinocytes and BHK cells transfected with
TF show a large potential in PKB phosphorylation, other cell lines do not respond with PKB activation. Thus, not every TF-expressing cell type responds to FVIIa exposure with a significant phosphorylation of PKB, and it might prove interesting to investigate the effects on FVIIa on the translation machinery in such cell types as it may contribute to our understanding as to the relative importance of the PKB and MAP kinase pathway for FVIIa-induced protein synthesis.

It is tempting to speculate on the possible function of FVIIa-dependent stimulation of protein production in physiology. Earlier studies have demonstrated a TF/FVIIa-dependent transcription of mRNAs encoding pro-inflammatory cytokines such as IL-8, IL-1β and the IL-6 like cytokine LIF, within 1.5 hours (6). In addition, FVIIa-treatment has been shown to induce upregulation of mRNAs encoding the growth factors hbEGF, FGF-5, Cyr61 and CTGF (6,22); and these proteins may form intermediates for TF-driven neo-angiogenesis. Adequate subsequent production of protein from these mRNAs may well be dependent on the signal transduction phenomena described in the present study. Hence it may prove interesting to investigate the effects of inhibitors of these pathways in experimental in vivo models of pathophysiology known to involve TF/FVIIa interaction. Studies examining this possibility are currently in progress.

In conclusion, we have shown here that FVIIa/TF interaction does not lead to cell proliferation, but induces signal transduction, associated with protein synthesis, as well as upregulation of the translational machinery, suggesting that FVIIa/TF may induce pathophysiological events such as angiogenesis, through production of proteins, relevant for these processes.
Chapter 4

References


