Intracellular signaling induced by the coagulation factors (F) VIIa and Xa is poorly understood. We report here studies on these processes in a human keratinocyte line (HaCaT), which is a constitutive producer of tissue factor (TF) and responds to both FVIIa and FXa with elevation of cytosolic Ca\(^{2+}\), phosphorylation of extracellular signal-regulated kinase (Erk) 1/2, p38\(^{\text{MAPK}}\), and c-Jun N-terminal kinase, and up-regulation of transcription of the early growth response gene-1 (egr-1). Using egr-1 as end point, we observed with both agonists that phosphatidylinositol-specific phospholipase C and the mitogen-activated protein kinase/Erk kinase/Erk pathway were mediators of the responses. The responses to FVIIa were TF-dependent and up-regulation of egr-1 mRNA did not require presence of the TF cytoplasmic domain. Antibodies to EPR-1 and factor V had no effect on the response to FXa. We have provided evidence that TF is not the sole component of the FVIIa receptor. The requirement for proteolytic activity of both FVIIa and FXa suggests that protease-activated receptors may be involved. We now report evidence suggesting that protease-activated receptor 2 or a close homologue may be a necessary but not sufficient component of this particular signal transduction pathway. The up-regulation of egr-1 describes one way by which the initiation of blood coagulation may influence gene transcription. The ability of these coagulation proteases to induce intracellular signals at concentrations at or below the plasma concentrations of their zymogen precursors suggests that these processes may occur also in vivo.

The vitamin K-dependent serine protease clotting factors have traditionally been thought to exert their effects in the fluid phase of the body fluids or at the surface of cells in contact with such fluids. Increasing evidence indicates that activation of these clotting factors also elicits numerous and profound alterations in the biology of the cells on whose surface the activation takes place or where the activated factor is bound. Direct intracellular signaling in cells exposed to the coagulation factor VIIa (FVIIa)\(^{1}\) was first reported in 1995 (1). It was found to be entirely dependent on the presence of tissue factor (TF), the factor VII- and VIIa-binding trigger of blood coagulation (2) on the cell surface. More recently, FVIIa has been shown to induce phosphorylation of the extracellular signal-regulated kinases 1 and 2 (Erk 1/2) (3) and to up-regulate three mRNA species: poly(A) polymerase (4) and vascular endothelial growth factor (5) in fibroblasts and urokinase type plasminogen activator receptor in pancreatic cancer cell lines (6). Two recent studies demonstrate the involvement of TF in cell adhesion/motility (7, 8). These effects may be mediated by interactions of the TF cytoplasmic tail with cytoskeletal adaptor proteins (7), and probably reflect another aspect of TF biology.

In the case of the coagulation factor Xa (FXa), Gajdusek et al. (9) reported induction of release of growth factors from the endothelium. Gasic et al. (10), and later Ko et al. (11), observed FXa-induced vascular smooth muscle cell proliferation. Signaling was suggested to involve rapid release of platelet-derived growth factor (PDGF), followed by PDGF receptor-mediated activation of the p21ras/p74src pathway (11). We showed that FXa triggered an increase in cytosolic free Ca\(^{2+}\) in Madin-Darby canine kidney cells (12). In endothelial cells addition of FXa can activate NO synthase (13, 14) and induce synthesis of cytokines and expression of adhesion molecules (14, 15). Antibodies to PDGF (11, 16, 17) or effector cell protease receptor-1 (EPR-1) (17, 18) both attenuate mitogenic responses to FXa in endothelial cells and in vascular smooth muscle cells. Factor V (FV), the cellular cofactor for FXa in formation of the prothrombinase complex (2), has to our knowledge not been shown to participate in any of these events.

Both FVIIa and FXa must be proteolytically active to induce the signaling process(es) (12). In the case of FVIIa, TF serves as its binding receptor but does not undergo any proteolytic cleavage in the process. This suggests that another molecule is the substrate for cleavage by the indispensable protease activity of FVIIa, and that this cleavage may trigger the intracellular signaling. The presence of TF on the cell surface is, however, an absolute requirement for the FVIIa induced intracellular changes to occur. Our hypothesis is thus that TF efficiently binds FVII/FVIIa present in plasma and other body fluids, and

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\(^{‡}\) The abbreviations used are: FVIIa, factor VIIa; [Ca\(^{2+}\)]\(_{i}\), cytosolic free Ca\(^{2+}\) concentration; DMEM, Dulbecco's modified Eagle's medium; EPR-1, effector cell protease receptor-1; Erk, extracellular signal-regulated kinase; MeK, mitogen-activated protein kinase/extracellular signal-regulated kinase; FVIIai, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone-inhibited FVIIa; FVa, factor Va; FXa, factor Xa; FXai, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone-inhibited FXa; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hapes-buffered salt solution; JNK, c-Jun N-terminal kinase; MDCK, Madin-Darby canine kidney; PAR, protease-activated receptor; PDGF, platelet-derived growth factor; PI-PLC, phosphatidylinositol-specific phospholipase C; PTK, pertussis toxin; MAPK, mitogen-activated protein kinase; TFPI, human tissue factor pathway inhibitor 1; TF, tissue factor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; RI, relative intensity; MOPS, 4-morpholinepropanesulfonic acid.

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Coagulation Factors VIIa and Xa Induce Cell Signaling Leading to Up-regulation of the egr-1 Gene* (Received for publication, April 7, 1999, and in revised form, August 30, 1999)

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egr-1 Induction by FVIIa and FXa

The constitutively TF-expressing keratinocyte line HaCaT (26) was kindly provided by Dr. U. Birk Jensen, Institute of Human Genetics, University of Aarhus, Aarhus, Denmark. HaCaT cells were cultured in keratinocyte-SFM supplemented with recombinant epidermal growth factor (0.5 ng/ml) and bovine pituitary extract (25 μg/ml). After cell detachment during culture, trypsin was inactivated with FCS, which was subsequently removed by repeated washes in keratinocyte SFM. Prior to Northern and Western experiments starvation (2 h prior to agonist addition) and stimulation was done in DMEM without additives. The keratinocyte SFM was low in calcium (<0.1 mM) in order to keep the cells in an undifferentiated state. The change to DMEM prior to stimulation brought the calcium levels up to ~1.8 mM. This change was done to facilitate Ca²⁺-dependent binding of the clotting factors to TF and possibly other surface receptors, and to allow calcium influx. Great care was taken to establish correct controls for the possible effects of this shift-up.

The human kidney epithelial line HK-2 (ATCC) was cultured as described for the HaCaT cells. CHO cells expressing PAR2 were a kind gift from Johan Sundelin (University of Lund, Lund, Sweden) (27). These were cultured in α-minimal essential medium without nucleosides, supplemented with 10% dialyzed FCS, l-glutamine, antibiotics, and methotrexate (20 μM). COS-1 cells were maintained in DMEM supplemented with 5% inactivated FCS, l-glutamine, and antibiotics.

Cell Culture and Transfection

Trypsin, hirudin, ATP, bradykinin, actinomycin D, and Hepes were obtained from Sigma; U73122 and U73343 were from BIOMOL (PA); pertussis toxin (PTX), FD98059, and SB203580 were from Calbiochem (San Diego, CA); keratinocyte SFM with additives, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, l-glutamine, and fetal calf serum (FCS) were from Life Technologies, Inc. (Paisley, Scotland); recombinant human factor VIIa (FVIIa), DEGRck-inactivated human FVIIa (FVIIaI), and recombinant human tissue factor pathway inhibitor (rTFPI) were all kind gifts from Novo-Nordisk (Bagsværd, Denmark); substrate FXa-1 was from Nycomed (Oslo, Norway); purified, BVV-activated human factor X was from Enzyme Research Laboratories (South Bend, IN); the fluorescent calcium indicator fura-2/AM and the surfactant Pluronic P-127 were from Molecular Probes (Eugene, OR); Bacto-dextrose was from Difco; Colorrapid was from Lucerna-Chem (Lucerne, Switzerland); monoclonal mouse anti-human TF (htf1) antibody and monoclonal anti-phospho-Erk 1/2 antibody were from Clontech (Lucerne, Switzerland); polyclonal anti-MAPK and anti-phospho-MAPK (Erk 1/2, p38MAPK, and JNK) antibodies and monoclonal anti-phospho-Erk 1/2 were from New England Biolabs (Beverly, MA); the anti-ΕPR-1 monoclonal antibody (B6) was kindly provided by Dr. D. Altiere (Yale University School of Medicine, New Haven, CT); anti-platelet-derived growth factor BB (PDGFBβ) was from R&D Systems (Oxon, UK); anti-FV and DEGRck-inactivated human FXa (FXai) were from Hema-globin (San Diego, CA); keratinocyte SFM with additives, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, l-glutamine, and fetal calf serum (FCS) were from Life Technologies, Inc. (Paisley, Scotland); recombinant human factor VIIa (FVIIa), DEGRck-inactivated human FVIIa (FVIIaI), and recombinant human tissue factor pathway inhibitor (rTFPI) were all kind gifts from Novo-Nordisk (Bagsværd, Denmark); the PAR2 agonist peptide SLIGRL (50 μM) or thrombin (1 unit/ml). Panel A, average responses in 330–580 cells. Panel B, examples of single cell responses to FVIIa and FXa. Different cells are shown in the 0–15-min and in the 30–40-min time windows. Arrows indicate addition of agonist.

Isolation of mRNA and Northern Blot Analysis

In these experiments medium was always changed to DMEM without additives for 2 h prior to addition of agonist to the cells. Unless otherwise stated, pretreatment with inhibitor or vehicle was done in this 2-h period. Agonist or vehicle was made up to 20% of the final volume, preheated to 37 °C, and added gently to the cells. Cells were harvested by washing in ice-cold PBS, then scraped off into 0.4 ml of lysis buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol) and sheared with a 21-gauge syringe. For isolation of mRNA, oligo(dT)-conjugated magnetic beads (Dynal, Oslo, Norway) were used according to the manufacturer's instructions. Samples were run on agarose/formaldehyde gels in MOPS buffer and blotted. Prehybridization and hybridization were performed in ExpressHyb™ solution from CLONTECH. Complete cDNAs were used to generate probes for egr-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and TF as described by Feinberg and Vogelstein (29, 30). The oligonucleotide probe used to detect the TF cytoplasmic domain had the sequence 5'-CTGGGGAGTCTCTCTTCAGCTCAGCTGCACACTCTGCCG-3' with a modified 3′-end.
FIG. 2. Up-regulation of egr-1 mRNA in HaCaT cells. Changes in egr-1 mRNA levels with time in response to FVIIa (100 nM), FXa (174 nM), or thrombin (1 unit/ml) in HaCaT cells. Northern blots were hybridized with 32P-labeled probes for egr-1 and GAPDH. The results are representative of three independent experiments.

FIG. 3. Effects of various concentrations of FVIIa and FXa on induced levels of egr-1 mRNA and [Ca2+]c in HaCaT cells. Upper panels show the effect of increasing concentrations of FVIIa and FXa on levels of egr-1 mRNA. Cells were harvested 45 min after agonist addition. The lower panels show average responses with their standard errors in logarithmic plots. The egr-1 intensities were normalized to GAPDH intensities for the same sample and further normalized to cells treated with vehicle alone. For the egr-1 data (closed circles), each point is based on four independent mRNA isolations from cells harvested 45 min after agonist addition. Concentration-dependent increases in maximal Ca2+ response during the first 210 s after addition of protease are plotted in the same graph (open circles, n = 65–280). Results are presented as average values and their standard error.

3'. For reprobing, the filters were stripped in 0.5% SDS at 100 °C for 5–10 min. Quantitation was done using a PhosphorImager (Molecular Dynamics).

Immunoblotting
Cells were washed as for mRNA isolation, and lysed directly in a reducing SDS sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue). Cell lysates were sonicated to shear DNA, denatured for 5 min at 100 °C and resolved by SDS-polyacrylamide gel electrophoresis, blotted onto Immobilon P membranes (Millipore), and the membranes blocked for 1 h in Tris-buffered saline with Tween (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween) with 5% BSA. Immunoblotting with anti-phospho-MAPK antibody and anti-MAPK antibody was used in the control cultures. The Ca2+ imaging and registration software has been developed in our laboratory (31). The cytosolic Ca2+ concentration was calculated using the equation [Ca2+]c = Kd · β (R - Rmin)/(Rmax - R) (32). Calibrations were done as described previously (33). The experiments were carried out at 37 °C.

Quantitative Analysis

Calcium—The maximum and average Ca2+ increases were calculated as the difference between the values after application of agonist and the average Ca2+ levels before. The average reflects the integral of the Ca2+ response. Cells with spontaneous responses before addition of agonist (ranging from 0 to 13% in different experiments) were excluded by not considering cells that during the first 60 s of observation had either a higher absolute Ca2+ level than 400 nM or a difference of more than 75 nM between the maximal and minimal Ca2+ levels. All other cells, both responders and non-responders, were included in the calcul-
**TABLE I**

Effect of extracellular inhibitors on increased levels of [Ca\(^{2+}\)], and egr-1 mRNA induced by FVIIa and FXa in HaCaT cells

| Pretreatment | FVIIa (100 nM) | FXa (174 nM) |
|--------------|---------------|---------------|
| Ca\(^{2+}\) | egr-1 | Ca\(^{2+}\) | egr-1 |
| Inhibition \(\pm\) S.E. (%) | n | Inhibition \(\pm\) S.E. (%) | n |
| FVIIai 100 nM | 87 \(\pm\) 4*** | 105 | 95 \(\pm\) 5** | 10 |
| FXa 174 nM | -7 \(\pm\) 7 (NS) | 66 | -14 \(\pm\) 4 (NS) | 103 |
| hirF-1 25 \(\mu\)g/ml | 83 \(\pm\) 3*** | 100 | 9 \(\pm\) 8 (NS) | 54 |
| Anti-PDGFB 10 \(\mu\)g/ml | -1 \(\pm\) 5 (NS) | 72 | 9 \(\pm\) 13 (NS) | 3 |
| Anti-FV 10 \(\mu\)g/ml | 24 \(\pm\) 3 (NS) | 196 | 11 \(\pm\) 10 (NS) | 4 |
| Hirudin 5 units/ml | 91 \(\pm\) 5** | 72 | 9 \(\pm\) 109 (NS) | 4 |

* 10 \(\mu\)g/ml B6 was used in mRNA studies.

** 2:1 mixture of TFPI and FXa (87 nM), FXa (174 nM), and thrombin (0.25 unit/ml) as indicated. Cells were harvested for mRNA isolation 45 min after agonist addition. Northern filters were hybridized with \(^{32}P\)-labeled probes to egr-1 and GAPDH. Quantitation of data in Table I.

Inhibition was calculated as described under “Experimental Procedures.” In each experiment agonist response without the inhibitor was taken as 100%. For [Ca\(^{2+}\)]\(_c\), calculations were based on the average Ca\(^{2+}\) values during the first 210 s after addition of agonist; cells were washed after pretreatment. Pretreatment did not affect the percentage of excluded cells. For egr-1 mRNA, no wash was introduced between pretreatment and agonist addition; cells were harvested 15 min after addition of agonist. RNA blots were hybridized with egr-1 and GAPDH probes.

**RESULTS**

Choice of Cell Types—We have previously reported that FVIIa triggers Ca\(^{2+}\) responses in MDCK cells, in COS-1 cells transfected to express TF, in J82 cells and in human umbilical vein endothelial cells (HUVEC) (1), and that FXa induced responses similar to FVIIa in MDCK cells (12). The signals varied between cell types and between individual cells within a population (1, 12). The response rate in MDCK was close to 100% (1, 12), but their canine origin served to limit our access to antibodies and other useful reagents. The spontaneously immortalized, non-tumorigenic keratinocyte human cell line (HaCaT) is a high level constitutive producer of TF. HaCaT cells responded with increased Ca\(^{2+}\) levels to both FVIIa and FXa, as well as to thrombin and SLIGRL, showing that this line also expresses PAR2 and thrombin receptors (Fig. 1, panel A). FVIIa and FXa both triggered a sustained elevation of [Ca\(^{2+}\)]\(_c\) (Fig. 1, panel B) in almost all cells tested. Their response pattern differed from that of MDCK cells (12). In HaCaT cells the Ca\(^{2+}\) oscillations were less prominent and non-synchronous and were replaced by a continuous elevation of [Ca\(^{2+}\)]\(_c\), generally failing to reach baseline during the initial minutes of response. The responses lasted approximately as long as the

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*Fig. 5. Effect of some inhibitors and antibodies on the induction of egr-1 mRNA by FXa. Cells were pretreated (2 h) with DMEM (vehicle) and an inhibitor or anti-PDGFBB (10 \(\mu\)g/ml) for 2 h prior to addition of vehicle (b) or FVIIai (174 nM), B6 (anti-EPR-1; 10 \(\mu\)g/ml), anti-PDGFBB (10 \(\mu\)g/ml), anti-FVa (10 \(\mu\)g/ml), or hirudin (5 units/ml) prior to addition of vehicle (c), FXai (87 nM), a preincubated 3:1 mixture of TFPI and FXa (87 nM), FXai (174 nM), and thrombin (0.25 unit/ml) as indicated. Cells were harvested for mRNA isolation 45 min after agonist addition. Northern filters were hybridized with \(^{32}P\)-labeled probes to egr-1 and GAPDH. Quantitation of data in Table I.

*Fig. 6. FVIIa-induced egr-1 up-regulation in TF-transfected COS-1 cells. COS-1 cells were transfected to express TF (wild type or truncated 2445). Controls were transfected with empty vector. Stable transfecants were pretreated with DMEM (vehicle) or FVIIai (100 nM) for 2 h prior to addition of vehicle (b) or FVIIai (50 nM). Cells were harvested for mRNA isolation 45 min after agonist addition. The same blot was hybridized with \(^{32}P\)-labeled probes to egr-1, TF, and GAPDH, and after stripping, with an oligonucleotide probe corresponding to the sequence deleted from the TF cytoplasmic domain. The bars (upper part) show the average increase of egr-1 mRNA relative to GAPDH with their standard errors from four experiments.

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*Fig. 5. Effect of some inhibitors and antibodies on the induction of egr-1 mRNA by FXa. Cells were pretreated (2 h) with DMEM (vehicle) and an inhibitor or anti-PDGFBB (10 \(\mu\)g/ml) for 2 h prior to addition of vehicle (b) or FVIIai (174 nM), B6 (anti-EPR-1; 10 \(\mu\)g/ml), anti-PDGFBB (10 \(\mu\)g/ml), anti-FVa (10 \(\mu\)g/ml), or hirudin (5 units/ml) prior to addition of vehicle (c), FXai (87 nM), a preincubated 3:1 mixture of TFPI and FXa (87 nM), FXai (174 nM), and thrombin (0.25 unit/ml) as indicated. Cells were harvested for mRNA isolation 45 min after agonist addition. Northern filters were hybridized with \(^{32}P\)-labeled probes to egr-1 and GAPDH. Quantitation of data in Table I.

*Fig. 6. FVIIa-induced egr-1 up-regulation in TF-transfected COS-1 cells. COS-1 cells were transfected to express TF (wild type or truncated 2445). Controls were transfected with empty vector. Stable transfecants were pretreated with DMEM (vehicle) or FVIIai (100 nM) for 2 h prior to addition of vehicle (b) or FVIIai (50 nM). Cells were harvested for mRNA isolation 45 min after agonist addition. The same blot was hybridized with \(^{32}P\)-labeled probes to egr-1, TF, and GAPDH, and after stripping, with an oligonucleotide probe corresponding to the sequence deleted from the TF cytoplasmic domain. The bars (upper part) show the average increase of egr-1 mRNA relative to GAPDH with their standard errors from four experiments.
**Table II**

| Pretreatment | FVIIa | FXa | SLIGRL | Thrombin | Bradykinin |
|--------------|-------|-----|--------|----------|------------|
| HSS          | 0 ± 8 | 0 ± 2 | 0 ± 2 | 0 ± 4 | 0 ± 2 |
| FVIIa        | 99 ± 5 | 69 ± 2 | 18 ± 7 (NS) | 20 ± 7 (NS) | ND |
| FXa          | 91 ± 2 | 99 ± 1 | 59 ± 2 | 63 ± 3 | ND |
| SLIGRL       | 57 ± 2 | 55 ± 2 | 88 ± 1 | 37 ± 3 | ND |
| Thrombin     | 7 ± 5 (NS) | 21 ± 3 (NS) | ND | 95 ± 1 | ND |
| Bradykinin   | 16 ± 4 (NS) | 7 ± 2 (NS) | 19 ± 7 (NS) | ND | 67 ± 2 |
| Trypsin      | 81 ± 1 | 96 ± 1 | 74 ± 2 | 81 ± 2 | -22 ± 3 (NS) |

*See Footnote c to Table I. ND, not determined.*

MDCK cell responses (12), although this has not been explored in detail. If not otherwise stated, the experiments reported have been carried out with the HaCaT cell line.

**Transcriptional End Point—**To facilitate dissection of the receptor/signaling system(s) engaged by FVIIa and FXa, we looked for a rapidly expressed, abundant mRNA regulated by these factors. The early growth response gene egr-1 showed rapid (30–60 min), transient (back to basal level within 2 h) increase in the HaCaT cells (Fig. 2). Inhibition of transcription by actinomycin D (10 μg/ml) added 5 min prior to stimulation abolished the induction of egr-1 by both proteases (p < 0.001; data not shown). The increase in egr-1 mRNA levels is thus highly likely to be a result of induced transcription rather than mRNA stabilization. This is the first demonstration of a transcription factor induced by these two clotting factors.

Both [Ca^{2+}]_i and egr-1 mRNA responses were concentration-dependent. EC50 values were an order of magnitude lower for the egr-1 mRNA response than for the calcium response (3 and 54 nM, respectively for FVIIa; 13 and 130 nM, respectively, for FXa) (Fig. 3). Both proteases gave a maximum egr-1 response at concentrations below the plasma concentrations of the corresponding zymogen precursor (10 nM for FVII and 178 nM for FX) (34).

The Ca^{2+} responses in MDCK cells to FVIIa and FXa were previously shown to be specific (i.e., not caused by contaminants in the agonist preparations) and to require their intact proteolytic activity (12). These experiments were repeated and expanded to ascertain that the Ca^{2+} and egr-1 mRNA responses seen in the HaCaT cells were specifically caused by the same factors in their activated state. Active site-inhibited FVIIa (FVIIai) did not induce responses in [Ca^{2+}]_i (data not shown) or egr-1 mRNA (Fig. 4, panel A; Table I). A marked inhibition of both egr-1 up-regulation and Ca^{2+} increase was seen when pretreating the cells with a neutralizing monoclonal antibody to TF (htf1) (Fig. 4, panels A and B; Table I). This excluded involvement of endotoxin and other contaminants, as well as demonstrating also in the HaCaT cell line the absolute requirement for TF and for proteolytic activity of the agonists. Pretreatment of the cells with FVIIai inhibited the effect of FXa. This inhibition was to a large extent reversible when cells were washed at pH 4 to remove FVIIai (Fig. 4, panel C) (28).

The binding site for FXa on HaCaT cells is unknown. The two main cellular components known to bind FXa are EPR-1 and FVa. Antibodies expected to block FXa binding to either of these were tested, and none had any effect on the egr-1 mRNA or [Ca^{2+}]_i, changes in response to FXa (Fig. 5, Table I). Neither rPDGFBB directly (data not shown) nor antibodies to PDGFBB had any effect in our system (Fig. 5, Table I). HaCaT cells may not express PDGF receptors. Active site-inhibited FXa (FXai) did not induce responses in [Ca^{2+}]_i or egr-1 mRNA (Fig. 5, Table I). In contrast to the effect of FVIIai on the response to FXa, pretreatment with FXai had no inhibitory effect on a subsequent incubation with FXa (Fig. 5, Table I), either when the cells were washed at pH 7.4 prior to addition of FXa (Ca^{2+} response) or when left unwashed (egr-1 response).

Specificity was demonstrated by the fact that pretreatment of FXa with a recombinant preparation of human tissue factor...
pathway inhibitor (rTFPI), which is the main physiological inhibitor of FXa, abolished both [Ca$^{2+}$], and egr-1 mRNA responses (Fig. 5, Table I). TFPI is also an inhibitor of FVIIa, so the effects of FXa could still be attributable to contaminating FVIIa in the FXa preparation. FXa stimulation, however, was unaffected by preincubation of the cells with FVIIai, which would block the response to contaminating FVIIa (Table I). Finally, hirudin was used to exclude generation of trace amounts of thrombin as a source for the activation (Fig. 5, Table I).

**Transducing Receptors**—The active site requirement of FVIIa and the lack of proteolysis of TF when in complex with FVIIa suggest that the receptor may be more complex. A dual receptor system with one binding and one proteolytically activated transducing receptor may seem likely. The lack of direct correlation between TF expression and FVIIa responsiveness seen in CHO and HK-2 cells supports this view (see below). If TF was not directly involved in signaling but rather in binding of the agonist, one might expect removal of the cytoplasmic tail of TF to have little or no effect. Extracellular binding of FVIIa of the agonist, one might expect removal of the cytoplasmic tail of TF was not directly involved in signaling but rather in binding with a construct encoding wild type human TF (hTF1–263), a correlation between TF expression and FVIIa responsiveness should be unaltered (12, 35). We therefore compared the egr-1 up-regulation in response to FVIIa in COS-1 cells transfected with a construct encoding wild type human TF (hTF1–263), a construct encoding human TF lacking the 18 C-terminal amino acids (hTF1–245, "tailless" TF) or vector control. The egr-1 response in cells expressing the tailless TF construct was at least as high as in cells expressing wild type TF (Fig. 6). Vector-transfected cells, which had very low TF activity, did not respond. Hybridization of the same Northern blots with an oligonucleotide probe corresponding to the sequence deleted from TF confirmed that the cells did indeed express tailless TF (Fig. 6). For both TF constructs only a limited number (10–20%) of the transfected cells expressed TF, which probably explains the low level of egr-1 mRNA increase relative to that seen in HaCaT cells.

The four cloned PARs are candidate transducing receptor components for both FVIIa and FXa. We have previously excluded the candidacy of receptors that can be down-regulated by thrombin in MDCK cells. This leaves PAR2 as the only presently known candidate for being the FVIIa or FXa protease-activated receptor. PAR2 is activated by trypsin and by mast cell tryptase and may have additional, unidentified activators in the vasculature (36). Factor Xa has been reported to activate PAR2 to some degree (37). In heterologous desensitization of the Ca$^{2+}$ responses (Table II), pretreatment with low concentrations of trypsin or with the PAR2 agonist SLIGRL for 45 min down-regulated the response to FVIIa by 81% and 57%, respectively. The FXa effect was similarly reduced by 98% and 58%. Tryptsin inhibited all the protease-inducible Ca$^{2+}$ signals tested, but not the response to bradykinin (Table II). Under the conditions used, trypsin treatment did not reduce cell surface TF (data not shown). Taken together, these results indicated the trypsin sensitivity of the signaling receptors for FVIIa and FXa. However, various cell surface substrates may have been cleaved by trypsin, which makes these data difficult to interpret. In addition, the desensitization by preincubation with SLIGRL may suggest that PAR2 or a close homologue was involved (Table II).

The question of a role for PAR2 was therefore approached more directly in a transfected CHO cell model. Neither FVIIa nor SLIGRL had any effect on the Ca$^{2+}$ levels of untransfected CHO cells. PAR2-transfected CHO cells were highly responsive to the PAR2 peptide agonist but gave no significant Ca$^{2+}$ response to the two coagulation factors. The construct pCDNA3bTF1–263 was then transfected into CHO cells expressing PAR2. The resulting cells expressed the PAR2 receptor, and all cells responded well to SLIGRL. In addition, more than 50% of the cells expressed high amounts of TF on their surface, fully active in triggering blood coagulation. These cells thus carried high levels of functional TF as well as PAR2, but gave no Ca$^{2+}$ signal when exposed to FVIIa or FXa (Fig. 7A). In parallel experiments, human HK-2 cells were shown to respond to the SLIGRL peptide (and thus to carry PAR2), to thrombin and (less pronounced) to FXa, but not at all to FVIIa, although the HK-2 line also expresses FXa (Fig. 7B). Increasing TF expression by induction with tumor necrosis factor-α did not alter the result. Thus, two different cell lines, both carrying PAR2 and TF on their surfaces, were unresponsive to FVIIa, clearly demonstrating that TF and PAR2 are not sufficient for this response. The desensitization of the FVIIa response by SLIGRL suggested that PAR2 or a close homologue may be involved, although factor VIIa did not down-regulate the SLIGRL response. In signaling triggered by FXa desensitization experiments suggested a possible involvement of PAR2 or a close homologue, but PAR2 alone is clearly not sufficient to mediate this signal.
Acting on the circumstantial evidence that the signaling receptor components for FVIIa and FXa belonged to the protease-activated subfamily of G protein-coupled receptors, the effect of pertussis toxin on the responses to the coagulation factors was compared with the effects on signals induced by the other agonists: thrombin, SLIGRL, bradykinin, and ATP. Significantly reduced but not abolished Ca\(^{2+}\) signaling was seen with ATP, thrombin, and SLIGRL when HaCaT cells were incubated with PTX (500 ng/ml) for 16–20 h prior to agonist addition (Fig. 8, A and B). The response to FVIIa was not altered, whereas the response to FVIIa was markedly reduced over the first few minutes, indicating that these two factors utilize different signaling receptors. [Ca\(^{2+}\)]\(_i\) in the cells treated with PTX and FVIIa reached the level of control cells (treated with agonist only) after 3–4 min and remained elevated. In Northern blots of egr-1 mRNA, essentially no differences caused by pretreatment with PTX were observed (Table III), showing that the initial part of the Ca\(^{2+}\) signal was not necessary for the egr-1 response.

**Intracellular Signaling Pathways Activated by FVIIa and FXa**—Signaling pathways activated upon binding of FVIIa and FXa are insufficiently described. Besides our work on Ca\(^{2+}\) signaling (1, 12), phosphorylation of Erk 1/2 has been observed (3). Addition of agonist, be it FVIIa or FXa, leads within seconds to Ca\(^{2+}\) release through a mechanism mediated by phosphatidylinositol-specific phospholipase C (PI-PLC) in both MDCK (12) and HaCaT cells (data not shown), as evidenced by the effect of its commonly used but not entirely specific inhibitor U73122 (38). Its most important unspecific effect is to cause release of Ca\(^{2+}\) from intracellular stores (39). No such effect was seen in our experiments. The Ca\(^{2+}\) signal most probably lies on the pathway to induction of egr-1 expression, since U73122 rapidly inhibited also this end point (Table III).

We confirmed in HaCaT cells the earlier report of the phosphorylation of Erk 1/2 by FVIIa (3) (Fig. 9). In contrast to that report (3), we also observed increased phosphorylation of key components of two other MAPK pathways investigated (p38MAPK, and C-Jun N-terminal kinase (JNK), Fig. 9). In addition, FXa induced increased phosphorylation in the same three MAP kinases (Fig. 9). In all cases preincubation with FVIIai abrogated the phosphorylation induced by FVIIa. An extra 44-kDa band seen in the phospho-JNK (p-JNK) blot was probably pErk 1, as it was absent in samples pretreated with PD 98059 (Fig. 9), and its detection in immunoblots was blocked by a monoclonal antibody to pERK (data not shown). Consistent with a role for Erk 1/2 phosphorylation in the signaling leading to enhanced egr-1 transcription, inhibition of Erk kinases Mek 1/2 by PD 98059 (Fig. 9) also inhibited up-regulation of egr-1 mRNA by both coagulation factors (Table III). Another inhibitor, SB 203580, with specificity for p38MAPK, did not inhibit the egr-1 response to either factor (Table III).

**DISCUSSION**

We have previously demonstrated that coagulation factors VIIa and Xa induce intracellular Ca\(^{2+}\) signals in various cell types, although not in all (1, 12). Signal transduction initiated by these factors is poorly understood. Most of our previous work was carried out using a MDCK cell line, which, being of canine origin, limited the access to useful antibodies and other reagents. In initial experiments we therefore screened various human cell lines and found that the constitutively TF-producing keratinocyte cell line HaCaT responded with marked Ca\(^{2+}\) elevation when exposed to FVIIa or FXa.

Using this cell line we have confirmed the absolute requirement of TF for FVIIa-induced signaling as well as the absolute necessity for both factors being in their proteolytically activated state. We then proceeded to establish a new end point for studies of the transduction pathway(s) in that we discovered that mRNA for the transcription factor Egr-1 was markedly (up to 12-fold) up-regulated when HaCaT cells were exposed to FVIIa or FXa. This is the first description of a link between regulation of a transcription factor and the initiation of the clotting cascade. The increase of the level of egr-1 mRNA required the same conditions as the Ca\(^{2+}\) response (i.e. FVIIa and FXa in their proteolytically active state, and an absolute requirement for availability of TF binding in the case of FVIIa). The difference in the EC\(_{50}\) values of the clotting factors for the two responses (Fig. 3), being approximately an order of magnitude lower for the egr-1 response, may be explained by the different time windows of observation. For both proteases (FVIIa and FXa), the EC\(_{50}\) values for the egr-1 response were well below the levels of the corresponding circulating (unactivated) clotting factors in plasma. The average egr-1 mRNA level induced by FVIIa was higher than that induced by FXa, for the Ca\(^{2+}\) signals the opposite was the case, and the FXa-induced response did not plateau.

Using the Ca\(^{2+}\) response and up-regulation of egr-1 mRNA as end points, we addressed the questions of what receptors were engaged by the two proteases and what signaling pathways they activated. TF is required for FVIIa signaling (1, 12), and we have suggested that TF acts as a co-factor, concentrating FVII/FVIIa at the cell surface, rather than as a signal-transducing receptor. The evidence for this has been the lack of Ca\(^{2+}\) signals in several cell lines that express TF constitutively and the lack of ability of active site inhibited FVIIa to induce signals when binding to TF. FVIIai binds TF with even higher affinity than FVIIa (3). The evidence for this has been the lack of Ca\(^{2+}\) signals in several cell lines that express TF constitutively and the lack of ability of active site inhibited FVIIa to induce signals when binding to TF. FVIIai binds TF with even higher affinity than FVIIa (3). Using this cell line we have confirmed the absolute requirement of TF for FVIIa-induced signaling as well as the absolute necessity for both factors being in their proteolytically activated state. We then proceeded to establish a new end point for studies of the transduction pathway(s) in that we discovered that mRNA for the transcription factor Egr-1 was markedly (up to 12-fold) up-regulated when HaCaT cells were exposed to FVIIa or FXa. This is the first description of a link between regulation of a transcription factor and the initiation of the clotting cascade. The increase of the level of egr-1 mRNA required the same conditions as the Ca\(^{2+}\) response (i.e. FVIIa and FXa in their proteolytically active state, and an absolute requirement for availability of TF binding in the case of FVIIa). The difference in the EC\(_{50}\) values of the clotting factors for the two responses (Fig. 3), being approximately an order of magnitude lower for the egr-1 response, may be explained by the different time windows of observation. For both proteases (FVIIa and FXa), the EC\(_{50}\) values for the egr-1 response were well below the levels of the corresponding circulating (unactivated) clotting factors in plasma. The average egr-1 mRNA level induced by FVIIa was higher than that induced by FXa, for the Ca\(^{2+}\) signals the opposite was the case, and the FXa-induced response did not plateau.

**TABLE III**

**Effects of various intracellular inhibitors on FVIIa- and FXa-induced up-regulation of egr-1 mRNA in HaCaT cells**

| Inhibitor | FVIIa | FXa |
|-----------|-------|-----|
| PTX 500 ng/ml | -22 ± 15 (NS)* | -29 ± 15 (NS) |
| U73122 5 mM | 72 ± 11** | 57 ± 7*** |
| SB203580 20 μM | -24 ± 14 (NS) | -49 ± 11 |
| PD98059 1 μM | -1 ± 5 (NS) | -25 ± 8* |
| PD98059 5 μM | 26 ± 8 (NS) | -5 ± 13 (NS) |
| PD98059 50 μM | 93 ± 4** | 94 ± 8** |

*See Footnote c to Table I.

Discussion

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In search for signaling receptors activated by FVIIa or FXa, the PARs were evident candidates, being the only receptors known to be activated by proteolysis. Heterologous desensitization experiments with the PAR agonists thrombin, trypsin, and SLIGRL in HaCaT cells confirmed our previous experiments in MDCK cells (12), demonstrating that receptors downregulated by thrombin (PAR1, -3, and -4) were not involved. Both PAR2 agonists (trypsin and SLIGRL) desensitized HaCaT cells to the effect of FVIIa and FXa while leaving TF intact, thus demonstrating the trypsin sensitivity of the putative signaling receptors. The facts that FXa has a moderate effect on PAR2 and that SLIGRL has an effect on the putative FXa signaling receptor suggest that there may be structural similarities between these two receptors.

However, in direct transfection experiments, the line of CHO cells carrying functional PAR2 as well as TF on their surface did not respond to FVIIa. Similar results were obtained using HK-2 cells, which express PAR2 and TF constitutively. Unless there are even more components to the initial binding/signaling receptor complex, we conclude that the signaling receptors are not yet found.

We have previously shown in MDCK cells, and confirmed in HaCaT cells here (data not shown) that the PI-PLC inhibitor U73122 completely abrogated the [Ca\textsuperscript{2+}] changes in response to either of the proteases. We show here that this compound strongly inhibited the egr-1 response as well, indicating that PI-PLC is a common mediator of the two responses (Table III), and consistent with the egr-1 response being on the same pathway and downstream of the Ca\textsuperscript{2+} signal. It has been reported (3), and we confirm here, that in certain cell types Erk 1/2 become phosphorylated when FVIIa binds to TF. We expand this observation by showing that exposure of HaCaT cells to FXa also leads to phosphorylation of Erk 1/2, and that both proteases induce significant phosphorylation of p38MAPK and JNK (Fig. 9). Induction of egr-1 by either of the two coagulation factors was abrogated by PD 98059 (Table III), an inhibitor of the Erk 1/2 kinases Mek 1/2. An inhibitor of p38MAPK, SB203580, had essentially no effect. This suggests a route to egr-1 induction via PI-PLC through Mek and Erk.

egr-1 is a zinc-finger transcription factor that recognizes the sequence GCGGGGCGC, which overlaps with the Sp1 consensus sequence. It is an immediate early growth response gene induced by cytokines, certain growth factors, DNA damaging agents, and heat shock, to mention but a few. Of particular interest in the present context is its role in the regulation of inducible transcription of the TF gene (41) and its increased DNA binding upon phosphorylation (42), which may be important for its binding in preference to Sp1. This may constitute a positive feedback cycle leading to increased levels of TF when coagulation is initiated. Induction of other genes by FVIIa or FXa in addition to egr-1 and TF has been reported, indicating that several physiological pathways may be affected. It remains to be seen what will be the physiological impact of these in vitro observations.

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