FVIIa:TF Induces Cell Survival via G\textsubscript{12}/G\textsubscript{13}-Dependent Jak/STAT Activation and Bcl\textsubscript{XL} Production

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Abstract—Tissue factor (TF), apart from activating the extrinsic pathway of the blood coagulation, is a principal regulator of embryonic and oncogenic angiogenesis, inflammation, leukocyte reverse transmigration, and tumor progression. It has become clear that these events are mediated by intracellular signal transduction elicited by TF/factor VIIa (FVIIa) interaction, but the details of this signaling remain largely obscure. In this study, we show that FVIIa/TF-interaction produces STAT5 phosphorylation, STAT5 nuclear translocation and transactivation of a STAT5 reporter construct. FVIIa-dependent STAT5 activation was dependent on FVIIa proteolytic activity but not on generation of the downstream coagulation factors Xa and thrombin, nor on the TF cytoplasmic domain. FVIIa-induced STAT5 phosphorylation was dependent on functional G\textsubscript{12}/G\textsubscript{13} class G proteins and Jak2 activity, but not Jak1 or Tyk2. Finally, we show that FVIIa leads to cell survival through a Jak2/STAT5-dependent production of the antiapoptotic STAT5 target Bcl\textsubscript{XL}, as well as Jak2-dependent activation of the antiapoptotic protein PKB. In conclusion, our results show that FVIIa induces cell survival through STAT5-dependent Bcl\textsubscript{XL} production and Jak2-dependent activation of PKB. Finally, we demonstrated for the first time that TF/FVIIa-signal transduction is dependent on G\textsubscript{12}/G\textsubscript{13} class G proteins. (Circ Res. 2004;94:1032-1040.)

Key Words: inflammation ■ survival ■ coagulation ■ signal transduction

Tissue factor (TF) is the main initiator of the coagulation cascade. On vessel rupture, this transmembrane protein, normally not present on cells that are in contact with the bloodstream, binds with high affinity to the zymogen factor VII (FVII). The subsequent generation of factor Xa, thrombin, and fibrin deposition finally results in the formation of a blood clot.\textsuperscript{1} In addition, a large variety of coagulation-independent functions for FVIIa/TF have been described. TF is absolutely required for embryonic blood vessel formation, because TF knockout mice are not viable due to an underdeveloped vascular system.\textsuperscript{2} Furthermore, both TF and its ligand FVIIa have been shown to play a role in tumor metastasis, whereas TF has been shown to be an independent risk factor for hepatic metastasis in patients suffering from colon cancer.\textsuperscript{3–5} Finally, the TF:FVIIa complex plays a role in sepsis and inflammation, because inhibition of this complex inhibits sepsis-induced mortality.\textsuperscript{6,7}

It is generally accepted that TF:FVIIa interactions provokes intracellular signal transduction in TF-expressing cells. Among the cellular signaling cascades targeted by this interaction are the MAP kinase pathway, the PI3 kinase pathway, Ca\textsuperscript{2+} signaling and activation of small GTPases, but such pathways are not a hallmark for inflammatory signaling per se.\textsuperscript{8–12} In 1990, Bazan noted the high homology between the extracellular part of TF and the proinflammatory interferon class II type receptors, including the interferon \(\gamma\) receptor.\textsuperscript{13} Indeed, recent in silico analysis has shown that all current members of the cytokine receptor class II family are derived from an ancient TF gene, as TF is the only member of the protein family found in teleosts.\textsuperscript{14} However, the intracellular part of TF does not resemble a cytokine receptor, and thus a cytokine receptor–like mechanism is unlikely. Rather, TF serves as a docking site for FVIIa, the latter cleaving a protease-activated receptor similar to the thrombin receptor thus triggering signal transduction.\textsuperscript{15} Nevertheless, we explored the possibility of TF:FVIIa-induced activation of signal transduction that is normally associated with cytokine receptors, the Jak/STAT pathway.

We report that FVIIa induces STAT5 activation, subsequent nuclear translocation and transactivation of a STAT5-dependent reporter construct, as well as production of the STAT5 target Bcl\textsubscript{XL}. This activation was dependent on FVIIa proteolytic activity, G\textsubscript{12}/G\textsubscript{13} GTPase activity, and Jak2, but not on the TF cytoplasmic tail. Finally, TF/FVIIa induced Jak2-dependent cell survival via activation of both STAT5 and PKB. Thus, our results show that FVIIa/TF complex formation results in activation of the Jak/STAT pathway. However, this pathway rather plays a role in FVIIa-induced...
cell survival than in inflammation. Thus, the activation of STAT transcription factors provides an obvious link between TF:FVIIa and their role in (patho)physiology along with cell survival and cancerous processes.

Materials and Methods

Materials

The antibodies raised against p-STAT5 (Tyr694), p-PKB, p-MAPK, and p-Tyk2 (Tyr1007/1008) were purchased from Cell Signaling Technologies. Antibodies against p-Jak1 (Tyr1022/1023) and p-Jak2 (Tyr1007/1008) were from Biosource, and antibodies against total Jak1 and 2 were from Upstate Biotech Inc. Antibodies against Tyk2 and BclXL were from Transduction Laboratories. Anti-FLAG and MTT were from Sigma. Total STAT 5 (directed against both amino acid change (Lys3) deleted Tyk2 construct was kindly provided by Dr Sandra Pellegrini (St Jude’s Children’s Hospital, Memphis, TN), G12 and G13 were from Drs L. Petersen, Novo Nordisk (United de Biologie des Interactions Cellulaires, Institut Pasteur, Paris). This construct encodes a protein containing amino acids 1 to 895 of Tyk2. FLAG-tagged STAT5A and B were from Dr James Ihle (St Jude’s Children’s Hospital, Memphis, TN), G12 and G13, dominant-negative constructs (G228A and G225A) were from Dr Stefan Offermanns (Institute of Pharmacology, University of Heidelberg), and the STAT5-responsive NTCP-luciferase construct was obtained from Dr P. Coffer (Graduate Center of Toxicology, University of Kentucky). The dominant-negative pXM-STAT5A (truncated at residue 750) construct was obtained from Dr P. Coffer (Utrecht University), and this construct serves as an inhibitor to both STAT5A and STAT5B. The insert was excised HindIII/XhoI and inserted into pcDNA3.1 (Invitrogen).

Constructs

Both of the DNA constructs encoding kinase-dead Jak1 and Jak2 were generous gifts from Dr David Levy (Department of Pathology, New York University School of Medicine, New York). The dominant-negative mutant of Jak1 contains a 3-amino acid change (Lys3) deleted Tyk2 construct was kindly provided by Dr Sandra Pellegrini (St Jude’s Children’s Hospital, Memphis, TN), G12 and G13, dominant-negative constructs (G228A and G225A) were from Dr Stefan Offermanns (Institute of Pharmacology, University of Heidelberg), and the STAT5-responsive NTCP-luciferase construct was obtained from Dr P. Coffer (Graduate Center of Toxicology, University of Kentucky). The dominant-negative pXM-STAT5A

Figure 1. Proteolytically active FVIIa induces STAT5 activation via TF but not its cytoplasmic domain. A, BHKT7 cells (black circles) or BHKTF/cyto cells (white circles) were serum-starved and treated with 100 nmol/L FVIIa. BHKT7 cells were also treated with 1 μg/mL insulin for the times indicated (white squares). As a control, BHK cells were also stimulated with FVIIa (black squares). STAT5 phosphorylation was assessed, using phospho-STAT5 antibodies on Western blot and the protein bands were quantified. The immunocomplex was precipitated with 20 μL 50% protein A-Sepharose for 1 hour. The lysate was then incubated overnight with 1 μg antibody. The immunocomplex was precipitated with 20 μL 50% protein A-Sepharose for 1 hour. The

Cell Culture and Transfection

Baby hamster kidney cells, either stably transfected with full-length TF or with a TF cytoplasmic domain-deleted mutant truncated at residue 247 (all a generous gift from Dr L. Petersen, Novo Nordisk), were maintained in Dulbecco’s Modified Eagle’s Medium, supplemented with 10% fetal calf serum (FCS, Gibco) and penicillin/streptomycin, at 37°C and 5% CO2 in a humidified environment. Transient transfections were performed as follows: for FLAG-tagged STAT5A, FLAG-tagged STAT5B or the luciferase expression constructs, cells were grown in 6-wells plates until 50% confluence and transfected with 0.4 μg construct using 1 μL Enhancer and 5 μL Effectene transfection reagent (Qiagen). For transient transfections using dominant-negative constructs, 2.5 μg construct, 21 μg of Enhancer reagent, and 133 μL of Effectene reagent were used. Using GFP-expression constructs, the transfection efficiency was determined to be at least 75%.

Stable dominant-negative STAT5-expressing BHKT7 cells were generated as follows: cells were grown in 6-wells plates until 50% confluence and transfected with 0.4 μg pcDNA3.1, using Effectene reagent. Cells were grown in DMEM containing 700 μg/mL G418, and positive clones were selected and screened for mutant STAT5 expression. Three clones (Nos. 1, 7, and 10) were selected for the experiments based on mutant STAT5 expression.

Immunoprecipitations

Treated cells were lysed in 0.5 mL lysis buffer (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na3VO4, and 1 μg/mL leupeptin) and were collected by scraping. Subsequently, the lysate was centrifuged at 14 000 rpm for 2 minutes, and the supernatant was precleared with 7.5, 150 mmol/L NaCl, 1 mmol/L Na2 EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na3VO4, and 1 μg/mL leupeptin) and were collected by scraping. Subsequently, the lysate was centrifuged at 14 000 rpm for 2 minutes, and the supernatant was precleared with 100 nmol/L FVIIa. BHKT7 cells were also treated with 1 μg/mL insulin for the times indicated (white squares). As a control, BHK cells were also stimulated with FVIIa (black squares). STAT5 phosphorylation was assessed, using phospho-STAT5 antibodies on Western blot and the protein bands were quantified. The immunocomplex was precipitated with 20 μL 50% protein A-Sepharose for 1 hour. The lysate was then incubated overnight with 1 μg antibody. The immunocomplex was precipitated with 20 μL 50% protein A-Sepharose for 1 hour. The
buffer. The supernatants (cytosolic fraction) were further diluted to 10^6 and washed cells (1 to 7 × 10^6) were scraped in 200 μL of the specific Fxa inhibitor tick anti-coagulant protein (TAP) or 25 U/mL of the thrombin inhibitor hirudin (Hir). Subsequently, cells were stimulated with 100 nmol/L FVIIa, 1 U/mL FXa, or 1 U/mL thrombin. STAT5 phosphorylation and total amounts of STAT5 were determined by Western blotting. 8,17 We determined FVIIa-induced phosphorylation states of STAT1, STAT3, and STAT5. Although we did not observe FVIIa-induced phosphorylation of STAT1, however, are not much alike but an intriguing question remains whether TF together with its ligand, FVIIa, activates Jak/STAT pathways. Therefore, we set out to investigate the possible involvement of STATs in TF-dependent signal transduction and whether the intracellular tail is involved in such signaling. To this end, BHK cells either expressing full-length TF (BHKTF) or a cytoplasmic domain deletion mutant (BHK TFΔcyto) were stimulated with recombinant FVIIa and STAT phosphorylation was determined using phosphospecific antibodies and Western blotting. 3,17 We determined FVIIa-induced phosphorylation states of STAT1, STAT3, and STAT5. Although we did not observe FVIIa-induced phosphorylation of STAT1 and STAT3 (results not shown), BHK TFΔcyto cells reacted to FVIIa with a transient increase in phospho-STAT5 immunoreactivity, which was already detectable after 5 minutes, reached a maximum effect 10 minutes after stimulation and returned to basal levels after approximately 60 minutes, whereas pan-STAT5 immunoreactivity was not affected (Figure 1A). Importantly, BHK TFΔcyto cells responded to FVIIa stimulation with transient STAT5 phosphorylation indistinguishable of that observed in BHK cells transfected with full-length TF, and low-fat milk powder. The bands were visualized, using Lumilight plus ECL substrate from Roche and a chemiluminescence detector with a cooled CCD camera from Syngene.

Luciferase Assay
Cells were grown in 24-wells and transfected with either the STAT5-responsive construct NTCP-luciferase, or a CMV-driven luciferase construct for 16 hours. Transfections were performed as described above. After transfection, the cells were serum-starved for another 16 hours. Subsequently, cells were stimulated with 100 nmol/L FVIIa for 3 hours. Cells were lysed in 100 μL luciferase assay buffer (25 mmol/L glycylglycine pH 7.9, 15 mmol/L MgSO_4, 4 mmol/L EGTA, 1% Triton X-100, 1 mmol/L dithiothreitol) of which 50 μL was used for the detection of luciferase activity on a Packard Topcount microplate scintillation counter.

Survival Assay
Cell viability was assessed using an MTT reduction assay (a colorimetric assay in which a tetrazolium compound is bioreduced by cells into a colored formazan product in direct proportion to the number of living cells in culture) in 24-well plates. Shortly, cells were starved for 1 day, after which FVIIa was added. Viability was measured at the times indicated in the graphs. The control was set at 100%.

Nuclear Isolation
Cell lysates were prepared and separated into nuclear and cytosolic fractions, as described before, with some modifications: stimulated and washed cells (1 to 7 × 10^6) were scraped in 200 μL ice cold hypotonic lysis buffer (20 mmol/L HEPES, pH 7.9, 1.5 mmol/L MgCl_2, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.5 mmol/L pefabloc, and 0.5 μg/mL of leupeptin and aprotinin). The lysate was passed 10 times through a 27-gauche needle. After centrifugation at 600 g at 60 °C for 10 minutes, the supernatant (cytosolic fraction) was further diluted to 300 μL with 3× sample buffer. The supernatants (cytosolic fraction) were further diluted to 300 μL with 3× sample buffer.

Western Blotting
After treatment, cells were rapidly harvested by adding 100 μL of heated (95°C) sample buffer and the lysates were collected by scraping. After 5 minutes of 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 supplemented with 0.1% Tween (wash buffer) and 2% low-fat milk powder, and incubated with primary antibody overnight at 4°C, diluted 1:1000 in wash buffer containing 2% BSA. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody in wash buffer, containing 2%

Statistical Analysis and Quantification of the Western Blots
In all graphs, means and standard errors of at least triplicates are shown. When Western blots were analyzed for phosphorylation of STAT5, MAP kinase, or PKB, equal loading was determined by blotting for total protein as well. Antibody bands were quantified using Genetools from Syngene.
therefore, we concluded that cytoplasmic domain of TF is not involved in this phosphorylation. Interestingly, this FVIIa-induced phosphorylation was much more transient than that observed after stimulation with insulin, an established inducer of STAT5 phosphorylation (Figure 1A).19 We also assessed the importance of FVIIa proteolytic activity using active site-inhibited FVIIa (FFR-VIIa or FVIIai). We observed that although a 10-minute stimulation with FVIIa potently induced STAT5 activation, FVIIai was ineffective in this respect (Figure 1B). We concluded that the FVIIa-dependent STAT5 phosphorylation is mediated by the extracellular domain of TF and FVIIa proteolytic activity. Dose-response analysis of FVIIa-induced STAT5 phosphorylation revealed that enhanced STAT5 phosphorylation in response to this protease was already detectable at concentrations as low as 1 nmol/L (Figure 1C), lying well within the physiological ranges of FVII in plasma (10 nmol/L). Finally, we assessed which isoform of STAT5, STAT5A, or STAT5B is/are activated on FVIIa stimulation. To this end, we transiently transfected BHKTF with expression constructs encoding either FLAG-tagged STAT5A or FLAG-tagged STAT5B. 20 Subsequently, cells were stimulated with FVIIa for various times and FLAG-tagged STAT5 was immunoprecipitated using a FLAG antibody. Finally, phosphorylation of STAT5 was induced STAT5 phosphorylation is dependent on G12/G13 but not on G. A, BHKTF cells were pretreated for 6 hours with 100 ng/mL pertussis toxin (PTX) and subsequently stimulated with LPA or FVIIa. MAP kinase phosphorylation or STAT5 phosphorylation was determined on Western blot. B, Cells were transiently transfected with dominant-negative G12α- or G13α-expressing constructs, subsequently serum-starved, and stimulated with 100 nmol/L FVIIa. Phosphorylated STAT5 as well as total levels of STAT5, G12, and G13 were determined on Western blot.

Figure 3. FVIIa-induced STAT5 phosphorylation is dependent on G12/G13 but not on G. A, BHKTF cells were pretreated for 6 hours with 100 ng/mL pertussis toxin (PTX) and subsequently stimulated with LPA or FVIIa. MAP kinase phosphorylation or STAT5 phosphorylation was determined on Western blot. B, Cells were transiently transfected with dominant-negative G12α- or G13α-expressing constructs, subsequently serum-starved, and stimulated with 100 nmol/L FVIIa. Phosphorylated STAT5 as well as total levels of STAT5, G12, and G13 were determined on Western blot.

Figure 4. Jak2, but not Jak1 or Tyk2, is required for FVIIa-induced STAT5 phosphorylation. A, BHKTF cells were stimulated for the indicated times with 100 nmol/L FVIIa. Subsequently, Jak1 (black circles), Jak2 (white circles), and Tyk2 (black squares) were analyzed for phosphorylation on Western blot and bands were quantified. B, In a similar experiment, phosphorylation of Jak1 (white bars), Jak2 (gray bars), and Tyk2 (black bars) was determined after 10 minutes of stimulation with the indicated FVIIa concentrations. C, BHKTF cells were transfected with kinase-dead Jak1, Jak2, and Tyk2 mutants, after which cells were starved and stimulated with 100 nmol/L FVIIa. Cell lysates were analyzed on Western blot for STAT5 phosphorylation, total amounts of STAT5, and total amounts of Jak kinases. D, Cells were preincubated with various concentrations of the specific Jak2 inhibitor AG490 and subsequently stimulated mock or with 100 nmol/L FVIIa. STAT5 phosphorylation as well as total amounts of this protein were determined by Western blot.
determined using a phosphospecific antibody for STAT5. We observed that, whereas total levels of FLAG-STAT5A and FLAG-STAT5B remained unchanged, the phosho-STAT5 immunoreactivity was transiently increased with respect to both STAT5A and STAT5B (Figure 1D), and hence, both STAT5 isoforms are a target for FVIIa-dependent signaling.

FVIIa-Induced STAT Phosphorylation Is not Dependent on Generation of Thrombin or FXa

After exposure of TF to the bloodstream, interaction of TF with FVII will take place, leading to FVII activation. The thus-generated FVIIa converts FX to FXa, and in turn, FXa activates thrombin. Hence, addition of FVIIa to cells may lead to FXa and thrombin generation. In order to investigate a possible role of FXa or thrombin in FVIIa-dependent STAT5 activation, we tested the effect of the FXa inhibitor TAP and the thrombin inhibitor hirudin on FVIIa-evoked STAT5 phosphorylation. As is obvious from Figure 2, both FXa and thrombin are capable of inducing STAT5 phosphorylation, and the effects of FXa and thrombin are sensitive to 200 nmol/L of TAP and 25 U/mL of hirudin, respectively. These inhibitors, however, did not influence FVIIa-induced STAT5 phosphorylation (Figure 2). Thus, although multiple factors of the coagulation cascade have the capacity to activate STAT5, the FVIIa-mediated STAT5 phosphorylation does not rely on either FXa or thrombin generation.

FVIIa-Mediated STAT5 Phosphorylation Is Dependent on G12/G13, but not on G1 Heterotrimeric G Proteins

FVIIa is believed to induce signaling through a protease-activated receptor (PAR), a heterotrimeric G protein–coupled receptor that has high homology with the thrombin receptor. However, until now, the nature of the G proteins involved is highly unclear. Therefore, we decided to investigate the involvement of two classes of G proteins that are frequently activated on thrombin stimulation; G\(_i\) and G\(_{12}/G_{13}\). To investigate the role of G\(_i\), BHK-TF cells were preincubated for 6 hours with the G\(_i\) inhibitor pertussis toxin (PTX). Whereas this toxin potently inhibited MAP kinase phosphorylation induced by lysophosphatidic acid (LPA), a well-known G\(_i\) activator, PTX did not inhibit FVIIa-induced STAT5 phosphorylation (Figure 3A), ruling out a role for G\(_i\) in FVIIa-induced STAT5 phosphorylation. Next, we determined the influence of G\(_{12}/G_{13}\) in this system, through transient transfection of BHK-TF cells with inactive G\(_{12a}\) or G\(_{13}\)/H9251 expression vectors. The transfection efficiency in our system was at least 75% as determined with GFP-expression constructs. As is clear from Figure 3B, both inactive G\(_{12a}\) or G\(_{13}\)/H9251 inhibited FVIIa-induced STAT5 phosphorylation. However, the use of dominant-negative G\(_{12a}\) or G\(_{13}\)/H9251 did not inhibit, but rather enhanced insulin-induced STAT5 phosphorylation. This demonstrates that functional G\(_{12}\) and G\(_{13}\) proteins are absolutely required for FVIIa-induced STAT5 phosphorylation.

FVIIa Induces STAT Phosphorylation via Activation of Jak2

The upstream kinases activating STATs are known as Jaks (Janus kinases) of which four forms exist: Jak1, Jak2, Jak3, and Tyk2. Using phosphospecific antibodies, the phosphorylation states of Jak1, Jak2, and Tyk2 were determined. Figure 4A shows that Jak1, Jak2, and Tyk2 phosphorylation are transiently upregulated after FVIIa stimulation. Whereas Jak2 and Tyk2 phosphorylation return to basal levels within 15 minutes, Jak1 phosphorylation was more persistent; phosphorylation was still observed after 45 minutes (not shown). Furthermore, phosphorylation of these kinases was already observed at FVIIa concentrations as low as 1 nmol/L (Figure 4B).

To establish the nature of Janus kinases in the phosphorylation of STAT5, we explored the role of these kinases in
this system by transiently transfecting BHK\textsuperscript{TF} cells with DNA-constructs, encoding kinase-dead variants of Jak1, Jak2, and Tyk2. These proteins have previously been shown to potently inhibit endogenous Jak1, Jak2, and Tyk2 enzymatic activity.\textsuperscript{21,22} As can be seen in Figure 4C, transfection of cells with kinase-dead Jak2 abrogated FVIIa-induced STAT5 phosphorylation, whereas the use of a kinase-dead Jak1 or Tyk2 mutant did not have this effect. We also preincubated BHK\textsuperscript{TF} cells with the specific Jak2 inhibitor AG490. As can be seen in Figure 4D, a 25 \( \mu \)mol/L preincubation with this inhibitor abrogates FVIIa-induced STAT5 phosphorylation. We concluded that, although FVIIa induces activation of Jak1, Jak2, and Tyk2, only Jak2 is essential in FVIIa-dependent STAT5 phosphorylation.

**Jak2 Activity Is Essential for FVIIa-Induced PKB Phosphorylation**

To check whether the effects of the Jak2 inhibitor AG490 on STAT5 phosphorylation were specific, the effect of AG490 incubation on other FVIIa targets such as MAP kinase and the anti-apoptotic protein PKB was also investigated. AG490 incubation showed no effect on MAP kinase phosphorylation (Figure 5A); however, it did inhibit PKB phosphorylation (Figure 5B). Transfection of BHK\textsuperscript{TF} cells with dominant-negative Jak2 showed similar effects (Figure 5C), pointing out a role for Jak2 in FVIIa-induced PKB activation.

**FVIIa Induces STAT5 Translocation and Transactivation**

STAT5 translocation to the nucleus is essential for STAT5 function, and thus if FVIIa-dependent STAT5 phosphorylation is relevant, it should result in such a translocation. Hence, BHK\textsuperscript{TF} were subjected to FVIIa for various times, and subsequently, cell fractionation was used to produce nucleusensured and cytosol-enriched fractions. FVIIa treatment produced a marked increase in STAT5 phosphorylation in both the cytosolic and the nuclear fraction (Figure 6A) that was observed at concentrations as low as 1 nmol/L (Figure 6B). Strikingly, STAT5 phosphorylation in the cytosol was enhanced compared with STAT5 phosphorylation in the nucleus. The latter might be due to the presence of nuclear-localized STAT phosphatases, such as TC-PTP, which have been shown to dephosphorylate STAT5 in the nucleus.\textsuperscript{23} To further establish the relevance of FVIIa-induced STAT5 phosphorylation, we made use of a STAT5-responsive luciferase reporter construct. Cells were transfected with either this construct or a constitutive luciferase-expressing construct. After starvation, cells were stimulated with 100 nmol/L FVIIa for 3 hours, and luciferase activity was determined. Figure 6C shows that a 3-hour treatment with FVIIa strongly enhances luciferase activity. Thus, FVIIa provokes Jak2-dependent STAT5 phosphorylation, nuclear translocation, and transactivation.

**FVIIa Induces Jak2/STAT5-Dependent Expression of Bcl\textsubscript{XL}**

Bcl\textsubscript{XL} is an antiapoptotic protein that has been shown to be upregulated as a result of STAT5 activation.\textsuperscript{24,25} Because FVIIa in our systems activates STAT5, we decided to investigate FVIIa-induced Bcl\textsubscript{XL} expression. Therefore, we starved BHK\textsuperscript{TF} cells for 2 hours and stimulated them with 100 nmol/L of FVIIa. As can be seen in Figure 7A, stimulation of these cells results in an increase in Bcl\textsubscript{XL} in time that showed to be concentration-dependent (Figure 7B). This expression could not be reversed with 10 \( \mu \)mol/L of AG490, but Bcl\textsubscript{XL}
expression could be inhibited with the same concentration of Jak2 inhibitor (25 μmol/L) that also potently inhibited STAT5 phosphorylation (Figure 7C).

To investigate the role of STAT5 in FVIIa-induced BclXL expression, we constructed BHK<sup>T</sup> cells stably expressing dominant-negative STAT5 (Figure 7D). Whereas wild-type cells considerably upregulate Bcl<sub>XL</sub> after FVIIa stimulation, dominant-negative STAT5-expressing cells show only a moderate upregulation of Bcl<sub>XL</sub> (Figure 7E), whereas activation of FVIIa targets such as MAP kinase, PKB, and Jak2 were not affected (Figure 7F). Therefore, we concluded that FVIIa-induced Bcl<sub>XL</sub> expression is mediated, at least partially, via STAT5.

**Jak2/STAT5 Are Involved in FVIIa-Induced Cell Survival**

Because Jak2 and STAT5 were shown to be involved in FVIIa-driven Bcl<sub>XL</sub> expression and Jak2 activity was shown to be essential for PKB phosphorylation in BHK<sup>T</sup> cells, we decided to investigate the role of Jak2 and STAT5 in FVIIa-induced cell survival, a recently described phenomenon.<sup>26,27</sup> Therefore, cells were starved and preincubated with 10 μmol/L and 25 μmol/L AG490 before stimulation with FVIIa. FVIIa (100 nmol/L) efficiently promoted cell survival in serum-starved cells (Figure 8A) and 10 μmol/L of AG490 did not inhibit this effect. However, 25 μmol/L of AG490, the same concentration that efficiently inhibited STAT5 phosphorylation and Bcl<sub>XL</sub> expression, also inhibited FVIIa-induced survival. Finally, to address the role of STAT5, wild-type BHK<sup>T</sup> cells and STAT5 mutant cells were starved and incubated with FVIIa. Whereas wild-type cells show a considerable survival after 1 and 2 days of FVIIa stimulation, STAT5 mutant cells show a greatly diminished cell survival (Figure 8B). Therefore, we conclude that FVIIa induces cell survival via Jak2 and STAT5 activation.

**Discussion**

It is now generally recognized that TF, apart from its role in coagulation, is an important mediator in inflammation and cancerous processes.<sup>3-7</sup> The molecular basis, however, of this action remains unclear. In the present report, we provide evidence that FVIIa provokes Jak2-dependent phosphorylation of STAT5 via G<sub>12</sub>/G<sub>13</sub> followed by nuclear translocation and transactivation by this transcription factor. Furthermore,
show that FVIIa induces survival in cells that are stimulated to go into apoptosis, but this survival was inhibited by the Jak2 inhibitor AG490 or expression of dominant-negative STAT5. Moreover, Jak2 activity in our system proved essential for activation of the antiapoptotic kinase PKB, the latter being a key player in FVIIa-induced cell survival.26,27 Indeed, Jak2 has been found to be an upstream activator of PKB before.29 Therefore, we hypothesize that Jak2 exerts its antiapoptotic effects through activation of both STAT5 and PKB, thus positively influencing tumor growth and possibly metastasis. Currently, it is unknown whether Jak2 influences PKB activity directly, or via PKB’s upstream kinase PI-3 kinase, but previous work suggests that PI-3 kinase may be an intermediate in Jak2-dependent PKB activation.10,11

Induction of STAT5, Jak1, and Jak2 phosphorylation was already observed at concentrations as low as 1 nmol/L. Because plasma levels of FVII are normally maintained at 10 nmol/L, this concentration of FVIIa appears to be physiologically relevant. FVIIa-induced activation of proteins such as c-Akt/PKB occurs at slightly higher concentrations (10 nmol/L), suggesting that FVIIa-induced STAT5 phosphorylation is a relatively efficient process. It is possible that this difference might be due to activation of different G proteins coupling to the FVIIa proteolytic target. We indeed show for the first time that FVIIa-induced Jak2/STAT5 activation occurs via the heterotrimeric G proteins G_{12/G_{13}}, whereas G_s played no role. Both the G_{12/G_{13}} and G_s classes of G proteins were investigated because these signal transducers are also frequently activated by thrombin. A third class of G proteins, also activated by thrombin is G_{ai}. Activation of the latter G protein leads to transient calcium release and subsequent calcium-dependent signal transduction. However, in contrast to thrombin, FVIIa does not elicit calcium signals in BHK cells, ruling out G_{ai} activity.28

In conclusion, FVIIa induces activation of the Jak/STAT pathway via G proteins, leading to enhanced cell survival.

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