Opposite and independent actions of cyclic AMP and transforming growth factor β in the regulation of type 1 plasminogen activator inhibitor expression

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We have investigated the mechanisms by which type 1 plasminogen activator inhibitor (PAI-1) is regulated by transforming growth factor β (TGF- β) and by epidermal growth factor (EGF) in CCL64 mink lung epithelial cells, BSC-1 monkey kidney epithelial cells, mouse embryo fibroblast (AKR-2B 84A) cells and normal rat kidney fibroblasts (NRK). TGF- β increases PAI-1 expression in all four cell lines, and EGF acts synergistically with TGF- β to increase PAI-1 expression in CCL64 cells but not in the other three cell lines. Here we show that PAI-1 expression can be regulated independently through two different signal transduction pathways. One pathway involves protein kinase C and is stimulated by the tumour promoter phorbol myristate acetate (PMA). Whereas preincubation with PMA completely eliminated PMA-induced PAI-1 synthesis and secretion in both CCL64 and BSC-1 cells, this treatment had no effect on TGF- β - and EGF-induced PAI-1 levels. Therefore we conclude that protein kinase C does not mediate the effects of either EGF or TGF- β on PAI-1 expression. The expression of PAI-1 was decreased by agents increasing intracellular cyclic AMP: (cAMP) cholera toxin, forskolin and dibutyryl cAMP lowered both the basal level and the TGF- β - and PMA-induced levels of PAI-1 expression. These effects of cAMP-elevating agents and of TGF- β on PAI-1 protein synthesis were also reflected in changes in TGF- β -induced PAI-1 gene transcription, as measured by nuclear run-on. These results show that PAI-1 gene expression is sensitive to high levels of intracellular cAMP and that this effect occurs at the transcriptional level. Although increased intracellular cAMP concentrations decrease the absolute level of PAI-1 expression, the ability of TGF- β and EGF to induce PAI-1 gene expression is unchanged. These results are discussed in relation to the observation that sensitivity to cAMP is a common feature of TGF- β -regulated genes.

INTRODUCTION

Control of plasminogen activator by its inhibitors is a key regulatory step in the conversion of plasminogen to the broad-spectrum proteinase, plasmin. In addition to playing a well-known role in the fibrinolytic pathway, plasmin degrades the protein components of the tissue extracellular matrix. There are at least three immunologically distinct plasminogen activator inhibitors [1–3], one of which is designated type 1 plasminogen activator inhibitor (PAI-1). First purified by van Mourik *et al.* [4], PAI-1 is a secreted serine proteinase inhibitor (M_r 48000–55000) and is ubiquitous in mammalian systems. Especially high concentrations of PAI-1 are found in serum and in platelets. The complete sequences both of human and rat PAI-1 cDNAs and of the human PAI-1 gene are known [5–7]. Although the role of PAI-1 in the regulation of fibrinolysis and tissue remodelling is becoming increasingly clear, little is known of the signal transduction pathway(s) regulating PAI-1 production.

Production of PAI-1 by cultured cells can be increased by a variety of compounds, such as transforming growth factor β (TGF- β) [8–10], glucocorticoids [11], interleukin-1 [12] and phorbol myristate acetate (PMA) [10,13]. Every cell we have thus far tested responds to TGF- β with increased PAI-1 production.

The increase in PAI-1 production in response to TGF- β and glucocorticoids is associated with an increase in PAI-1 mRNA [7,9,14]. The induction of PAI-1 by TGF- β is immediate and is unaffected by cycloheximide [8,10].

The cellular signals used by TGF- β to activate expression of PAI-1 are not known. Evidence suggests that the ability of TGF- β to induce *c-sis* is inhibited by high concentrations of cellular cyclic AMP (cAMP) [15] and involves the G-protein, G_i [16,17]. Our results reported here suggest that a G_i -like protein is not involved in the induction of PAI-1.

In this paper we describe studies on the mechanism by which TGF- β regulates PAI-1 protein expression in epithelial and fibroblast cell lines. We have studied near-term mink lung epithelial (CCL64) cells, African green monkey kidney epithelial (BSC-1) cells, normal rat kidney fibroblast (NRK 49F) cells, and mouse embryo fibroblast (AKR-2B 84A) cells. All four cell lines gave similar results. We found that PAI-1 expression can be regulated by two separate signal transduction pathways, one of which involves protein kinase C (PKC). However, PKC does not mediate the cellular response to TGF- β or epidermal growth factor (EGF). The intracellular cAMP concentration is an important determinant of the basal and induced levels of PAI-1, but the signal transduction pathway through which TGF- β

Abbreviations used: AKR-2B 84A, mouse embryo fibroblast cells; BSC-1, African monkey kidney epithelial cells; cAMP, cyclic AMP; CCL64, mink lung epithelial cells; CRE, cAMP-responsive element; dbcAMP, dibutyryl cAMP; DMEM, Dulbecco–Vogt's modified Eagle's medium; EGF, epidermal growth factor; TGF- β , transforming growth factor type β ; pTGF- β , porcine TGF- β ; GI/TGF- β , monkey type-2 TGF- β ; MLEM, mink lung epithelial mutant cells lacking the TGF- β receptor; NRK, normal rat kidney fibroblasts; PAI-1, type 1 plasminogen activator inhibitor; PKC, protein kinase C; PMA, phorbol myristate acetate; PIP62/65, PMA-induced proteins of M_r 62000/65000; R1B, TGF- β type I receptor-negative cells; TRE, 'TPA' (PMA)-responsive element.

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regulates the expression of PAI-1 is not influenced by cAMP-regulated functions.

MATERIALS AND METHODS

Materials

Porcine TGF- β (pTGF- β) was purified from porcine plasma according to the method of Assoian *et al.* [18]. Its activity was measured by its ability to inhibit the incorporation of thymidine by CCL64 cells, as previously described [10]. The concentration of TGF- β producing 99% inhibition of thymidine incorporation was designated 1 unit/ml. Monkey TGF- β type-2 (GI/TGF- β) was a gift from Dr. Robert Holley (Salk Institute for Biological Studies, La Jolla, CA, U.S.A.). Human platelet TGF- β type 1 was a gift from Dr. H. Moses (Vanderbilt University, Nashville, TN, U.S.A.). The following cDNAs were also gifts: human PAI-1 cDNA from Dr. Keld Danø (Rigshospitalet, Copenhagen, Denmark) [19] and rat PAI-1 cDNA from Dr. R. Zeheb and Dr. T. Gelehrter (University of Michigan Medical School, Ann Arbor, MI, U.S.A.) [7]. All chemicals and reagents were from Fisher Scientific or Sigma, with the exception of the following: EGF (Collaborative Research), PMA (Sigma or CCR, Inc. Bedford, MA, U.S.A.), 4-*O*-methyl-PMA (CCR), isoprenaline (Calbiochem), random primer kits (NEN or Amersham), Zeta-Probe nylon membranes (Bio-Rad), radioisotopes (New England Nuclear), RNasin and RNAase-free DNAase (Promega), yeast tRNA (Boehringer-Mannheim) and non-fat dry milk (Carnation).

Cell culture and cell lines

All cells were cultured in Dulbecco-Vogt's modified Eagle's medium (DMEM), which contained 0.45% glucose and no sodium pyruvate (Gibco), plus 5–10% calf serum, 10 units of penicillin/ml and 10 μ g of streptomycin/ml. The stock cell cultures were grown as monolayers on plastic tissue-culture dishes at 37 °C in a water-saturated atmosphere of 10% CO₂ in air. The CCL64 cells were from the American Type Culture Collection; Dr. Robert Holley (The Salk Institute for Biological Studies, La Jolla, CA, U.S.A.) donated the BSC-1 cell line; Dr. Harold Moses (Vanderbilt University, Nashville, TN, U.S.A.) donated the AKR-2B (clone 84A) cell line; and Dr. Duane Enger (Iowa State University, Ames, IA, U.S.A.) provided the NRK (clone 49F) cell line. Dr. Phillip Howe (Vanderbilt University, Nashville, TN, U.S.A.) donated the mink lung epithelial mutant (MLEM) cell line [20], and Dr. F. T. Boyd and Dr. J. Massagué (Sloan Kettering Cancer Center, New York, NY, U.S.A.) donated the R1B (TGF- β type I receptor-negative) cell line [21].

Labelling with [³⁵S]methionine

The [³⁵S]methionine metabolic labelling method is described fully in previous publications [10,22]. In brief, cells were suspended in DMEM containing 0.2–1.0% calf serum and plated in 24-well tissue-culture dishes. At 1–2 days after the cells had attached to the dish the medium was removed, and fresh DMEM containing 0.2–0.5% calf serum was added. About 24 h later, TGF- β , PMA or other agents were added as indicated for specific experiments. After the indicated incubation time the cells were labelled with [³⁵S]methionine for between 1 and 6 h in the presence of the same additions. Under these conditions, incorporation of [³⁵S]methionine is linear for at least 8 h. The labelled proteins were resolved on SDS/PAGE (7.5–15% gels) and visualized by fluorography.

SDS/PAGE and densitometry

SDS/PAGE, fluorography and densitometric measurement of [³⁵S]methionine in the protein bands were performed as pre-

viously described [10,22]. All densitometer scans were within the linear range of densitometer sensitivity, which was determined from serial dilutions of samples containing radioactive proteins. Internal standards were also included on each gel to allow comparison of the results between fluorograms. The results from densitometer scans were normalized to the rate of [³⁵S]methionine incorporation into cellular proteins by dividing the value of the area under the peak by the amount of acid-insoluble [³⁵S]methionine radioactivity in the Nonidet P-40-soluble fraction of the cell preparation.

Immunoprecipitation

Samples of medium were immunoprecipitated with polyclonal antisera raised against purified proteins. Dead *Staphylococcus aureus* (Pansorbin, Calbiochem) were used as a carrier, according to the procedure outlined previously [23].

[³H]Thymidine incorporation and labelling index

Cells grown on coverslips were labelled with a 4 h pulse of [³H]thymidine 24–30 h after adding growth factors. The time of labelling was determined for each cell line as the time of maximal response after the addition of growth factor. The cells were prepared as previously described and the acid-insoluble incorporated ³H was analysed by liquid scintillation counting and by determining the labelling index [24].

In vitro transcription/nuclear run-on

In vitro transcription assays were performed with NRK and BSC-1 cells according to the method of Greenberg & Ziff [25,26], with modifications described by Celano *et al.* [27]. For each assay point, four 15 cm plates of cells were treated for 2 h under the conditions indicated for each experiment, washed with phosphate-buffered saline, scraped from the dish and pooled. [³²P]UTP (125 μ Ci/tube) was used with each set of nuclei.

The recovered *in vitro*-transcribed ³²P-labelled RNA was dissolved in hybridization buffer [50% formamide, 5 $\times$ SSPE (1 $\times$ SSPE = 0.18 M-NaCl, 0.01 M-Na₂HPO₄, 0.001 M-EDTA), 1% SDS, 0.5% non-fat dry milk, 0.1 mg of yeast tRNA/ml, 0.5 mg of carrier salmon sperm DNA/ml] at 42 °C for 4 days and hybridized with cDNAs absorbed to Bio-Rad Zeta-probe nylon membranes with a slot-blot apparatus. The plasmids containing the indicated cDNA had been linearized with appropriate restriction enzymes before being blotted to the membrane. After hybridization, the membranes were washed for 60 min with four changes of 2 $\times$ SSC/0.1% SDS (1 $\times$ SSC = 0.15 M-NaCl, 0.015 M-trisodium citrate) at 22 °C, then with 0.2 $\times$ SSC/1% SDS for 1 h at 42 °C. The washed membranes were exposed to X-ray film between two enhancing screens for 2 days, and the resulting autoradiograms were scanned using a densitometer.

RNA isolation and Northern blot analysis

Cells were treated with TGF- β for 3–4 h, washed with phosphate-buffered saline, then lysed with 4 M-guanidinium isothiocyanate. Total RNA was isolated according to the method of Chirgwin *et al.* [28]. The RNA was resolved on a 1% agarose/formaldehyde gel. After electrophoresis, the RNA was transferred to Zeta-Probe membranes (Bio-Rad) in 50 mM-NaOH and hybridized with ³²P-labelled cDNA in 40–50% formamide, 0.36 M-NaCl, 0.02 M-Na₂HPO₄, 7% SDS, 1% poly(ethylene glycol) (20000-*M_r*), 0.05% non-fat dry milk and 0.33 mg of salmon sperm DNA/ml (hybridization buffer) at 50 °C in a Hybrid-Ease chamber (Hofer Scientific), or in heat-sealed bags, for 18–24 h. The membranes were washed as described for *in vitro* transcription, and then washed with 0.5 $\times$ SSC (22 °C) and exposed to X-ray film between two

Fig. 1. Effects of TGF- β and PMA on the induction of secreted proteins in AKR-2B, BSC-1, CCL64 and NRK cells

AKR-2B cells were preincubated for 2.8 h with or without 1 unit of pTGF- β /ml or 10 ng of PMA/ml and then labelled for 4 h with [35 S]methionine. BSC-1 cells were preincubated for 0 h with or without 1 unit of pTGF- β /ml or 10 ng of PMA/ml and then labelled for 6 h with [35 S]methionine. CCL64 cells were preincubated for 2.5 h with or without 1 unit of pTGF- β /ml or 10 ng of PMA/ml and then labelled for 4 h with [35 S]methionine. NRK cells were preincubated for 0.5 h with or without 1 unit of pTGF- β /ml or 10 ng of PMA/ml and then labelled for 6.25 h with [35 S]methionine. Shown are fluorograms of SDS/polyacrylamide gels through which [35 S]methionine-labelled proteins secreted by these cells had been resolved. PAI-1, procathepsin L (CL) and PIP62/65 are marked by arrows. The numbers on the side of the figure show the positions of the M_r standards used on this and other gels. They were (from top to bottom): β -galactosidase (116000), BSA (67000), ovalbumin (45000) and carbonic anhydrase (30000). Lanes: -, control; T, pTGF- β ; P, PMA.

intensifying screens. The autoradiograms were quantified by scanning densitometry.

Reverse zymography

Reverse zymography was performed on the medium from AKR-2B, BSC-1, CCL64, and NRK cells treated or not with TGF- β , PMA or EGF [29]. The samples were resolved by electrophoresis as described above, but were not exposed to reducing agents. The acrylamide gels were then soaked twice in 2.5% Triton X-100 for 30 min each. The gels were blotted thoroughly to remove excess liquid and then overlaid on to the surface of freshly prepared fibrin-agar indicator gels. Fibrin-agar gels contained 1% agarose, 25 μ g of plasminogen/ml, 0.2 unit of bovine thrombin/ml, 2.5 mg of human fibrinogen/ml and 0.05 unit of urokinase plasminogen activator/ml. Plastic film was placed over the acrylamide gel, and 'enzymography' was carried out at 37 $^{\circ}$ C in a humidified tissue culture incubator. At appropriate times (usually 2–4 h after the start of the incubation), the acrylamide gels were removed and the fibrin-agar indicator gels were photographed. All cell lines used in this study secreted PAI-1 that could be measured by this assay.

RESULTS AND DISCUSSION

Effects of TGF- β and PMA on the induction of PAI-1 and PAI-1 mRNA in fibroblast and epithelial cells

Previously we showed that TGF- β and PMA induced PAI-1 expression in CCL64 near-term mink lung epithelial cells [10] and in BSC-1 African green monkey kidney epithelial cells [8]. In this study, the effects of TGF- β and of PMA on the expression of PAI-1 and other secreted proteins were analysed in four cell lines: AKR-2B, BSC-1, CCL64 and NRK cells (Fig. 1). In all cell lines TGF- β induced PAI-1. The average increases are shown in Table 1. The range of increase in PAI-1 expression in response to TGF- β in these experiments varied from 1.3-fold (lowest BSC-1 cell value) to over 1000-fold (highest CCL64 cell value). For all cell lines except the CCL64 cells the basal level of PAI-1 expression could be measured in every experiment. Although low, the basal level of PAI-1 expression was measurable in 78% of experiments with CCL64 cells. The other 22% showed the same increases in PAI-1 expression, but the level of PAI-1 could

not be expressed as a value relative to the negative control. The results from these latter experiments were not included in the statistical analysis in Table 1.

The response of PAI-1 expression to PMA was not the same in all cells. Treatment with PMA increased PAI-1 to levels similar to or higher than those with TGF- β in the two epithelial cell lines, CCL64 and BSC-1, and to a lesser extent in NRK cells. By contrast, treatment with PMA decreased PAI-1 expression in AKR-2B cells.

The protein that we identify as PAI-1 can be immunoprecipitated with an antibody to bovine endothelial cell plasminogen activator inhibitor [10]. In addition, all four cell lines secrete increased amounts of PAI-1 in response to TGF- β , as identified by reverse zymography (results not shown). Because reverse zymography is a semi-quantitative assay, no quantification was attempted.

The effects of TGF- β and PMA on PAI-1 mRNA levels were also analysed in BSC-1 and NRK cells. Unlike human cells [30,31], NRK cells possess only one mature PAI-1 transcript of 3 kb. As demonstrated for NRK cells in Fig. 2, the level of PAI-1 mRNA changed in parallel with the level of PAI-1 protein synthesis. Similar results were found for BSC-1 cells and CCL64 cells (T.-H. Chuang & M. Nilsen-Hamilton, unpublished work). Thus increases in PAI-1 mRNA are at least partly responsible for the higher level of PAI-1 secretion by TGF- β - and PMA-treated cells compared with untreated controls. This is consistent with similar results reported for bovine and human endothelial cells [14,32] and for WI38 lung cells [14].

cAMP regulation of PAI-1 gene expression

Santell & Levin [33] demonstrated that increased cAMP levels inhibit both basal and PMA-induced secretion of PAI-1 from human endothelial cells. Here we have extended these studies to several species of fibroblast and epithelial origin and have explored the mechanistic basis for this effect of cAMP. We treated AKR-2B, BSC-1, CCL64 and NRK cells with various factors that can alter the intracellular cAMP concentration. When added simultaneously with TGF- β and PMA, forskolin, an activator of adenylate cyclase, blocked or significantly reduced the expression of PAI-1 in all cell lines, as shown in Fig. 3(a) for CCL64 cells and in Fig. 3(b) for NRK cells. Forskolin also

Table 1. Effects of TGF- β , PMA, forskolin and cholera toxin on the levels of secretion of PAI-1 by AKR-2B, BSC-1, CCL64 and NRK cells

All cells were grown as described in the Materials and methods section. Some cells were preincubated with cholera toxin (CT) for 4–5 h before adding TGF- β , PMA or forskolin (Forsk.). After these additions, the cells were incubated for 2–3 h and then labelled with [35 S]methionine for another 4 h in the presence of the same additions. Concentrations of the additions were: TGF- β , 10 ng of human platelet TGF- β /ml or 1 unit of pTGF- β /ml; PMA, 10 ng/ml; forskolin, 50 μ M; cholera toxin, 100 ng/ml. The amount of acid-insoluble [35 S]methionine incorporated by the cells was measured as described in the Materials and methods section. Each value is the average of 2–30 independent estimates; each estimate was derived from a single well of cells. In all experiments the values for the treatments were normalized to the average of the control values. Student's *t* test was used for statistical analysis. When two variables were present, the significance of the difference between the samples treated with forskolin or cholera toxin and the control samples without these treatments was tested. The numbers in parentheses are the numbers of independent estimates used to derive the average values shown. Significant differences: ****P* < 0.005, ***P* < 0.01, **P* < 0.025. No asterisk indicates that the *P* value was > 0.025 and that the difference was not considered significant. The absolute values of the controls (cm²) were: AKR-2B (average 1.55; range 0.48–4.27); BSC-1 (average 1.60; range 0.42–2.65); CCL64 (average 0.72; range 0.03–2.19); NRK (average 1.57; range 0.25–5.81).

Additions	Secreted PAI-1 (relative units)			
	AKR-2B	BSC-1	CCL64	NRK
Control	1.0 (16)	1.0 (28)	1.0 (25)	1.0 (22)
TGF- β	13.8 (15)***	5.5 (30)***	12.8 (28)***	9.5 (24)***
PMA	0.52 (14)***	5.0 (27)***	109.5 (26)*	3.2 (22)***
Forsk.	0.69 (5)	0.59 (10)***	1.1 (9)	0.39 (14)***
TGF- β	5.4 (6)***	2.3 (12)***	6.1 (5)***	2.9 (14)***
+ forsk.				
PMA	0.73 (4)	3.0 (8)***	19.7 (2)	0.99 (12)***
+ forsk.				
CT	0.5 (5)***	0.40 (6)***	1.0 (7)	0.44 (5)***
TGF- β	3.5 (6)***	3.3 (6)***	11.5 (9)	3.4 (6)***
+ CT				
PMA + CT	0.60 (4)	2.8 (4)*	35.0 (8)***	2.0 (4)***

Fig. 2. Effect of TGF- β and PMA on the level of PAI-1 mRNA in NRK cells

NRK cells were incubated for 3 h with or without 1 unit of pTGF- β /ml or 10 ng of PMA/ml, then lysed with guanidinium isothiocyanate, and total RNA was isolated and Northern blots performed as described in the Materials and methods section using a rat PAI-1 cDNA. —, Control; T, pTGF- β ; P, PMA.

lowered the basal level of expression of PAI-1 in all but CCL64 cells. This effect on the basal level was statistically significant for BSC-1 and NRK cells. Similarly, cholera toxin significantly reduced the TGF- β -induced expression of PAI-1 in all but CCL64 cells (Table 1). One reason for the lack of an observed

Fig. 3. Effects of forskolin on the induction of secreted proteins

Cells were treated with 50 μ M-forskolin with the indicated growth regulators 2.5 h before labelling for 4 h with [35 S]methionine. All treatments included 2 mM-acetic acid and 0.1 % dimethyl sulphoxide besides the additions described. Fluorograms are shown: (a) CCL64 cells; (b) NRK cells. PAI-1 is marked with an arrow. In (b), the upper arrow marks the position of the 62/65 kDa doublet that is induced by PMA in NRK cells. —, Control; T, pTGF- β ; P, PMA; Forsk, forskolin.

effect on the basal level in CCL64 cells may be that the basal level of PAI-1 production by CCL64 cells is at the lower end of the range of sensitivity of the densitometer. Consequently, the quantitative results for the basal level of secretion of PAI-1 by CCL64 cells in the presence and the absence of forskolin were sufficiently variable that we were unable to observe significant differences, although in some individual experiments there was a substantial decrease in the basal level of PAI-1 expression in response to either cholera toxin or forskolin.

Treatment of the cells with dibutyl cyclic AMP (dbcAMP) also decreased the expression of PAI-1 (Table 2). As with forskolin and cholera toxin, dbcAMP inhibited PAI-1 expression under all conditions in BSC-1 cells, but did not have a large inhibitory effect on the basal level of PAI-1 expression in CCL64 cells.

The results in Tables 1 and 2 also demonstrate that the average increase in PAI-1 expression in response to TGF- β is the same in the presence and the absence of agents that increase intracellular

Table 2. Effect of dbcAMP on PAI-1 production by BSC-1 and CCL64 cells

BSC-1 and CCL64 cells were grown as described in the Materials and methods section. pTGF- β (1 unit/ml), PMA (10 ng/ml) and dbcAMP (1 mM) were added 3.75 h before the cells were labelled with [35 S]methionine for an additional 4.5 h. The medium was then collected and analysed for the presence of secreted PAI-1. The TGF- β , PMA and dbcAMP were present during the entire 8.25 h. Duplicate samples were evaluated and the results are presented as means $\pm$ s.d. The results were analysed by analysis of variance (SE) and least significant difference (LSD; $P < 0.05$). In this analysis, the effect of dbcAMP was found to be significant in both experiments. For the experiment with BSC-1 cells, LSD = 1.45 and SE = 0.42. For the experiment with CCL64 cells, LSD = 1.4 and SE = 0.35.

Treatment	[35 S]Methionine incorporated into PAI-1 (relative units)	
	BSC-1	CCL64
Control	1.0 $\pm$ 0.23	1.0 $\pm$ 0.78
TGF- β	8.0 $\pm$ 0.93	19.0 $\pm$ 0.16
PMA	4.6 $\pm$ 0.06	915 $\pm$ 203
dbcAMP	0.33 $\pm$ 0.02	3.0 $\pm$ 1.2
TGF- β + dbcAMP	2.4 $\pm$ 0.83	13.0 $\pm$ 1.7
PMA + dbcAMP	0.35 $\pm$ 0.06	471 $\pm$ 116

Fig. 4. PAI-1 expression in response to TGF- β in the presence and absence of forskolin

PAI-1 expression by BSC-1 and NRK cells was measured as described for Fig. 3. The amount of PAI-1 produced in response to TGF- β in the presence and absence of forskolin was estimated for each experiment by dividing the average (of duplicate values) of the TGF- β treated samples by the average of the control samples. The same calculation was done for the TGF- β and control samples in cells that were also treated with forskolin. The average increase in response to TGF- β was determined from five experiments. —, No forskolin added; F, 50 μ M-forskolin present.

cAMP. This result was consistent for all except CCL64 cells. For NRK cells and BSC-1 cells, a Student's *t*-test was used to examine the differences between the response to TGF- β (fold over control) in the presence and absence of forskolin. The results shown in Fig. 4 are from the analysis of five separate experiments for each cell line. In each experiment, each treatment was tested with independent duplicates. The *t*-test showed that the response to TGF- β was not significantly different in the presence and absence of treatments that increase intracellular cAMP ($P < 0.1$). Therefore the ability of TGF- β to induce PAI-1 is not altered by forskolin. However, forskolin severely decreased the absolute level of expression of PAI-1.

Both basal and TGF- β -induced PAI-1 mRNA levels in NRK cells were also reduced by either forskolin or cholera toxin. The

Table 3. Effect of TGF- β , PMA, forskolin and cholera toxin on the expression of secreted PIP62/65 and procathepsin

The cells were grown as described in the legend to Fig. 1. Cells were preincubated with cholera toxin (CT) for 4–5 h before TGF- β , PMA or forskolin (forsk.) was added. They were then incubated and labelled with [35 S]methionine as described for Table 1. The amount of acid-insoluble [35 S]methionine incorporated by the cells was measured as described in the Materials and methods section. Student's *t* test was used for statistical analysis. The *t* tests for the forskolin or cholera toxin combinations with TGF- β or PMA were performed by comparing these samples with the appropriate TGF- β - or PMA-treated samples. The numbers in parentheses are the numbers of estimates. Significant differences: *** $P < 0.005$, ** $P < 0.01$, * $P < 0.025$; no asterisk indicates that the *P* value was > 0.025 and that the difference was not considered significant. The statistical finding of no significance of the effect of forskolin on the production of procathepsin L (proCL) is due to the great variation in the response of these cells to forskolin and the relatively small number of values. The range of control values was from 0.7 to 1.3, whereas the range of values from forskolin-treated cells was 4.3–61.8. Similar variation was observed in the results of treatment with TGF- β , forskolin and their combinations. In these studies the controls ranged from 0.69 to 1.3 and the values from treated cells range from 3.0 to 26.0.

Additions	Expression (relative units)	
	PIP62/65 (NRK cells)	ProCL (AKR-2B cells)
SXSS		
Control	1.0 (24)	1.0 (12)
TGF- β	0.95 (23)	1.2 (12)
PMA	24.9 (22)***	18.5 (8)***
Forsk.	1.0 (10)	21.6 (6)
TGF- β + forsk.	1.2 (10)	16.1 (5)*
PMA + forsk.	5.7 (8)***	50.0 (4)
CT	1.4 (5)	7.7 (6)***
TGF- β + CT	1.7 (5)	7.7 (6)**
PMA + CT	20.7 (4)	47.1 (4)***

decreases in mRNA level, as measured by densitometry from Northern blots, were consistent with the decreases in the level of PAI-1 secreted protein (results not shown). TGF- β increased PAI-1 mRNA 5-fold, whereas forskolin and cholera toxin decreased basal and TGF- β -stimulated PAI-1 mRNA to 40 % of the levels in untreated cells.

The effects of forskolin and cholera toxin on the overall rate of protein synthesis were analysed, to determine whether these agents inhibit protein synthesis or cause cell death. The results of a representative experiment show that treatment of NRK cells with forskolin, cholera toxin or dbcAMP had no effect on the rate of protein synthesis or on the number of cells on the dish (results not shown). The same results were found with all of the cell lines examined in this study.

Effect of cAMP on other secreted proteins

To investigate the possibility that the effect of cAMP-elevating agents on PAI-1 was a general response of genes regulated by TGF- β and PMA, we analysed the effects of forskolin and cholera toxin on the expression of other secreted proteins. In NRK clone 49F cells, we analysed the effect of cAMP on a doublet of 62000/65000- M_r that is also induced by PMA, which we have called 'PMA-induced proteins' (PIP62/65) (Figs. 1 and 3b). These proteins are not induced by TGF- β . As with PAI-1, forskolin lowered the PMA-induced levels of PIP62/65 by greater than 80 % (Table 3; Figs. 1 and 3b). In contrast with their effects

Fig. 5. Effect of long-term exposure to high concentrations of PMA on induction of PAI-1

CCL64 cells were treated with (a) 0.1 % dimethyl sulphoxide or (b) PMA (100 ng/ml) in 0.1 % dimethyl sulphoxide for 48 h. At the end of this preincubation period the medium was removed and replaced with [³⁵S]methionine labelling medium with 0.2 % calf serum and the indicated additions: E, 5 ng of EGF/ml; T, 1 unit of pTGF- β /ml; TE, EGF and pTGF- β in combination; P, 10 ng of PMA/ml. Present with all treatments and in the controls (–) were 2 mM-acetic acid, 0.005 % BSA and 0.1 % dimethyl sulphoxide. The cells were labelled with [³⁵S]methionine for 6 h. Samples of medium were run on SDS/PAGE (7.5–15 % gels) as described in the Materials and methods section. PAI-1 is marked with an arrow.

Fig. 6. Transcriptional analysis of PAI-1 by nuclear run-on

NRK cells were treated for 2 h with monkey TGF- β , type 2 (1 unit/ml) or PMA (10 ng/ml) with or without 50 μ M-forskolin. All treatments included 2 mM-acetic acid and 0.1 % dimethyl sulphoxide. Nuclei were isolated and used for *in vitro* transcription/nuclear run-on as described in the Materials and methods section. PAI-1, 2 μ g of rat PAI-1 cDNA [17], actin, 2 μ g of mouse actin; FN, 2 μ g of mouse fibronectin; pBR322, 2 μ g of pBR322; –, control; T, TGF- β , P, PMA; Forsk, forskolin.

on the expression of PAI-1, cAMP-elevating agents did not significantly lower the basal level of PIP62/65 expression.

The expression of procathepsin L [also called major excreted protein (MEP)] is negatively regulated by TGF- β after 16–20 h of incubation [23]. After 4–8 h of incubation with TGF- β , there was no significant decrease in the expression of procathepsin L by AKR-2B cells (Table 3). In contrast with their effect on the expression of PAI-1 and PIP62/65, cAMP-elevating agents increased the expression of procathepsin L.

Effect of PKC down-regulation on TGF- β induction of PAI-1

PMA and other phorbol tumour promoters act through PKC to increase PAI-1 expression. Because TGF- β activates PKC in microvessels from brain [34], we hypothesized that TGF- β induces PAI-1 by activating PKC. If so, then down-regulation of PKC should diminish the responsiveness of PAI-1 to TGF- β induction.

We treated CCL64 cells with high concentrations of PMA (100 ng/ml) for 48 h and then challenged them with TGF- β or

PMA. The results (Fig. 5) show that EGF and TGF- β , but not PMA, were able to induce PAI-1 in cells that had been pre-incubated with PMA. Quantitative analysis of the results in Fig. 5 confirmed that there was no significant increase in the expression of secreted PAI-1 after the cells had been preincubated with PMA. Therefore, although induction of PAI-1 by PMA is mediated by PKC, induction by TGF- β of PAI-1 does not depend upon activation of PKC.

Analysis of transcription rates by nuclear run-on

To determine the relative changes in transcriptional activity in response to TGF- β , PMA and forskolin, nuclear run-on transcription assays were performed. Several gene activities were measured in NRK cells, including those of PAI-1, α -actin and fibronectin (Fig. 6). A striking finding in these studies was that the level of expression of PAI-1 mRNA from TGF- β -stimulated cells was extremely high compared with the levels of expression of other genes such as actin and fibronectin. Despite the apparent high rate of transcription of the PAI-1 gene, PAI-1 mRNA levels were low and difficult to detect by Northern analysis compared with the readily detectable actin mRNA (results not shown). These results suggest that TGF- β regulates the PAI-1 level by altering the rate of transcription and that the intracellular level of PAI-1 mRNA is markedly suppressed relative to its transcription rate by the high rate of its turnover. PAI-1 mRNA has been reported to have a relatively short half-life of 2.2–3 h [35].

Quantification of the PAI-1 transcript signal by densitometric analysis showed that PAI-1 gene activity in NRK clone 49F cells was significantly increased over the control levels in both TGF- β - and PMA-treated cells. Forskolin lowered the rate of PAI-1 transcription in TGF- β -stimulated NRK cells to 20–40 % of the control value ($n = 3$ experiments). Analysis of the basal level of transcriptional activity of the PAI-1 gene was not possible because it was too low to be detected in this assay.

From these results, it appears that the main effect of cAMP on PAI-1 expression is at the transcriptional level. The extent of inhibition of PAI-1 gene transcription observed in the nuclear run-on assay was comparable with that observed in PAI-1 protein expression in cells with elevated cAMP.

Sensitivity to regulation by cAMP can be observed for many genes regulated by TGF- β . In most cases the direction of regulation by cAMP and TGF- β is opposite. For example, levels of procathepsin L, collagenase, PIP62/65 and laminin are decreased by TGF- β and increased by elevating intracellular cAMP ([23,36–39]; the present paper). Levels of PAI-1, *sis* and *myc* are increased by TGF- β and decreased by raising intracellular cAMP ([8,10,14,15,32,33,40–43]; the present paper). Plasminogen activator expression is decreased in some cell types and increased in others in response to TGF- β [31,43–45] and agents increasing intracellular cAMP [33,46–48]. Thus it seems that a common feature of TGF- β -regulated genes is that they are also regulated by cAMP. Exceptions to the observation of opposite regulation by cAMP and TGF- β are the genes for fibronectin [49] and stromelysin [50], which are regulated in the same direction by TGF- β and cAMP. Where it has been determined, cAMP regulates the expression of these TGF- β -regulated genes at the level of transcription ([40,47,49,50]; the present paper).

Our results suggest that the cAMP-mediated and TGF- β -initiated signal transduction pathways are independent and non-interacting. However, the ability of cAMP to affect the expression of many TGF- β -regulated genes suggests that cAMP influences the level of activity of some common component(s) required for transcription of all TGF- β -regulated genes. Alternatively, the activity of one or more transcription factors required for the expression of many TGF- β -regulated genes may be controlled by

cAMP, or a cAMP-regulated transcription factor may act as a negative transcriptional regulator of these genes. In this regard, it is relevant that the proto-oncogene *fos* has been demonstrated to be important for the expression of transin in response to TGF- β [51]. Recent results have suggested that protein kinase A mediates the activation of AP-1, the *fos*-*jun* heterodimer, by tumour promoters [52], and that AP-1 can inhibit expression of the osteocalcin gene by occupying the TREs (AP-1 recognition sequences) flanking the vitamin D-responsive CCAAT box in the promoter region [53]. Also, as well as binding the TRE, AP-1 recognizes the CRE (cAMP-responsive element). A requirement for *fos* in gene regulation by TGF- β and the consistent association between the sensitivity of genes to regulation by cAMP and TGF- β suggest that the regulatory element recognized by *fos* in these TGF- β -regulated genes may be a CRE.

The TGF- β receptor mediates induction of PAI-1 by TGF- β , but not by PMA

To determine whether the TGF- β receptor mediates the induction of PAI-1 by both TGF- β and PMA, we tested two cell lines, MLEM and R1B, lacking functional TGF- β receptors [20,21]. The cells were treated with TGF- β or PMA for 2 h and then labelled with [35 S]methionine for 5 h in the presence of the same factors. Samples of medium from cells treated with TGF- β or PMA were immunoprecipitated with antibody raised against purified bovine endothelial cell plasminogen activator inhibitor. This step was particularly important in analysing the results from the R1B mutants, because for these cells PAI-1 was not electrophoretically resolved from another secreted protein that is not induced by TGF- β . TGF- β did not induce PAI-1 in either mutant (Fig. 7). In contrast, PMA induced PAI-1 in both MLEM and R1B cells to levels approximately equivalent to PMA-induced PAI-1 levels in the CCL64 cells (Fig. 7). The protein kinase inhibitor H7 also lowered PMA-induced levels of PAI-1, but not TGF- β -induced production of PAI-1 (results not shown). These data show that activation of the TGF- β receptors is necessary for TGF- β induction of PAI-1, but that induction of PAI-1 by PMA is independent of the TGF- β receptor.

Effect of pertussis toxin on TGF- β -effected signal transduction

Several reports have provided evidence that the signal transduction pathway leading to TGF- β -stimulated DNA synthesis in AKR-2B cells might be mediated by a pertussis-toxin-sensitive GTP-binding protein [16,17]. Thus we tested the effect of pertussis toxin on PAI-1 synthesis. Under the conditions used for these studies, pertussis toxin had no significant effect on TGF- β - or PMA-induced expression of PAI-1 or on the expression of any other secreted protein. Pertussis toxin did, however, inhibit DNA synthesis by 50% in BSC-1 cells and by 20% in AKR-2B cells within 20 h of its addition (results not shown).

TGF- β stimulates DNA synthesis in quiescent AKR-2B cells, but with a long lag time [54]. The stimulation is effected by the induction of *c-sis* by TGF- β , followed by autocrine stimulation through the platelet-derived growth factor receptor [55]. Pertussis toxin prevented stimulation of DNA synthesis by EGF or platelet-derived growth factor in mouse fibroblasts only if added before the growth factors [36,56]. Thus, although a G_i -like protein may be involved in the events leading to proliferation of quiescent AKR-2B cells initiated by *sis*, we have no evidence to suggest the involvement of a G_i -like protein in TGF- β -induced synthesis of PAI-1.

Summary

Here we have shown that PAI-1 gene transcription is regulated by TGF- β via a mechanism that is independent of PKC and pertussis-toxin-sensitive G proteins. The gene can also be

Fig. 7. Effect of TGF- β and PMA on induction of PAI-1 in CCL64, MLEM and R1B cells

CCL64, MLEM and R1B cells were plated in DMEM with 1% calf serum. At 24 h later, the medium was changed to fresh DMEM with 0.5% calf serum. After a further 36 h, monkey TGF- β type 2 (T) or PMA (P) was added. All cells also received 2 mM-acetic acid and 0.1% dimethyl sulphoxide. The cells were incubated for 2 h, then the medium was replaced with [35 S]methionine medium with the appropriate additions and the cells were labelled for 5 h. Medium from each treatment was immunoprecipitated with anti-PAI-1 antibody. The original media and the immunoprecipitates were resolved by SDS/PAGE. PAI-1 is indicated with the arrow. (a) Original medium, (b) immunoprecipitates.

regulated by phorbol esters via what seems to be a PKC-dependent mechanism. The phorbol ester-driven pathway to activating PAI-1 gene transcription is independent of the TGF- β pathway and does not involve the activation of TGF- β production.

The PAI-1 gene is negatively regulated by cAMP. A common feature of genes regulated by TGF- β is that they are also regulated by cAMP. Here we show that co-regulation of PAI-1 gene expression by cAMP and TGF- β is through independent and non-interacting signal transduction pathways. The TGF- β signal transduction pathway is not sensitive to intracellular cAMP, although the target gene is inhibited by cAMP. These results suggest that regulation of gene expression by cAMP and TGF- β may involve one or more common DNA regulatory elements.

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