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Factor VIIa:TF induces activation of the JAK/STAT pathway; inflammatory signaling via non-inflammatory receptors

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Abstract

In addition to acting as the primary initiating event in the blood coagulation, binding of factor VIIa (FVIIa) to tissue factor (TF) also provokes signal transduction in tissue factor-expressing cells. Although, this signal transduction plays an important role in a variety of pathophysiological events, in particular inflammation and angiogenesis, the details of this signaling remain largely obscure. Interestingly, the extracellular domain of TF is highly homologous to the cytokine class II receptors and especially the interferon γ receptor, which is known to mediate downstream signaling via Jak-mediated phosphorylation of STAT transcription factors. Here we show that FVIIa:TF-interaction produces STAT5 phosphorylation, STAT5 nuclear translocation and transactivation of STAT5 reporter constructs. FVIIa dependent STAT5 activation was dependent on FVIIa proteolytic activity but was insensitive to inhibitors of the downstream coagulation factors and Xa and thrombin. Also the TF cytoplasmic domain was not required for STAT activation. Finally FVIIa incubation induces activation of Jak1, Jak2 and Tyk2, and functional Jak2 kinase was required for FVIIa-induced STAT5 phosphorylation. Thus our results show that the sequence homology between the cytokine class II receptors and TF is reflected in similar downstream signal transduction. Furthermore, the activation of STAT transcription factors provides an obvious link between FVIIa:TF and the associated pro-inflammatory events.
Introduction

Tissue factor (TF) is the main initiator of the coagulation cascade; upon 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 leading to the subsequent generation of factor Xa, thrombin and fibrin deposition, finally resulting in the formation of a blood clot. In addition, a large variety of coagulation independent functions for FVIIa:TF interaction have been described; in particular it is absolutely required for embryonic blood vessel formation (1-3) and one of the keyplayers in atherosclerosis (4) and its prominently implicated in inflammatory processes. The latter is especially demonstrated by the observation that inhibition of the FVIIa:TF complex, using either TF antibodies or active site-inhibited FVIIa was demonstrated to dramatically decreases mortality in baboons infused with a LD100 E. coli, whereas more downstream inhibitors did not have this effect (5-7). On a more cellular level, TF was shown to mediate leukocyte reverse transmigration (8) and proves pivotal for FVIIa-induced synthesis of proinflammatory mediators, among which, IL-1β, IL-8 and the prostaglandin E2 receptor (9). How FVIIa:TF interaction is linked to inflammatory processes remains, however, poorly understood.

It is now generally accepted that FVIIa:TF interactions provokes intracellular signal transduction in TF expressing cells. Among the cellular signaling cascades targeted by this signaling are the MAP kinase pathway (10,11), the PI3 kinase pathway (12), Ca2+ signaling (13,14) and activation of small GTPases (12), but such pathways are not a hallmark for inflammatory signaling per se. Interestingly, already in 1990, Bazan noted the high homology between the extracellular moieties of TF and the interferon class II type receptors (15), which are closely associated with proinflammatory cytokine signaling. Signaling via interferon receptors is well established to be mediated the sequential activation of Jak kinases followed by phosphorylation of STAT (Signal Transducers and Activators of Transcription) proteins by these kinases. STAT phosphorylation in turn produces dimerization and nuclear translocation of these transcription factors, finally culminating in altered gene expression. Based on the sequence homology between TF and interferon receptors and their shared involvement in inflammatory responses, we decided to investigate the possible involvement of the Jak/STAT system in TF-dependent signaling.
Chapter 5

Here we report that FVIIa induces STAT5 activation. This activation was dependent on FVIIa proteolytic activity, but not on the TF cytoplasmic tail. Thus our results show that the sequence homology between the cytokine class II receptors and TF is reflected in similar downstream signal transduction. Furthermore, the activation of STAT transcription factors provides an obvious link between FVIIa:TF and the associated pro-inflammatory events.
Experimental Procedures

Materials: The antibodies raised against phosphorylated STAT5 and phosphorylated Tyk2 were purchased from Cell Signaling Technologies (Beverly, MA, USA). Antibodies against phosphorylated Jak1 (Tyr 1022/1023) and Jak2 (Tyr 1007/1008) were from Biosource International (Camarillo, CA, USA). Antibodies against Jak1 and Jak2 were from Upstate Biotech Inc. (Waltham, MA, USA) and antibodies against Tyk2 was from Transduction Laboratories (Mississauga, ON, Canada). Anti-FLAG was from Sigma (St. Louis, MO, USA). Tissue Culture material was from Greiner Bio-one (Alphen a/d Rijn, The Netherlands).

Cell culture: Baby hamster kidney cells, either stably transfected with full length TF or with a TF cytoplasmic domain-deleted mutant, were maintained in Dulbecco’s Modified Eagles Medium, supplemented with 10% Fetal Calf Serum (FCS, Gibco) and penicillin/streptomycin, at 37°C (10,16), and 5% CO₂ in a humidified environment. The cells were routinely passaged three times per week. Starvation was carried out in DMEM deprived of FCS, for 20 hours. AG490 was used for 16 hours in the concentrations indicated.

Constructs: Both the DNA constructs encoding kinase-dead Jak1 and Jak2 were generous gifts from Dr. David Levy. The kinase-deleted Tyk2 construct was kindly provided by Dr. Sandra Pellegrini. FLAG-tagged STAT5A and B were from Dr. James Ihle and the STAT5-responsive NTCP-luciferase construct was provided by Mary Vore.

Immunoprecipitations: BHK²⁵ cells were transfected with 0.4 mg FLAG-tagged STAT5A or FLAG-tagged STAT5B construct, 3.2 ml enhancer and 10 ml Effectene for 16 h. After starvation for 16 h and stimulation with FVIIa for various times, the cells were lysed in 0.5 ml lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin) and were collected by scraping. Subsequently, the lysate was centrifuged at 14,000 rpm for 2 min, and the supernatant was precleared with 20 μl 50% protein A-sepharose for 1h. The lysate was then incubated overnight with 1 μg FLAG antibody. The immuno complex was precipitated with 20 μl 50% protein A-sepharose for 1h. The beads were washed three times with lysis buffer and were subsequently resuspended in 40 μl denaturing sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue).
Mutant Jak kinase analysis- For the experiments using kinase-dead Jak1, Jak2 or Tyk2 constructs, cells were transfected using Effectene from Qiagen. Transfections with kinase-dead constructs were carried out as follows; cells were grown until approximately 50% confluency in 6-well plates. 2.5 μg DNA was diluted in 100 μl Tris-EDTA, and incubated with 21 μg of enhancer reagent for 5 min. Subsequently, 133 μl of Effectene reagent was added and the mixture was incubated for 10 min. After this, the solution was dropwise added to the cells and these were incubated with the DNA complexes for 18 hours. After serum starvation, cells were stimulated with 100 nM FVIIa for 10'.

Nuclear isolation- Cell lysates were prepared and separated into nuclear and cytosolic fractions, essentially as described before (17), with some modifications; stimulated and washed cells (1-7 × 10^6) were scraped in 200 μl icecold hypotonic lysis buffer (20 mM Heps, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM pefabloc, and 0.5 μg/ml of leupeptin and aprotinin). The lysate was passed 10 times through a 27 gauge needle. After centrifugation at 600 × g, the resultant pellets (nuclear fraction) were washed again in lysis buffer and were resuspended in 300 μl 1x sample buffer. The supernatants (cytosolic fraction) were further diluted to 300 μl with 3x sample buffer.

Western Blotting- 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 and the 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 supplemented with 0.1% tween (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 ECL substrate from Roche and a chemiluminescence detector with a cooled CCD-camera (Genegnome) from Syngene. Antibody bands were quantified using Genetools from Syngene.

Luciferase assay- Cells were grown in 24-wells and transfected with either the STAT5-responsive construct ntep-luciferase, or a CMV-driven luciferase construct for 16 h.
Transfections were carried out, using 0.2 μg construct, 1.6 μl enhancer and 5 μl Effectene transfection reagent. After transfection, the cells were serum starved for another 16 h. Subsequently, cells were stimulated with 100 nM FVIIa for 3 h. Cells were lysed in 100 μl luciferase assay buffer (25 mM glycylglycine pH 7.9, 15 mM MgSO₄, 4 mM EGTA, 1% Triton X-100 1 mM dithiotreitol) and luciferase activity was measured, using the Packard Topcount microplate scintillation counter.
Results

FVIIa:TF interaction induces phosphorylation of STAT5- There is substantial sequence homology between the extracellular domains of TF and the interferon receptors. The intracellular domains of TF and the interferon receptors, however, are not much alike and it is still unclear whether TF employs interferon receptor-like signal transduction mechanisms. Therefore we set out to investigate possible involvement of STATs in TF-dependent signal transduction in Baby Hamster Kidney cells stably transfected with TF (BHK\textsuperscript{TF}) (10). To this end the effects of recombinant FVIIa on STAT phosphorylation were determined using phosphospecific antibodies and Western blotting. As can be seen in Fig. 1A, BHK\textsuperscript{TF} cells reacted to FVIIa with a transient increase in phospho-STAT5 immunoreactivity, which was already detectable after 5 min, reached a maximum effect 10 min after stimulation and returned to basal levels after approximately 60 min. In contrast, pan-STAT5 immunoreactivity was not affected within this time frame. Interestingly, this FVIIa-induced phosphorylation was much more transient than that observed after stimulation with insulin, an established inducer of STAT5 phosphorylation (Fig. 1B) (18). 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 nM (Fig. 2), lying well within the physiological ranges of FVII in plasma (10 nM). We concluded that TF, like other members of the cytokine class II receptors has the ability to provoke STAT phosphorylation.
FVIIa proteolytic activity but not the cytoplasmic domain of TF mediate STAT5 phosphorylation- To further investigate the role of TF in FVIIa-induced STAT phosphorylation, we used a BHK cell line, stably transfected with a cytoplasmic domain-deleted TF (16). As these cells responded to FVIIa stimulation with transient STAT5
phosphorylation indistinguishable of that observed in BHK cells transfected with full length TF, we concluded that cytoplasmic domain of TF is not involved in this phosphorylation (Fig. 3A). As a control, we also stimulated the parental BHK cells with FVIIa, but we did not detect any FVIIa-dependent STAT5 phosphorylation in these cells (Fig. 3B). Therefore, only the extracellular part of TF is required for FVIIa-induced STAT5 phosphorylation. We also assessed the importance of FVIIa proteolytic activity employing active site-inhibited FVIIa (FFR-VIIa or FVIIai). We observed that although a 10 min-stimulation with FVIIa potently induced STAT5 activation, FVIIai was ineffective in this respect (Fig 3C). We concluded that the FVIIa-dependent STAT phosphorylation is mediated by extracellular domain and TF and FVIIa proteolytic activity.

FVIIa-induced STAT phosphorylation is not dependent on generation of thrombin or factor Xa- After exposure of TF to the bloodstream, interaction of TF with factor VII will take place, leading to factor VII activation. The thus-generated FVIIa converts factor X to

\[
\begin{array}{c|c|c|c}
FVIIa & FVIIa/hirudin & FVIIa/TAP \\
\hline
0 & 10 & 20 \\
\hline
\text{phospho-STAT5} \\
\hline
\text{STAT5} \\
\end{array}
\]

\[
\begin{array}{c|c|c|c}
FXa & FXa/TAP & \text{thrombin} & \text{thrombin/hirudin} \\
\hline
0 & 10 & 20 & 0 & 10 & 20 \\
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\text{phospho-STAT5} \\
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\text{STAT5} \\
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Figure 4: FVIIa-induced STAT5 phosphorylation is not dependent on FXa or thrombin formation. BHK cells were preincubated with 200 nM 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 nM FVIIa, 1 U/ml FXa or 1 U/ml thrombin. STAT5 phosphorylation and total amounts of STAT5 were determined by Western Blot.
factor Xa and in turn factor Xa activates thrombin. Hence addition of FVIIa to cells may lead to factor Xa and thrombin generation. In order to investigate a possible role of factor Xa or thrombin in FVIIa-dependent STAT5 activation, we tested the effect of the factor Xa inhibitor TAP and the thrombin inhibitor hirudin on FVIIa-evoked STAT5 phosphorylation. As obvious from Fig. 4B, both factor Xa and thrombin are capable of inducing STAT5 phosphorylation, and the effects of factor Xa and thrombin are sensitive to 100 nM of TAP and 25 U of hirudin respectively. These inhibitors, however, did however not influence FVIIa-induced STAT5 phosphorylation (Fig. 4A). 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 induces both STAT5A and STAT5B phosphorylation- STAT5 consists of two isoforms; STAT5A and STAT5B. To address the question as to which isoform is targeted by FVIIa, we transfected BHK^TF with expression constructs encoding either FLAG-tagged STAT5A or FLAG-tagged STAT5B (19). Subsequently, cells were stimulated with FVIIa for various times and FLAG-tagged STAT5 was immunoprecipitated using a FLAG antibody. Finally, phosphorylation of STAT5 was determined using a phosphospecific antibody for STAT5. We observed that, whereas total levels of FLAG-STAT5A and FLAG-STAT5B remained unchanged, the phosphoSTAT5 immunoreactivity was transiently increased with respect to both STAT5A and STAT5B and hence both STAT5 isoforms are a target for FVIIa-dependent signaling.

**IP: α-FLAG**

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**Figure 5:** Both STAT5A and STAT5B are activated by FVIIa. BHK^TF cells were transfected with FLAG-tagged STAT5A and FLAG-tagged STAT5B constructs. After starvation, the cells were stimulated and FLAG-tagged STAT5 was immunoprecipitated. Subsequently, the phosphorylation status of the immunoprecipitated STATs were assessed on Western Blot, using a phosphospecific STAT5 antibody. Total FLAG-STAT was determined using a FLAG antibody.
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. Fig. 6 shows that Jak1, Jak2 and Tyk2 phosphorylation are transiently upregulated after FVIIa stimulation, a maximum being reached after 5 min. Whereas Jak2 and Tyk2 phosphorylation return to basal levels within 15 min, Jak1 phosphorylation was much more persistent. To establish the role of these Janus kinases in the phosphorylation of STAT5, we preincubated BHK cells with the specific Jak inhibitor AG-490.

Figure 6: FVIIa stimulation induces activation of Jak kinases. BHK cells were stimulated for the indicated times. Subsequently, Jak1, Jak2 and Tyk2 were analyzed for phosphorylation on Western Blot. Total amounts of Jak1, Jak2 and Tyk2 were determined, using antibodies directed against total Jak1, Jak2 and Tyk2.

Figure 7: STAT5 phosphorylation, but not MAP kinase phosphorylation is mediated by Jak kinases. Cells were preincubated with various concentrations of the specific Jak-inhibitor AG-490 and subsequently stimulated with 100 nM FVIIa. STAT5 and MAP kinase phosphorylation as well as total amounts of these proteins were determined by Western Blot.
AG490. As can be seen in Fig. 7 a 25 mM preincubation with this inhibitor abrogates FVIIa-induced STAT5 phosphorylation, without affecting other FVIIa-induced signaling pathways such as the p42/p44 MAP kinase pathway. We further explored the role of Jak kinases in this system by transiently transfecting BHK\textsuperscript{TTF} cells with DNA-constructs, encoding kinase-dead variants of Jak1, Jak2 and Tyk2 (20,21). As can be seen in Fig. 8, 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 concluded that, although FVIIa induces activation of Jak1, Jak2 and Tyk2, only Jak2 is essential in FVIIa-dependent STAT5 phosphorylation.

![Figure 8: Jak2, but not Jak1 or Tyk2 is required for FVIIa-induced STAT5 phosphorylation. BHK cells were transfected with kinase-dead Jak1, Jak2 and Tyk2 mutants, after which cells were starved and stimulated with 100 nM FVIIa. Cell lysates were analyzed on Western Blot for STAT5 phosphorylation, total amounts of STAT5 and total amounts of Jak kinases.](image)

**Figure 8:** Jak2, but not Jak1 or Tyk2 is required for FVIIa-induced STAT5 phosphorylation. BHK\textsuperscript{TTF} cells were transfected with kinase-dead Jak1, Jak2 and Tyk2 mutants, after which cells were starved and stimulated with 100 nM FVIIa. Cell lysates were analyzed on Western Blot for STAT5 phosphorylation, total amounts of STAT5 and total amounts of Jak kinases.

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{TTF} were subjected to FVIIa for various times and subsequently, cell fractionation was employed to produce nucleus-enriched and cytosol-enriched fractions. As can be seen in Fig. 9A, FVIIa treatment produces a marked increase in STAT5 phosphorylation in both the cytosolic and the nuclear fraction. Strikingly,
STAT5 phosphorylation in the cytosol was still evident after 60 min whereas phosphorylation of STAT5 in the nucleus was transient and returned to basal levels at 30 min. The latter might be due to the presence of nuclear-localized STAT phosphatases, such as TC-PTP, which has been shown to dephosphorylate STAT5 in the nucleus (22). 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 nM FVIIa for 3 hrs, and luciferase activity was determined. Fig. 9B shows that a 3 hrs treatment with FVIIa strongly enhances luciferase activity. Thus FVIIa provokes Jak2-dependent STAT5 phosphorylation, nuclear translocation and transactivation.

Figure 9: FVIIa stimulates STAT5 translocation to the nucleus and STAT5-dependent gene transcription. (A) Cells were starved and stimulated with FVIIa for various times. Subsequently, cells were fractionated into cytosolic and nuclear fractions and these fractions were analyzed for phosphorylated STAT5 contents by Western Blot. Total β-actin contents were also determined as a measure for the purity of the fractions. (B) Cells were transfected with NTCP-luciferase or CMV-luciferase. After serum starvation, cells were stimulated with FVIIa for 3h and luciferase activity was determined. To exclude aspecific signals, the normalized luciferase activity was obtained by dividing the signal from cells transfected with NTCP-luciferase, by the signal obtained from cells transfected with CMV-luciferase.
Discussion

It is now generally recognized that TF, apart from its role in coagulation, is an important mediator in inflammatory processes (5,6,8,23). The molecular basis, however, of this role remains unclear. Since TF bears striking sequence homology with the IFN α,β and γ receptor (15), it is possible that FVIIa:TF binding leads to signaling similar to that evoked by interferon receptors. In the present report we provide evidence that FVIIa provokes Jak2-dependent phosphorylation of STAT5, followed by nuclear translocation and transactivation by this transcription factor. An important implication of this work is that the sequence homology of TF with the cytokine class II receptors is reflected in the activation of similar downstream effector mechanism, i.e. Jak/STAT signaling. As Jak/STAT responses are firmly linked to pro-inflammatory responses, Jak/STAT activation may well be implicated in TF action in inflammation.

Figure 11: Proposed mechanism for FVIIa:TF-induced Jak-STAT signaling in BHK7T cells. The FVIIa:TF complex targets a still unknown transmembrane protein, leading to activation of Jak1, Jak2 and Tyk2. Jak2 subsequently phosphorylates STAT5A and B, inducing nuclear translocation. Once in the nucleus, STAT5 possibly as a dimer or in complex with another STAT induces gene transcription.
However, despite the fact that TF resembles a class II cytokine receptor, the nature of the signaling cascade leading to activation of the Jak2/STAT5 pathway completely differs from that observed after activation of class II cytokine receptors, which directly recruit Jaks to the intracellular domain of the receptors. We showed that STAT5 phosphorylation is independent of the TF cytoplasmic tail, but relies on the proteolytic activity of FVIIa. These data are most consistent with the often-proposed platform hypothesis, in which TF serves as a docking site for FVIIa, which in turn proteolytically cleaves and thereby activates a protease activated G protein-coupled receptor. One of the proteins that has frequently been implicated in FVIIa:TF-dependent signaling is Protease-activated Receptor 2 (PAR-2) (24). However, the nature of the transmembrane receptor in BHK\textsuperscript{TF} cells remains obscure (25). Thus TF may resemble a cytokine receptor, acts as a cytokine receptor via the activation of STATs, but may achieve this employing atypical signaling mechanisms.

Using recombinant FVIIa and a BHK cell line expressing full length TF, we observed transient phosphorylation of STAT5 coinciding with activation of Jak1, Jak2 and Tyk2. Although only functional Jak2 was required for STAT5 responses, it is to be expected that the activation of the other Janus kinases will have downstream consequences as well, leading to for instance phosphorylation of other members of the STAT family of signal transducers. Indeed in preliminary experiments we have seen enhanced phosphorylation of STAT6 in response to TF, although this response is less robust than the STAT5 responses observed in the present study (S. Slofstra, unpublished observations). Nevertheless, it is tempting to speculate that different Jak kinases may target different STATs, but obviously further work is needed to substantiate this notion.

The functioning of STAT5 in pathophysiology has not been very well investigated but we feel that FVIIa-dependent STAT5 activation may well be important in the various functions of TF in physiology. It has been reported that STAT5 mediates gene expression of CIS and Oncostatin M (26,27), which are important mediators of cytokine signaling and inflammatory processes. This could very well correlate with a role for TF, observed in inflammation. Importantly, induction of this STAT5 phosphorylation was already observed at concentrations as low as 1 nM. Since plasma levels of factor VII are normally maintained at 10 nM, this concentration of FVIIa appears to be a physiological relevant concentration. We reported earlier that FVIIa-induced activation of proteins such as c-Akt/PKB occurs at slightly higher concentrations of approximately 10 nM, suggesting that FVIIa-induced STAT5
phosphorylation is a relatively efficient process. We currently do not know the nature of the differences in concentration-dependency observed between PKB activation and STAT5 activation, but it is possible that this difference might be due to activation of different G-proteins coupling to the FVIIa proteolytic target. Disregarding the molecular mechanism, in the present study we have shown that STAT5 activation is a major target for FVIIa-dependent signal transduction and future experiments should address the importance of this activation for pathophysiology.
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References


Chapter 5


