Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2174–2179, March 1996
Medical Sciences

Factor XII-induced mitogenesis is mediated via a distinct signal transduction pathway that activates a mitogen-activated protein kinase

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Communicated by Oscar Ratnoff, University Hospitals of Cleveland, Cleveland, OH, October 9, 1995

ABSTRACT **Clotting factor XII (Hageman factor) contains epidermal growth factor (EGF)-homologous domains and is reported to be a potent mitogen for human hepatoma (HepG2) cells. In this study, we tested whether factor XII exhibits growth factor activity on several other EGF-sensitive target cells, including fetal hepatocytes, endothelial cells, alveolar type II cells, and aortic smooth muscle cells. We found that factor XII significantly enhanced [^{3}H]thymidine incorporation in aortic smooth muscle cells (SMCs) and all other cells tested. Tyrphostin, a growth factor receptor/tyrosine kinase antagonist, inhibited both EGF- and factor XII-induced responses. However, differences in the levels of magnitude of DNA synthesis, the observed synergism between EGF and factor XII, and the differential sensitivity to tyrphostin suggest that the EGF receptor and the factor XII receptor may be nonidentical. The factor XII-induced mitogenic response was achieved at concentrations that were 1/10th the physiologic range for the circulating factor and was reduced by popcorn inhibitor, a specific factor XII protease inhibitor. Treatment of aortic SMCs with factor XII, as well as activated factor XII, resulted in a rapid and transient activation of a mitogen-activated/extracellular signal-regulated protein kinase with peak activity/tyrosine phosphorylation observed at 5 to 10 min of exposure. Taken together, these data (*i*) confirm that clotting factor XII functions as a mitogenic growth factor and (*ii*) demonstrate that factor XII activates a signal transduction pathway, which includes a mitogen-activated protein kinase.**

Growth factors modulate mammalian tissue growth by inducing cell division or by activating cell cycle control elements or signal transduction events that ultimately lead to cell proliferation. Epidermal growth factor (EGF), a known mitogen for a variety of cells, mainly epithelial, stimulates pleiotropic responses in target cells, including increased protein and DNA synthesis (1–3). In many cases, the effects of peptide growth factors are more varied and complex than mitogenesis *per se*, including alterations in the overall patterns of gene expression (4, 5). Therefore, Sporn and Roberts (4) suggested that the term "peptide signaling molecule" may be more aptly descriptive. Some proteins with EGF-like activity, including the 19-kDa protein from vaccinia virus and transforming growth factor type α, have been found to contain regions with structural homology to EGF (2, 3). Recently, coagulation factors exhibiting EGF-like repeats have been shown to exhibit growth factor activity on liver-derived hepatocytes (HepG2) (6) and smooth muscle cells (7). Therefore, factor XII, in addition to its role in the blood coagulation cascade, may act as a peptide signaling molecule for other growth factor-sensitive target cells to regulate cell growth, proliferation, and/or differentiation processes under physiologic conditions (6).

The primary structure of factor XII (8–11) includes two EGF-like domains in its N-terminal region. Located within this same region are fibronectin-homologous domains, which represent possible binding sites for collagen exposed during tissue injury. The C terminus contains the catalytic region of the serine protease, which, upon surface-mediated factor XII activation, initiates clotting. While factor XII exhibits mitogenic activity on HepG2 cells (6), the biochemical mechanism(s) by which this putative signaling molecule stimulates cellular proliferation has yet to be determined.

The mechanism of EGF action (2) is characterized by EGF receptor binding and autophosphorylation of the EGF receptor kinase, followed by activation of signal transduction pathways that include phosphorylation of tyrosine residues in intracellular proteins. Recently, the EGF family of cell surface receptors has been expanded to include at least four distinct receptor species, each with different ligand specificities (12–14). EGF-enhanced DNA synthesis is abrogated by tyrphostins (15), a relatively but not entirely specific antagonist for EGF-class receptors (16). Accordingly, the mitogenic effects of peptide signaling molecules that act via stimulation of EGF-class receptors may also be inhibited by tyrphostins.

In this study, we tested the hypotheses that factor XII exhibits EGF-like activity on target cells via distinct receptor-mediated mechanisms. The results indicate that the mitogenic response is extended to additional cell types, is specific for factor XII, is distinguished from EGF-induced mitogenesis, and is mediated by a signal transduction pathway, which includes the phosphorylation and activation of a mitogen-activated/extracellular signal-regulated protein kinase (MAPK/ERK).

MATERIALS AND METHODS

Materials. Purified human factor XII (specific activity, 123 units per mg of protein) was supplied by Enzyme Research Laboratories (South Bend, IN), and murine EGF was supplied by Collaborative Research. Tyrphostin (RG50864) was kindly provided by Rhône Poulenc-Rorer Central Research, Collegeville, PA. Popcorn inhibitor (PCI) was a gift from Oscar D. Ratnoff (Case Western Reserve University School of Medicine, Cleveland, OH). At a concentration of 10 μg/ml, PCI inhibits half of the factor XII coagulant activity. Using a Sigma limulus amebocyte lysate kit, endotoxin was detected in un-

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Abbreviations: EGF, epidermal growth factor; MAPK/ERK, mitogen-activated/extracellular signal-regulated protein kinase; PCI, popcorn inhibitor; SMC, smooth muscle cell; TCA, trichloroacetic acid; PDGF, platelet-derived growth factor.

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diluted PCI (0.06 endotoxin unit/ml), while no endotoxin was detected in any PCI dilution.

Antibodies. The antibody to sea star $p44^{mpk}$ was kindly provided by S. L. Pelech (University of British Columbia, BC Canada). The α-$p44^{mpk}$, is an affinity-purified rabbit polyclonal antibody that was generated against $p44^{mpk}$ using purified protein from sea star oocytes. This antibody preferentially recognizes active forms of mammalian MAPK/ERKs. A rabbit anti-peptide antibody directed against subdomain I of human/rat ERK1 containing the sequence QLQYIGEGAYGMVSSA was also generated (F. Hall and D. Warburton, University of Southern California). This antibody (anti-ERK) recognizes both ERK1 and ERK2. A mouse monoclonal anti-phosphotyrosine (α-PY-20) antibody was purchased from ICN.

Animals. Timed pregnant Sprague–Dawley rats (supplied by Charles River Breeding Laboratories) were sacrificed at 19 days of gestation with an excess of chloroform in an exposure chamber placed under a fume hood. The fetuses were removed under sterile conditions at 4°C and weighed to confirm their gestation (2.2 g at day 19; term = day 22).

Cells and Culture Conditions. Primary rat aortic smooth muscle (A10) cells (American Type Culture Collection) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; HyClone). Adherent cells were dissociated with trypsin/EDTA; 1×10^5 cells per well were then subcultured in 24-well plates (each well, 16-mm diameter), in serum-free DMEM supplemented with 0.1% bovine serum albumin for 24 hr before treatment with specific agents. Treated and untreated cells were then maintained at 37°C in 95% air/5% CO_2 (17).

To test the specificity of the factor XII mitogenic effect on aortic smooth muscle cells (SMCs), [^{3}H]thymidine incorporation was measured in cells that were treated with factor XII alone or in combination with PCI, a known specific factor XII inhibitor. The cells were cultured for 20 hr with or without factor XII, or with PCI added simultaneously or 14 hr after addition of factor XII (Table 1).

Fetal hepatocytes were isolated by exposure to collagenase as described (18). Hepatocytes were sedimented at $50 \times g$ for 5 min to separate them from other hepatic epithelial cells; 1×10^5 hepatocytes per well were plated in six-well plates (each well, 35-mm diameter) for 48 hr in medium consisting of an equal mixture of Williams E and Ham's F-12 medium (GIBCO), penicillin (50 units/ml), streptomycin (50 μg/ml), transferrin (10 μg/ml), insulin (0.1 μg/ml), hydrocortisone (1.0 μM), triiodothyromine (1.0 μM), and 10% FBS. Cells were exposed to serum-free medium (without FBS) for 15 hr before addition of specific agents. After treatment, cultures were maintained for an additional 24 hr at 37°C in 95% air/5% CO_2.

Fetal alveolar type II pneumocytes were isolated as described (19). Briefly, cells were isolated by protease digestion and sedimented by centrifugation at $420 \times g$ for 5 min. Cells were resuspended in DMEM with 10% FBS and subjected to differential adherence on plastic for 1 hr, followed by centrifugation at $120 \times g$ for 3 min. The final epithelial cell pellet was resuspended in DMEM containing penicillin (10 units per 100 ml), gentamicin (4 mg per 100 ml), and 10% FBS and plated at a density of 2×10^4 cells per well in 24-well plates. Following adherence in the presence of 10% FBS, attached cells were incubated in DMEM without FBS for 24 hr before treatment with specific agents. Treated and untreated cultures were incubated for 24 hr at 37°C, in 95% air/5% CO_2 in high humidity.

Bovine newborn aortic endothelial cells (EC-12; a gift from Walter Laug, Childrens Hospital Los Angeles, Los Angeles) and A431 human epitheloid carcinoma cells (American Type Culture Collection) cells were plated at an initial density of 1×10^6 cells per well in six-well plates. Cultures were grown to confluence for 1–3 days in DMEM supplemented with 10% FBS. Cells were washed with DMEM without FBS and then resuspended in fresh serum-free medium for 48 hr before treatment with specific agents. Treated and untreated cultures were further maintained at 37°C, in 95% air/5% CO_2.

Treatment of Cell Cultures with Specific Agents. Cultures of aortic SMCs, fetal hepatocytes, fetal alveolar type II cells, EC12 cells, and A431 cells were treated with various doses of factor XII, EGF, or solvent controls. Specific test agents were dissolved in the serum-free medium used for the respective cultures as described above. Cultures were treated with serum-free medium containing factor XII (3–6 μg/ml), EGF (20 ng/ml), PCI (3 μg/ml), tyrphostin (25 μM), or combinations of these agents as indicated for each experiment.

[^{3}H]Thymidine Incorporation into DNA. After serum starvation and exposure to specific agents and to [^{3}H]thymidine (1 μCi per well; specific activity, 6.7 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) for 24 hr, cultures were placed on ice, rinsed twice with cold phosphate-buffered saline (PBS), and then rinsed three times with ice-cold 5% trichloroacetic acid (TCA). The final TCA rinse was removed and the TCA-insoluble material was solubilized with 0.2 ml of 1M sodium hydroxide followed by neutralization with an equal volume of 1M acetic acid. Part of the solubilized material was used for protein determinations and the rest was quantified by liquid scintillation counting. [^{3}H]Thymidine incorporation into cellular macromolecules was expressed as absolute radioactivity (dpm per well) (20). For endothelial and A431 cells, [^{3}H]thymidine was added for a 2-hr pulse-labeling period.

[^{3}H]Leucine Incorporation Assay. Leucine incorporation into macromolecules was measured in selected endothelial and A431 as described (6). [^{3}H]Leucine was added for a 2-hr pulse-labeling period. Then cells were washed extensively with fresh serum-free medium and then dissociated with trypsin/EDTA. Total [^{3}H]leucine incorporated into TCA-precipitable polypeptides was measured by liquid scintillation counting.

Lactate Dehydrogenase (LD) Assay. To determine possible cytotoxicity induced by specific agents, LD activity was measured in conditioned medium during exposure of cells to EGF, factor XII, or tyrphostin, using the Sigma LD-L kit. One unit of LD activity is defined as that amount of enzyme that catalyzes formation of 1 μmol of NADH per min under the conditions of the assay procedure.

Western Blotting of MAPK/ERK. Rat aortic SMCs were grown to 80% confluency in T75 flasks in DMEM containing 10% FBS. The cell monolayer was rinsed twice with serum-free medium and, following incubation for 1 hr in serum-free medium, the cells were exposed to factor XII or activated

Table 1. Inhibition of factor XII-induced growth in aortic SMC cultures by PCI

Factor XII, μg/ml	0 at 0 hr	3 at 0 hr	3 at 0 hr	3 at 0 hr	0 at 0 hr
PCI, μg/ml	0 at 0 hr	0 at 0 hr	3 at 14 hr	3 at 0 hr	3 at 0 hr
Total incubation time, hr	20	20	20	20	20
n	4	4	5	5	5
Mean [^{3}H]thymidine incorporation ± SD, dpm per 10^5 cells	1413 ± 224	2441 ± 627	2822 ± 421	1786 ± 203	1391 ± 293
P		<0.01	<0.001	<0.05	NS

P value represents comparisons of factor XII- and PCI-treated SMC cultures with buffer control cultures; *n*, number of determinations in a single representative experiment; NS, not significant.

factor XII for 5, 10, and 30 min. At the end of the treatment, the cell monolayer was rapidly rinsed twice with ice-cold medium without serum and lysed in 1 ml of ice-cold lysis buffer. The lysis buffer contained 0.1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 25 mM sodium fluoride, 5 μg of leupeptin per ml, 0.2% Triton X-100, and 0.3% Nonidet P-40 in 50 mM Tris·HCl/150 mM NaCl, pH 7.5. The lysates were centrifuged at 12,000 × *g* at 4°C for 5 min and the supernatants were saved. Aliquots of supernatant containing 10 μg of protein were subjected to SDS/PAGE in 12% gels under reducing conditions and proteins electrotransferred to PVDF membranes (Millipore). After blocking nonspecific binding by incubation with 5% nonfat dry milk or 1% bovine serum albumin (for phosphotyrosine detection) in PBS, the membranes were incubated with primary antibody at 4°C overnight. Antibody detection was performed using the appropriate alkaline phosphatase conjugated secondary antibodies.

Statistical Analysis. Analysis of variance was used to evaluate the significance of differences between the experimental cultures and their controls (21).

RESULTS

Mitogenic Effects of Factor XII on Aortic SMCs. Fig. 1 demonstrates a 5-fold increase in [^{3}H]thymidine incorporation in factor XII-treated aortic SMC cultures [5229 ± 459 (SD) dpm per 10^5 cells] in comparison to control untreated cultures (945 ± 111 dpm per 10^5 cells; $P < 0.001$). The concentration of factor XII used in this experiment is ≈1/6th the physiologic range for the circulating protease (35–40 μg/ml) (22). The [^{3}H]thymidine incorporation was greater in cultures treated with maximal concentrations of factor XII than in EGF-treated cultures (1717 ± 99 dpm per 10^5 cells; $P < 0.001$), although DNA synthesis was also significantly enhanced in EGF-treated cultures compared to untreated cultures ($P <$ .001) under these conditions. When the effects of factor XII and EGF on proliferation of cultured aortic SMCs were examined under low (0.1% FBS) serum conditions, both factor XII and EGF were found to be sufficient for mitogenesis in a small (≈25%) but significant ($P < .05$) population of the cells (data not shown). Tyrphostin (25 μM) inhibited DNA synthesis in both factor XII-treated (3326 ± 291 dpm per 10^5 cells; $P < 0.001$) and EGF-treated (1291 ± 147 dpm per 10^5 cells; $P < 0.001$) cultures. However, factor XII-induced mitogenesis was inhibited to a somewhat lesser extent. DNA synthesis in tyrphostin-treated cultures was slightly reduced ($P < 0.05$) compared to control untreated cultures.

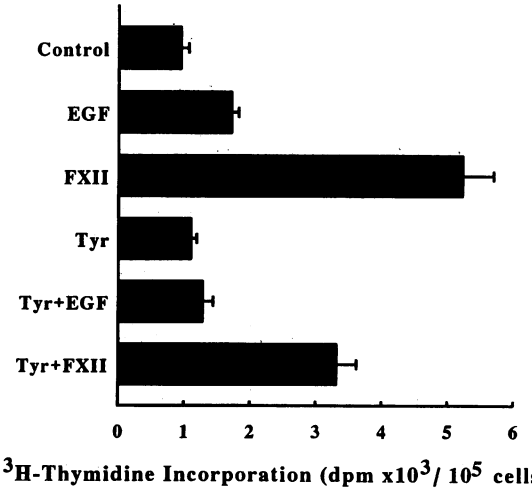

FIG. 1. [^{3}H]Thymidine incorporation in aortic smooth muscle cell cultures exposed to factor XII (6 μg/ml; $n = 6$), EGF (20 ng/ml; $n = 6$), tyrphostin (25 μM; $n = 6$), and combinations of these specific agents for 24 hr ($n = 6$). Absolute radioactivity is expressed as dpm per 10^5 cells.

A lower concentration of factor XII was used to further test the specificity of the observed mitogenic effect. Table 1 shows that factor XII enhanced [^{3}H]thymidine incorporation in cultured aortic SMCs at a concentration that was only 1/10th the physiologic range for the circulating protease. This response was inhibited by PCI, a known inhibitor of coagulation factor XII (23). Inhibition of mitogenesis occurred when factor XII and PCI were added simultaneously to the cultures at the start of a 20-hr incubation period ($P < 0.05$). In contrast, no inhibition of growth was detected in factor XII-treated cultures when PCI was added 14 hr later. These data attest to the specificity of factor XII-mediated growth factor activity on aortic smooth muscle cells, since PCI inhibition is directed specifically against factor XII.

Mitogenic Effects of Factor XII on Fetal Hepatocytes and Alveolar Type II Cells. Table 2 shows enhanced incorporation of [^{3}H]thymidine in factor XII-treated fetal hepatocyte and alveolar type II cell cultures to levels approximately the same as those observed in EGF-treated cell cultures. For comparison, factor XII was added to these cultures at ≈1/6th the physiologic (circulating) concentration (22). The concentration of EGF varies considerably among different tissues, ranging from about 0.2 to 60 ng/ml (5). Thus, the amount added to these cultures was well within the physiologic ranges. Abrogation of the increased thymidine incorporation was noted in factor XII-treated hepatocyte and alveolar cell cultures in the presence of tyrphostin, a growth factor receptor/tyrosine kinase antagonist. The amount of lactate dehydrogenase was not significantly different in treated and untreated cultures, indicating that cellular integrity was preserved during the culture period. The similar levels of mitogenesis, as well as the inhibitory effects of tyrphostin observed in these cells, is consistent with the possibility that factor XII growth-promoting activity may be mediated via one or more members of the EGF-receptor family of cellular transmembrane receptors.

Fig. 2 confirms that factor XII and EGF independently enhanced DNA synthesis in fetal hepatocyte cultures (33,445 ± 5680 dpm per 10^5 cells; $P < 0.001$; 40,236 ± 9736 dpm per 10^5 cells; $P < 0.001$ respectively) and that tyrphostin abrogates the EGF response (21,055 ± 2399 dpm per 10^5 cells). Additionally, [^{3}H]thymidine incorporation was significantly greater in fetal hepatocyte cultures treated with both factor XII and EGF (54,225 ± 10,356 dpm per 10^5 cells; $P < 0.001$) than in those cultures treated with either factor XII or EGF alone. These data indicate that factor XII and EGF exhibit synergistic action on fetal hepatocyte growth, which further suggests that

Table 2. [^{3}H]Thymidine incorporation in factor XII-treated fetal hepatocytes and alveolar type II cell cultures

	Hepatocytes, dpm per 10^5 cells	Alveolar type II, dpm per 10^4 cells
Control untreated	2020 ± 234	4722 ± 102
Factor XII (6 μg/ml)	2796 ± 214 $P < 0.01$	6768 ± 32 $P < 0.001$
Factor XII (6 μg/ml) and tyrphostin (25 μM)	2129 ± 163 NS	2956 ± 26 $P < 0.001$
EGF (20 ng/ml)	2456 ± 153 $P < 0.05$	6078 ± 10 $P < 0.001$

P value represents comparisons between treated and untreated (control) cultures; $n = 3$ in each group. Results are expressed as means ± SD.

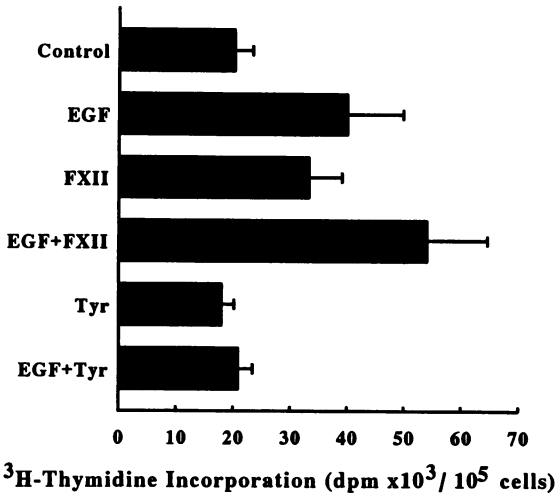

FIG. 2. [^{3}H]Thymidine incorporation in primary fetal rat hepatocyte cultures exposed to factor XII (6 μg/ml; n = 6), EGF (20 ng/ml; n = 6), tyrphostin (25 μM; n = 6), and combinations of these specific agents (n = 6 in each group). Absolute radioactivity is expressed as dpm per 10^5 cells.

the putative receptor for factor XII may be nonidentical to that of EGF.

Mitogenic Effects of Factor XII on EC-12 Endothelial Cells and A431 Epithelioid Carcinoma Cells. To determine whether the mitogenic activity of factor XII could be further generalized, two additional known EGF-responsive target cell types were also tested. Factor XII treatment stimulated an increase in [^{3}H]thymidine incorporation both by EC-12 endothelial and A431 epitheloid cells. The increase was significant at several peptide concentrations: A431 cells were sensitive to factor XII at 0.3 μg/ml ($P < 0.04$) and 3.0 μg/ml ($P < 0.005$), and EC-12 cells showed a significant increase at 0.3 μg/ml ($P < 0.02$). As expected, EGF itself, like factor XII, enhanced thymidine incorporation in both EC-12 and A431 cells (Table 3). A significant increase in leucine incorporation (i.e., protein synthesis) was observed in A431 cells in response to both factor XII ($P < 0.04$ at 0.3 μg/ml; $P < 0.005$ at 3.0 μg/ml) and EGF ($P < 0.005$ at 5 ng/ml; $P < 0.001$ at 10 ng/ml; $P < 0.01$ at 20 ng/ml), while leucine incorporation by EC-12 cells was not significantly increased by either polypeptide. Thus, both of these cell types responded to exogenous factor XII in a manner that is similar to their EGF responses.

Rapid and Transient Activation of a MAPK/ERK Pathway in Factor XII-Treated Aortic SMCs. To further investigate the molecular signaling mechanisms of factor XII mitogenesis, we examined the status of the MAPK/ERK protein kinase(s), which is activated by a wide range of growth factors by receptor-mediated mechanisms (24, 25) that include direct tyrosine phosphorylation of the kinase. Treatment of aortic SMCs with either unactivated or activated factor XII resulted in a rapid (within 5 min) and transient phosphorylation and activation of a 42-kDa MAPK/ERK isoform, as determined by increased immunoreactivity with the anti-MPK antibody (Fig. 3), an antibody that selectively recognizes active forms of these enzymes (26, 27). While the levels of both p44^{ERK1} and p42^{ERK2} proteins remained constant over these time periods (Fig. 3*A*), the activation and tyrosine phosphorylation of a prominent MAPK/ERK species (presumably p42^{ERK2}) was evident at 5–10 min (Fig. 3*B*). The activation of the ≈42-kDa MAPK, as determined by anti-MPK immunoreactivity, was confirmed by the appearance of phosphotyrosine immunoreactivity detected by anti-phosphotyrosine antibodies (Fig. 3*C*). The same activation/tyrosine phosphorylation of p42^{ERK2} and time course was observed whether native or proteolytically activated factor XII was used in these studies.

DISCUSSION

Several novel aspects of factor XII function and specificity have been described in this report. Since the primary structure of factor XII includes EGF-like domains (8–11), we hypothesized that factor XII might influence cell growth via activation of the EGF receptor or a closely related transmembrane receptor/tyrosine kinase. Therefore, tyrphostin was used to test the possible involvement of receptor-mediated tyrosine phosphorylations in the observed cellular responses. Tyrphostins are synthetic compounds that inhibit both autophosphorylation of the EGF receptor kinase and phosphorylation of tyrosine residues in cytosolic proteins (15). Abrogation of EGF receptor kinase activity by tyrphostin is relatively selective for EGF-induced growth since tyrphostins have considerably less effects on platelet-derived growth factor (PDGF)- or serum-induced cell growth (15). Tyrphostins do not directly affect EGF receptor binding by ligands or by synthesis and degradation of EGF receptors. Thus, tyrphostins have been used as pharmacological probes to clarify the mechanism of action of proposed mitogens (15).

The present study demonstrates that both EGF and factor XII stimulate thymidine incorporation in all growth factor-responsive cells tested. Tyrphostin inhibited the EGF responses markedly, without any indication of cytotoxicity. In aortic SMCs, factor XII activity was also inhibited, but to a considerably lesser degree. The observed inhibition of DNA

Table 3. [^{3}H]Thymidine and [^{3}H]leucine incorporation in factor XII-treated A431 and EC12 cell cultures

	A431 cells		EC-12 cells	
	[^{3}H]Thymidine	[^{3}H]Leucine	[^{3}H]Thymidine	[^{3}H]Leucine
Factor XII control (buffer)	9769 ± 1059	40,667 ± 1158	10,161 ± 2172	8212 ± 2115
Factor XII (0.3 μg/ml)	12,782 ± 1774 $P < 0.04$	45,093 ± 2521 $P < 0.04$	15,933 ± 2528 $P < 0.02$	8437 ± 1115 NS
Factor XII (3.0 μg/ml)	18,042 ± 2914 $P < 0.005$	44,014 ± 1149 $P < 0.02$	ND	ND
EGF control (buffer)	6446 ± 2482	3340 ± 980	1812 ± 84	89,747 ± 457
EGF (5 ng/ml)	12,281 ± 3543 $P < 0.02$	8589 ± 2628 $P < 0.005$	2215 ± 162 $P < 0.01$	92,487 ± 667 NS
EGF (10 ng/ml)	12,060 ± 1903 $P < 0.006$	10,373 ± 193 $P < 0.001$	2371 ± 395 $P < 0.04$	84,309 ± 172 NS
EGF (20 ng/ml)	15,157 ± 357 $P < 0.0002$	11,948 ± 280 $P < 0.01$	ND	ND

NS, not significant; ND, not done. Values are means (dpm per 10^5 cells) of duplicate samples ± SD.

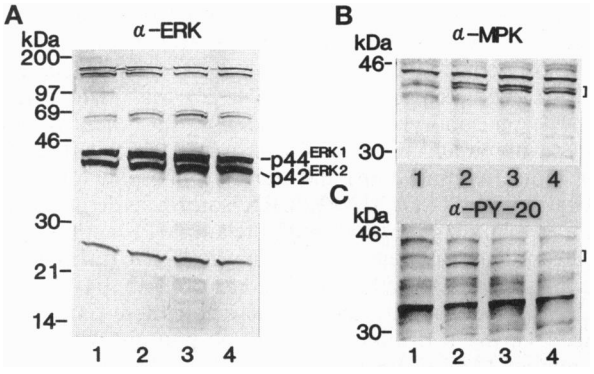

FIG. 3. Western blotting of MAP/ERK. Protein (10 μg) from A10 cell lysates was subjected to SDS/PAGE in 12% gels under reducing conditions; the proteins were transferred electrophoretically to PVDF membranes, blocked with 5% nonfat dry milk or 1% bovine serum albumin (for phosphotyrosine detection) in PBS, and incubated with anti-ERK antibody (*A*), which recognizes $p44^{ERK1}$ and $p42^{ERK2}$, anti-MPK antibody (*B*), which selectively recognizes activated forms of these enzymes, and α-PY-20 (*C*), which specifically recognizes phosphotyrosine residues in proteins. Lanes: 1, control; 2, 5-min exposure; 3, 10-min exposure; 4, 30-min exposure to intact factor XII. Similar results were noted upon exposure to activated factor XII.

and protein synthesis by tyrphostin in factor XII-treated cells suggests that activation of a transmembrane receptor/tyrosine kinase is a plausible mechanism by which factor XII induces cell growth. However, we observed differential levels of cell stimulation and inhibitor potency, which suggest that the site of action for factor XII may be nonidentical to the EGF receptor. Interestingly, the mitogenic response of aortic SMCs to factor XII was much greater than to EGF, further suggesting that the cellular receptors may be different. Moreover, in fetal hepatocytes, we observed a pronounced synergistic effect between EGF and factor XII, which supports the evidence that a distinct receptor system may be involved.

Factor XII coagulant activity is specifically inhibited by PCI (28). Therefore, we used PCI to demonstrate that the observed EGF-like peptide signaling activity of the factor XII preparations used was due specifically to factor XII rather than to either a nonspecific trophic effect or to the activity of an undetected trace contaminant. In all cases tested, PCI inhibited factor XII growth factor activity. Furthermore, when PCI was added 14 hr after factor XII exposure, PCI had no inhibitory effect on mitogenesis. Thus, PCI specifically blocks the factor XII mitogenic responses and is not acting as a cytotoxic agent that independently prevents an increase in thymidine incorporation. The finding that the mitogenic effects of factor XII were elicited within the physiological ranges of factor XII concentration further indicates that the observed mitogenic effects are factor XII specific. While the mechanism of PCI inhibition is not known, it can be used as a tight-binding affinity substrate to isolate and purify factor XII (28). Since factor XII can bind PCI and then be released without permanently altering its biological activity, it might be that PCI binding reversibly alters factor XII conformation in such a way as to inhibit its coagulant activity. Presumably, this altered conformation also inhibits factor XII growth factor activity. Functional relationships between EGF-like domains and coagulant activity have been shown for several related coagulation factors, including thrombin, thrombomodulin (29), factor IX (30, 31), factor X (32), and protein S (33).

Two EGF-like repeats within the primary structure (10) of factor XII may be responsible for its mitogenic activity. Similar EGF domains are found in other coagulation peptides (29–33), such as factors X and IX, and protein S, which could also account for their reported mitogenicity. However, these EGF domain-bearing coagulation factors vary markedly in their degree of growth factor activity. This may be due to conformational differences affecting the accessibility of active sites for binding to cellular receptors. At this point, it is pertinent to note that thrombin, in addition to its role in hemostasis, exerts profound cellular effects by binding to specific cell surface receptors. Fibronectin type I and II domains alternate with EGF repeats in the N terminus of the factor XII molecule (10). Conceivably, these fibronectin homologous domains facilitate factor XII binding to receptors on responsive cells by anchoring the factor XII molecules on the cell surface.

MAPKs, otherwise known as ERKs, are key intermediates in a signal transduction pathway that links many types of cell surface receptors with cytoplasmic and nuclear events that initiate mitogenesis (24). The MAPK/ERK signal transduction pathway, activated by a number of diverse mitogens, is a direct protein kinase cascade involving a MAP kinase kinase kinase (i.e., Raf-1) and a MAP kinase kinase (i.e., MEK) as proximal components (25). Enzymatic activation of the MAPK/ERK themselves (i.e., $p44^{ERK1}$ and $p42^{ERK2}$) requires phosphorylation of both threonine and tyrosine residues within the phosphoregulatory subdomain VIII of these protein kinases.

The results of the present study demonstrate that factor XII exposure leads to rapid activation and tyrosine phosphorylation of a MAPK/ERK isoform with an apparent molecular mass of 42 kDa. The results are substantiated by the increased immunoreactivity of anti-MPK antibodies, which selectively recognize activated forms of these enzymes (26, 27), and by the presence of phosphotyrosine as determined by Western blotting with anti-phosphotyrosine antibodies (Fig. 3*C*). The rapid and transient activation of a prominent MAPK/ERK isoform observed in aortic SMCs provides potentially important insights into the mechanisms of factor XII-induced mitogenesis as well as a factor XII signal transduction pathway that is amenable to future experimentation.

While the precise nature of the factor XII receptor and its signal transduction pathway remains to be determined by future study, the biological and clinical significance of factor XII growth factor activity may relate to several potential roles. Factor XII may play a role in fetal lung development (34, 35) and fetal liver development or liver regeneration (36), since we have shown that primary cultures of fetal hepatocytes and fetal alveolar type II cells are both responsive to factor XII. Factor XII mitogenic activity could also play a role in localized wound healing, or potentially in a systemic response to trauma, since several other cell types are also responsive to factor XII growth factor activity. Factor XII is a liver-derived glycoprotein with procoagulant properties activated upon direct contact with subendothelial collagen. The amount of factor XII required to induce growth (3–6 μg/ml) is 1/6th–1/10th the normal titer of factor XII in plasma (35–40 μg/ml) (22). Thus, the concentration of factor XII that promotes cell growth is much less than that required for induction of clotting *in vitro*. In fetal hepatocytes, EGF and factor XII appear to act synergistically, which raises the possibility that factor XII may act as a cofactor or potentiator of EGF responses. Additionally, hepatocytes secrete factor XII (37) and regulate its secretion rate (38), suggesting a possible autocrine function. These present studies were conducted with purified systems, and the mitogenic activity of factor XII may be greatly modified in a physiologic milieu that includes other growth factors and coagulation factors with known growth factor activities.

Finally, factor XII may play a role in the pathogenesis of atheroocclusive disease. Endothelial cell proliferation in response to damage is known to be accompanied by increased secretion of PDGF, which can itself induce myointimal proliferation (39). Factor XII, a normal component of plasma, may cause endothelial cells or SMCs (40) to increase PDGF secretion, which could promote vessel occlusion. Thus, factor XII may have a local paracrine role in the endothelial response

to injury. Factor XII has a direct mitogenic effect on aortic SMCs, and this effect is appreciably greater than that of EGF. Furthermore, factor XII and EGF exhibit synergistic effects on cell proliferation when presented concomitantly. An appealing concept is that circulating factor XII, which is elevated in persons at statistical risk of thrombosis, could participate in the induction of endothelial and smooth muscle cell growth and possibly stimulate these cells to alter their cellular programs and secretory products.

First reported in human hepatoma (HepG2) cells (6), the observed mitogenic activity of factor XII has now been extended to include fetal hepatocytes, aortic SMCs, fetal alveolar type II cells, endothelial (EC-12) cells, and epithelioid carcinoma (A431) cells. The mitogenic effects of factor XII were blocked by PCI, which attests to the specificity of the peptide signaling molecule, and by tyrphostin, which is a selective growth factor receptor/tyrosine kinase antagonist. While a number of cellular responses to factor XII are similar to those of EGF, critical differences in the magnitude of responses, the observed synergism between EGF and factor XII, and a differential sensitivity to tyrphostin suggests that the cellular receptor for factor XII may be nonidentical to the classical EGF receptor. While the nature of the cellular receptor for factor XII remains to be identified by further study, factor XII activates a signal transduction pathway that includes a MAPK/ERK, providing a mechanistic link between the putative extracellular receptor, cytoplasmic signaling enzymes, and characteristic nuclear events that underlie the mitogenic responses of cells to peptide growth factors.

This work was supported in part by Grant GM4915 from the National Institutes of Health (to F.L.H.) and a grant from Armour Pharmaceutical Co., Collegeville, PA (to E.M.G.).

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