Factor VIIa and Thrombin Induce the Expression of Cyr61 and Connective Tissue Growth Factor, Extracellular Matrix Signaling Proteins That Could Act as Possible Downstream Mediators in Factor VIIa-Tissue Factor-induced Signal Transduction*

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Extracellular interactions of plasma clotting factor VIIa (FVIIa) with tissue factor (TF) on cell surfaces trigger the intracellular signaling events. At present, it is unclear how these signals influence phenotype. To elucidate this, we have used cDNA microarray technology to examine changes in transcriptional program in human fibroblasts in response to exposure to FVIIa. cDNA microarrays revealed that FVIIa binding to TF up-regulated the expression of Cyr61 and CTGF (connective tissue growth factor), the genes that encode extracellular matrix signaling proteins Cyr61 and CTGF, respectively. Northern blot analysis confirmed that FVIIa binding to TF markedly increased the expression of Cyr61 and CTGF in a time- and dose-dependent manner. FVIIa catalytic activity is required for the gene induction. In addition to FVIIa, thrombin also induced the expression of Cyr61 and CTGF. Hirudin abolished the thrombin-induced expression of these mRNAs but not the FVIIa-induced expression. FVIIa-induced expression of Cyr61 appears not to involve the currently known protease-activated receptors (PARs), whereas thrombin-induced expression involves the activation of PAR1 and possibly an additional PAR. Various intracellular signaling pathway inhibitors exhibited different inhibitory pattern on FVIIa and thrombin-induced up-regulation of Cyr61. Cyr61 and CTGF could act as downstream mediators of FVIIa-TF in affecting various biological processes.

The primary function of plasma clotting factor VIIa (FVIIa), upon its binding to TF on the cell surface, is to initiate blood coagulation (1). However, a number of recent studies suggest that FVIIa binding to TF influences an array of important biological functions other than coagulation, such as angiogenesis (2, 3), embryo vascularization (4), and tumor metastasis (2, 5, 6). At present, it is unclear how FVIIa-TF contributes to these biological processes. Recent studies have shown that FVIIa binding to TF induces various intracellular signals (7, 8). The cumulative data so far suggest that FVIIa-TF mediates cell signaling by two distinct mechanisms, TF cytoplasmic domain-dependent (independent of the proteolytic activity of FVIIa) and FVIIa-TF proteolytic activity-dependent (independent of the cytoplasmic domain of TF) (7). A growing number of recent reports suggest that the later mechanism is a predominant mechanism. For example, binding of FVIIa to cell surface TF was shown to induce intracellular Ca2+ oscillations in a number of TF expressing cells (9, 10), transient phosphorylation of tyrosine in monocytes (11), activation of MAP kinase (12, 13), alteration in gene expression in fibroblasts (14), up-regulation of urokinase receptor in tumor cells (15), and Egr-1 expression in HaCaT cells (16). Because active site-inactivated factor VIIa (FVIIai) fails to induce many of the above signaling responses, from Ca2+ oscillations (10) to MAP kinase activation (12) and gene induction (12, 16), it appears that the catalytic activity of FVIIa is required for FVIIa-TF-mediated signal transduction. Furthermore, specific inhibitors for FXa and/or thrombin failed to inhibit the FVIIa-induced signal transduction (12, 13, 16), suggesting that the FVIIa-induced signal transduction arises directly from FVIIa proteolytic activity and not from possible generation of downstream activated coagulation factors, such as FXa and thrombin. TF cytoplasmic domain is not required for FVIIa-TF-induced proteolytic signaling because cells transfected with TF mutant lacking the cytoplasmic domain were fully capable of mediating FVIIa-induced p44/42 MAP kinase phosphorylation (13) and up-regulation of Egr-1 (16).

At present, it is unclear how various intracellular signals induced by proteolytically active FVIIa contribute to pathophysiological functions, such as angiogenesis and tumor metastasis. Studies have shown that the FVIIa-TF complex has a strong effect on migration of cultured smooth muscle cells (17) and pancreatic cancer cells (15). The catalytic activity of FVIIa was essential for the increased cell migration in these cells. In preliminary studies, Seigbahn et al. (18) showed that incubation of fibroblasts with FVIIa reduces the platelet-derived growth factor concentration required to stimulate fibroblast migration by a 100-fold. Overall, all of the above data suggest that a signaling cascade involving the proteolytic function of FVIIa can result in phenotypic changes that are crucial for angiogenesis and tumor metastasis. However, so far, no clear connection has been established among the various FVIIa-induced intracellular changes. Furthermore, it is yet to be shown how FVIIa-induced intracellular changes lead to phenotypic changes. One possibility is that FVIIa could induce the expression of growth regulators that act downstream to induce cellular processes.

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To investigate the above possibility, in the present study, we have examined alterations in gene expression in human fibroblasts in response to FVIIa using cDNA microarray technology. Our data reveal that the cellular expression of only very few genes was detectably altered in fibroblasts upon exposure to FVIIa. Further studies confirmed that FVIIa up-regulated the expression of two related genes, Cyr61 and CTGF, which are growth factor-inducible immediate early genes, the products of which were shown to promote cell adhesion, augment growth factor-induced DNA synthesis, and stimulate cell migration in fibroblasts and endothelial cells.

MATERIALS AND METHODS

Cell Culture—A fibroblast cell line derived from normal embryonic lung tissue (WI-38, obtained from ATCC, Manassas, VA) was grown in Dulbecco’s modified Eagle’s medium (GLUTAMAX with high glucose from Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Celsior, Herndon, VA), 1% penicillin and streptomycin (BioWhittaker, Walkersville, MD), and 1% l-glutamine (Bio-Whittaker). When the cells reached about 80% confluency, the serum-containing growth medium was removed and the cells were washed once with Dulbecco’s modified Eagle’s medium and then incubated with Dulbecco’s modified Eagle’s medium for 20–24 h to make the cells quiescent.

Proteins—Recombinant human FVIIa, a gift from Novo Nordisk (Gentofte, Denmark), was reconstituted in sterile water at a concentration of 1–1.3 mg/ml. The stock FVIIa solutions were checked for contaminating trace levels of endotoxin using limulus amebocyte lysate (BioWhittaker), and none was detected (detection level, 30 pg). Active site-inactivated factor FVIIa was prepared by incubating recombinant human FVIIa with a peptidyl inhibitor, d-Phe-t-Phe-Arg chloromerthyl ketone, as described earlier (20). Human factor VII was purified as described earlier (21). Factor VII preparations contained 0.03–0.4% (w/w) FVIIa in them. Human plasma FXa was purified as described previously (22) or obtained from Enzyme Research Laboratories (South Bend, IN). Thrombin was obtained from Enzyme Research Laboratories. Recombinant tick anticoagulant protein (TAP) was kindly provided by George Vlasuk (Corvas, San Diego, CA) and recombinant hirudin was obtained from either Sigma or Calbiochem (San Diego, CA). Preparation of monospecific, polyclonal rabbit anti-human TF IgG was described earlier (23). Monospecific polyclonal rabbit anti-human factor X antiserum was prepared by immunizing a rabbit with purified homologous human factor Xa and IgG fractions from the antiserum were obtained by precipitation with 40% ammonium sulfate saturation followed by DEAE-Affil-Blue chromatography.

cDNA Microarray—WI-38 cells were cultured to 80% confluency in T-75 flasks and serum-deprived for 24 h to cause them to enter the quiescent state as described above. The culture medium was replaced with fresh serum-free Dulbecco’s modified Eagle’s medium (supplemented with 5 mM NACl) and allowed to stabilize for 2 h in tissue culture incubator. Then, the cells were treated with a control medium or the medium supplemented with recombinant FVIIa (5 μg/ml, 100 nm) for 90 min at 37 °C in a culture incubator. At the end of 90 min of treatment, total RNA was isolated from the control and FVIIa-treated cells using TRIZOL (Life Technologies, Inc.). Poly(A) RNA was purified by a double pass over Oligo Tex mRNA isolation columns as described in manufacturer’s technical bulletin (Qiagen, Valencia, CA). Eight hundred ng of highly purified poly(A) RNA from the control and FVIIa-treated cells were sent for cDNA microarray analysis service (UniGEM Human V microarray, Genome Systems Inc, St. Louis, MO).

Northern Blot Analysis—Total RNA was prepared using TRIZOL reagent from quiescent monolayers of WI-38 cells that were exposed to FVIIa and other materials as described under “Results.” Northern blot analysis was carried out using a standard procedure. Briefly, 10 μg of total RNA was size-fractionated by gel electrophoresis in 1% agarose/8% formaldehyde gels and transferred onto the nitrocellulose membrane by a capillary blot method. Northern blots were prehybridized at 42 °C for 2 h with a solution containing 50% formamide, 5 × SSC, 50 mM Tris·HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, 100 μg/ml denatured salmon sperm DNA, and 0.2% bovine serum albumin before hybridizing with either 32P-labeled Cyr61 or CTGF cDNA probes (10 cmap/ml) in the same solution for overnight. The hybridized membranes were exposed to either DuPont NEN or Fuji RX x-ray film. For quantification purposes, the membranes were exposed to phosphor screens for 1–4 h, and the exposed screens were analyzed in a PhosphorImager (Molecular Dynamics) using Image-Quant NT software. The units (counts) obtained in various treatments were normalized to counts present in control-treated sample and/or controls present in a positive control, such as FVIIa-treated cells.

FVIIa Binding to Cell Surface TF—125I-FVIIa binding to cell surface TF was determined as described earlier (24). Briefly, confluent monolayers of WI-38 in 12-well culture plates were incubated for 2 h at 4 °C with varying concentrations of 125I-FVIIa in a calcium-containing Hepes buffer (10 mM Hepes, pH 7.5, 150 mM NaCl, pH 7.5, buffer supplemented with 5 mM CaCl2 and 1 mg/ml fatty acid-free bovine serum albumin). Then, unbound 125I-FVIIa was removed, and the monolayers were washed four times with the buffer. The cells were removed from the well by trypsin digestion and counted for the radioactivity to determine the amount of cell-associated FVIIa. Parallel experiments were carried out in which the monolayers were first incubated for 30 min with rabbit anti-human TF IgG (100 μg/ml) before radioactive ligand was added to cells to obtain nonspecific FVIIa binding to cell surfaces. TF-specific binding was determined by subtracting the nonspecific FVIIa binding values from the corresponding binding values obtained in the absence of anti-TF antibody. In general, TFSpecific binding of 125I-FVIIa in WI-38 cells was in the range of 70–80%.

Factor VII Activation—Activation of factor VII bound to WI-38 cells was evaluated as described earlier (25). Briefly, monolayers of WI-38 cells were incubated at 4 °C for 90 min with 100 nM Factor VII (10 nM) in a buffer containing benzamidine·HCl (0.1 M) and calcium ions (5 mM) to allow factor VII binding to TF with minimal activation of factor VII. Then, the unbound factor VII was removed, and the monolayers were washed three times and overlaid with calcium-containing buffer. At indicated time periods, the supernatant was removed from the dish, and cell-bound 125I-Factor VII was eluted by incubating cells for 5 min with EDTA (5 mM). The eluates were reduced with 10% (v/v) 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Chromogenic Assay—WI-38 cells were cultured in 96-well culture plates and made quiescent as described above. After washing the cells, 5 μg/ml (100 nm) FVIIa in 100 μl of calcium-containing buffer was added to the wells containing cells or to the control wells coated with buffer alone (controls). After 45 min of incubation, 25 μl of chromogenic substrates for FXa or thrombin, i.e., Chromozym X and Chromozym TH (1.25 nmol), were added to the wells and allowed to stand for 3 h to develop color. The plate was read in a microplate reader (Molecular Devices) at 405 nm to obtain the sensitivity of the assay to detect the generation of traces of FXa and thrombin, trace concentrations of factor Xa, 0.1–2 ng/well (20 pm to 0.4 nm), or thrombin, 0.2–10 milliunits/well (17 pm to 0.87 nm) were added directly to cells cultured in parallel wells on the same plate before Chromozym X or Chromozym TH was added to the wells to develop color.

RESULTS

cDNA Microarray—The quiescent human fibroblasts (WI-38) were exposed to a control serum-free medium or the serum-free medium supplemented with recombinant human FVIIa (5 μg/ml, 100 nm) for 90 min. Poly(A) RNA was purified from these samples, and 600 ng of mRNA was labeled with either Cy3 or Cy5 fluorescence and then hybridized to the UniGem Human V chip containing 8000 sequence-verified expressed sequence tags, representing up to 5000 known human genes (service performed by Genome System Inc. for a fee). As controls, to measure sensitivity, monitor the reverse transcription reaction and purification, determine hybridization efficiency, and gain an overall view of the quality and performance of the assay, several known concentrations of reference cDNA were spiked into mRNAs during the probe generation reaction. Analysis of these controls indicated the success of hybridization process (balance coefficient, 1.69).

Global analysis of experimental data revealed minimal differential expression in hybridization signals between the control and FVIIa-treated samples. Only a very small number of genes showed moderate differential expression. We found up-regulation of five genes (2–3.5-fold higher in FVIIa treatment), whereas one gene was down-regulated upon FVIIa treatment (2.4-fold lower) (∆ 2 is a conservative estimate for determining the minimum magnitude of real ratios). One of the FVIIa up-regulated genes (2.5-fold higher in FVIIa-treated cells),
FVIIa-induced Expression of Cyr61 and CTGF in Fibroblasts

Cyr61, encodes an extracellular matrix signaling protein that was recently shown to promote cell proliferation, migration and tumor metastasis (19). It is interesting to note that CTGF, a gene belonging to the same family as Cyr61, was at a level 1.8-fold higher in FVIIa-treated cells than in control cells. A repeat of cDNA microarray with mRNA samples obtained in cells exposed to FVIIa for 30 min confirmed the up-regulation of both Cyr61 and CTGF but not the other genes.

To validate the data obtained in microarray, we have subjected the RNA samples from the control and FVIIa-treated cells that were originally used for microarray analysis to Northern blot analysis and probed with radiolabeled Cyr61 cDNA. The data show that Cyr61 cDNA probe hybridized to a single transcript (approximately 2.0 kilobases) of RNA isolated from the control and FVIIa-treated cells. The hybridization signal was very faint for the RNA isolated from the control-treated cells, and the intensity of hybridization signal was higher for the RNA isolated from FVIIa-treated cells (data not shown). Quantification of the signal revealed that the expression of Cyr61 was 2.8-fold higher in cells exposed to FVIIa over the control-treated cells, which correlates well with the data of the microarray.

Kinetics of FVIIa-induced Expression of Cyr61—To determine the kinetics of Cyr61 expression, quiescent fibroblasts were treated for varying time periods with 100 nM FVIIa. Total RNA was extracted and subjected to Northern blot analysis. As shown in Fig. 1, Cyr61 expression was markedly increased in a time-dependent manner in FVIIa-treated cells. The expression was peaked at about 45 min and thereafter declined close to base levels. Cyr61 mRNA levels in cells treated with FVIIa for 45 min were 5-15-fold higher than in cells treated with a control vehicle.

To determine dose dependence of FVIIa, quiescent fibroblasts were treated with varying doses of FVIIa (0.1–5 μg/ml, 2–100 nM) for 45 min, and then total RNA samples from the cells were subjected to Northern blot analysis. As shown in Fig. 2A, treatment of fibroblasts with concentrations as low as 2 nM FVIIa was sufficient to induce the expression of Cyr61. Treatment of fibroblasts with 10 nM FVIIa, a concentration equivalent to the plasma concentration of factor VII, resulted in a prominent response, close to the maximal. The kinetics of FVIIa-induced dose-dependent expression of Cyr61 was quite similar to the kinetics of FVIIa binding to TF receptor on WI-38 cells (Fig. 2B). FVIIa-induced expression of Cyr61 was fully dependent on FVIIa binding to cell surface TF because preincubation of WI-38 cells with rabbit anti-human TF IgG (100 μg/ml) for 30 min before the addition of FVIIa completely blocked the FVIIa-induced expression of Cyr61 (see Fig. 11).

In an additional experiment, we investigated whether a plasma concentration of factor VII is sufficient to induce the expression of Cyr61. Quiescent fibroblasts were treated with 10 nM factor VII for varying time periods, and the induction of Cyr61 gene expression was evaluated by Northern blot analysis. The data show that a plasma concentration of factor VII effectively induced the expression of Cyr61 (Fig. 3A). However, it requires at least 2 h of incubation with factor VII to induce the expression of Cyr61. This is consistent with the observation that factor VII bound to cell surface TF was slowly autoactivated to FVIIa, and a 60-min time period was required for the conversion of a substantial amount of factor VII to FVIIa (Fig. 3B).

Factor VIIa-induced Expression of CTGF—CTGF, a molecule structurally related to Cyr61, exhibits biological responses similar to those of Cyr61. Because the relative ratio of CTGF expression in FVIIa-treated sample versus the control sample in the initial cDNA microarray was 1.8, which was lower than a conservative estimate of the lowest possible real magnitude, we did not initially choose this for further examination. However, after knowing the kinetics of FVIIa-induced expression of Cyr61 (i.e. FVIIa-stimulated expression of Cyr61 reached a peak at 45 min and thereafter was repressed to base levels within 2 h after the stimulation), we thought it possible that FVIIa could also induce the expression of CTGF and that the minor difference in CTGF expression that we observed in the cDNA microarray analysis between the control and FVIIa-treated cells might be the result of a 90-min treatment period selected for the microarray assay. Therefore, we have tested the kinetics of FVIIa-induced expression of CTGF by Northern blot analysis. As shown in Fig. 4, FVIIa treatment markedly induced the expression of CTGF mRNA steady state levels in a time- and dose-dependent manner. The kinetics of FVIIa-induced expression of CTGF were very similar to the kinetics of FVIIa-induced expression of Cyr61.

Involvement of Transcriptional Mechanism in Accumulation of Cyr61 and CTGF mRNA in FVIIa-treated Fibroblasts—To
investigate whether transcription is involved in FVIIa-mediated increase in Cyr61 mRNA steady-state levels, quiescent WI-38 cells were incubated with actinomycin-D (10 μg/ml) for 30 min before the addition of FVIIa for 45 min. As shown in Fig. 5, actinomycin-D treatment completely blocked the FVIIa-induced expression of Cyr61. This finding indicates a transcriptional mechanism for induction of Cyr61. To investigate whether de novo protein synthesis is required for the induction of Cyr61 mRNA by FVIIa, WI-38 cells were pretreated with a protein synthesis inhibitor, cycloheximide, before the cells were exposed to FVIIa for 45 min. As shown in Fig. 5, the stimulatory effect of FVIIa was not blocked by cycloheximide. In fact, cycloheximide markedly increased the FVIIa-induced Cyr61 mRNA accumulation, indicating a rapid degradation of mRNA involving a labile protein, a hallmark property of immediate-early genes. Similar results were obtained with FVIIa-induced expression of CTGF (data not shown).

Factor VIIa Catalytic Activity Is Required for Cyr61 Induction—To test whether FVIIa catalytic activity is required for the induction of Cyr61, WI-38 cells were treated with FVIIa and FVIIai for 45 min and the expression of Cyr61 was evaluated by Northern blot analysis. In contrast to FVIIa, FVIIai failed to induce the expression of Cyr61 (Fig. 6). These data suggest that FVIIa proteolytic activity is required for the induction of Cyr61. In this context, it may be important to point out that FVIIai was shown to bind to cell surface TF with the same or higher affinity than FVIIa (20, 26). Furthermore, the specific inhibitors of FXa and thrombin, i.e. TAP and hirudin, respectively, failed to abolish the FVIIa-induced expression of Cyr61 (Fig. 7). Quantitation of the hybridization signals from several independent experiments showed that TAP partially reduced the FVIIa-induced expression of Cyr61, whereas hirudin had only a minimal effect on the FVIIa-induced gene expression. Data for Cyr61 expression were as follows (percentages normalized to basal expression found in untreated cells): FVIIa, 684 ± 94%; TAP + FVIIa, 438 ± 135%; hirudin + FVIIa, 571 ± 109% (mean ± S.E., n = 5). Similar data were obtained with FVIIa-induced expression of CTGF. Although the difference between FVIIa-induced expression of Cyr61 in the presence of inhibitors was not statistically significant from Cyr61 expression in the absence of inhibitors (p = 0.173 for FVIIa versus TAP + FVIIa and p = 0.46, FVIIa versus FVIIai + hirudin), we cannot entirely discount the inhibitory effect of TAP on FVIIa-induced gene expression. At present, it is un-
WI-38 cells express various PARs by Northern blot analysis. Quiescent monolayers of WI-38 cells were preincubated with a control medium or the medium containing 200 nM recombinant TAP or 5 units/ml hirudin for 30 min before their exposure to FVIIa (100 nM), which was preincubated correspondingly with either a control medium, TAP, or hirudin. At the end of 45 min of treatment with FVIIa, total RNA was harvested and subjected to Northern blot analysis using radiolabeled 

Factor Xa and Thrombin Induce the Expression of Cyr61 and CTGF—In addition to FVIIa, we also tested the effect of purified plasma FXa and thrombin for their ability to induce the expression of Cyr61 and CTGF. Preliminary experiments revealed that both FXa (1 μg/ml, 20 nM) and thrombin (1 units/ml, 8.6 nM) effectively increased (10- and 25-fold, respectively) the steady state levels of both Cyr61 and CTGF mRNA (data not shown). In additional experiments, we used varying concentrations of factor Xa and thrombin and found that concentrations as low as 0.2 nM FXa and 0.008 nM thrombin were sufficient to induce detectable expression of Cyr61 (Fig. 8) and CTGF (data not shown).

In further experiments, we tested the effect of TAP and hirudin on FXa- and thrombin-induced expression of Cyr61. As shown in Fig. 9, TAP, as expected, completely inhibited the FXa-induced response. It is important to note that hirudin, a specific inhibitor of thrombin, also fully inhibited the FXa-induced response. These data raise a possibility that traces of thrombin, most probably generated on the cell surface by FXa, may be responsible for the FXa-induced response. Thrombin-induced expression of Cyr61 is fully inhibited by hirudin and TAP had no effect on the thrombin-induced gene expression (Fig. 9).

PAR Agonist Peptide-induced Expression of Cyr61—The above data clearly establish that thrombin induces the expression of Cyr61. Thrombin was known to activate a number of protease-activated receptors, such as PAR1, PAR3, and PAR4. To determine whether one or more PARs are involved in the induction of Cyr61 gene expression, first we examined whether WI-38 cells express various PARs by Northern blot analysis. These experiments revealed that WI-38 cells express mRNAs for all known PARs, PAR1, PAR2, PAR3, and PAR4 (data not shown). Next, we evaluated the ability of various PAR agonist peptides to induce the expression of Cyr61. As shown in Fig. 10, PAR1 agonist peptide markedly enhanced, by about 10-fold, the expression of Cyr61. PAR2 agonist peptide substantially enhanced (4-fold increase) the expression of Cyr61. PAR3 and PAR4 agonist peptides have only a minimal effect on the expression of Cyr61. These data suggest that thrombin-induced activation of Cyr61 gene expression, at least partly, is the result of activation of PAR1 by thrombin.

FVIIa-induced Expression of Cyr61 and CTGF Was Independent of FXa- and Thrombin-induced Response—Because our data show that both FXa and thrombin, in addition to FVIIa, would also induce the expression of Cyr61 and CTGF and that the FVIIa-induced response could be partially inhibited by the specific inhibitor of FXa, TAP, it is important for us to address whether FVIIa-induced expression of Cyr61 and CTGF stems from a direct action of FVIIa/TF or an indirect effect, mediated by generation of FXa and thrombin. We have used multiple independent approaches to address this.

First, we measured for possible generation of FXa and thrombin in our experimental system by using sensitive chromogenic assays. WI-38 cells were cultured in 96-well culture plates and then made quiescent using the same protocol that was used to measure FVIIa-induced expression of Cyr61. FVIIa (100 nM) was added to the wells containing the cells or to control wells that were allowed to incubate for 45 min. The generation of FXa and thrombin in the
peptide pretreated cells compared with the cells that were not
increase in PAR1 agonist peptide for 3 h (Fig. 13). However, the fold
and 6-fold, respectively, in cells that were pretreated with
FVIIa and thrombin increased the expression of PAR1 peptide agonist
did not make the cells fully refractory to PAR1 peptide revealed that preexposure of cells to
FVIIa-induced response but also desensitized the FVIIa-
preexposure of cells to thrombin not only desensitized the
FVIIa but not to FXa or thrombin. In reciprocal experiments,
completely desensitized the cells to the subsequently added
. As shown in Fig. 12
A,
Cyr61
expression was substantially lower in PAR1
agonsists and that thrombin up-regulates the expres-
activation results in cleavage of the receptor and the subsequent
internalization and degradation of the receptor. This makes
cells refractory to a subsequent protease-induced response via
activation of PAR1.
Desensitization of Transducing Receptors—The active site
requirement of FVIIa to induce gene expression suggests that
FVIIa induces signaling through proteolysis of a membrane-
associated protein, possibly a PAR. Protease-induced PAR ac-
activity results in cleavage of the receptor and the subsequent
internalization and degradation of the receptor. This makes
cells refractory to a subsequent protease-induced response via
the same receptor. We have used this property to test whether
FVIIa-induced signaling involves the activation of the same
receptor(s) that was activated by thrombin. Quiescent WI-38
30 min. Similarly, FVIIa (100 nM) and FXa (20 nM) were also incubated with antibodies before they
were added to the cells. Cells were treated with FVIIa and FXa with or
without antibodies for 45 min. Total RNA (10 µg) was subjected to
Northern blot analysis and probed with radiolabeled
Cyr61. A shows a representative autoradiograph, and B shows quantitative data.

Additional desensitization experiments conducted with
PAR1 peptide agonist revealed that preexposure of cells to
PAR1 peptide agonist did not make the cells fully refractory to
the subsequent stimulus with FVIIa or thrombin. For example,
FVIIa and thrombin increased the expression of Cyr61 by 3-
and 6-fold, respectively, in cells that were pretreated with
PAR1 agonist peptide for 3 h (Fig. 13). However, the fold
increase in Cyr61 expression was substantially lower in PAR1
peptide pretreated cells compared with the cells that were not
pretreated with PAR1 peptide agonist. In a reciprocal experi-
ment, preexposure of cells to FVIIa had no effect on PAR1
peptide agonist peptide-induced expression of Cyr61.

In further experiments, to assess the involvement of PAR2,
quiescent fibroblasts were treated with PAR2 peptide agonist
(25 µM) for 3 h before they were stimulated with FVIIa for 45
min. The data show that PAR2 desensitization had no signifi-
cant effect on FVIIa-induced expression of Cyr61. The mean
data from two experiments as follow (shown as a percentage of
control): PAR2 peptide for 45 min, 51%; PAR2 peptide for 3 h,
88%; PAR2 peptide for 3 h + PAR2 peptide for 45 min, 186%;
PAR2 peptide for 3 h + FVIIa for 45 min, 763%; FVIIa for 45
min, 1085%.

Overall, the above desensitization studies with FVIIa,
thrombin, and PAR peptide agonists suggest that FVIIa-
duced expression of Cyr61 does not involve the activation of
PAR1 and PAR2 and that thrombin up-regulates the expres-
sion Cyr61 through more than one pathway, one being the
activation of PAR1.

Effect of Various Intracellular Inhibitors on FVIIa- and
Thrombin-induced Up-regulation of Cyr61—To further
strengthen the observation that FVIIa and thrombin up-regu-
late the expression of Cyr61 through activation of different
signaling pathways, we have investigated the effect of various intracellular inhibitors on FVIIa- and thrombin-induced expression of Cyr61. As shown in Fig. 14, cholera toxin significantly inhibited both the FVIIa- and thrombin-enhanced expression of Cyr61, by 96 and 66%, respectively. MAP kinase inhibitor, PD 98059, markedly impaired (87% inhibition) the FVIIa-mediated increased expression of Cyr61, whereas it had a minimal effect (25% inhibition) on the thrombin-induced expression of Cyr61. Similarly, a protein tyrosine kinase inhibitor, herbimycin, and a phospholipase C inhibitor, U73122, suppressed the FVIIa-induced response by 52% and 80%, respectively, whereas they minimally inhibited (less than 25%) the thrombin-induced expression of Cyr61. The slight inhibition in the thrombin-induced expression of Cyr61 observed in cells treated with PD98059, herbimycin, and U73122 was not statistically significant (p values were 0.35 or higher when compared with thrombin-induced expression of Cyr61 in the absence of inhibitors), whereas their effect on FVIIa-induced expression was highly (p < 0.01 for PD 98059 and U73122) or moderately (p = 0.07 for herbimycin) significant. In contrast to the above inhibitors, a specific PI3-kinase inhibitor, LY 294002, markedly impaired both the FVIIa- and thrombin-induced expression of Cyr61 (100% and 80%, respectively). These data suggest that FVIIa and thrombin, predominantly, signal through the activation of two different signaling pathways, which could merge and/or overlap downstream.

**FIG. 12.** Desensitization of protease-mediated Cyr61 gene expression. Quiescent monolayers of WI-38 cells were treated with FVIIa (100 nM) (A) or thrombin (0.85 nM) (B) for 45 or 180 min. The monolayers that were exposed to FVIIa or thrombin for 180 min were subsequently treated for 45 min with a fresh addition of FVIIa (100 nM), FXa (20 nM), or thrombin (0.85 nM). Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled Cyr61. The top panels show autoradiographs from a representative experiment, and the bottom panels show quantitative data (n = 4, mean ± S.E.). For quantitative purposes, Cyr61 mRNA levels measured in quiescent cells treated with a control serum-free medium in the same experiment was taken as 100%.

**FIG. 13.** Effect of PAR1 peptide pretreatment on FVIIa- and thrombin-induced expression of Cyr61. Quiescent monolayers of WI-38 cells were treated with PAR1 peptide (25 μM) for 45 min or 3 h. The cells that were treated with PAR1 peptide for 3 h were restimulated for 45 min with the subsequent addition of FVIIa (100 nM) or thrombin (0.85 nM). In parallel, cells were treated with FVIIa (100 nM) for 3 h followed by PAR1 peptide (25 μM) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled Cyr61, and hybridization signals were quantitated using a Phosphoimager (n = 4, mean ± S.E.).

**DISCUSSION**

The primary function of plasma clotting FVIIa, upon its binding to TF on the cell surface, is to initiate blood coagulation. A number of recent studies suggest that FVIIa binding to TF not only triggers the coagulation cascade but also leads to other cellular processes such as angiogenesis and tumor metastasis (2). It is not yet clear how FVIIa-TF influences such complex biological processes. Although a number of recent reports (see Ref. 7) show that binding of FVIIa to TF induces intracellular signaling, it is unknown how these specific cellular events influence biological functions. One possibility is that FVIIa-induced signaling could lead to expression of growth regulators that act downstream to induce cellular processes. To investigate this possibility, in the present study, we have exposed human fibroblasts to FVIIa and examined changes in the transcriptional program using a cDNA microarray. We chose fibroblasts because these cells normally encounter serum, which contain growth factors and activated clotting factors in the context of vascular injury due to physical (e.g. surgery) or pathophysiological conditions. Furthermore, the temporal program of gene expression observed in response to serum suggests that fibroblasts are programmed to interpret the abrupt exposure to serum not as a general mitogenic stimulus but as a specific physiological signal (27). Characterization of transcriptional activation in response to serum and growth factors also suggests that fibroblasts are active participants in a conversation among the diverse cells that collectively control inflammation, angiogenesis, and wound healing (27).

cDNA microarray analysis with mRNA isolated from fibroblasts exposed to FVIIa for 90 min suggested a possible up-regulation of Cyr61. Northern blot analysis confirmed these data. In addition to Cyr61, the cDNA microarray also showed differential expression of four other genes, but the differential expression ratio was very close to the borderline significance, and in subsequent preliminary experiments, we could not confirm their differential expression by Northern blot analysis. In addition to Cyr61, FVIIa also up-regulated the expression of
Cyr61, a structurally related molecule, which elicits biological responses similar to those of Cyr61. FVIIa-induced expression of Cyr61 and CTGF was transient and FVIIa dose-dependent. The expression levels peaked between 30 and 60 min and thereafter reduced close to base levels in 2–3 h (Figs. 1 and 4). The kinetics of FVIIa dose-dependent expression of Cyr61 was quite similar to the kinetics of FVIIa binding to TF. A low concentration of FVIIa (2 nM), well below the level of the corresponding circulating zymogen, is sufficient to induce the expression of Cyr61, and the expression levels reached a peak close to maximal expression at 10 nM FVIIa (Fig. 2).

Induction of both Cyr61 and CTGF expression was dependent on FVIIa binding to cell surface TF because anti-TF IgG was shown to completely block the FVIIa-induced expression of both Cyr61 and CTGF. The catalytic activity of FVIIa is essential for its ability to induce the expression of Cyr61 and CTGF, as evidenced by the inability of active site-inactivated FVIIa, which binds to TF with the same or a higher affinity than FVIIa (20), to up-regulate the gene expression. Although FXa and thrombin could also induce the expression of Cyr61, it is unlikely that they are involved in the FVIIa-induced expression of Cyr61. We found no evidence for the generation of traces of FXa and thrombin in our experimental system. Furthermore, hirudin, a specific inhibitor of thrombin, failed to suppress the FVIIa-induced expression of Cyr61. Although TAP, a specific inhibitor of FXa, partially inhibited the FVIIa-induced response, this cannot be viewed simply as FXa being responsible for the FVIIa-induced response, because all of the remaining experimental data provide support to a contrary notion. These other data are as follows: (i) no traces of FXa generation in our experimental system (detection limit, 20 pM, a concentration well below the concentration of FXa required to induce Cyr61); (ii) neutralizing anti-FX antibodies failed to suppress the FVIIa-induced response, whereas they effectively blocked the FXa-induced response (Fig. 11); and (iii) hirudin completely suppressed the FXa-induced response (Fig. 9), whereas it had no significant effect on the FVIIa-induced response (Fig. 7). These data suggest that FVIIa-induced expression of Cyr61 is independent of FXa and that the FXa-induced expression of Cyr61 might have stemmed from generation of undetectable levels of thrombin in our experimental system. In this context, it may be pertinent to point out that FXa used in present study, although highly purified and apparently homogeneous, is derived from plasma and as low as 8 pM thrombin (which is below our detection limit) is sufficient to induce the expression of Cyr61.

The observation that FVIIa proteolytic activity is required for FVIIa-induced expression of Cyr61 and CTGF suggests two possible mechanisms by which FVIIa could act. One possibility is that FVIIa could release, by its proteolytic action, a growth factor-like substance (which might have been sequestered on the cell surface or the extracellular matrix) that directly links to signal transducing receptor system. Factor Xa (28) and thrombin (29) have been shown to cause an autocrine release/expression of growth factors. However, this possibility is unlikely because exposure of cells that were preincubated with anti-TF IgG (to block the direct action of FVIIa) to FVIIa conditioned medium did not lead to up-regulation of Cyr61 expression (data not shown). Rapid induction of Cyr61 by FVIIa precludes the possible involvement of de novo synthesized growth factors in FVIIa-induced response. Furthermore, cycloheximide, the protein synthesis inhibitor, failed to block the FVIIa-induced expression of both Cyr61 and CTGF. These data are consistent with our recent observation that FVIIa-conditioned medium failed to induce MAP kinase activation in baby hamster kidney cells transfected with TF (30).

A second and more likely possibility is that FVIIa proteolytically activates a cell surface receptor, akin to a PAR. Earlier studies, using Ca\(^{2+}\) release (10, 30), up-regulation of Egr-1 mRNA (16), and MAP kinase activation (13) as markers for FVIIa-induced signaling, suggested this possibility. However, a direct evidence is lacking for this. So far, four PARs (PAR1, PAR2, PAR3, and PAR4) have been identified. Northern blot analysis of RNA isolated from WI-38 cells with various PAR-specific cDNA probes demonstrated that WI-38 cells express all known PARs (data not shown). Therefore, all four known PARs are potential candidate substrates for FVIIa. Thrombin activates PAR1, PAR3, and PAR4 and thus is likely to desensitize these receptors (31). Pretreatment of the cells with thrombin down-regulated the ability of FVIIa to induce the expression of Cyr61, raising the possibility that FVIIa may be activating one of the thrombin-activable PARs. Experiments with PAR-specific peptide agonists revealed that PAR1 and PAR2 peptide agonists, and not other peptide agonists, substantially up-regulated the expression of Cyr61 (Fig. 10) and CTGF (data not shown). However, unlike thrombin, PAR1 peptide agonist pretreatment did not fully abolish the FVIIa-induced response. Similarly, desensitization with PAR2 peptide agonist pre-treatment did not fully abolish the FVIIa-induced response. The cumulative data of these experiments suggest, although they do not prove, that FVIIa-induced expression of Cyr61 does not involve any known PARs. Although such a conclusion is in full agreement with the conclusion reached in recent studies (16, 30), our
data differ from the data of these studies in one main aspect. Earlier studies, which used different cell types, showed that receptors down regulated by thrombin were not involved in FVIIa-induced signaling events (16, 30), whereas the present data show that thrombin treatment down regulated the FVIIa-induced response. A possible explanation for these differences could be that FVIIa may be activating more than one type of PAR, the expression of which is cell-specific. Alternatively, in our experiments, thrombin desensitized the FVIIa-induced response not because they share a common receptor but because the thrombin-induced signal pathways consumed one or more of downstream mediators that are necessary for the FVIIa-induced response. The latter possibility may also explain why PAR1 peptide pretreatment partially reduced the FVIIa-induced response. The observation that PAR1 peptide pretreatment did not completely abolish the thrombin-induced expression of Cyr61 suggests that thrombin may also up-regulate the expression of Cyr61 via an additional pathway.

Induction of Cyr61 by both FVIIa and thrombin was abrogated by cholera toxin, which supports the involvement of G-protein, Gs, in FVIIa and thrombin-mediated signal transduction in WI-38 cells. PLC-inhibitor U73122 and MAP kinase inhibitor PD98059 markedly suppressed the FVIIa-induced expression of Cyr61 but had only minimal effect on thrombin-induced expression of Cyr61. These data suggest that FVIIa induces the expression of Cyr61 via PLC through the activation of MAP kinase. These observations are consistent with the earlier observations made with FVIIa-induced expression of Egr-1 (16) and the fact that FVIIa induces the activation of MAP kinase in many cell types expressing TF (12). LY 294002, the specific PI 3-kinase inhibitor, inhibited both the FVIIa- and thrombin-induced response, indicating that the second messenger, IP3, is a common mediator of the two responses. The differential effect of various cellular inhibitors on FVIIa- and thrombin-induced up-regulation of Cyr61 further strengthens our conclusion that FVIIa-induced up-regulation of Cyr61 does not involve the action of thrombin.

At present, it is unknown whether TF cytoplasmic tail plays any role in FVIIa-induced expression of Cyr61 and CTGF. Experiments conducted with baby hamster kidney cells transfected with full-length TF and cytoplasmic tail deleted TF (13) did not provide any meaningful data. Human Cyr61 cDNA probe hybridized to multiple RNA species in both control and FVIIa-treated baby hamster kidney cells. However, based on earlier studies, which showed FVIIa-induced activation of MAP kinase (13) and Egr-1 induction (16) were independent of TF intracellular domain, it seems unlikely that TF cytoplasmic tail plays a role in FVIIa-induced gene expression in fibroblasts. Although TF cytoplasmic domain was shown to be required in mediating some specific cellular events (32, 33), it should be noted that these events were independent of FVIIa.

Cyr61 is an immediate-early gene that is transcriptionally activated by serum growth factors in fibroblasts (34). It encodes a secreted 40-kDa, cysteine-rich and heparin-binding protein that associates with extracellular matrix and cell surfaces (35). Cyr61 is a member of an emerging gene family of conserved and modular proteins characterized by the presence of an N-terminal secretory signal, followed by four modular structural domains and 38 cysteine residues that are largely conserved among members of the family (19). The Cyr61 protein is shown to (i) promote the attachment and spreading of endothelial cells in a manner similar to that of fibronectin, (ii) enhance the effects of basic fibroblast growth factor and platelet-derived growth factor on the rate of DNA synthesis of fibroblasts and vascular endothelial cells, and (iii) promote cell migration in both fibroblasts and endothelial cells (36). Recent studies show that Cyr61 acts as a ligand to integrin α5β1 (37), an adhesion receptor known to be involved in signaling that regulates a number of cellular processes including angiogenesis and tumor metastasis (38, 39). Expression of Cyr61 in tumor cells was shown to promote tumor growth and vascularization (40). Thus, it is possible that the FVIIa-induced expression of Cyr61, acting through integrin α5β1, may play an important role as a downstream mediator in mediating FVIIa-TF-induced effect, such as promotion of angiogenesis and tumor metastasis. The observations that FVIIa catalytic activity is required for migration of various cell types (15, 17, 18) and tumor metastasis (6, 41) are consistent with the requirement of FVIIa catalytic activity for the induction of Cyr61.

Although CTGF behaves very similar to Cyr61, subtle differences exist between them (see Refs. 19 and 42). For example, (i) CTGF has shown to be mitogenic in itself, whereas Cyr61 has no intrinsic mitogenic activity but augments growth factor-induced DNA synthesis; (ii) Cyr61 stimulates chemotaxis, whereas CTGF stimulates both chemotaxis and chemokinesis; and (iii) although both Cyr61 and CTGF are extracellular matrix-associated signaling molecules, CTGF is shown to secrete into culture medium (43). Thus, it is possible that FVIIa could regulate cellular functions locally via Cyr61, whereas FVIIa acts at a distance from its site through the secretion of CTGF. It is interesting to note that, similar to TF expression (44–46), CTGF mRNA is undetectable in normal blood vessels but overexpressed in atherosclerotic lesions (47). In atherosclerosis, high levels of CTGF expression may be responsible for extracellular matrix accumulation and thus progression of atherosclerotic lesions (48). It is possible that overexpression of CTGF in atherosclerotic plaque could have been the result of increased expression of TF in atherosclerotic plaque. Activation of TF-mediated coagulation pathway not only plays a major role in determining plaque thrombogenicity but may also have other effects on the vessel wall (49). For example, thrombin (50, 51) and FXa (52), the intermediatory byproducts FVIIa-TF coagulation pathway, are shown to promote vascular smooth cell proliferation and thus may play a role in the development of intimal hyperplasia. More importantly, recent studies suggest that FVIIa-TF may have direct effect on aortic smooth cell migration and neonatal aortic smooth cell proliferation (17, 53, 54). It is possible that some, if not all, of these events are mediated by FVIIa-TF-induced expression of CTGF. If so, CTGF may represent the downstream effector for FVIIa-TF. Currently, we are in the process of developing the necessary reagents (recombinant Cyr61 and CTGF proteins and antibodies against them) to test the above possibilities.

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Factor VIIa and Thrombin Induce the Expression of Cyr61 and Connective Tissue Growth Factor, Extracellular Matrix Signaling Proteins That Could Act as Possible Downstream Mediators in Factor VIIa-Tissue Factor-induced Signal Transduction

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