SYNERGISTIC AND MULTIDIMENSIONAL REGULATION OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 EXPRESSION BY TRANSFORMING GROWTH FACTOR TYPE β AND EPIDERMAL GROWTH FACTOR

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Background: TGFβ and EGF co-regulate important cellular responses including proliferation, EMT and PAI-1 expression.

Results: TGFβ and EGF synergistically stimulate PAI-1 transcription through Smads and AP-1 combined with mRNA stabilization.

Conclusion: TGFβ and EGF coordinate multiple cellular responses to rapidly achieve large increases in PAI-1 expression.

Significance: Synergism increases the sensitivity, precision, rapidity and range of change in specific gene expression.

The major physiological inhibitor of plasminogen activator, type I plasminogen activator inhibitor (PAI-1) controls blood clotting and tissue remodeling events that involve cell migration. Transforming growth factor type β (TGFβ) and epidermal growth factor (EGF) interact synergistically to increase PAI-1 mRNA and protein levels in human HepG2 and mink Mv1Lu cells. Other growth factors that activate tyrosine kinase receptors can substitute for EGF. EGF and TGFβ regulate PAI-1 by synergistically activating transcription, which is further amplified by a decrease in the rate of mRNA degradation, the latter being regulated only by EGF. The combined effect of transcriptional activation and mRNA stabilization results in a rapid two-order of magnitude increase in the level of PAI-1. TGFβ also increases the sensitivity of the cells to EGF, thereby recruiting the cooperation of EGF at lower than normally effective concentrations. The contribution of EGF to the regulation of PAI-1 involves the MAPK pathway and the synergistic interface with the TGFβ pathway is downstream of Mek1/2 and involves neither phosphorylation of Erk1/2 nor Smad2/3. Synergism requires the presence of both Smad and AP-1 recognition sites in the promoter. This work demonstrates the existence of a multidimensional cellular mechanism by which EGF and TGFβ are able to promote large and rapid changes in PAI-1 expression.

INTRODUCTION

Cells are finely tuned to their external environment with many response modes. Most mammalian cell responses are initiated at the level of receptors that recognize hormones, growth factors, cytokines and other currencies of intercellular communication. The means by which these signals are integrated guides the speed and intensity of the cell’s response. In many instances the integration is additive, with both signals contributing a certain quantitative
change in the target cellular function. However, in some cases the signals are integrated so as to be synergistic where the sum of the two signals provides a larger increase than expected if they were additive. These synergistic responses provide opportunities for a broader range of response that can tightly controlled over a larger range of intensity or time span. The requirement of two or more regulators for increased gene expression also minimizes the probability of adventitious gene activation that could be deleterious to the organism.

Plasminogen activator inhibitor (PAI-1), a secreted glycoprotein, is the major plasma inhibitor of plasminogen activator (PA) (1). By controlling the plasminogen activator-plasmin proteolytic cascade, PAI-1 is crucial to the regulation of haemostasis and to many biological processes that involve remodeling of the extracellular matrix (ECM, 2,3-6). Due to its critical function in normal physiology, PAI-1 requires tight control over a large range of its gene and protein expression.

The need for tight regulation of PAI-1 expression levels and time of production is reflected in the many hormones, cytokines and growth factors that regulate it, including insulin, transforming growth factor type beta (TGFβ), epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2), insulin-like growth factor I (IGF-I) and tumor necrosis factor alpha (TNFα) (7-12). TGFβ and EGF cooperatively regulate PAI-1 expression (9,13).

Here we demonstrate that EGF and TGFβ cooperate synergistically to regulate PAI-1 expression at the level of transcription and that EGF alone stabilizes PAI-1 mRNA with the result that the mRNA level increases rapidly by two orders of magnitude higher after addition of TGFβ and EGF. In addition, TGFβ recruits the cooperation of EGF even when it is at suboptimal concentrations by increasing the cells’ sensitivity to EGF. The mechanism by which synergism is achieved is unique to the combination of the two growth factors and the same level of expression cannot be achieved by either growth factor alone. These studies demonstrate a powerful synergistic regulation of PAI-1 gene expression between two growth factors that are frequently present at elevated levels in diseased tissues such as cancer and during an inflammatory response.

**EXPERIMENTAL PROCEDURES**

**Materials.** Human TGFβ1 and recombinant epidermal growth factor were from R&D systems (Minneapolis, MN). Reagents were: cordycepin and 4-thiouridine (4SU) (Sigma, St. Louis, MO), Trizol, DNase I and reverse transcriptase (Invitrogen (Carlsbad, CA), EZ-Link biotin-HPDP (Thermo Scientific (Rockford, IL), biotin-16-UTP (Epigene Biotechnologies, Madison, WI), Curcumin (Enzo® Life Science, Plymouth Meeting, PA), kinase specific inhibitors (U0126, SB202190, PP2, LY294002, SP600125) and their negative controls (U0124, SB20474, PP3, LY303511) (Calbiochem, EMD Chemicals Inc., San Diego, CA). Radioisotopes were α32P-dCTP (New England Nuclear, Boston, MA), α32P-UTP (ICN, Irvine, CA), 35S-methionine and 35S-Trans-label (Invitrogen). All reagents were analytical grade or better.

Antibodies were rabbit monoclonal antibodies to phospho-p44/42 MAP Kinase (Thr202|Tyr204) (Cell Signaling Technology, Inc., Danvers, MA), rabbit polyclonal antibodies to phospho-ser467-Smad2 (GenScript USA Inc., Piscataway, NJ), phospho-Fos (ser32|ser362|thr232) and phospho-c-Jun (ser63|ser73) (Assay Biotech Inc., Sunnyvale, CA), mouse HRP conjugated monoclonal antibody (C4) to β-actin (Santa Cruz Biotechnology Inc., CA). PAI-1 antiserum (α-Sp46) was from JG Rheinwald (14).

Plasmids were: p3TP-lux (#11767), (15) CMV-Fast-1 WT (#16521) (16) and SBE4-Luc (#16495) (17) (Addgene, Inc., Cambridge, MA), pRL-SV40 (Promega, Madison, WI).
The plasmids ΔFosB and empty vector were from Y Nakabeppu (18).

**Cell Culture.** The mink lung epithelial (Mv1Lu) cells (ATCC, CCL64, Manassas, VA) were maintained as monolayer cultures in Dulbecco-Vogt's medium (DMEM) with 0.45 % glucose, 10 % calf serum, 10 units/ml penicillin, 10 µg/ml streptomycin at 37 °C, in a water saturated atmosphere with 10 % CO2 in air. MLEC clone 32 cell line (Mv1Lu stably transfected with a human PAI-1 luciferase reporter (19)) was a gift from Daniel B Rifkin (New York University, NY). Human hepatocellular carcinoma HepG2 cells (ATCC, HB-8065) were maintained in Eagle's minimal essential medium (MEME, Sigma) supplemented with 10 % fetal calf serum, 1 % non-essential amino acids (Irvine Scientific, Santa Ana, CA), 10 µM sodium pyruvate (Gibco, Carlsbad, CA), 10 units/ml penicillin and 10 µg/ml streptomycin at 37 °C in a water-saturated atmosphere with 5 % CO2 in air. All cell lines were confirmed to be mycoplasma negative with regular testing by PCR analysis.

For experiments, cells were seeded and cultured until confluent. The medium was then changed to Dulbecco-Vogt's modified Eagle’s medium (DMEM) or minimal essential medium (MEME, Sigma) with or without 0.2 % calf serum for 24 h prior to addition of growth factors and other treatments. Growth factors were 1 ng/ml EGF and 5 ng/ml TGFβ unless otherwise stated.

**Transfection.** Mv1Lu cells were seeded one day before transfection. Cells were transfected with 1.6 ng/ml plasmids in DMEG medium in the presence of 0.4 % lipofectamine 2000 (Invitrogen). After 20 h of transfection, the cells were treated with 1 ng/ml EGF, 5 ng/ml TGFβ or their combination and incubated for the indicated time period. Samples were collected and analyzed for luciferase activity or by RT-qPCR. Both firefly and renilla luciferase activities were measured in a Glomax 20/20 Luminometer (Promega) using the Promega Dual-Glo protocol. The relative reporter activity for each treatment was calculated as the ratio of firefly to renilla luciferase activities.

**Metabolic labeling.** PAI-1 was metabolically labeling as previously described (9). Confluent Mv1Lu cells were treated with EGF and/or TGFβ for 2 h then 35S-methionine was added to the culture medium. After 4 h, the medium was collected and the amount of radiolabeled PAI-1 protein was determined by SDS-PAGE followed by autoradiography. After 8 h exposure to EGF and/or TGFβ, 35S-Trans-label was added to HepG2 cells to label newly synthesized protein. The amount of human PAI-1 protein was detected after immunoprecipitation by using PAI-1 antiserum (α-Sp46), followed by SDS-PAGE and autoradiography. For quantitative results the exposed film was scanned to determine the relative amount of cpm in each protein band and each value was divided by the total acid-precipitable 35S-cpm in the cell monolayer from which the sample of medium was removed. By this means the incorporation of 35S-amino acid into secreted proteins was normalized to the number of producing cells.

Newly transcribed RNA was metabolically labeled as previously described (20) with modifications. Fifteen minutes before the end of the treatment period, 500 µM 4-thiouridine was added to the cell culture medium. The reaction was stopped 15 min later and total cellular RNA was isolated using Trizol reagent. Newly transcribed RNA was biotinylated by incubation with 0.2 mg/mL biotin-HPDP in 10 mM Tris (pH 7.4), 1 mM EDTA for 1.5 h at room temperature. The biotinylated RNA was isolated on streptavidin coated beads, M270. The mRNAs were quantified by RT-qPCR.

**mRNA half-life.** Mv1Lu cells were treated with 5 ng/ml EGF and/or 1 ng/ml TGFβ, for 2 h then 15 µg/ml cordecypin was added and the cells
incubated for the identified time periods before collection and extraction of total RNA for analysis by qRT-PCR. At least five independent experiments were performed for each treatment.

*Western blots* were visualized by chemiluminescence using Enhanced Luminol Reagent: Oxidizing Reagent (PerkinElmer Life Sciences, Waltham, MA) for 1 min and then exposed to autoradiography film.

**Statistical and quantitative analysis.** Data from multiple experiments are summarized and represented as the mean ± standard error of the mean. The data was analyzed by the linear model of ANOVA and multiple comparisons were performed according to the least square means Student’s t test. The concentration dependence of PAI-1 expression in the presence of TGFβ with or without EGF was analyzed for the EC_{50} by using the online software BioDatafit 1.02 (Chang Bioscience, Inc., Castro Valley, CA; [http://www.changbioscience.com/stat/ec50.html](http://www.changbioscience.com/stat/ec50.html)). The four parameter (Hill-slope) model was used to fit the data, and calculate logEC_{50}. The effects of treatments on the PAI-1 mRNA half-life were assessed by linear regression analysis and analysis of covariance (ANCOVA). All statistical analyses were performed using the software JMP7.0 (SAS Institute Inc, Cary, NC) unless otherwise stated. Significant differences between samples are indicated as: * p<0.05, **: p<0.01 and ***: p<0.001. Synergism is calculated as: synergism = TE net effect / (T net effect + E net effect).

**RESULTS**

*Synergism between EGF and TGFβ in regulating PAI-1 mRNA and protein level.* EGF and TGFβ cooperate to stimulate PAI-1 protein production as demonstrated in mink lung Mv1Lu cells and human liver HepG2 cells (Fig. 1A,B). The synergistic increase in PAI-1 gene expression is also observed at the mRNA level (Fig. 1C,D). Synergism is defined as when the increase due to the combination of treatments is more than expected from the sum of the increases due to either treatment alone.

Unlike the large changes in gene expression observed for PAI-1, much smaller changes in mRNA levels were observed for other genes (Fig 1C,D). There was a slightly increase for urokinase (uPA) expression in response to EGF but there was no effect of TGFβ. A small (but not statistically significant) synergistic increase was observed in urokinase plasminogen activator receptor (uPAR) expression. Cyclooxygenase (COX2) mRNA was not altered by EGF, TGFβ, or their combination. Thus, the synergism between EGF and TGFβ is gene specific but not species specific.

*Synergism occurs with both growth factors at saturating concentrations.* If EGF and TGFβ utilize the same mechanism to increase PAI-1 gene expression they should only work synergistically at concentrations below their saturation. To test this hypothesis, we varied the concentration of each growth factor to reach saturation in the presence of a constant saturating concentration of the other (Supplementary Fig. 1). The results clearly show that neither growth factor can substitute for the other in raising the level of PAI-1 mRNA to the level observed with both together, which is about an order of magnitude beyond that achievable by either growth factor alone.

TGFβ increases the sensitivity of cells to EGF but not vice versa. Analysis of the EC_{50}s for PAI-1 mRNA changes in response to EGF and/or TGFβ also revealed that TGFβ increases the cells’ sensitivity to EGF by an order of magnitude but EGF does not change the EC_{50} for TGFβ responsiveness (Supplementary Table 1). Thus, TGFβ recruits EGF at suboptimal concentrations to a synergistic interaction.

The synergism with TGFβ is downstream from tyrosine kinases and PKC. To determine if a signal transduction pathway downstream from...
a tyrosine kinase is responsible for the EGF contribution to synergism with TGFβ, we examined the effect on TGFβ-activated PAI-1 expression of fibroblast growth factor 2 (FGF-2) insulin-like growth factor 1 (IGF-1), and tumor necrosis factor alpha, (TNFα). While the PAI-1 mRNA level was not significantly increased after treatment by IGF-1, FGF-2 or TNFα alone, it was dramatically increased when the cells were treated with the combination of any one of these stimuli and TGFβ (Fig. 2A,B). EGF and TGFβ exhibited the highest synergism, which elevated PAI-1 mRNA level about 4-fold over the additive effects expected from each growth factor acting independently.

Synergism is not uniquely downstream of tyrosine kinases. Phorbol myristate acetate (PMA), a PKC agonist, synergizes with TGFβ on PAI-1 expression (Fig. 2C). This effect was specific for PAI-1 and not observed for a 73 kDa TGFβ-induced protein secreted by the same cells (Fig 2D).

The MAPK pathway is required for synergism. The involvement of RTK receptors and PKC identified a subset of signal transduction pathways that might be involved in the synergistic mechanism. To identify the likely pathway(s), a series of kinase inhibitors and their inactive analogs were tested (Fig. 3A-E, Supplementary Table II). Although several inhibitors decreased the overall responses to EGF and TGFβ, only inhibition of the p38 MAPK and the Mek1/2 reduced synergism, with the largest effect being produced by the Mek1/2 inhibitor, U0126. Phosphorylation of Erk1/2, a major target of Mek, increased within 10 min of adding EGF but was not altered by TGFβ (Fig. 3F). Therefore, the synergism is a downstream of Mek.

De novo protein synthesis is not required for the synergism. To test if new translation were required for synergism, cells were treated with cycloheximide prior to growth factor treatment. Although blocking protein synthesis decreased the total PAI-1 mRNA levels induction (Fig. 4A), the calculated synergisms with and without cycloheximide treatment were $2.7 ± 0.2$ and $3.3 ± 0.8$ respectively, which were not significantly different by a two-tailed T test.

EGF stabilizes PAI-1 mRNA. We evaluated if enhanced mRNA stability might be the post-transcriptional step responsible for the period of persistently elevated PAI-1 mRNA level in response to the combination of EGF and TGFβ. Although the basal level of PAI-1 mRNA was too low to obtain sufficient quantitative values for a decay curve, we were able to determine the mRNA decay rates in cells treated with each growth factor alone and in combination (Fig. 4B). The PAI-1 mRNA half-life was 37 min in TGFβ-treated cells, 52 min in EGF-treated cells and 56 min in cells treated with EGF and TGFβ. A statistical analysis of the data verified that the PAI-1 mRNA half-life in cells treated with the combination of EGF and TGFβ was significantly different from that in cells treated with TGFβ alone ($p< 0.05$) but not different from that in cells treated with EGF. Thus, by stabilizing PAI-1 mRNA, EGF uniquely contributes to the magnitude of the increase in expression of PAI-1 mRNA in combined treatment with TGFβ and increases the time span of increased PAI-1 mRNA after growth factor stimulation.

Transcription of the PAI-1 gene is synergistically induced by TGFβ and EGF. To determine if EGF and TGFβ cooperate to increase the rate of initiation of PAI-1 transcription, we performed nuclear run-on experiments and found a synergistic effect of about 2-fold for newly transcribed PAI-1 mRNA (Fig. 5A).

A trivial explanation for the observed synergism is that, rather than increasing the maximum level of gene expression, the combination of the two growth factors changes the time course of expression to move the peak
expression level closer to the monitored time point. To test this question we performed a time course study where it was seen that PAI-1 transcription and the mRNA level was increased within 1 h of adding EGF and TGFβ and peaked at 1.5 and 2 h, respectively (Fig. 5B,D). Synergism was observed throughout the induction period. These results demonstrate that the response of PAI-1 gene expression is rapid and that synergism between EGF and TGFβ does not result from a temporal disparity.

**The mechanism of synergism between EGF and TGFβ does not intersect with the Smad pathway.** Crosstalk has been reported between the MAPK and Smad pathways (21,22). To determine if this type of interaction is the basis for the cooperation between EGF and TGFβ, we examined the phosphorylation of Smad 2/3 using an antibody that recognizes the phosphorylated c-terminal SS (V/M)S motif and found the Smads were phosphorylated only in response to TGFβ and EGF treatment did not affect Smad phosphorylation (Supplementary Fig. 2A).

To determine if nuclear Smad activity is synergistically increased by EGF and TGFβ, we utilized the plasmid pSBE4, which contains four Smad binding elements (SBE) that are specifically recognized by activated Smad3/4 (17) and the plasmid p3ARE that contains the activin responsive element that is regulated by Smad2/4 and FAST-1 (forkhead activin signal transducer-1; 23). TGFβ activated Smad3/4 but had little or no effect on Smad2/4, whereas EGF inhibited Smad3/4 and Smad2/3 activities regardless of TGFβ stimulation (Supplementary Fig 2B,C). Thus, synergism between EGF and TGFβ does not involve increasing the nuclear activities of the Smads.

**Synergism between EGF and TGFβ involves an interaction between AP-1 and Smads at the promoter.** When tested with the reporter plasmid p3TP, which contains adjacent AP-1 and Smad-binding elements, EGF and TGFβ increased expression with 3-fold synergism (Fig. 6A). These results suggest that cooperation between TGFβ and EGF involves interaction between AP-1 and Smad, both of which bind the PAI-1 promoter.

To confirm the involvement of AP-1 in the synergism between EGF and TGFβ, we tested two AP-1 antagonists, curcumin and dominant negative Fos (ΔFosB). Both antagonists significantly reduced 3TP activation in response to the combination of EGF and TGFβ but not to EGF or TGFβ alone (Fig. 6B,C). By contrast, curcumin did not affect activation of the pSBE4 promoter that contains only Smad binding elements (Fig. 6D). ΔFosB also decreased synergistic induction of PAI-1 mRNA in Mv1Lu cells, demonstrating that the same mechanism as is seen in the reporter constructs is likely to be occurring on the endogenous PAI-1 promoter (Fig. 6F, Supplementary Table III). Neither Fos nor Jun phosphorylation was regulated synergistically (Fig. 6E).

**DISCUSSION**

Synergism between cell regulators is a powerful means of increasing the strength of the response. EGF and TGFβ synergize to regulate several important cellular processes including colony formation in soft agar (24), the epithelial to mesenchymal transition (EMT) (25) and cell movement during tissue remodeling (26) The ability to tightly control gene expression by the synergistic interaction of extracellular regulators such as growth factors is likely to be an important aspect of gene regulation in vivo and has already been demonstrated for some systems (27-29).

Genes that are regulated synergistically include those encoding the EGF receptor, several matrix metalloproteinases, fibronectin, β and γ actin (30-32). Synergism was also concluded for leukemia-inhibitory factor (LIF) mRNA turnover based on an observed increase in LIF mRNA with no regulation of 6
transcription (33). Although the COX2 protein was reported to be synergistically induced by EGF and TGFβ in Mv1Lu cells (34), we observed no synergism in the regulation of COX2 mRNA in this study.

Our results, reported here and previously, show that EGF and TGFβ synergistically increase PAI-1 protein (9) and mRNA expression by an order of magnitude over the effect of either growth factor alone and by almost two orders of magnitude over the level in untreated cells. The combined increase in PAI-1 is about four times that expected from summing the individual responses and is the result of an integrated cellular response that includes 1) synergistic increase in transcription involving AP-1 and Smad, 2) stabilization of PAI-1 mRNA by EGF, 3) sensitization of EGF signaling by TGFβ.

The main target of the transcriptional cooperation between EGF and TGFβ is the interaction of AP-1 and Smad at the promoter. Cooperative interactions between exogenously expressed AP-1 and Smads in regulating gene expression have been demonstrated with several constructed promoters and endogenous PAI-1 expression (21,22).

We also found that the large increase in PAI-1 expression due to transcription was further amplified by stabilization of PAI-1 mRNA by EGF. This result is consistent with other reports of EGF stabilizing mRNAs, including those encoding β–tubulin, the EGF receptor, p21/CIP1, cyclin D1, TGFα, amphiregulin (AR), IL-1α, IL-1β and IL-6 LIF, TGFβRII and gastrin (11,33,35-41). Because PAI-1 mRNA is rapidly degraded, the regulation of its half-life can have a large impact on PAI-1 expression (42). The 2-fold synergism in transcriptional activity and 2-fold increase in stability of PAI-1 mRNA due to EGF calculates to about a 4-fold synergism between EGF and TGFβ.

PAI-1 plays an important role in the epithelial to mesenchymal transition (EMT) by modulating cell motility and facilitating conversion to an invasive phenotype, perhaps by modifying the cells’ environment (32,43). Therefore it may be relevant that both Mv1Lu and HepG2 cells are epithelial cell types. We have not observed synergism between EGF and TGFβ in PAI-1 expression in NRK and AKR-2B cells, both fibroblast cell types (data not shown).

With inhibition of Mek1/2 the synergism between EGF and TGFβ was lost, which suggests the MAPK pathway contributes to the synergistic regulation. Consistent with this conclusion is our observation and that of others of the synergism between TGFβ and growth factors that stimulate the MAPK pathway (44,45). Protein kinase C can also activate the MAPK pathway and in glioma cells, PKCδ Is activated by EGF via Src (46). However, in this study, we found that inhibiting Src did not prevent synergism between EGF and TGFβ. We also found that Erk1/2, the major target of Mek1/2 phosphorylation was not synergistically phosphorylated. Thus, Mek is required for synergism, but is not downstream from the synergistic event. By activating Mek1, EGF initiates phosphorylation of Smad2 at its C-terminal domain to activate Smad-mediated transcription (47). But, we found no synergism in Smad2 C-terminal phosphorylation or of June or Fos phosphorylation.

The complex cellular response to the combination of EGF and TGFβ also involves TGFβ lowering the cells’ sensitivity to EGF by about 10-fold. This effect of TGFβ is most likely not mediated by a change in the affinity of the EGF receptor for its ligand, which has been reported not to be influenced by TGFβ in Mv1Lu cells (31). Although the increased sensitivity to EGF does not contribute to the synergism between EGF and TGFβ on PAI-1 expression, its outcome is to make it more...
likely for cells to respond to the EGF in their environment. Thus, when TGFβ activates PAI-1 expression in the absence of concurrently added EGF, it might still act synergistically with low levels of EGF already present in the cells’ environment, which it effectively recruits to promote PAI-1 expression. This may explain why PAI-1 does not appear to be regulated synergistically by EGF and TGFβ in some cells that might synthesize enough EGF or other growth factor to prime the synergistic mechanism in an autocrine fashion such that only TGFβ is required to complete the synergistic signal. Modulating the efficiency of a cooperating growth factor is an effective way for a single growth factor to achieve a large change in gene expression.

If the pathways by which EGF and TGFβ stimulate PAI-1 transcription ultimately converge on the same event, such as to activate a protein kinase or a transcription factor, synergism might be observed at concentrations of each that are below saturating for the targeted response. But, once one each growth factor is present at saturating concentrations with the target event fully activated, the other growth factor should have no further stimulatory effect. If such were true for the effect of EGF and TGFβ on the regulation of PAI-1 expression then their synergism should be observed only when they are present at sub-saturating concentrations as observed for the synergism between EGF and insulin in stimulating S6 phosphorylation (48). By contrast the synergism between EGF and TGFβ is observed when either or both growth factors are present at saturating concentrations. This observation suggests that the combination of the two growth factors results in a signal that is different from those initiated by either growth factor alone. The involvement of an additional signal that depends on both growth factors is consistent with the published observation that the combined expression of recombinant Smad and AP-1 results in increased promoter activity that is further increased by TGFβ (22).

In summary, we have shown that TGFβ and EGF cooperation synergistically to regulate PAI-1 expression by an integrated and multidimensional mechanism involving effects on signal transduction, transcription, mRNA turnover, a change in cellular sensitivity to EGF. Such combined mechanisms of regulation may be how the local and systemic concentrations of PAI-1 are so closely controlled in vivo so as to appropriately regulate the level of plasminogen activator activity and plasmin production. This integrated regulatory mechanism may also control the expression of other genes and cellular processes that are synergistically regulated by EGF and TGFβ.
REFERENCES

FOOTNOTES

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LEGENDS TO FIGURES

Figure 1. Synergistic regulation of PAI-1 protein and mRNA in mink and human cells. A,B) PAI-1 protein: Mv1Lu and human HepG2 cells were labeled with a 4 h pulse of $^{35}$S-methionine (Mv1Lu) or $^{35}$S-Trans-label (HepG2) after incubating the cells for 2 h (Mv1Lu) or 8 h (HepG2) with growth factors. The amount of radiolabeled PAI-1 in the medium (Mv1Lu), or after immunoprecipitation with PAI-1 antiserum (HepG2), is shown after resolution by SDS-PAGE (duplicate samples shown for HepG2). The bands identified for Mv1Lu as PAI-1 were verified as such by immunoprecipitation (9). B) The effects of EGF and TGFβ on PAI-1 synthesis in Mv1Lu cells were quantified by scanning the identified bands (PAI-1 and 38 kDa) in the gel shown in the top left panel (Mv1Lu) and normalizing these values to the total cell acid-precipitable $^{35}$S-cpm. Duplicate independent samples were tested and the average results with standard errors are shown. C,D) PAI-1 mRNA: Cells were treated for 2 h with 5 ng/ml EGF, 1 ng/ml TGFβ or the combination of EGF and TGFβ at the same concentrations. PAI-1, uPAR, COX2 and uPA mRNAs were quantified by RT-qPCR for Mv1Lu (C) and HepG2 (D). -, vehicle control; E, EGF; T, TGFβ; TE, TGFβ and EGF.

Figure 2. TGFβ synergizes with various ligands of RTK receptors and with PMA. A,B) PAI-1 mRNA levels, A: Cells were treated for 2 h with or without 5 ng/ml EGF, 5 ng/ml FGF-2, 5 ng/ml TNFα or 150 ng/ml IGF-I in the presence or absence of 1 ng/ml TGFβ. PAI-1 mRNA was measured by RT-qPCR. B: The synergism observed in A is quantified. N: Number of independent experiments. C,D) Secreted protein levels. Mv1Lu were labeling with a 4 h pulse of $^{35}$S-methionine after 2 h incubation of the cells with growth factors. The amount of radiolabeled PAI-1 secreted into the medium was determined by autoradiography. The effects of TGFβ and PMA on PAI-1 synthesis in Mv1Lu cells were quantified by scanning the identified bands (C: PAI-1 and D: 73 kDa protein) in the gel and normalizing these values to the cell monolayer acid-precipitable $^{35}$S-cpm. More than 4 independent experiments were performed for each condition, and the data was shown as mean ± standard errors.

Figure 3. MAPK is involved in the synergism. A-E) Effects of blocking specific pathways by kinase inhibitors. Cells were treated for 1 h with inhibitors (or control analogs) prior to 2 h with growth factors. PAI-1 mRNA was measured by RT-qPCR. Empty bars: control analogs; filled bars: inhibitors. F) No synergism in phosphorylation of Erk1/2. Cells were treated for 1 h with 20 µM Mek inhibitor U0126 or its inactive analog U0124 then for 10 min with 1 ng/ml TGFβ, 5 ng/ml EGF or their combination. The phosphorylation of Erk1/2 was determined by western blot. -: vehicle control, E: EGF, T: TGFβ, TE: EGF and TGFβ.
Figure 4. A) Synergism does not require de novo protein synthesis. Cells were first treated with 50 μg/ml cycloheximide for 1 h and then with 1 ng/ml TGFβ, 5 ng/ml EGF, or their combination for 2 h. PAI-1 mRNA was measured with RT-qPCR. About 3-fold of synergism was observed in both cycloheximide-treated (■) and control (□) cells. B) EGF, but not TGFβ, increases the half life of PAI-1 mRNA. Mv1Lu cells cultured in DME medium with 0.2% calf serum were incubated for 2 h with 1 ng/ml TGFβ and 5 ng/ml EGF or their combination. Cordycepin (15 μg/ml) was then added (set as zero time) to block transcription. Total RNA was isolated at the indicated times and PAI-1 mRNA was quantified and normalized to the GAPDH values in the same samples. The results are from at least five independent experiments. The half life of PAI-1 mRNA in TGFβ-treated cells was calculated to be 36 min (regression coefficient from a least squares analysis = 0.90) and that in EGF-treated cells as 53 min (regression coefficient = 0.90). The half life of PAI-1 mRNA in TGFβ plus EGF treated cells is 58 min (regression coefficient = 0.80). When tested by using analysis of covariance (JMP 7.0) the difference between treatment with TGFβ alone and treatment with the combination of EGF and TGFβ was significant to the level of p < 0.05.

Figure 5. A,C) EGF and TGFβ cooperate to increase PAI-1 transcription. Cells were treated for 2 h with 5 ng/ml EGF, 1 ng/ml TGFβ or their combination. Newly transcribed PAI-1 mRNA (A) and total mRNA (C) were quantified by the nuclear run-on assay. Shown are the average results with the SEM from two independent experiments. B,D) The synergistic mechanism is activated soon after growth factor addition. Cells were treated with EGF and TGFβ or their combination for various time periods then 4SU was added for the last 15 min of each incubation. Newly synthesized (B) and total (D) RNAs were isolated and quantified for PAI-1 and GAPDH by RT-qPCR and the PAI-1 value was normalized to the GAPDH value for the same sample. -, vehicle control; E, EGF; T, TGFβ; TE, TGFβ and EGF.

Figure 6. AP-1 is involved in the synergism between EGF and TGFβ. A: Transcriptional activities mediated by Smad2/3 and AP-1 respond synergistically to EGF and TGFβ. Cells were transfected with the 3TP luciferase reporter for 17 h then for 6 h with growth factors B, C: Synergism is inhibited by curcumin and ΔFosB. Cells were transfected with 3TP with and without ΔFosB for 17 h then for 6 h with growth factors with or without 20 μM curcumin, which was added 1 h prior D: Curcumin has no effect on Smad activity. Cells were transfected with pSBE4 and treated with curcumin and growth factors. Promoter activities were measured with luciferase assay. E: No synergism on phosphorylation of Fos and Jun. Cells were treated with growth factors for 40 min. Western blots showing the phosphorylation of Fos and Jun in the same samples. The lower portion of the gel (not transferred to nitrocellulose) was stained with Coomassie blue and is shown as the loading control. F: Synergistic induction of PAI-1 mRNA is inhibited by ΔFosB: Cells were transiently transfected with ΔFosB for 17 h then for 2 h with growth factors then PAI-1 mRNA was quantified relative to GAPDH -. vehicle control, E: EGF, T: TGFβ, TE: EGF and TGFβ. Empty bars: control; Filled bars: inhibitors.
Figure 2
Figure 3
Figure 6

3TP (Smad and AP-1)

A

pSBE4 (Smad)

promoter activity

B

curcumin

C

ΔFosB

3TP (Smad and AP-1)

D

curcumin

E

loading control

F

PAI-1 mRNA

- E T TE - E T TE - E T TE
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