1992

Cloning and characterization of mink plasminogen activator inhibitor type 1 (PAI-1) cDNA and the regulation of mink PAI-1 expression at mRNA level

Tsung-Hsien Chuang
Iowa State University

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Cloning and characterization of mink plasminogen activator inhibitor type 1 (PAI-1) cDNA and the regulation of mink PAI-1 expression at mRNA level

Chuang, Tsung-Hsien, Ph.D.

Iowa State University, 1992
Cloning and characterization of mink plasminogen activator inhibitor type 1 (PAI-1) cDNA and the regulation of mink PAI-1 expression at mRNA level

by

Tsung-Hsien Chuang

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GENERAL SUMMARY

LITERATURE CITED
INTRODUCTION

At least four immunologically distinct plasminogen activator inhibitors (PAIs) have been found. Plasminogen activator inhibitor type 1 (PAI-1) formerly called the endothelial cell inhibitor (1,2) is found in the α-granules of blood platelets (3,4) and in plasma (5,6). Plasminogen activator inhibitor type 2 (PAI-2) was originally named placental type inhibitor (7,8) because of the original source for its purification. Plasminogen activator inhibitor type 3 (PAI-3) is detected in urine (9) and also found in blood (10). Protease nexin was purified from culture medium of human foreskin fibroblast cells (11).

These PAIs are members of the SERPIN (serine protease inhibitor) family. The function of these PAIs is to act as binding proteins of plasminogen activators (PAs). Two distinct types of plasminogen activators that have been found are tissue type plasminogen activator (t-PA), and urokinase type plasminogen activator (u-PA) (12). Plasminogen activators are serine proteases that convert plasminogen to plasmin. Plasmin is a broad spectrum protease that degrades fibrin as well as several extracellular matrix proteins (13,14). Thus, by controlling the plasminogen and plasmin cascade, the PAIs regulate several biological processes such as vascular fibrinolysis, cellular migration and tissue remodeling (12,15,16).
Of the PAIs, PAI-1 is the major inhibitor of PAs. The interaction between PAI-1 and PAs is rapid, with a second order rate constant between $10^7 - 10^8 \text{ M}^{-1}\text{s}^{-1}$ (17) whereas the rate constants for interaction between PAI-2 (18), PAI-3 (19) and protease nexin (20) is only between $10^3 - 10^5 \text{ M}^{-1}\text{s}^{-1}$.

Plasminogen activator inhibitor type 1 is a glycoprotein with molecular weight 50 kD. Complementary DNAs of PAI-1 have been cloned from human cells (21), rat cells (22) and bovine cells (23). The deduced amino acid sequence of human PAI-1 revealed that this protein contains a signal peptide with 23 amino acids, a mature peptide with 379 amino acids and three potential N-glycosylation sites. Synthesis of PAI-1 in cultured cells is highly regulated. Several agents such as lipopolysaccharide, interleukin-1, serum, transforming growth factor-β, basic fibroblast growth factor, tumor necrosis factor, thrombin, dexamethasone and phorbol ester are known to induce PAI-1 synthesis in cultured cells (24).

Transforming growth factor-βs (TGF-βs) are a group of bifunctional growth factors that can control cell growth and differentiation. Distinct molecular forms of TGF-β have been identified in mammals that are designated TGF-β1, TGF-β2 and TGF-β3 (25,26,27,28). Sources for isolating TGF-β are platelets, placenta and kidney. TGF-β isolated from these sources is a 25 kD protein with two 12.5 kD peptides linked together by interchain disulfide bonds. As a growth activator, TGF-β
stimulates anchorage independent growth of rat NRK-49F fibroblast cell and mouse embryo AKR-2B fibroblast cell (29). Anchorage independent growth of NRK-49F cells is stimulated synergistically with EGF. As a growth inhibitor, TGF-β inhibits the growth of cultured monkey kidney BSC-1 cells, mink lung CCL64 cells and other cell lines, both neoplastic and nonneoplastic (30). In cultured cells, TGF-β regulates the synthesis of many proteins which are important in extracellular matrix construction and remodeling. Those proteins include collagenase (31), plasminogen activator (32), plasminogen activator inhibitor (33,34), fibronectin and fibronectin receptor (35,36). The mechanism by which TGF-β regulates the synthesis of these proteins is not well understood. However, cellular responses to TGF-β are mediated by its receptors. Three different TGF-β receptors have been discovered (37,38) with molecular weight of 50-80 kD (type I), 115-140 kD (type II) and 280-330 kD (type III). Transforming growth factor-β1, TGF-β2 and TGF-β3 bind to all three receptors (39). The dissociation constant between TGF-β and these receptors is about 10-200 pM (37). The receptors do not possess tyrosine kinase activity (40). The ability of type I receptor to bind TGF-β is increased by expression of type II receptor (41). The ability of type II receptor to bind TGF-β is increased by expression of type III receptor (42).

Epidermal growth factor (EGF) is a growth factor originally discovered because of its ability to stimulate the
opening of eyelids of newborn mice and to accelerate eruption of mouse teeth. This ability is due to the stimulation of epidermal growth and keratinization by EGF (43). Male mouse salivary glands and human urine are good sources for isolating EGF. Epidermal growth factor from human was known as urogastrone. Both mouse EGF and urogastrone are single peptide chain of 53 amino acids with 70% homology to each other (44,45). In cultured cells EGF stimulates the synthesis of plasminogen activator (46,47), plasminogen activator inhibitor (35,48), ornithine decarboxylase (49), c-jun and c-fos gene products (50,51). The effects of EGF are mediated by the EGF receptor which is a 170 kD transmembrane glycoprotein with 1180 amino acids. The EGF receptor contains an intracellular tyrosine kinase domain. Stimulation of this kinase activity by EGF leads to its autophosphorylation and to the phosphorylation of various other cellular substrates.

12-O-tetradecanoylphorbol-13-acetate (TPA) is one of a group of chemical compounds known as tumor-promoting phorbol esters. The biological activity of phorbol esters includes influencing the state of cell differentiation, proliferation and the expression of specific genes (52,53,54). As an analogue of diaceylglycerol, TPA activates the calcium and phospholipid dependent protein kinase C (55). A TPA response element (TRE) in the 5'-regulatory region of genes has been identified (56,57,58) with a consensus sequence 5'-
TGA(C/G)TCA-3'. Activation of genes through this element is mediated by binding of the AP-1 complex, which is a dimer of the protooncogene products of c-fos and c-jun. The human PAI-1 gene has no TRE consensus sequence (59,60) but has a sequence in which six of seven nucleotides are conserved (GGAGTCA). This sequence is located at position -672 to -666 upstream of the PAI-1 CAP sites. Two segments which confer TGF-β inducibility on the PAI-1 gene are -791 to -546 and -328 to -187 upstream of the CAP sites (61). The TRE-like sequence is localized in the former region.

Previous studies from this laboratory showed that the synthesis of PAI-1 is induced by TGF-β and TPA in different cell lines such as African green monkey kidney epithelial BSC-1 cells, rat kidney NRK cells, mouse embryo AKR-2B cells and mink lung epithelial CCL64 cells (34). In mink lung CCL64 cells PAI-1 is induced by EGF, TGF-β and TPA. Transforming growth factor-β and EGF act synergistically to increase the level of PAI-1. The synergistic effect was also observed in human hepatoma Hep G2 cells (62).

To study the synergistic effect and the mechanisms by which the PAI-1 gene is regulated in mink lung CCL64 cells, I tested the following hypothesis: the expression of PAI-1 gene is regulated at the mRNA level by altering the transcriptional activity of the PAI-1 gene and by altering the stability of PAI-1 mRNA.

Growth factors and tumor promoters can increase tran-
scriptional activity and mRNA stability of inducible genes in cultured cells. For example, epidermal growth factor stabilizes the mRNA of the EGF receptor, β-tubulin and β-actin in cultured human KB epidermal carcinoma cells (63). Transforming growth factor-β increases the steady state level of type I procollagen and fibronectin mRNA by acting at the post-transcriptional level in cultured human dermal fibroblasts (64). An increase in transcriptional activity is responsible for the induction of PAI-1 in bovine aortic endothelial cells (65), human rhabdomyosarcoma cells (66).

In the present studies, the accumulation of PAI-1 mRNA, the half life of PAI-1 mRNA, and the transcriptional activity of the PAI-1 gene were determined in mink lung CCL64 epithelial cells treated with EGF, TGF-β, EGF plus TGF-β or TPA by using a cloned mink PAI-1 cDNA as a probe. The results indicated that TGF-β increases the rate of PAI-1 gene transcription and EGF stabilizes PAI-1 mRNA. The combination of the effect of TGF-β and EGF result in a synergistic effect on PAI-1 expression. The TPA induced PAI-1 mRNA has a fast turnover rate with half life about 25 min. The fast degradation rate causes a rapid decrease of accumulation in PAI-1 mRNA and PAI-1 protein in these cells after the transcription rate decreases.
EXPLANATION OF THESIS/DISSERTATION FORMAT

An alternate format as outlined in the Iowa State University Graduate College Thesis Manual is used for this dissertation. This dissertation is separated into four parts: Introduction, Section I, Section II and Summary. The introduction gives a brief description of those proteins and growth factors that were studied in this research project and provides a background for the research. Section I, "Cloning and post-transcriptional regulation of mink plasminogen activator inhibitor type 1 gene in mink lung CCL64 cells" describes the procedures for mink PAI-1 cDNA cloning and the characterization of the mink PAI-1 clone. The post-transcriptional regulation of 12-0-tetradecanoylphorbol-13-acetate induced PAI-1 mRNA is also described in this section. Section II, "A synergistic effect of transforming growth factor-beta and epidermal growth factor on the induction of plasminogen activator inhibitor type 1 in mink lung CCL64 epithelial cells" provides an insight into the mechanism by which induction of PAI-1 in mink lung CCL64 cells is synergistically regulated. Coauthor of section I, Dr. Richard T. Hamilton, is a faculty of the Department of Zoology and Genetics of Iowa State University. He was responsible for helping me in the cloning of the PAI-1 cDNA. Coauthor of section I and II, Dr. Frederic W. Thalacker, was a graduate student in our
laboratory. He contributed the results showing the regulation of the PAI-1 protein. The summary discusses the conclusions of my dissertation. The references in each section of the dissertation follow the format of the journal to which the paper will be submitted.
SECTION I.

CLONING AND POST-TRANSCRIPTIONAL REGULATION OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 IN MINK LUNG CCL64 EPITHELIAL CELLS
Cloning and Post-transcriptional Regulation of Plasminogen Activator Inhibitor Type 1 gene in Mink Lung CCL64 Cells

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INTRODUCTION

Proteolytic degradation of the extracellular matrix that surrounds cells is associated with biological processes such as fibrinolysis, cellular migration, tissue remodeling, and tumor metastasis (10, 12, 30). One of the mechanisms which has been implicated in the proteolytic process is the plasminogen activator/plasmin system (44). Two distinct groups of plasminogen activators, urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), convert the inactive zymogen plasminogen into plasmin. A broad spectrum serine protease, plasmin degrades fibrin and extracellular matrix proteins. Both u-PA and t-PA are negatively regulated by plasminogen activator inhibitor type 1 (PAI-1). The inhibition is rapid with a second-order rate constant between $10^{-10}$ M$^{-1}$s$^{-1}$ (17). Therefore, the regulation of PAI-1 expression is likely to be important in controlling these biological processes.

PAI-1 is a glycoprotein with a molecular weight of 50 kD that is present in plasma and platelets (8, 14). In a variety of cultured cells, the production of PAI-1 is modulated by different agents. For example transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), lipopolysaccharide (LPS) and interleukin induce the synthesis of PAI-1 in endothelial cells (8, 13, 33, 37). Transforming growth factor-
β, dexamethasone and hydrocortisone stimulate the production of PAI-1 in fibroblast cells (9, 46, 20). TGF-β, epidermal growth factor (EGF) and 12-O-tetradecanoylphorbol-13-acetate (TPA) increase the secretion of PAI-1 in epithelial cells and hepatoma cells (46, 20, 22). Synthesis of PAI-1 in these cells is generally preceded by an increase in the steady state level of PAI-1 mRNA. Evidence has been provided that transcriptional regulation is responsible for the stimulatory effect on PAI-1 induction. Transforming growth factor-β was also found to stabilize PAI-1 mRNA in human hepatoma cells (48).

In previous studies (46), we found that the synthesis of PAI-1 is regulated by TPA, TGF-β, and EGF in mink lung CCL64 epithelial cells. The rate of production of secreted PAI-1 reaches a maximum between 5 to 7 hours after the cells are treated with TGF-β and then declines rapidly. Compared to that reported in bovine aortic endothelial cells (36), human rhabdomyosarcoma cells (24) and human WI-38 lung fibroblast cells (23), the rate of accumulation and decline of PAI-1 mRNA in mink lung CCL64 epithelial cells is rapid.

In these studies, it was found that PAI-1 mRNA accumulates rapidly after the addition of TPA and reaches a maximum at 3 hours after TPA addition. The PAI-1 mRNA level then declines rapidly to the basal level at 7 hours after TPA addition. The brief period of PAI-1 gene expression reflects
an increase in gene expression and a fast turn over rate of PAI-1 mRNA. The half-life of PAI-1 mRNA was measured to be 25 min. Degradation of PAI-1 mRNA was retarded by cycloheximide and actinomycin D. Messenger RNA of PAI-1 in mink lung CCL64 cells shares several common features with mRNA of proto-oncogene c-fos and c-myc (41, 49). These mRNAs are inducible, rapidly degraded and stabilized by cycloheximide and actinomycin D. A GU-rich sequence was identified from the 3'-untranslated region of the PAI-1 mRNA. This sequence in 3' LTR of type 1 human T cell leukemia virus was reported to directed the cleavage of viral pre-mRNA (1). Another region of 120 bp that is highly conserved between PAI-1 mRNAs from different species was also identified. It is expected that these regions might be important in regulating turnover of PAI-1 mRNA.
MATERIALS AND METHODS

Cell culture: Mink lung CCL64 epithelial cells (a gift from Dr. Robert Holley) were routinely cultured in Dulbecco-Vogt's modified Eagle's medium (DME) containing 0.45% glucose, 10% calf serum, 10 unit/ml penicillin and 10 μg/ml streptomycin at 37°C in water saturated atmosphere with 13% CO₂ in air. For experiments, cells were plated in DME with 10% calf serum. Once confluent, the medium was changed to DME with 0.2% calf serum and the cells incubated for another 24 hours before addition of TPA (10 ng/ml; Sigma).

Preparation of RNA: For harvest of cells, cell culture medium was aspirated and cells were washed twice in Tris-buffered salts (0.14 M NaCl, 5.0 mM KCl, 0.68 mM CaCl₂, 0.49 mM MgCl₂, 0.70 mM Na₂HPO₄, 25 mM Tris-HCl pH 7.1). After scraping the cells into 4 M guanidinium isocyanate, 25 mM sodium acetate pH 6, the total cellular RNA was separated from the cell lysate by centrifugation through a cushion of 5.7 M cesium chloride, 25 mM sodium acetate pH 6 (6), at 195,000 x g for 18 hours. Poly(A⁺)-mRNA was prepared by affinity chromatography on oligo(dT)-cellulose (4). All the solutions for RNA preparation were made with diethylpyrocarbonate treated water.

cDNA Cloning and Sequencing: A mink cDNA library with approximately 1 x 10⁶ individual clones was constructed in the Zap
II vector (Stratagene) with poly(A\textsuperscript{+}) mRNA prepared from cultured TPA stimulated mink lung epithelial CCL64 cells. After amplification, the library was screened with radiolabeled human PAI-1 cDNA (3) (EcoRI-Bgl II fragment; specific activity $1 \times 10^9$ cpm/μg) in a hybridization buffer (40% formamide, 7% SDS, 1% polyethylene glycol MW=20000, 0.25% non-fat milk, 0.6 M NaCl, 4 mM EDTA-Na\textsubscript{2}, 50 μg/ml salmon sperm DNA, 40 mM NaH\textsubscript{2}PO\textsubscript{4} pH 7.5). Positive phage clones were converted into the phagemid form in the Bluescript SK(-) vector by in vivo excision (40). Plasmids were isolated and the size of the cDNA inserts were determined by restriction enzyme digestion and analysis by agarose gel electrophoresis. Nested deletions from both strands were constructed using exonuclease III and mung bean nuclease digestions (Stratagene protocol). Sequencing was performed from double stranded plasmid DNA by the dideoxy chain termination method (34) and the data was analyzed with the aid of computer sequence analysis program (GCG package) designed by Genetics Computer Group of the University of Wisconsin (11).

[^35]S-Methionine labeling and SDS-polyacrylamide gel electrophoresis: Cells were plated on 24 wells (1.6 cm diameter/well) tissue culture dishes with DME medium containing 0.2% calf serum. One or two days later, after cell attachment, the medium was replaced with fresh medium and TPA was added about 24 hours later. After the indicated incubation time, the
cells were labeled with $[^{35}S]$-methionine (60-200 $\mu$Ci/ml, Amersham). The labeling medium was collected, the cell were rinsed once with Tris-buffered salts and lysed with NP-40 buffer (0.5% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 100 Kallikrein inactivator units of aprotinin/ml, 10 mM Tris-HCl pH 7.1). The cell lysate was separated into NP-40 soluble and insoluble fractions by centrifugation at 3000 X g for 20 min. The $[^{35}S]$-methionine labeled samples were resolved by 7.5% - 15% gradient SDS polyacrylamide gel electrophoresis (27). Fluorography and densitometric measurements were used to determine the amount of $[^{35}S]$-methionine incorporated into each protein band. The value of the area under the peak was divided by the amount of acid insoluble $[^{35}S]$-methionine radioactivity in the NP-40 soluble fraction so that the results from the densitometer were normalized with the rate of $[^{35}S]$-methionine incorporation into cellular proteins.

Northern Blot and Hybridization: Fractionation of RNA on 1% agarose-formaldehyde gel was performed by using MOPS buffer (20 mM 3-[N-morpholino]propanesulfonic acid, 1 mM EDTA, 5 mM sodium acetate pH 6) as a running buffer. Northern blots were performed by transferring the RNA to nylon membranes (Zeta probe, from Bio-Rad) with 50 mM sodium hydroxide solution. RNA on the blots was hybridized with radiolabeled mink PAI-1 probe for 18 hours at 47°C in hybridization buffer. The blots were washed to a final stringency 30 mM NaCl, 3 mM sodium citrate, 0.2% SDS twice for 15 min at 47°C and exposed to
Kodak XAR film at -75°C with intensifying screens. To evaluate the relative loading of RNA samples, the blots were first stripped by incubating with 0.1 % SDS, 15 mM NaCl, 1.5 mM sodium citrate, 30 min at 80° twice and then rehybridized with radiolabelled 18 S ribosomal RNA probe (15). The densities of autoradiographic signals were quantified by scanning densitometry (Zenith model 504 XL). The relative densities of individual bands from PAI-1 mRNA were normalized with the relative densities of 18 S ribosomal RNA in each samples. **Nucleotide sequence accession number:** The mink PAI-1 cDNA sequence has been submitted to the GenBank/EMBL data bank under accession number X58541.
RESULTS

Cloning of mink PAI-1 cDNA: Fourteen positive clones were isolated by screening $4 \times 10^4$ phages from the amplified mink cDNA library with radiolabeled human PAI-1 cDNA fragment. A single clone with an insert size of 3 kb was chosen for further analysis. The nucleotide sequence of the mink PAI-1 cDNA is shown in figure 1. This cDNA codes for a 68 bp 5'-nontranslated sequence, a 1200 bp translational open reading frame, and a 1720 bp 3'-nontranslated region. The in-frame coding region of mink PAI-1 cDNA is translated into a 400 amino acid sequence (Fig. 1) which includes a leader peptide of 21 amino acids and a mature peptide of 379 amino acids. A comparison of the leader peptides of mink PAI-1 with those of human, bovine, rat and mouse PAI-1, showed that mink PAI-1 lacks two amino acids found in PAI-1 of the other species (Fig. 2). Because mink PAI-1 is secreted into culture medium by mink lung CCL64 cells (46), the missing two amino acids are obviously not essential for secretion of PAI-1. As with the other PAI-1s, there is no cysteine in the mature mink PAI-1 peptide. Mink PAI-1 has three potential N-glycosylation sites (Fig. 1) which are located at the same positions in the peptide sequence as in the human PAI-1 sequence. The entire amino acid sequence of mink PAI-1 is 86%, 87%, 79%, 77% identical to human (16), bovine (25), mouse (28), and rat
(50) PAI-1, respectively.

In the 1.72 kb 3'-nontranslated region, a 120-bp sequence was identified that is highly conserved in PAI-1 mRNAs from different species (Table 1). This region in the 3'-untranslated segment of PAI-1 mRNA has been previously reported to be more than 90% identical in nucleotide sequence between rat and human PAI-1 mRNAs (50). When the comparison of these regions is extended to bovine, mouse, human, and rat PAI-1 mRNAs the sequence reported shows between 88% and 95% identity to the species listed on Table 1 with the equivalent mink sequence. A GU-rich region (2769-GUUCUUUGUUUGUUUGUUU-2787) was also identified in the 3' end of the PAI-1 mRNA which is not conserved in PAI-1 mRNA from other species but is highly conserved with the GU-rich region (605-GUCUUUGUUUGUUUGUUU-623) on type 1 human T cell leukemia virus 3' LTR. The T-cell leukemia virus LTR GU-rich region directs its pre-mRNA cleavage and facilitates polyadenylation (1).

**Regulation of PAI-1 expression:** TPA stimulation of PAI-1 synthesis in CCL64 cells was studied with $[^{35}S]$-methionine labeling and analysis by SDS-polyacrylamide electrophoresis. A 48 kD protein was recognized as PAI-1 by its molecular weight and its ability to be immunoprecipitated with an antibody made against bovine PAI-1 (46). The results show that the production of secreted PAI-1 protein increased shortly after TPA stimulation and reached a maximum at 6-10
hours. The production of PAI-1 then declined rapidly and returned to the basal level after 18 hours (Figure 3). The profile of TPA induced PAI-1 production is similar to the profile for TGF-β induced PAI-1 synthesis (46) in which the maximum production rate occurred about 6 hour after induction with the rate declining to basal level after 18 hour. The time course of PAI-1 mRNA accumulation was determined (Figure 3). The PAI-1 transcripts were barely detectable 0.5 hours after TPA addition and reached maximal accumulation 3 hours after TPA addition. The level of PAI-1 mRNA then declined rapidly and reached its basal level after 7 hour.

Degradation of PAI-1 mRNA: CCL64 cells were incubated with TPA for two hours to induce the transcription of PAI-1 gene. After blocking further transcription with cordycepin, total RNA was isolated at different times and analyzed by Northern blotting. The result (Figure 4) shows that PAI-1 mRNA in the CCL64 cells has a half-life of 25 min. Cycloheximide treatment was observed to stabilize PAI-1 mRNA in the cordycepin treated cells. Actinomycin D, an agent usually used to block transcription by its ability to intercalate with GC base pairs of DNA, also stabilized PAI-1 mRNA (Figure 4). The stabilization effect of actinomycin D was also observed when in combination with cordycepin (Figure 4).
**TABLE 1**

Comparison of the nucleotide sequence of the high conserved 3'-nontranslation region of PAI-1 mRNA from different species.

The number in parentheses indicate the starting and termination nucleotide numbers of the conserved sequence. Identity of the sequences were analyzed by the computer program (11).

<table>
<thead>
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<th>Identity</th>
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<tr>
<td>mink PAI-1 (2413-2532)</td>
<td></td>
<td>93.3%</td>
</tr>
<tr>
<td>bovine PAI-1 (2446-2565)</td>
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<td>95.0%</td>
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<tr>
<td>human PAI-1 (2437-2556)</td>
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<td>89.2%</td>
</tr>
<tr>
<td>mouse PAI-1 (2446-2565)</td>
<td></td>
<td>88.1%</td>
</tr>
<tr>
<td>rat PAI-1 (2504-2623)</td>
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Figure 1. The nucleotide sequence and deduced amino acid sequence of mink PAI-1 cDNA: The single letter code is used for the amino acid sequence. The arrow indicates the leader peptide cleavage site. The asterisks(*) indicates potential N-glycosylation site. The active center is boxed. The region with 120 bp high conserved nucleotide sequence is underlined. The GT rich region is doubly underlined and the polyadenylation signal is indicated by five asterisks above the letter code for the bases.
Figure 2. Comparison of the leader peptides of the PAI-1 from different species. The numbers indicate the amino acid position.
mink PAI-1  1 MQMST..VCLALGLALVFGEASA  21
human PAI-1  1 MQMSPALTCLVLGLALVFGEASA  23
bovine PAI-1  1 MRMSPVFACLALGLALIFGEASA  23
rat PAI-1  1 MQMSSALTCLTLGLVLVFGKGFA  23
mouse PAI-1  1 MQMSSALACLILGLVLVSGKGFT  23
Figure 3. Regulation of PAI-1 expression by TPA.

A: Time course of PAI-1 protein synthesis: CCL64 cells were plated in DME medium with 0.2% calf serum. Twenty four hours later TPA (10 ng/ml) was added to the cells. The cells were labeled with $[^{35}\text{S}]$-methionine for 4 hrs, beginning from 0 to 18 hrs after addition of TPA. The symbols in the figure are located at the middle point of the labeling periods.

Time course of PAI-1 mRNA accumulation: CCL64 cells were incubulated in DME medium with 0.2% calf serum. After treated with 10 ng/ml TPA, total RNA was isolated from cells at various times. Total RNA (10 $\mu$g) from specific time points was resolved by formaldehyde-agarose gel electrophoresis. After blotting to nylon membranes, the RNAs were probed with $^{32}\text{P}$-labeled PAI-1 and $^{32}\text{P}$-labeled 18S ribosomal RNA probes. The relative intensities of the PAI-1 mRNA and 18S ribosomal RNA bands on autoradiograms were analyzed by laser densitometry. To control for the loading of total RNA, the intensity of PAI-1 mRNA was normalized to the intensity of 18S ribosomal RNA. The results are representative of two separate experiments.

Protein (-A-); mRNA (-O-).

B: Northern blot exposures that were used to obtain the data shown in 3A.
Figure 4. Degradation of PAI-1 mRNA.
CCL 64 cells were incubated in DME with 0.2% calf serum. After incubation with 10 ng/ml TPA for two hours, cordycepin (15 μg/ml), actinomycin D (5 μg/ml), or cordycepin plus cycloheximide (10 μg/ml) were added (zero time). Total cellular RNAs were isolated at the specified times after zero time, resolved by agarose gel electrophoresis, and analyzed by Northern blot (10 μg RNA/lane). The probes were 32P-labeled PAI-1 and 18S ribosomal RNA probes. Autoradiograms were analyzed by laser densitometry. To control for loading of total RNA, the intensity of the signal from PAI-1 mRNA was normalized with intensity of signal from 18S ribosomal RNA. The results are from two separate experiments. Top panel: Effects of actinomycin D, cycloheximide, cordycepin on PAI-1 mRNA degradation within 2 hours of their addition. Bottom panel: Effects of actinomycin D, cycloheximide on PAI-1 mRNA degradation during a 7 hour period after their addition. Cord./CHX. (---); Act D (-O-); Cord. (-V-); Cord./Act D (-A-).
DISCUSSION

Biosynthesis of PAI-1 is regulated in cultured cells by growth factors, tumor promoters and hormones. These agents are known to stimulate PAI-1 synthesis by increasing PAI-1 gene transcription. For example, TGF-β, TNF-α and LPS increase PAI-1 gene transcription in bovine aortic endothelial cells (36), TPA increase PAI-1 gene transcription in human rhabdomyosarcoma cells (24) and dexamethanone activate PAI-1 gene in human fibrosarcoma cells (31). As well as gene transcription, regulation of mRNA stability is a very important means of controlling the rate of protein accumulation and the steady state levels of mRNAs in cells (38, 7, 32). Here we show that the expression of PAI-1 in mink lung CCL64 epithelial cells is governed both by increased synthesis after induction and the high turnover rate of its mRNA.

We have found that the TPA induced PAI-1 mRNA in CCL64 cells has a half life of about 25 min. Thus PAI-1 mRNA in CCL64 cells has a faster turnover rate than most mRNA in eukaryotic cells (43). The high turnover rate is an important factor that determines the brief time span of accumulation of PAI-1 mRNA in CCL64 cells. In these cells the PAI-1 mRNA rapidly accumulates to its maximum 3 hour after induction and declines to basal levels after 7 hour. The profile of mRNA accumulation is followed in time by a similar profile of the
production of secreted PAI-1 protein. The production of secreted PAI-1 increases to a maximum at 6-10 hour and returns to its basal level about 18 hour after the cells were treated with TPA.

The rate of PAI-1 mRNA turnover is decreased by cycloheximide and actinomycin D. These effects have been reported for other mRNAs. For example, actinomycin D stabilizes the mRNA of a cytokine gene, gro (45). Actinomycin D and cycloheximide prevent the decay of mRNA encoding the transferrin receptor (26), truncated c-fos (41), and c-myc (49). A model that has been proposed to explain the effects of actinomycin D and cycloheximide (45, 26, 41) involves a labile factor encoded by a rapidly turning over mRNA that is involved in regulating degradation of selected mRNAs. In this model Actinomycin D and cycloheximide stabilize specific mRNAs by blocking synthesis of the labile factor. Our results can not support this model for PAI-1 mRNA because cordycepin, another transcriptional inhibitor, does not stabilize the PAI-1 mRNA. An alternate model (5) which has been postulated to explain the ability of cycloheximide to stabilize the mRNA of inducible early response genes was that the degradation of these mRNAs is associated with translation. In this model Cycloheximide blocks translational elongation by inhibiting the peptidyl transferase activity of the 60S ribosomal subunit and prevents degradation of the mRNA. We propose that actino-
mycin D may also stabilize PAI-1 mRNA by blocking translation. Actinomycin D is reported to inhibit binding of mRNAs to ribosomes (43, 42). Actinomycin D is known to bind mRNAs in vitro and in vivo (47). Therefore it is possible that the PAI-1 mRNA is prevented from binding to ribosomes after intercalation of actinomycin D into the secondary structure of PAI-1 mRNA.

To test the possibility that cordycepin promotes PAI-1 mRNA degradation by facilitating the removal of the polyadenylated tail, the stabilization effect of actinomycin D was tested in the presence of cordycepin. That the same stabilization effect of actinomycin D was observed indicated that 1) cordycepin does not decrease the stability of PAI-1 mRNA, and 2) the stabilizing effect of actinomycin D is posttranscriptional. In bovine aortic endothelial cells (36) the half-life of LPS, TGF-β and EGF induced PAI-1 mRNA was determined to be 2.2 hour. In human hepatoma cells (48) the half-lives of TGF-β and EGF induced PAI-1 mRNA were reported to be 3.1 hour and 2.3 hour respectively. However, in these experiments actinomycin D was used to block PAI-1 gene transcription. Results reported in this work indicate that PAI-1 mRNA has a shorter half-life in mink lung CCL64 cells compared to that in bovine aortic cells and human hepatoma cells or that the half-life of PAI-1 mRNA was misinterpreted by the previous investigators because they used actinomycin D as the transcriptional blocking agent.
Our results describe several interesting features of PAI-1 mRNA including its inducibility, high turnover rate and stabilization by cycloheximide and actinomycin D. A group of mRNAs from transiently expressed genes of cytokines and oncogenes also share these features. A conserved AUUUA sequence at the 3' untranslated region of these mRNAs was found to mediate mRNA degradation (39). Although this AU-rich sequence is not found in PAI-1 mRNA, a related GU-rich sequence is localized in the 3' untranslated region. A similar GU-rich sequence was found in the 3' LTR of type 1 human T cell leukemia virus. This region is believed to direct cleavage of the viral pre-mRNA (1). The GU-rich sequence in PAI-1 mRNA may also direct an endonucleolytic cleavage that renders the mRNA susceptible to further degradation. Besides the GU-rich sequence, a previously identified 120 bp, highly conserved sequence was found in the 3'-untranslated region. The significance of this high conservation is not yet clear but it is expected that this region must participate in some specific function because it has been maintained so faithfully through mammalian evolution.

In summary, we have demonstrated that PAI-1 mRNA has a very short half-life in mink lung CCL64 epithelial cells. The fast turnover rate of PAI-1 mRNA is consistent with the brief period of expression of the PAI-1 gene after the cells are stimulated by TPA. Analysis of the PAI-1 cDNA sequence iden-
tified regions that may be important for the regulation of mRNA degradation. These results also show that PAI-1 mRNA turns over considerably faster than previously reported. The rapid rate of mRNA degradation allows rapid changes in the amount of PAI-1 secreted by cells in response to specific stimuli during events such as fibrinolysis, cellular migration, and tissue remodeling.
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SECTION II.

A SYNERGISTIC EFFECT OF TRANSFORMING GROWTH FACTOR-BETA AND EPIDERMAL GROWTH FACTOR ON THE INDUCTION OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 IN MINK LUNG CCL64 CELLS
A synergistic effect of transforming growth factor-beta and epidermal growth factor on the induction of plasminogen activator inhibitor type 1 in mink lung CCL64 epithelial cells.

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Plasminogen activator inhibitor type 1 (PAI-1) is a serine protease inhibitor with a molecular weight about 50 kD. Because of its ability to control the activity of plasminogen activator (PA) and therefore the activation of plasmin, PAI-1 is proposed to mediate functions of growth factors.

The production of secreted PAI-1 is induced by transforming growth factor-beta (TGF-β) in mink lung CCL64 epithelial cells. Epidermal growth factor (EGF) interacts synergistically with TGF-β to increase PAI-1 expression in these cells to a level that is significantly higher than after stimulation by TGF-β alone. The individual and synergistic effects of EGF and TGF-β are observed at the mRNA level. Transforming growth factor-β increased the rate of PAI-1 gene transcription. However, TGF-β and EGF did not activate PAI-1 gene transcription synergistically. The half life of PAI-1 mRNA is 30 min in cells treated with TGF-β and 46 min in cells treated with TGF-β and EGF. It is concluded that the synergistic effect of TGF-β and EGF on PAI-1 induction is due to a combination of transcriptional activation by TGF-β and stabilization of PAI-1 mRNA by EGF.
INTRODUCTION

Plasminogen activator inhibitor type 1 (PAI-1) is a glycoprotein with molecular weight of 50 kD and is present in plasma and platelets (1,2). Plasminogen activator inhibitory activity is increased after major surgery (3), severe trauma (3) and myocardial infarction (4). In healthy individuals the PA-inhibitory activity in plasma fluctuates diurnally (5). Clearance of PA-inhibitory activity from the circulation is rapid with a half life of 3.5 minutes and 7 minutes in rat (6) and rabbits (7), respectively.

PAI-1 is a negative regulator of plasminogen activators. Interaction between PAI-1 and two distinct groups of plasminogen activator (urokinase-type plasminogen activator, and tissue type plasminogen activator) is rapid with a second order rate constant between $10^7-10^8 \text{ M}^{-1}\text{s}^{-1}$ (8). Plasminogen activators are serine proteases that convert the zymogen plasminogen into plasmin. Plasmin, a broad spectrum serine protease, degrades fibrin and extracellular matrix proteins. Therefore by controlling the PA-plasmin proteolytic cascade, PAI-1 is a crucial component in the regulation of many biological processes which involve the construction of extracellular matrices (9,10). Because of this property PAI-1 is proposed to mediate some of the effects of growth factors (11).
Transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) can influence the differentiation and proliferation of cells (12,13). A cellular response to a growth factor may be modified by the presence of another growth factor. For example, anchorage independent growth of rat kidney NRK cells is stimulated synergistically by TGF-β and EGF (14).

We found that the production of secreted PAI-1 is increased by TGF-β in mink lung CCL64 epithelial cells (15). Epidermal growth factor increases PAI-1 expression in mink CCL64 cells to a level that is significantly higher than after stimulation by TGF-β alone. Synergistic induction of PAI-1 by TGF-β and EGF was also found in human hepatoma cells (16). Synergism allows cells to achieve a response which is not possible by stimulation with one growth factor alone.

We conducted experiments to examine the mechanisms that govern the synergism between EGF and TGF-β on PAI-1 induction in CCL64 cells. The results indicate that a combination of transcriptional activation by TGF-β and stabilization of PAI-1 mRNA by EGF explains the synergistic effect.
MATERIALS AND METHODS

Materials: TGF-βs were purified from porcine platelets according to the method of Assoian et al. (17) and from bovine platelets with a modified method (manuscript in preparation). The concentration of TGF-β that produced 99% inhibition of \(^3\)H-thymidine incorporation by mink lung CCL64 Cells was defined as 1 unit/ml. Cells have equal sensitivity in PAI-1 induction to 1 unit/ml TGF-β from either porcine or bovine. Epidermal growth factor was from Collaborative Research. 12-O-tetradecanoylphorbol-13-acetate (TPA) and cordycepin were from Sigma. \(\alpha^{32}\)P-dCTP was from New England Nuclear. \(\alpha^{32}\)P-UTP was from ICN. The Random Primer Labeling Kit was from Amersham and the Zeta-probe nylon membrane was from Bio-Rad. All reagents were analytical grade or better.

Cell Culture: The mink lung CCL64 epithelial cell line, was obtained from Dr. Robert Holley. Stock cell cultures were grown as monolayer on tissue culture dishes with Dulbecco-Vogt's medium (DMEM) containing 0.45% glucose, 10% calf serum, 10 units/ml penicillin and 10 μg/ml streptomycin, at 37°C, in a water saturated atmosphere with 13% CO₂ in air.

Preparation of cDNA probes: A 1.5 kb mink PAI-1 cDNA (manuscript in preparation) fragment corresponding to bases 150-1691 was generated by digesting mink PAI-1 cDNA with Sma I. The fragment was resolved by agarose gel electrophoresis, and isolated by the glass bead affinity adsorption method (Gene-
To make the probe, the isolated cDNA fragment was radiolabeled with $\alpha^{32}$P-dCTP to a specific radioactivity about $1 \times 10^9$ cpm/µg using the random primer extension method.

**Preparation of total RNA, Northern blot, and hybridization:**

Mink lung CCL64 cells were grown in DME medium with 0.2% calf serum for 24 hours. The cells were stimulated for two hours with different agents as indicated for individual experiments. In some experiments, cordycepin was added to determine the decay rate of the induced mRNA. The cells were then washed twice with Tris-buffered salts (0.14 M NaCl, 5 mM KCl, 0.68 mM CaCl$_2$, 0.49 mM MgCl$_2$, 0.70 mM Na$_2$HPO$_4$, 25 mM Tris-HCl pH 7.1) and lysed with 4 M guanidinium isocyanate, 25 mM sodium acetate pH 6. The total RNA was separated from the cell lysate by isopycnic centrifugation in 5.7 M cesium chloride, 25 mM sodium acetate (18). After several rinses with 70% ethanol to remove the cesium chloride, the RNA was resuspended in 0.3 M sodium acetate pH 6, and preserved at -75°C. Electrophoresis of RNA through 1% agarose-formaldehyde gels was performed by using 1 mM EDTA, 5 mM sodium acetate, 20 mM MOPS (3-[N-morpholino] propanesulfonic acid), pH 6 as running buffer. Northern blots were performed by transferring the RNA to nylon membranes in 50 mM sodium hydroxide. The RNA on the blots was hybridized with radiolabeled mink PAI-1 probe for 18 h at 47°C in hybridization buffer (40% forma-
mide, 7% SDS, 1% polyethylene glycol MW=20000, 0.25% non-fat milk, 0.6 M NaCl, 4 mM EDTA-Na$_2$, 50 µg/ml salmon sperm DNA, 40 mM NaH$_2$PO$_4$, pH 7.5). The blots were washed in a solution of 30 mM NaCl, 0.2% SDS, 3 mM sodium citrate, pH 7 twice for 15 min at 47°C. The blots were then exposed to Kodak XAR film at -75°C with two intensifying screens. To control the loading of RNA samples, the blots were stripped in 0.1% SDS, 10 mM NaCl, 1.5 mM sodium citrate, at 80°C for 30 min twice and rehybridized with radiolabelled 18 S ribosomal RNA probe (19). The densities of autoradiographic signals were quantified by scanning densitometry (Zeinth model 504 XL). The density of signal from PAI-1 mRNA was normalized to the density of signal from 18 S ribosomal RNA in each sample. Nuclear run-on assay: Nuclear run-on was performed as described by Greenberg and Ziff (20). Briefly, confluent mink lung CCL64 cells were grown in DME medium with 0.2% calf serum in two 15 cm diameter plates for 24 hours. The cells were treated for two hours with growth factors and other reagents as described in individual experiments. Nuclei from stimulated and control cells were isolated by lysing the cell pellet with NP-40 lysis buffer II (10 mM NaCl, 3 mM MgCl$_2$, 0.5% NP-40, 10 mM Tris HCl, pH 7.4) and centrifuged at 2000 x g for 5 min. The supernatant was removed and the lysis procedure was repeated once. The nuclei were suspended in 200 µl glycerol
storage buffer (5 mM MgCl₂, 0.1 mM EDTA, 40% glycerol, 50 mM Tris HCl pH 8.3), mixed with an equal volume of 2 x reaction buffer (5 mM MgCl₂, 0.3 M KCl, 1 mM each of ATP, CTP, GTP, 0.165 mM (500 μCi/ml) ³²P-UTP (3000 mCi/mmole), 50 mM Tris HCl pH 8.0) and incubated 30 min at 30°C to continue transcription in vitro. The radiolabelled RNA was isolated and 1x10⁶ cpm were used for hybridization with 10 μg linearized mink PAI-1 cDNA fixed on nylon membranes. The hybridization solution was 40% formamide, 7% SDS, 1% polyethylene glycol MW=20000, 0.25% non-fat milk, 0.6M NaCl, 40 mM NaH₂PO₄, 4 mM EDTA and 100 μg/ml wheat germ tRNA. Hybridization performed at 47°C for 4 days. The membrane was washed to a final stringency 0.2 X SSC, 0.2% SDS twice for 15 min at 47°C, and analyzed with a Phosphorimager (Molecular Dynamics). The signal intensity of PAI-1 was normalized to the signal intensity from P65D5 cDNA (21) which was used as a loading control after the signal intensity of an equal amount of linearized pBluescript SK vector was substracted as background.
RESULTS

EGF and TGF-β synergistically regulate PAI-1 production: Growth factor- and mitogen-induced PAI-1 synthesis in cultured cells is generally preceded by an increase in the steady state level of PAI-1 mRNA (22,23,24). To determine whether the synergism between TGF-β and EGF is also evident at the level of mRNA, mink lung CCl64 cells were treated with TPA, EGF, TGF-β or EGF plus TGF-β for 2 hours. Total RNAs were then isolated and a Northern blot was performed. Figure 1 shows that the accumulation of PAI-1 mRNA changes in parallel with the level of the secreted PAI-1 protein. These results also show that the synergistic effect of EGF and TGF-β occurs at the mRNA level.

Transcriptional activity of the PAI-1 gene is not synergistically induced by TGF-β and EGF: To determine whether the synergism between EGF and TGF-β in increasing the level of PAI-1 mRNA occurs at the transcriptional level, the activation of the PAI-1 gene was studied with nuclear run-on experiments. The results showed that TGF-β and EGF increased the transcriptional activity of PAI-1 gene but although EGF caused a significant increase in PAI-1 transcription in TGF-β stimulated cells there was no synergism between EGF and TGF-β in stimulating the transcription. (Figure 2)

EGF stabilizes PAI-1 mRNA: The observation that TGF-β and EGF
do not act synergistically to increase the transcriptional activity of the PAI-1 gene suggested that the TGF-β induced PAI-1 mRNA is stabilized in response to EGF. We tested this possibility by determining the effect of EGF on the decay rate of PAI-1 mRNA from CCL64 cells. The PAI-1 mRNA has a half-life of 30 min in cells treated with TGF-β whereas its half-life is 46 min in cells treated with EGF and TGF-β (Figure 3). Statistical test by using Analysis of Variance (SAS, 1986) with P < .05 indicates that the difference between these two half-lives are significant. It is concluded that EGF stabilizes PAI-1 mRNA in CCL64 cells.
Figure 1. Induction of PAI-1 mRNA in CCL64 cells.
A: CCL64 cells were cultured in DME medium with 0.2% calf serum for 24 hours, then stimulated with 1 unit/ml TGF-β, 5ng/ml EGF, 10 ng/ml TPA or EGF plus TGF-β for 2 hours. After incubation, total RNA were isolated. Equal amounts of total RNA (10 μg) were resolved by formaldehyde-agarose gel electrophoresis. After blotting to a nylon membrane, the blot was hybridized with radiolabeled PAI-1 and 18S ribosomal RNA probes. After exposure to X-ray film, the fluorogram was analyzed by laser densitometry. To control for loading of total RNA, the intensity of signal from PAI-1 mRNA was normalized with the intensity of signal from 18S ribosomal RNA in each sample.

The result of PAI-1 mRNA is from an average of two separate experiments. The two experiments are normalized with the value from TGF-β plus EGF induced PAI-1 mRNA in each experiment. The data for PAI-1 protein production is taken from previously published results (15).

C, control; E, EGF; T, TGF-β.

Protein (□□□□□); mRNA (□□□□□).

B: Northern blot exposures that were used to obtain the data.
FIGURE 1B
Figure 2. The effect of EGF and TGF-β on PAI-1 gene transcription.

Transcriptional activity of the PAI-1 gene was determined by the nuclear runoff assay. CCL64 cells were incubated in DME medium with 0.2% calf serum. Nuclei were isolated 2 hours after the addition of 10 ng/ml TPA, 1 unit/ml TGF-β, 5 ng/ml EGF or combination of TGF-β and EGF. Transcriptional runoff was performed and the nascent RNA transcripts were isolated. Nylon filters with absorbed linearized plasmid were hybridized to the radiolabeled runoff transcripts. The amount of radioactivity associated with each DNA plasmid was analyzed with Phosphoimager. The signal intensity of PAI-1 was normalized to signal intensity from P65D5 cDNA (21) after subtracting the signal intensity from pBluescript SK vector which was as background.

Table: The results is from an average of three separate experiments. These experiments are normalized with the value of TGF-β plus EGF induced PAI-1 gene transcriptional activity in each experiments.

A statistical comparison was done by the Student's T test with P < .05. A significant difference was shown between transcriptional activity of the PAI-1 gene induced by TGF-β and by TGF-β+EGF.

Figure: Results from one experiment in which induction of PAI-1 mRNA and PAI-1 gene transcriptional activity were measured simultaneously. C, control; E, EGF; T, TGF-β.

mRNA ( ); Gene transcriptional Activity ( ).
### Additions vs. fold increase in PAI-1 gene transcriptional activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fold Increase in PAI-1 Gene Transcriptional Activity</th>
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<tr>
<td></td>
<td>1.00 ± 1.00</td>
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<tr>
<td>EGF</td>
<td>2.33 ± 1.22</td>
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<tr>
<td>TGF-β</td>
<td>3.14 ± 0.08</td>
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<tr>
<td>EGF+TGF-β</td>
<td>4.00 ± 0.00</td>
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<tr>
<td>TPA</td>
<td>8.00 ± 2.60</td>
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![Graph showing fold increase in PAI-1 gene transcriptional activity](image-url)
Figure 3. The half life of PAI-1 mRNA in cells induced by TGF-β and EGF plus TGF-β.
CCL64 cells cultured in DME medium with 0.2% calf serum were incubated with 1 unit/ml TGF-β and EGF (5 ng/ml) plus TGF-β for 2 hours. Cordycepin (15 µg/ml) was then added (zero time) to block transcription. Total RNA was isolated at specific times and 10 µg total RNA loaded in each lane of 1% agarose-formaldehyde gel. The Northern blots were probed with radiolabelled PAI-1 and 18 S ribosomal RNA probe. After exposure to X-ray film, the autoradiograms were analyzed with laser densitometry. The intensity of signal from PAI-1 mRNA was normalized with the intensity of signal from 18S ribosomal RNA. The results were from two separate experiments. The half life of PAI-1 mRNA in TGF-β treated cells is 30 min (regression coefficient from a least squares analysis = 0.991). The half life of PAI-1 mRNA in TGF-β plus EGF treated cells is 46 min (regression coefficient from a least squares analysis = 0.993).
When tested by using Analysis of Variance (SAS, 1986) the difference between these two lines were found to be significant to the level of P < 0.05. TGF-β + EGF (-○-); TGF-β (-▲-).
DISCUSSION

Epidermal growth factor and TGF-β synergistically induce PAI-1 synthesis in mink lung CCL64 cells (15). Synergism between TGF-β and EGF has been observed in other instances. For example, rat NRK cells are stimulated to grow in soft agar only in the presence of both EGF and TGF-β but not by TGF-β alone (14).

TGF-β alters the number of high affinity EGF receptors in cultured rat heart endothelial cells (25). Expression of EGF induced c-myc, JE and KC genes are decreased by TGF-β treatment in cultured mink BALB/MK cells (26). TGF-β and EGF synergistically increase the transcriptional activity of the EGF receptor gene in human mammary carcinoma MDA 468 cells (27) and the fibronectin, cytoskeletal-β and γ-actin genes in mouse AKR-2B cells (28). Increased EGF binding affinity is not responsible for the synergistic effect of TGF-β and EGF on PAI-1 induction in mink lung CCL64 cells, since TGF-β has no influence on the EGF binding affinity in these cells (29).

In our study, it was found that the level of PAI-1 mRNA increased in parallel with the level of PAI-1 protein after mink CCL 64 cells are treated with EGF, TGF-β, and their combination. Further investigation with nuclear runon assays showed that transcriptional activity of the PAI-1 gene was 1.3 fold increased in cells treated with EGF plus TGF-β compared to cells treated with TGF-β alone. The half life of
REFERENCES


Previous studies from this laboratory indicate that the synthesis of plasminogen activator inhibitor type 1 (PAI-1), is highly regulated in cultured mink lung CCL64 epithelial cells. Transforming growth factor-β (TGF-β) increases the production of secreted PAI-1 to its maximal level at 5 to 7 hours after induction. The secretion then returns to its basal level by 18 hours after addition of TGF-β. Epidermal growth factor (EGF) and TGF-β synergistically regulate the synthesis of PAI-1 (34). This study was conducted to understand the mechanism by which PAI-1 is regulated in these cells and to reveal the mechanism of synergistic effect.

Mink PAI-1 cDNA was cloned for the following reason. To compare with PAI-1 cDNA from different species, to search the potential determinant regions for mRNA turnover and for using as a probe to detect PAI-1 mRNA and PAI-1 genomic clone.

A mink PAI-1 cDNA library with $1 \times 10^6$ original clones was constructed in Zap II vector from poly(A$^+$)-mRNA. The mRNA was isolated from TPA stimulated mink lung CCL64 cells. A mink PAI-1 clone with 2.97 kb insert was isolated by screening the library with radiolabeled human PAI-1 cDNA probe. The isolated clone was sequenced and characterized by analysis with the aid of computer program (68). A 68 bp 5′-nontranslated leader sequence, 1200 bp coding region and 1720 bp 3′-nontranslated region are coded by the cDNA. The coding region can be trans-
lated into a peptide with 400 amino acids. A 379 amino acid mature peptide is preceded with a 21 amino acid signal peptide. Compared to the PAI-1 sequence from other species, the entire amino acid sequence of mink PAI-1 is 86%, 87%, 79% and 77% identical to human (21), bovine (23), mouse (67) and rat (22) respectively. Two amino acids in the signal peptide of mink PAI-1 were missing. Residues of the active center (Arg367-Met368) and three potential N-glycosylation sites are conserved. The missing two amino acids in the signal peptide obviously are not essential for secretion of mink PAI-1, because mink PAI-1 is secreted into the culture medium by mink lung CCL64 epithelial cells (34).

Two potential important regions from the 3'-untranslated region of mink PAI-1 mRNA were identified. One is a 120 bp highly conserved region, the other is a GU-rich region. The highly conserved region has a 88-95% identical compared to the equivalent regions in the bovine (23), mouse (67), human (21) and rat (22) cDNAs. The significance of this high conservation in the 3'-untranslated region is unclear but it can be expected that this region contains some specific functions for the sequence to be maintained so faithfully through mammalian evolution. The second region, the GU-rich sequence, may play an important role in mRNA degradation since this sequence shows high identity to the GT-rich sequence on type 1 human T cell leukemia virus 3' LTR (69). The T cell leuke-
mia virus LTR GT rich region directs its pre-mRNA cleavage and facilitates polyadenylation.

The post-transcriptional regulation of PAI-1 gene in mink lung CCL64 cells was studied. TPA induces the production of secreted PAI-1 to its maximum at 6-10 hours and the production declined rapidly to its basal level by 18 hours. TPA induced PAI-1 mRNA to its maximum at 3 hours and the amount of PAI-1 mRNA then decreased to its basal level by 7 hours. The parallel changes in the time courses of protein production and mRNA accumulation suggest that the level of PAI-1 mRNA determines the production of secreted PAI-1. The time span for PAI-1 mRNA accumulation in mink lung CCL64 cells is much shorter than was reported for bovine aortic cells (65). In bovine aortic cells, PAI-1 mRNA is induced by TGF-β, TNF-α and LPS to its maximum at 12 hours and returns to its basal level by 48 hours. The half life of PAI-1 mRNA is about 30 min in mink lung CCL64 cells treated with TPA or TGF-β. This is shorter than the average half-life of mRNA in eukaryotic cells (70).

Several features of PAI-1 mRNA are that it is inducible, has a high turnover rate and is stabilized by cycloheximide and actinomycin D. The same properties were found for the mRNA of truncated c-fos (71) and c-myc (72). One mechanism often cited to explain the stabilization effect of actinomycin D is that the degradation of mRNA involves a labile factor and actinomycin D blocks the transcription of the
factor therefore stabilizing the mRNA (73,74). However, our results can not support this model since cordycepin does not stabilize PAI-1 mRNA. Cordycepin is a inhibitor of transcription that should have the same effect as actinomycin D in blocking the synthesis of proposed labile factor. We proposed that the degradation of PAI-1 mRNA in mink lung CCL64 cells is translation associated (75). Actinomycin D is proposed to prevent degradation of PAI-1 mRNA by preventing its translation. It has been found that actinomycin D can bind mRNA in vitro and in vivo (76) and inhibit the binding of mRNA to ribosomes (77,78).

The synergistic effect of PAI-1 induction was studied. In mink lung CCL64 cells, TGF-β and EGF synergistically induced PAI-1 to a level that is significantly higher than expected from the sum of EGF and TGF-β alone.

The steady state level of PAI-1 mRNA increases in parallel with the production of secreted PAI-1 in cells treated with EGF, TGF-β, and the combination of both. This result indicates that the synergism occurred at the level of mRNA accumulation. The nuclear runon assays indicate that TGF-β and EGF increase the transcriptional activity of PAI-1 gene, however the PAI-1 gene is not synergistically induced by EGF plus TGF-β. The half-life of PAI-1 mRNA in cells treated with EGF and TGF-β is 46 min. The half-life of PAI-1 mRNA in cells treated with TGF-β is 30 min. These results indicate that the
synergistic effect of these growth factors in PAI-1 expression is the result of a combination of transcriptional activation of PAI-1 gene by TGF-β and the PAI-1 mRNA stabilization by EGF.

Mechanisms for controlling mRNA degradation in eukaryotic cells have been described. One example is the degradation of histone mRNA. Histone mRNA is degraded by a ribosome-associated exonuclease (79). This nuclease is activated by interaction with a factor that recognize the stem loop structure at the 3' terminus of histone mRNA. Stabilization of transferrin receptor mRNA presents another example. An iron response element (IRE) in the 3' untranslated region of this mRNA determines the stability of the mRNA (73). The IRE contains five approximately 30 bp imperfectly repeated sequences. Binding of a 90 kD protein to this element stabilizes the mRNA while dissociation of the 90 kD protein causes the destabilization on the transferrin receptor mRNA (80). Another example is the degradation of tubulin mRNA. The first 13 translated bases of this mRNA codes for a tetra peptide, Met-Arg-Glu-Ile. It is this region that autoregulates its own mRNA stability (81). The mode of tubulin mRNA degradation is that a ribonuclease is activated after binding of unassembled dimers of α- and β-tubulin to the nascent amino acid terminal of β-tubulin. The activated ribonuclease then degrades the ribosome-bound β-tubulin mRNA (82). An other mechanism which controls mRNA degradation is mediated by an AU-rich sequence
at the 3'-untranslated end of mRNA coding for proto-oncogenes, lymphokines and cytokines (83). Messenger RNA encoding proteins in these classes usually have short half lives of 15-30 min. A protein factor of 34 kD which binds to the AU-rich sequence has been isolated (84, 85). The AU sequence in c-fos and c-myc is necessary but not sufficient for mRNA degradation. Another sequence in the coding region is also necessary for the mRNA instability (71,72). One interesting aspect is that all the mRNA degradation mechanisms described above, except for the transferrin receptor mRNA, are believed to be translationally related.

Of these mRNAs described above, the c-myc and c-fos mRNA share many of the same features as PAI-1 mRNA. They are inducible, have high turnover rates and are stabilized by cycloheximide. Truncated fos and myc mRNAs are also stabilized by actinomycin D. The GU-rich region in PAI-1 mRNA may direct its mRNA degradation as AU-rich sequence does for c-fos and c-myc mRNAs.

The next step for this research is to identify the regions of PAI-1 mRNA that regulate its degradation. This can be done by making deletion constructions of mink PAI-1 cDNA in an expression vector, express the construct in cells and determine half-lives of the expressed mRNA from each construct.

In summary, these studies have revealed that the
control of PAI-1 mRNA degradation in mink lung CCL64 cells is crucial in determining the extent and profile of expression of the PAI-1 gene. The GU-rich region and the 120 bp high conserved region identified in the 3'‐untranslated region of mink PAI-1 mRNA might be important in the regulation of PAI-1 mRNA degradation. EGF stabilizes PAI-1 mRNA in mink lung CCL64 epithelial cells.
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