Signal Transduction via the Mitogen-activated Protein Kinase Pathway Induced by Binding of Coagulation Factor VIIa to Tissue Factor*

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The putative role of tissue factor (TF) as a receptor involved in signal transduction is indicated by its sequence homology to cytokine receptors (Bazan, J. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6934–6938). Signal transduction induced by binding of FVIIa to cells expressing TF was studied with baby hamster kidney (BHK) cells stably transfectected with TF and with a reporter gene construct encoding a luciferase gene under transcriptional control of tandem cassettes of signal transducer and activator of transcription (STAT) elements and one serum response element (SRE). FVIIa induced a significant luciferase response in cells expressing TF, BHK(+TF), but not in cells without TF. The BHK(+TF) cells responded to the addition of FVIIa in a dose-dependent manner, whereas no response was observed with active site-inhibited FVIIa, which also worked as an antagonist to FVIIa-induced signaling. Activation of the p44/42 MAPK pathway upon binding of FVIIa to TF was demonstrated by suppression of signaling with the specific kinase inhibitor PD98059 and demonstration of a transient p44/42 MAPK phosphorylation. No stimulation of p44/42 MAPK phosphorylation was observed with catalytically inactive FVIIa derivatives suggesting that the catalytic activity of FVIIa was obligatory for activation of the MAPK pathway. Signal transduction caused by a putative generation of FXa activity was excluded by experiments showing that FVIIa/FX-induced signaling was not quenched by tick anticoagulant protein, just as addition of FXa could not induce phosphorylation of p44/42 MAPK in BHK(+TF) cells. These results suggest a specific mechanism by which binding of FVIIa to cell surface TF independent of coagulation can modulate cellular functions and possibly play a role in angiogenesis and tumor metastasis as indicated by several recent observations.

The extrinsic pathway of blood coagulation is initiated when FVIIa circulating in plasma binds to the integral membrane protein, tissue factor (TF), exposed to the blood upon injury of the vessel wall. The role of TF in blood coagulation has been studied extensively (see Refs. 1 and 2 for reviews). Initiation of coagulation as a result of FVIIa/FX activity is an extracellular event confined to the outer leaflet of the plasma membrane on TF-expressing cells. This was firmly established when it was shown by Paborsky et al. (3) that truncation of the cytoplasmic C-terminal of TF did not affect its cofactor function in the coagulation process. Still a number of other observations suggested an intracellular function of TF. It was found that TF showed sequence homology to the cytokine receptor superfamily (4). All members of this family are involved in signal transduction. Subclass II, which includes the receptor for interleukin 10 and receptors for interferon α/β and γ, shows the highest homology to TF (5). The crystal structure of TF (6, 7) further substantiated its structural resemblance to the cytokine receptors. This homology might imply a possible functional role for TF as a receptor involved in signal transduction. Studies on a putative intracellular activity induced by FVII/FVIIa have been elusive. Two recent studies (8, 9) reported that FVIIa could induce oscillations in intracellular free calcium in various TF-expressing cells. The authors proposed that this involved activation of phospholipase C; however, they failed to demonstrate direct phosphorylation caused by activation of intracellular kinases. Other studies have provided evidence that serine residues of the cytoplasmic domain of TF can be phosphorylated in TF-transfected cells (10) and that this domain works as a substrate for protein kinase activity when exposed to cell lysates (11). Signal transduction was also indicated in studies with cultured human monocytes (12) showing that addition of FVIIa could induce a transient tyrosine phosphorylation of several polypeptides. Finally very recent results suggested that FVIIa induced alteration in gene expression in human fibroblasts (13). With these diverse observations it was of interest to characterize a putative FVIIa-induced signal transduction pathway in further detail.

Ligand-induced oligomerization of receptor subunits, which juxtaposes to engage an intracellular signaling machinery, is a common element in signal initiation from cytokine receptors (14). This may lead to rapid phosphorylation of a subset of intracellular receptor-associated proteins and mitogen-activated (Ser/Thr) kinases (MAPK) (15, 16). These kinases are arranged in several parallel signaling pathways, the induction of which may eventually activate the serum response element (SRE) and lead to transcription (17). Cytokine receptors, which like TF lack intrinsic kinase catalytic domains, may couple ligand binding to tyrosine phosphorylation by using noncovalently associated protein kinases, the Janus kinases, which phosphorylate signal transducers and activators of transcription (STATs) to induce binding to specific DNA elements and gene transcription. The JAK-STAT pathway may be engaged in

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‡ The abbreviations used are: FVIIa, activated FVII; TF, tissue factor; FVII, factor VII; FVIIa, FVIIa blocked in the active site with D-Phe-L-Arg chloromethyl ketone; FX, factor X; TAP, tick anticoagulant protein; BHK, baby hamster kidney; SRE, serum response element; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; FCS, fetal calf serum.

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cross-talk with the MAPK pathway (18). The present study demonstrates that binding of FVIIa to cell surface TF leads to gene transcription as a result of signal transduction via the p44/42 MAPK pathway and activation of SRE and/or STAT elements.

EXPERIMENTAL PROCEDURES

Cell Culture—The baby hamster kidney cell line BHK-21 tk- ts13 (ATCC CRL 1632) was cultured in Dulbecco's modified Eagle's medium containing 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin. A human endothelial cell line ECV-304 (ATCC CRL-1998) was cultured in Medium 199 with Earle's salts containing 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin. An epithelial-like canine kidney cell line MDCK-II was kindly provided by Professor Bo van Deurs, University of Copenhagen, Denmark and cultured in Dulbecco's modified medium supplemented with 5% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All cell lines were grown in T-75 or T-175 flasks and subcultured into 24-well tissue culture plates or 23.8-cm2 single wells.

Proteins—Human recombinant FVII and FVIIa was expressed and purified as described (19). FVIIa was obtained by blocking of FVIIa in the active site with d-Phe-L-Phe-L-Arg chloromethyl ketone as described previously (20). FVIIa and FVIIai were iodinated by the lactoperoxidase/H2O2 method (21) and purified by size exclusion chromatography on a Pharmacia Biotech Inc. NAP 5 Sephadex G-25 with 0.1 M NH4HCO3, 0.5% (w/v) human serum albumin as the mobile phase. FX was from Enzyme Research (Lafayette, IN). Recombinant tick anticoagulant protein (rTAP) was kindly provided by Dr. G. P. Vlasuk, Corvas (San Diego, CA). The inhibitor PD98059 and the phosphospecific antibody against p44/42 MAPK were from Cell Signaling Technology (Beverly, MA). The inhibitor PD98059 and the phosphospecific antibody against p44/42 MAPK was detected using a PhosphoPlus antibody kit (Biolabs) according to the manufacturer's protocol. BHK(+TF) cells were cultured in medium with 0.1% FCS for 24 h prior to the experiment. The experiment was performed by adding media with 0.1% FCS containing the various ligands for the indicated periods of time to the cells. As a positive control of cell signaling cells were treated with 15% FCS for 15 min in each experiment. Cells were lysed in 150 μl of SDS sample buffer (9.2% (w/v) SDS, 25 mM Tris-HCl, 40% (v/v) glycerol, 80 mM EDTA, 1.2% (w/v) bromphenol blue, pH 6.8) supplemented with 3 mM sodium orthovanadate and 24.2 mM dithiothreitol. Lysates were loaded on a 12% SDS-polyacrylamide gel. A biotinylated protein marker was loaded on each gel, and fully phosphorylated p44/42 MAPK was loaded as a positive control in some experiments.

RESULTS AND DISCUSSION

Characterization of Transfected BHK Cells—Baby hamster kidney (BHK) cells without detectable amounts of TF were stably transfected with the TF expression vector. These BHK(+TF) cells contained approximately 3 × 10⁵ TF/cell as revealed by a binding assay with ¹²⁵I-labeled FVIIa and expressed functionally active TF working as a cofactor for FVIIa-mediated activation of FX with an EC₅₀ = 1.0 nM. No significant activation was observed with untransfected BHK cells (results not shown). Subsequently the cells were stably transfected with the KZ136 reporter plasmid encoding a luciferase gene under transcriptional control of tandem cassettes of STAT1 and -3 and STAT4, -5, and -6 and one SRE. Serum contains a multitude of growth factors and activates several of these elements. Addition of 15% serum to starved cells transfected with the KZ136 reporter construct resulted in a 5–8-fold increase in luciferase activity independent of coexpression of TF (results not shown).

Effect of FVIIa, FVII, and FVIIai on BHK(+TF) Cells Transfected with the KZ136 Signaling Reporter Gene Construct—Fig. 1A shows that TF-transfected BHK cells, BHK(+TF), with the reporter construct responded to the addition of FVIIa, whereas a significant response was not observed when FVIIai was added to BHK cells without TF. This suggested that FVIIa was involved in TF-dependent signal transduction resulting in gene transcription. The FVIIa-induced response was saturable with an apparent EC₅₀ of approximately 20 nM as indicated by the results shown in Fig. 1B.

The mobilization of Ca²⁺ stores in TF-expressing cells observed by Rottingen et al. (8), as well as transient phosphorylation of intracellular monocyte polypeptides observed by Masuda et al. (12), appeared to require the participation of catalytically active FVIIa. Hence, we have investigated whether proteolytically active FVIIa was also mandatory for TF signal transduction monitored by the luciferase reporter system. As shown in Fig. 2 the addition ofzymogen, one-chain FVII, induced a luciferase response comparable with that of the
activity was measured as described under “Experimental Procedures.”

inhibited by binding of FVIIai to TF. Fig. 3 shows that addition of FXa or thrombin.

suggested that FVIIa/TF-induced signal transduction did not occur, although the addition of FXa was capable of stimulating the response induced by FVIIa, the response induced by FXa might occur via one of the known MAPK pathways. Fig. 4 demonstrates that the FVIIa-induced luciferase response in BHK(+TF) cells was completely inhibited by the specific inhibitor PD98059. This inhibitor binds to the inactive form of MAPKK (MEK) preventing its transformation into an active kinase (31). Phosphorylation of p44/42 MAPK and further downstream activation is thereby inhibited. The results shown in Fig. 4 thus suggested that stimulation of gene transcription by FVIIa/TF might occur via the MAPK pathway.

**FVIIa-induced Phosphorylation of p44/42 MAPK in BHK(+TF) Cells**—Using a specific antibody against the phosphorylated Thr202/Tyr204 residues of p44/42 MAPK it was possible to confirm this conclusion. BHK(+TF) cells were grown to 90% confluence and then starved in 0.1% FCS for 24 h. Fig. 5 (panels A and B) shows a Western blot of cell lysates from BHK(+TF) cells exposed to 100 nM FVIIa for 0, 3, 5, 7, 10, and 40 min (lanes 2–7). Specific antibodies were used to visualize the amount of activated MAPK (panel A) and of total MAPK (panel B). Fig. 5A demonstrates that the MAP kinase was transiently phosphorylated peaking at approximately 10 min, whereas the amount of total MAPK remained essentially constant (panel B).

When a similar experiment was performed with nontransfected BHK cells the MAPK was not activated by FVIIa, but a phosphorylated MAPK response was obtained with 15% serum (results not shown). The results shown in Fig. 5 (panels C and D) were obtained with BHK(+TF) cells exposed to 100 nM FVII, FVIIa, FVIIai, [Ala344]FVII, or FXa for 15 min. A profound activation was seen with FVIIa, less so with FVII, and no significant activation was induced by FVIIai or an inactive FVII variant, [Ala344]FVII, in which the active site serine was changed by site-directed mutagenesis (panel C, lanes 2–5). It was interesting to note that the addition of FXa (panel C, lane 6) also failed to induce
phosphorylation of p44/42 MAPK. No effect on the total amount of p44/42 MAPK level was observed (panel D). These results strongly suggested that FVIIa activity was needed for this response and since FXa did not induce phosphorylation, a putative FVIIa-mediated generation of FXa could not account for p44/42 MAPK activation with FVIIa.

Antibodies against phosphorylated SAPK or p38 MAPK were tested in a similar way in separate experiments; however, none of these pathways appeared to be stimulated by binding of FVIIa to TF (results not shown).

**FVIIa-induced Phosphorylation of p44/42 MAPK in ECV 304 and MDCK Cells—FVIIa/TF-induced stimulation of gene transcription was also studied in the human immortalized endothelial cell line, ECV 304.** After starvation for 24 h, ECV 304 cells expressed about 25,000 TF molecules per cell as estimated of the MAPK pathway inhibited the stimulation induced by FVIIa as well as the background activity. This indicates that the catalytic activity of FVIIa is mandatory for this gene transcription although the existence of parallel pathways or cross-talk between pathways cannot be excluded. Recent studies have provided circumstantial evidence that FVIIa/TF may be involved in signal transduction and gene transcription. The present work demonstrates that this is the case and that it occurs in a FVIIa- and TF-dependent reaction via the p44/42 MAPK pathway. The results also suggest that the catalytic activity of FVIIa is mandatory for this process and that an indirect signaling pathway via FX activation can be excluded. The target substrate for the FVIIa activity has, however, not been identified, just as it remains to be shown whether TF as such works as a transmembrane signal transducer either alone or in combination with a putative β-subunit. The experiments with the effect of PD98059 on the luciferase response (Fig. 4) showed that this specific inhibitor of the MAPK pathway inhibited the stimulation induced by FVIIa as well as the background activity. This indicates that MAPK phosphorylation is a probable route to FVIIa-induced gene transcription although the existence of parallel pathways or cross-talk between pathways cannot be excluded. Recent studies have suggested a role for TF in angiogenesis (32, 33), embryo vascularization (34), tumor metastasis (35–37), and smooth muscle cell migration (38). FVIIa/TF-induced signal transduction might provide a common molecular mechanism linking these cellular events together. The present work has uncovered important details about the signaling process and provided clues to further elucidate the mechanism.

**Conclusion**

Several recent studies have provided circumstantial evidence that FVIIa/TF may be involved in signal transduction and gene transcription. The present work demonstrates that this is the case and that it occurs in a FVIIa- and TF-dependent reaction via the p44/42 MAPK pathway. The results also suggest that the catalytic activity of FVIIa is mandatory for this process and that an indirect signaling pathway via FX activation can be excluded. The target substrate for the FVIIa activity has, however, not been identified, just as it remains to be shown whether TF as such works as a transmembrane signal transducer either alone or in combination with a putative β-subunit. The experiments with the effect of PD98059 on the luciferase response (Fig. 4) showed that this specific inhibitor of the MAPK pathway inhibited the stimulation induced by FVIIa as well as the background activity. This indicates that MAPK phosphorylation is a probable route to FVIIa-induced gene transcription although the existence of parallel pathways or cross-talk between pathways cannot be excluded. Recent studies have suggested a role for TF in angiogenesis (32, 33), embryo vascularization (34), tumor metastasis (35–37), and smooth muscle cell migration (38). FVIIa/TF-induced signal transduction might provide a common molecular mechanism linking these cellular events together. The present work has uncovered important details about the signaling process and provided clues to further elucidate the mechanism.

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