

# Regulation of the Synthesis and Activity of Urokinase Plasminogen Activator in A549 Human Lung Carcinoma Cells by Transforming Growth Factor- $\beta$

Jorma Keski-Oja, Francesco Blasi,\* Edward B. Leof, and Harold L. Moses

Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232; and \*International Institute of Genetics and Biophysics, Consiglio Nazionale delle Ricerche, 80125, Naples, Italy

**Abstract.** Transforming growth factor- $\beta$  (TGF $\beta$ ) is a regulator of cellular proliferation which can alter the proteolytic activity of cultured cells by enhancing the secretion of endothelial type plasminogen activator inhibitor and affecting the secretion of plasminogen activators (PAs) in cultured fibroblastic cells. We used the TGF $\beta$ -responsive malignant human lung adenocarcinoma cell line A549 to study the relationships between the known TGF $\beta$ -induced growth inhibition and the effects of TGF $\beta$  on the secretion of PA activity by A549 cells. PA activity was quantitated by caseinolysis assays, and characterized by urokinase mRNA analysis, immunoprecipitation, and zymography assays. PA-inhibitor production was observed in autoradiograms of

SDS-polyacrylamide gels and reverse zymography assays. It was found that TGF $\beta$  enhanced the production of PA activity by these cells, in accordance with an enhancement of urokinase mRNA levels. A concomitant stimulation of type 1 PA-inhibitor production was also observed in A549 cells in response to TGF $\beta$ . In contrast to the observations of A549 cells, TGF $\beta$  caused a decrease in the expression of both urokinase and the tissue-type PA mRNA in human embryonic WI-38 lung fibroblasts indicating opposite regulation of the expression of PAs in these cells. The results suggest that TGF $\beta$  may play a role in the regulation of the invasive, proteolytically active phenotype of certain lung carcinoma cells.

**M**ALIGNANT transformation of cultured cells is often associated with the production of specific growth factors (see 10, 16, 23, 34), some of which have been termed transforming growth factors because of their ability to induce anchorage-independent growth in non-malignant cells (34, 43). Transforming growth factor- $\beta$  (TGF $\beta$ )<sup>1</sup> is mainly responsible for the induction of anchorage-independent growth in fibroblastic cells, either alone (32) or in combination with epidermal growth factor or TGF $\alpha$  (43). TGF $\beta$  acts presumably as a mitogen for fibroblastic cells grown in adherent cultures by inducing the expression of the *c-sis* protooncogene (30). It appears, however, to have a dual role in the regulation of cell growth: while being stimulatory for cells of mesenchymal origin, it is a potent growth inhibitor for cultured epithelial cells (35, 44). The mechanism of this inhibitory activity of TGF $\beta$  is unclear at present.

J. Keski-Oja's present address is Department of Virology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki, Finland. F. Blasi's present address is Mikrobiologisk Institut, The University of Copenhagen, Øster Farimagsgade 2A, 1353 Copenhagen K, Denmark.

1. *Abbreviations used in this paper:* PA, plasminogen activator; PAI, plasminogen activator inhibitor; u-PA, endothelial-type plasminogen activator inhibitor; TGF $\beta$ , transforming growth factor- $\beta$ .

Plasminogen activators (PAs) are the major mediators of pericellular proteolysis. Enhanced proteolytic activity is frequently observed in fibroblastic cells transformed with viruses or chemicals, as well as in cultured tumor cells (9, 41, 46). Normal cells can evidently regulate the synthesis of plasminogen activators (18) whereas in cancer cell cultures elevated levels (see 46) and lack of response to growth factors (52) are frequently observed. At least two types of PAs exist in tissues: the urokinase- and tissue-type plasminogen activators (u-PA and t-PA, respectively). u-PA has specific cell surface receptors (50, 51, 56) and recently an autocrine model was proposed depicting features in the regulation of cell migration (51).

Several cell lines in culture also produce PA inhibitors (6, 46). Three well-defined, immunologically different types exist: protease nexin I (48, 49), endothelial-type (55) and placental-type (3) PA inhibitors (PAI-1 and PAI-2, respectively). The secretion of PA inhibitors as well as PAs from cells can be affected by mitogens, corticoids, and tumor promoters (1, 8, 12, 18, 27, 29, 52; see 9, 46). Simultaneous secretion of both PAs and the inhibitors complicates the analyses since it is necessary to use test systems which allow discrimination between total and inhibitor-modulated PA activity (see 6, 46). We have recently shown that TGF $\beta$  can modulate the proteolytic activity of cultured dermal fibro-

blasts by affecting the secretion from cells of both PAs and PA inhibitors. A dominant feature in the action of TGF $\beta$  is the induction of PAI-1 (26, 27).

To determine whether PA activity is affected in an epithelial lung cell line that is sensitive to TGF $\beta$  growth inhibition, we studied the effects of TGF $\beta$  on the proteolytic activity of A549 cells in culture and compared the responses with fibroblastic lung cells. We find that TGF $\beta$  enhances the level of u-PA mRNA in A549 cells while decreasing it in WI-38 human lung fibroblasts. In accordance with our previous observations (26, 27), enhanced secretion of the type-1 plasminogen activator inhibitor was also seen. However, quantification of the net PA activities by caseinolysis assays demonstrated that TGF $\beta$  enhanced the overall proteolytic activity of A549 cells. We report here that TGF $\beta$  regulates two of the three components of the u-PA system in human A549 cells controlling the synthesis and activity of u-PA and secretion of PAI-1.

## Materials and Methods

### Reagents

Human urokinase was purchased from Calbiochem Behring Corp. (La Jolla, CA), aprotinin from Sigma Chemical Co. (St. Louis, MO). TGF $\beta$  was purified from outdated human platelets as described (35). The TGF $\beta$  was shown to be homogeneous by silver staining of SDS-PAGE under reducing and nonreducing conditions and autoradiography of gels of reduced and nonreduced  $^{125}\text{I}$ -TGF $\beta$ . Its growth stimulatory and inhibitory properties were demonstrated using AKR-2B and A549 cells as described. TGF $\beta$  inhibits efficiently the soft agar growth of A549 cells at a concentration of 0.5 ng/ml, and totally prevents it at 5 ng/ml (35, 44).

### Cell Cultures

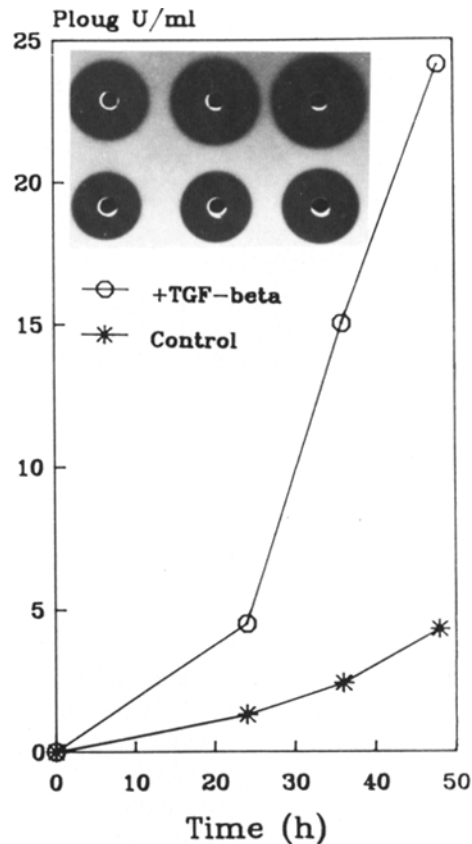
Human embryonic lung WI-38 fibroblasts (CCL-75; American Type Culture Collection, Rockville, MD), human HT-1080 sarcoma cells (CCL-121; American Type Culture Collection), and A549 lung adenocarcinoma cells (CCL-185; American Type Culture Collection) were cultivated in medium 199 containing 10% heat-inactivated FCS (Gibco, Chagrin Falls, OH), 100 IU/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin at 37°C in a humidified atmosphere containing 5% CO $_2$ .

### Stimulation by Growth Factors

Cells were seeded at a density of  $3.0 \times 10^4$  per Linbro well (16 mm diam; Flow Laboratories, Inc., McLean, VA). Upon confluency the medium was replaced with serum-free medium 199 and incubated at 37°C for an additional 6 h to remove most serum proteins. The medium was replaced by fresh medium 199 and the growth factors were added as indicated. The cultures were then incubated at 37°C for 2–48 h and labeled with [ $^{35}\text{S}$ ]methionine where indicated. The medium and the cells were collected and analyzed as described.

### Radioactive Labeling

Confluent cultures were washed and incubated in serum-free medium 199 for 6 h. The medium was changed and the cultures were labeled with 50  $\mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]methionine (1,390 Ci/mmol; Amersham International, Amersham, UK) in the presence of TGF $\beta$ , and aprotinin where indicated, at 37°C for 4–24 h. The medium was collected and clarified by centrifugation (600 g, 10 min). 1-ml aliquots of the supernatant were then incubated with 50- $\mu\text{l}$  aliquots (50% of suspension in PBS; 10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4) of concanavalin A-Sepharose (Con A-Sepharose; Pharmacia, Uppsala, Sweden) at 22°C in a rotary shaker for 2 h. Con A-Sepharose is known to bind PAI-1 efficiently (1, 26). The Sepharose particles were washed four times with 1 ml PBS/Tween-80 (0.05%), and the bound proteins were dissolved in Laemmli's gel sample buffer. The samples were analyzed by discontinuous SDS-PAGE.



**Figure 1.** Effect of TGF $\beta$  on the proteolytic activity of A549 cells. The cells were cultivated in plastic Linbro wells. Upon confluency, 2–3 d later, the cultures were changed to serum-free medium 199 for 24 h. New medium supplemented with TGF $\beta$  (5 ng/ml) was then added. At different times, the media were collected (duplicates at each point) and analyzed by caseinolysis assays (see Materials and Methods). The values are plotted as Ploug units (PU/ml) by comparison with a serial dilution of urokinase standard. (Inset) Results of the caseinolysis assays of the 24-, 36-, and 48-h time points; (upper line) TGF $\beta$ ; (lower line) control.

### Immunoprecipitation Analyses

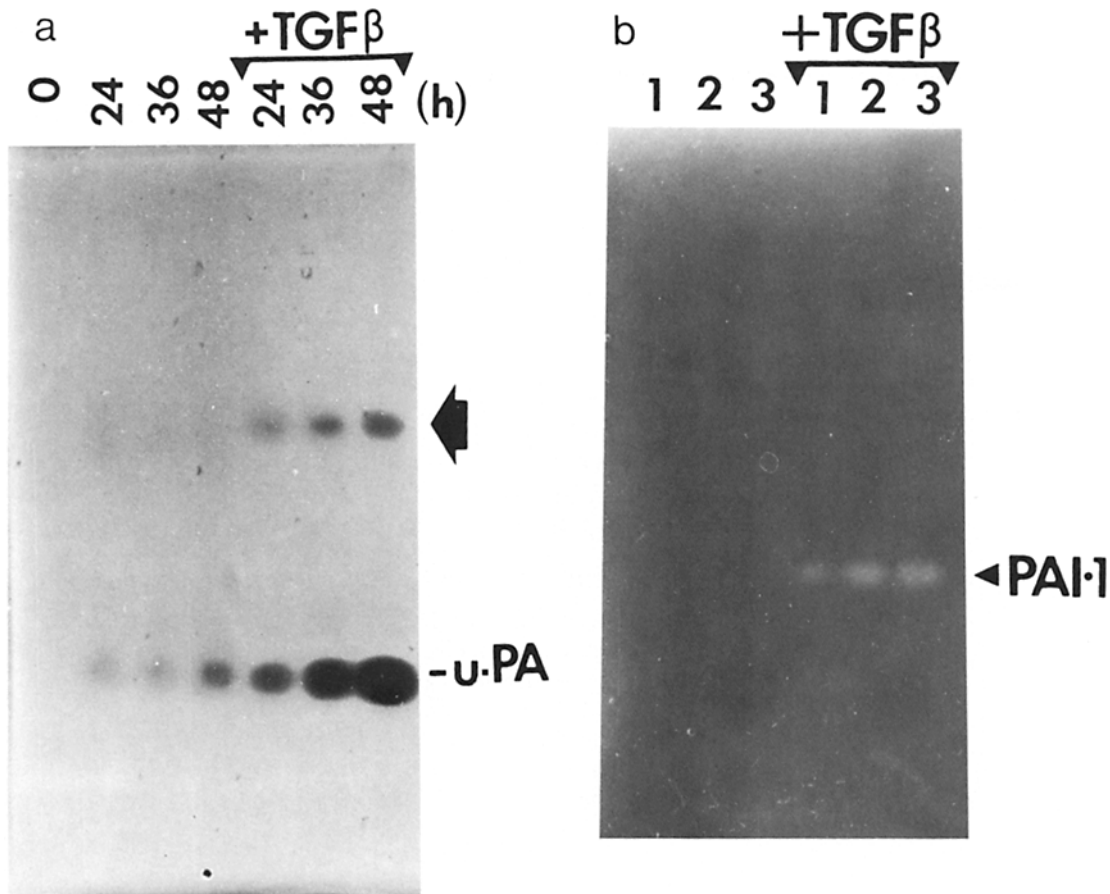
Rabbit antibodies against endothelial-type PA inhibitor (PAI-1) were obtained from Dr. D. J. Loskutoff (Department of Immunology, Scripps Institute and Research Foundation, La Jolla, CA). Human urokinase was immunoprecipitated using specific rabbit anti-u-PA IgG antibodies (14). The immunoprecipitates were separated using protein A-Sepharose as previously described (26), followed by SDS-PAGE analysis, and fluorography.

### SDS-PAGE

PAGE in the presence of SDS was performed according to Laemmli (25), using vertical slab gels. The samples were run nonreduced or reduced with 10% 2-mercaptoethanol in Laemmli's sample buffer. Radioactive molecular weight markers were purchased from Amersham International. After electrophoresis the gels were treated with Amplify (Amersham International) before drying for fluorography or processed for zymography assays (see below).

### Caseinolysis in Agarose Assays

The assays for the demonstration of the total PA activity were performed using agarose plates containing plasminogen and casein as described in detail (45). Plasminogen, when activated by PA present in the medium sample, degrades casein and forms a clear disk of caseinolysis in the gel proportional to the PA activity of the sample and time of diffusion. Human urokinase was



**Figure 2.** Zymographic assays of PA and PAI in the medium of TGF $\beta$ -treated A549 cells. (A) Zymographic detection of PA activity. Aliquots of medium (50  $\mu$ l) were analyzed in an 8% SDS-PAGE under nonreducing conditions, followed by zymography assay (see Materials and Methods). A casein-containing agarose gel was then placed onto the polyacrylamide gel. The gel was incubated at 37°C for 8 h and photographed under indirect illumination. Note the increase of both u-PA and higher molecular mass complexes ( $M_r$  100,000; upper arrow) in the media of TGF $\beta$ -treated cultures. The incubation times of the cultures are shown. (B) Reverse zymographic detection of PAI activity. Medium aliquots (50  $\mu$ l) were analyzed for the presence of PA inhibitors by reverse zymography (see Materials and Methods). The samples were analyzed under reducing conditions in an 8% SDS-PAGE, known to inactivate u-PA. Note the increase of PAIs in the medium from TGF $\beta$ -treated cultures (indicated in the figure). Incubation times of the cultures were (1) 24, (2) 36, (3) 48 h.

used as a standard, and PA activity was plotted in Ploug units (from 50 to 0.05 PU/ml). Plates without added plasminogen were included to control for non-PA proteolysis.

### Zymography Assays

Zymography and reverse zymography assays (13, 17) were used to demonstrate the migration of either PAs or the PA inhibitors. To observe the molecular forms of PA activity the nonreduced medium samples were first subjected to electrophoresis in NaDodSO<sub>4</sub>-polyacrylamide gels. NaDodSO<sub>4</sub> was removed by extensive washing (200 ml, three times, 4 h) with PBS/Triton X-100 (2.5%) followed by caseinolysis overlay assay as described earlier (27, 47). To observe the inhibitors (reverse zymography), the samples were reduced with 2-mercaptoethanol and analyzed in gels. This treatment inactivates PAs but does not affect PAI-1 that does not contain disulfide bridges (see reference 6). The gels were finally incubated in 200 ml of PBS/Triton X-100 (2.5%) containing 0.5 IU/ml urokinase at 22°C for 30 min (27). A casein-agarose gel containing plasminogen was then placed on the polyacrylamide gel and the inhibition of caseinolysis was observed.

### RNA Isolation and Northern Blot Analysis

Cytoplasmic poly(A<sup>+</sup>)-containing RNA was prepared by previously published procedures. Confluent cultures of cells were washed under serum-free conditions for 4–18 h, and exposed to TGF $\beta$  (5 ng/ml), epidermal

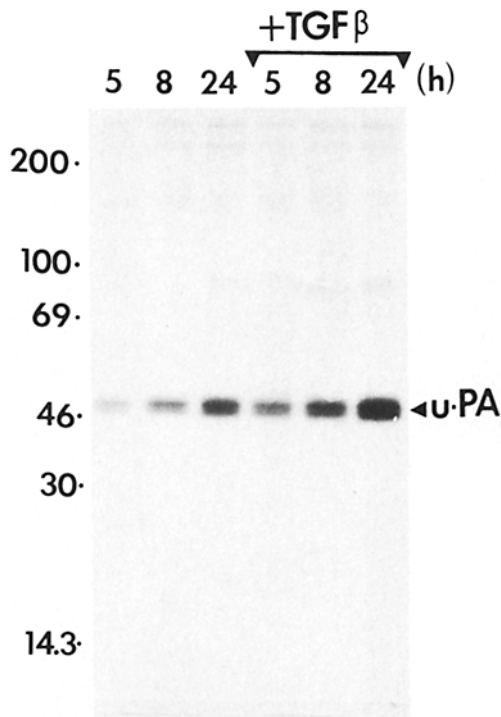
growth factor (5 ng/ml), or cycloheximide (1.5  $\mu$ g/ml) for time periods described above. At the termination of the incubation the cells were lysed with lysing buffer (0.14 M NaCl, 0.002 M EDTA in 0.01 M Tris-HCl buffer, pH 7.4), containing 1% SDS. The poly(A<sup>+</sup>)-containing RNA was purified by one cycle of oligo-dT cellulose chromatography.

For Northern analysis equivalent quantities of RNA, as determined by OD<sub>260/280</sub> and ethidium bromide staining, were subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. Conditions for electrophoresis, transfer to nitrocellulose, and hybridization at 43°C to nick-translated <sup>32</sup>P-labeled human urokinase cDNA (57) were performed as described (20).

## Results

### Effects of TGF $\beta$ on the Proteolytic Activity of A549 Lung Carcinoma Cells

Confluent cultures were placed in serum-free medium 199 for 24 h to assure quiescence and removal of residual serum factors. The medium was then replaced with fresh serum-free medium supplemented with TGF $\beta$  (1–20 ng/ml) and the proteolytic activity of the medium samples determined after 8 h by caseinolysis assays. It was found that TGF $\beta$  stimulated



**Figure 3.** Immunoprecipitation analysis of the effect of TGF $\beta$  on the level of urokinase in A549 cell medium: time dependency of TGF $\beta$  stimulation. Confluent cultures of A549 cells were labeled with [ $^{35}$ S]methionine in the presence of TGF $\beta$  (5 ng/ml) and aprotinin (200 U/ml) for the times shown. After labeling the media were clarified by centrifugation and radiolabeled urokinase precipitated with specific antibodies. The immunoprecipitates were analyzed by 8% SDS-PAGE followed by fluorography. Densitometric analysis of the proteins in the  $M_r$  50,000 region showed that after 24-h incubation the amount of radiolabeled urokinase antigen was increased over twofold (respective relative densitometric values for urokinase from controls: 1, 2, 3.7, and from TGF $\beta$ -treated cultures: 2.5, 4, 7.7).

proteolytic activity in the cultures by enhancing PA activity with submaximal effect at the concentration of 5 ng/ml (data not shown). A time course study showed that a sixfold enhancement of net PA activity over control is obtained after 48 h (Fig. 1). After treatment with SDS (0.2%) to inactivate SDS-sensitive PA inhibitors (for example protease-nexin I) (31, 48), the difference was of the same magnitude (data not shown). Thus, TGF $\beta$  induces a time- and dose-dependent increase in net PA activity in A549 cells.

#### Zymographic Analysis of PA Activity

Medium samples from TGF $\beta$ -treated cells were analyzed by SDS-gel electrophoresis to visualize the molecules contributing the PA activity (6, 17). Analysis of the caseinolysis overlay indicator gel showed a PA activity band in A549 cells that comigrates with u-PA marker ( $M_r$  52,000). The TGF $\beta$ -dependent increase in u-PA (Fig. 2 a) agrees with the results of the caseinolysis assays (Fig. 1). A higher molecular mass form of PA activity ( $\sim$ 100 kD) was also observed in the samples from TGF $\beta$ -treated cells (Fig. 2 a, arrow); this corresponds to previously described (5, 6) SDS-stable complexes between u-PA and PAI-1 (see also 26, 28). The fact that

u-PA/PAI-1 complex shows enzymatic activity is probably due to the proteolytic degradation of PAI-1 by u-PA (2, 38). Both the high molecular mass PA activity and the active 52-kD u-PA were increased in TGF $\beta$ -treated cells. No activity band was observed in the absence of plasminogen (not shown).

Reverse zymography assays of the unfractionated conditioned medium samples of the same experiment showed that a PA-inhibitory activity was demonstrable only in TGF $\beta$ -treated cultures. The inhibitor migrated in gels just behind u-PA and was not easily demonstrable because of high u-PA activity (gel not shown). However, when analyzed under reducing conditions where u-PA activity is destroyed, a time-dependent increase of PA inhibition in the PAI-1 region was clearly seen (Fig. 2 b). This result supports the conclusion in Fig. 2 a that the 100-kD band is a complex between u-PA and PAI. Immunoprecipitation experiments showed that this inhibitor is in fact PAI-1 (see Fig. 4).

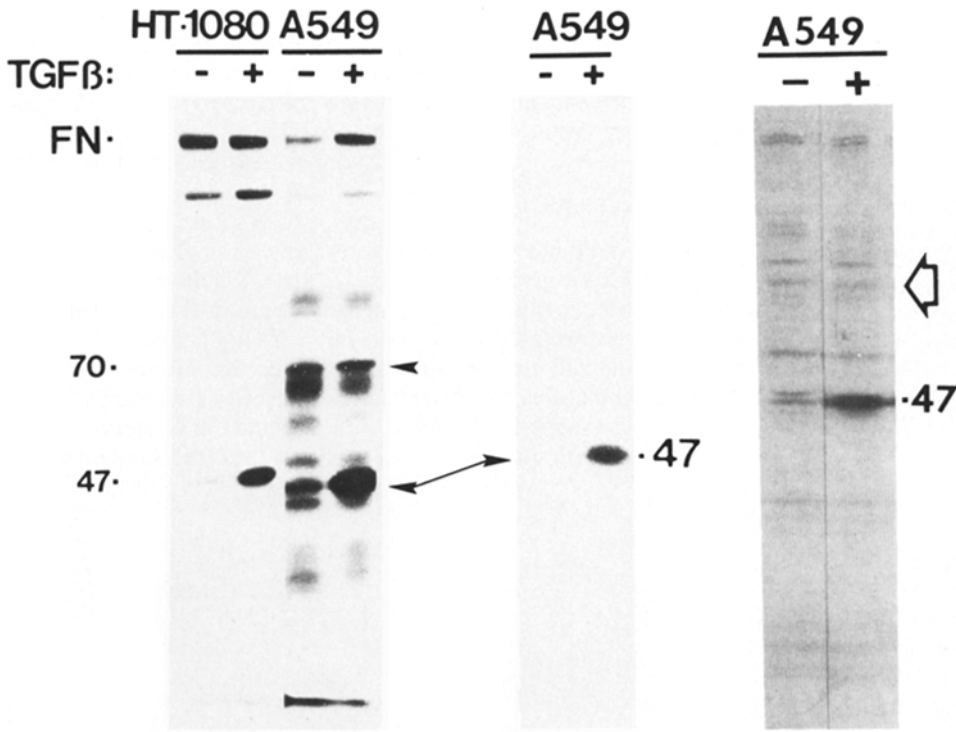
#### Detection of Urokinase by Immunoprecipitation

To study whether TGF $\beta$  affects the synthesis of urokinase, immunoprecipitation with anti-u-PA antibodies of the conditioned media of metabolically labeled A549 cells was performed. Confluent cultures of A549 cells were labeled with [ $^{35}$ S]methionine under serum-free conditions in the presence of TGF $\beta$  (5 ng/ml) and aprotinin (100 IU/ml). After different incubation times, the medium was collected and immunoprecipitation analysis was carried out from identical volumes using antibodies against human u-PA. Analysis of the autoradiograms of the immunoprecipitates by densitometric scanning showed that TGF $\beta$ -treated cells secreted about twofold more radiolabeled u-PA antigen than the control indicating enhanced synthesis and secretion (Fig. 3). The 100-kD complex was not recognized by anti-catalytic u-PA antibodies (Fig. 3), but was recognized by monoclonal antibodies against the A chain of u-PA (data not shown).

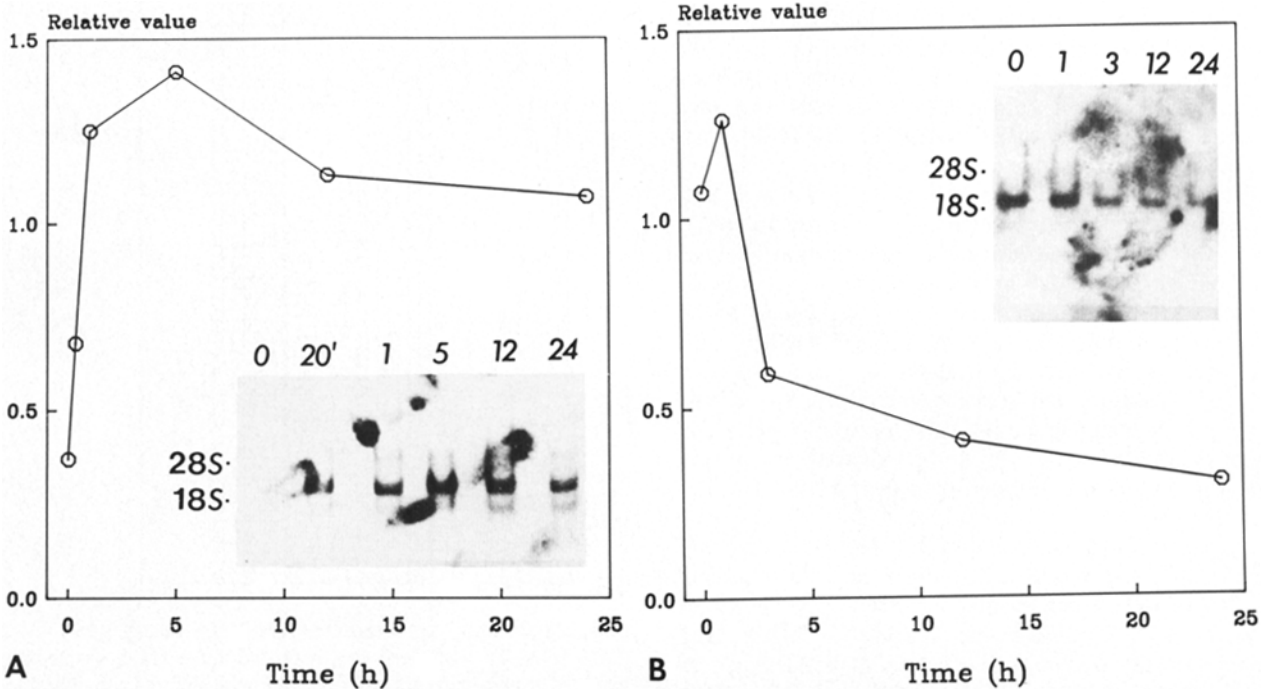
#### Effects of TGF $\beta$ on Plasminogen Activator Inhibitor Production

We have previously shown that treatment of cultured cells with TGF $\beta$  leads to enhanced production of PAI-1 in human fibroblastic (26) and sarcoma cells (28). The PAI-1 is known to bind to Con A-Sepharose and to migrate in SDS-polyacrylamide gels as a 47,000- $M_r$  protein (26). To study the effect of TGF $\beta$  on PAI-1 synthesis, A549 cells were labeled for 18 h with [ $^{35}$ S]methionine with or without TGF $\beta$ . Equivalent aliquots of the radiolabeled medium from A549 cells (and HT-1080 cells as a positive control; reference 28) were incubated with Con A-Sepharose and the bound polypeptides eluted with Laemmli's sample buffer and analyzed by SDS-PAGE. Analysis of the gel fluorograms indicated that the intensity of a 47,000- $M_r$  protein was enhanced in samples from TGF $\beta$ -treated cultures (Fig. 4). In addition, a prominent 70,000- $M_r$  protein was observed in the medium. The nature of the 70,000- $M_r$  protein will be object of further studies.

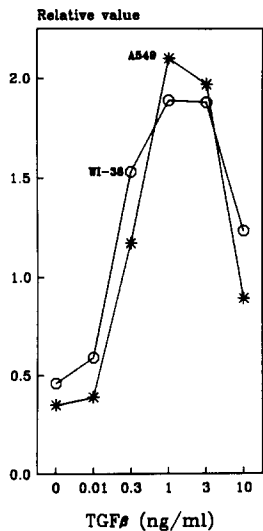
To analyze whether the A549 cell-derived radiolabeled 47,000- $M_r$  protein was associated with PA-inhibitory activity, a reverse zymography assay was performed. Con A-binding proteins were analyzed in an 8% SDS-polyacrylamide gel under reducing conditions which are known to inactivate



**Figure 4.** Effects of TGF $\beta$  on PA-inhibitor level in A549 cell medium. Confluent cultures of A549 cells were labeled with [ $^{35}$ S]methionine under serum-free conditions in the presence of TGF $\beta$  (5 ng/ml; - or +) as shown. (Left) The media were collected after an 18-h labeling and PAI-1 isolated from 1-ml aliquots by binding to Con A-Sepharose followed by SDS-PAGE analysis (see Materials and Methods). HT-1080 cells were used as a positive control (28). A fluorogram of an 8% SDS-PAGE is shown. Note the enhancement of a 47,000- $M_r$  protein (47, arrow) in samples from TGF $\beta$ -treated cells. (Center) Reverse zymographic analysis of the respective A549 medium samples indicating activity of the 47,000- $M_r$  protein (negative of the photograph is shown). (Right) Immunoprecipitation analysis of the medium at 12 h using anti-PAI-1 antibodies. Open arrow indicates the position of 70,000- $M_r$  protein(s) based on markers. The position of PAI-1 (47) is also shown.



**Figure 5.** Northern blot analysis of urokinase mRNA in A549 cells and WI-38 human lung fibroblasts. The cells were grown as described in the Materials and Methods section with TGF $\beta$  (5 ng/ml). At the times indicated poly(A $^+$ ) mRNA was isolated and analyzed by Northern blotting using a human urokinase cDNA probe (see Materials and Methods). The relative amounts of mRNA were estimated from the autoradiograms using laser scanning densitometry. *A*, A549 lung carcinoma cells; *B*, WI-38 lung fibroblasts. (Insets) The results of the Northern blot analyses. The mobilities of the marker RNAs are shown on the left.



**Figure 6.** Regulation of u-PA mRNA in A549 and WI-38 cells by TGF $\beta$ : dose dependency. The cells were treated with increasing concentrations of TGF $\beta$  (indicated on the figure) for 4, (A549) or 1 h (WI-38), followed by isolation of mRNA as described in Materials and Methods. Northern hybridization analysis was carried out using the human urokinase cDNA probe, and the levels of mRNA were estimated by densitometric scanning. The values are expressed as relative units, and therefore the amounts of mRNA are not comparable between the two cell lines. The designations of the cell lines are indicated.

protease nexin-I and PAs (6, 26). Reverse zymography indicated that the 47,000- $M_r$  polypeptide was associated with PA-inhibitory activity (Fig. 4) in a way similar to the samples from WI-38 fibroblastic cells (26), suggesting that the inhibitor is PAI-1. To further identify the PAI activity in TGF $\beta$ -stimulated cultures immunoprecipitation analysis using anti-PAI-1 antibodies was performed. A 47,000- $M_r$  protein was precipitated from the medium of TGF $\beta$ -treated cells as shown previously using WI-38 cells (26) (Fig. 4, right lanes).

#### Effect of TGF $\beta$ on the Expression of the Urokinase Gene

The previous data demonstrate that TGF $\beta$  can increase both the activity and synthesis of PA in A549 cells (Figs. 1-3). We have previously demonstrated that TGF $\beta$  decreases u-PA activity in WI-38 normal human fibroblasts (26). We have therefore determined whether this difference in TGF $\beta$  effect could be also visible at the level of urokinase mRNA accumulation. Cells were grown to confluency and treated with TGF $\beta$  under serum-free conditions. Cytoplasmic poly(A<sup>+</sup>)-mRNA was isolated and Northern hybridization analysis carried out using a human urokinase probe. In accordance with the data of Fig. 1, it was found that TGF $\beta$  enhanced the level of urokinase mRNA in A549 cells (Fig. 5 A). This u-PA mRNA increase is observed already at 20 min, peaks  $\sim$ 5 h after TGF $\beta$  addition, and remains unchanged for at least 24 h (Fig. 5 A, scanning curve). A TGF $\beta$  time-dependency study of the mRNAs from WI-38 cells showed an early increase of u-PA mRNA followed by a two- to threefold decrease (Fig. 5 B).

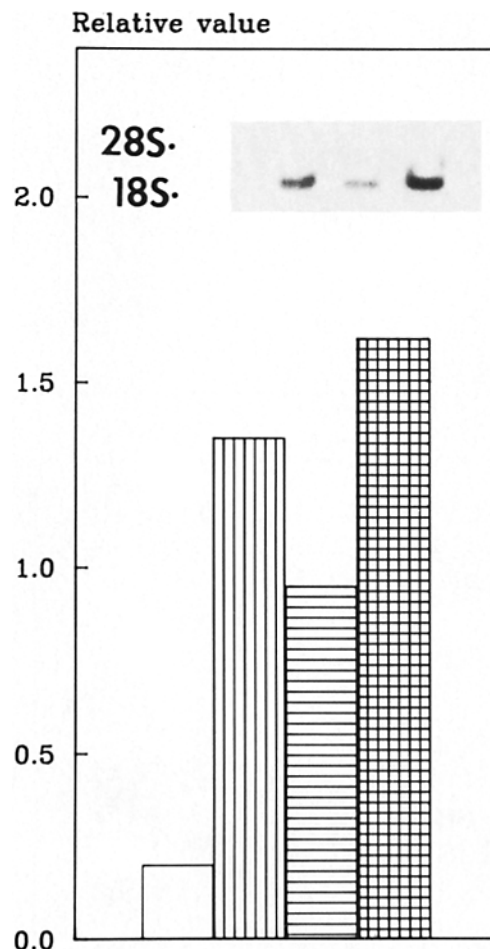
When the dose dependence of TGF $\beta$  induction was analyzed in A549 and WI-38 cells with 4- and 1-h treatment times, respectively, a bell-shaped response was obtained not only with A549 cells but also with WI-38 cells (Fig. 6). The maximal induction was reached at  $\sim$ 1 ng/ml of TGF $\beta$  in both cases. Obviously, TGF $\beta$  increases the level of u-PA mRNA in both cells. However, in WI-38 cells it also triggers another mechanism that leads to a fast disappearance of the u-PA mRNA.

To determine whether the induction of urokinase mRNA is a primary event not dependent on prior protein synthesis, A549 cells were treated with cycloheximide (1.5  $\mu$ g/ml) in

the presence or absence of TGF $\beta$  for 5 h. Northern blot analysis of the respective mRNAs shows that cycloheximide itself is able to enhance the expression of urokinase and that the inhibition of protein synthesis does not prevent the effect of TGF $\beta$  (Fig. 7). In WI-38 cells, cycloheximide also enhances the expression of urokinase mRNA, but its effect is antagonistic with TGF $\beta$  (not shown).

#### Discussion

TGF $\beta$  is a growth modulator, which can either stimulate or inhibit growth depending on the cell system used (35, 44, 54). Its effects are mediated via specific cell surface receptors that are as ubiquitous as TGF $\beta$  itself (11, 15, 33, 53). Both monomeric and disulfide-bonded multisubunit receptor complexes have been found at the cell surfaces in chemical cross-linking studies (7, 33). Many of the diverse biological activities of TGF $\beta$  may be indirect through stimulation, syn-



**Figure 7.** Effect of cycloheximide and TGF $\beta$  on u-PA mRNA in A549 cells. The cultures were incubated under serum-free conditions as described in Materials and Methods and treated with cycloheximide (1.5  $\mu$ g/ml) in the presence of TGF $\beta$  (5 ng/ml) for 5 h. The poly(A<sup>+</sup>) mRNA was isolated and analyzed by Northern hybridization. The relative amounts of the mRNA were estimated densitometrically from the autoradiogram. (□) Control, untreated; (▨) TGF $\beta$ -treatment; (▧) cycloheximide; (▩) treatment with both TGF $\beta$  and cycloheximide. Insert: The autoradiogram (samples in the respective order) with RNA markers (28S and 18S).

thesis, and release of growth factors (23) or extracellular matrix components (21). Here we have carried out a study on the effects of TGF $\beta$  on the synthesis and activity of u-PA in A549 human lung carcinoma cells.

In this paper we have shown that TGF $\beta$  enhances the overall fibrinolytic activity of A549 cells (Fig. 1). This is at variance with what was observed with WI-38 fibroblasts (26, 32). We have analyzed the molecular nature of the effect of TGF $\beta$  in A549 cells and have compared it with previously published (26) or new data (Figs. 5 and 6) from WI-38 cells. In A549 cells, the increase in plasminogen activator activity is due to an increase of u-PA synthesis as shown by immunoprecipitation experiments with metabolically labeled cells. This is in turn due to an elevated level of u-PA mRNA; the mRNA increase becomes evident by 20 min after TGF $\beta$  addition. The effect of TGF $\beta$  on u-PA mRNA does not require protein synthesis as it is not blocked by cycloheximide (Fig. 7). In WI-38 cells, however, the treatment with TGF $\beta$  was reported to reduce the overall plasminogen activator activity (26) due to a decrease in plasminogen activator activity and to a strong increase in the production of PAI-1 (26). We have now extended this investigation and measured the levels of u-PA mRNA in TGF $\beta$ -treated WI-38 cells (Figs. 5 B and 7). In WI-38 cells the level of u-PA mRNA, while being still increased by cycloheximide treatment (as in A549 cells), is also increased by TGF $\beta$  for short treatment times. However, this early increase is followed by a drastic decrease of u-PA mRNA level (Fig. 5 B). Therefore, in WI-38 cells the net effect will be a decrease of u-PA mRNA and hence of u-PA synthesis. We interpret these results so that the primary effect of TGF $\beta$  may be to increase u-PA mRNA level in both A549 and WI-38 cells. In WI-38 cells, however, a second mechanism is triggered that induces a drastic reduction of the u-PA mRNA level. This mechanism may be acting at the level of mRNA half-life. However, further studies are required to understand this point.

While TGF $\beta$  affects u-PA mRNA level in opposite directions in A549 and WI-38 cells, its effect on PAI-1 is similar in the two types of cells. In fact TGF $\beta$  induces PAI-1 in A549 cells (Fig. 4), an effect qualitatively similar to that reported for WI-38 cells (26, 32). However, the production of PAI-1 mRNA in response to TGF $\beta$  differs in these two cell types, being considerably less in A549 cells (24). The final effect on the fibrinolytic activity of A549 cells is therefore different in the two cells; TGF $\beta$  causes a net decrease in WI-38 cells, and a net increase in A549 cells. The latter is also evidenced by the increase in the u-PA/PAI-1 complex in TGF $\beta$ -treated A549 cells (see Fig. 2 a).

It is interesting to note the opposing effect of TGF $\beta$  on u-PA activity of cells of mesenchymal (decrease) and epithelial (increase) origin. TGF $\beta$  has opposing effects also on the growth properties of these two cell lines (35, 44). The intrinsic complexity of the growth and pericellular proteolytic pathway prevents a meaningful correlation between cellular growth and proteolytic activity in these cell lines. Obviously, the combined action of different endogenous and exogenous growth factors, proteases and protease inhibitors may have divergent effects on different cells, the final result depending on a carefully controlled balance between them. In any case, since the effect of TGF $\beta$  on u-PA mRNA has so far been studied only in A549 and WI-38 cells, any generalization will have to await for further studies on other epithelial and mesenchymal cell lines.

### ***Growth-promoting Factors in the Regulation of PA Activity***

Growth factors appear to play an important role in the regulation of the proteolytic activity of cultured cells (see references 9, 46). Various hormones and hormonelike substances can alter the secretion of both PAs and PAIs (1, 18, 36, 40, 42). Exposure of suitable cell types in culture to physiological concentrations of steroids, vasopressin, calcitonin, relaxin, and other polypeptide hormones from pituitary glands have been shown to elevate PA activity (see 9, 46). A role for EGF and phorbol esters in the regulation of PA activity has been suggested in a number of studies (12, 18, 19, 29, 39, 58). An increase of u-PA mRNA was observed in cultured human carcinoma cells treated with biologically active phorbol esters (52). The secretion of t-PA by cultured cells is also sensitive to effectors of cellular growth (26, 29, 39). Interestingly, TGF $\beta$  appears to have divergent direct effects on the u-PA level in different cells even though being in both cases a positive activator at the genetic level. This system may prove of great interest in elucidating the effects of TGF $\beta$  at the genetic level. The bell-shaped form of the dose-dependency curve suggests that alterations of TGF $\beta$  concentration may modify the u-PA responses in the microenvironment of the cells. Our results, however, cannot be explained on the basis of a different sensitivity of the two cells to TGF $\beta$ . In fact, induction of  $\beta$ -actin and *c-sis* mRNA follows the same time and concentration dependence in A549 and WI-38 cells (our unpublished results).

As another aspect we have recently found evidence for proteolytic activation of latent form of TGF $\beta$  (Lyons, R. M., J. Keski-Oja, and H. L. Moses, manuscript submitted for publication). This might offer the cells a regulatory mechanism to control the proteolytic balance in their microenvironment.

### ***Growth Regulation and Extracellular Matrix***

Enhanced proteolytic activity may cause degradation of the pericellular structures (40) required for anchorage-independent growth. If the induction of pericellular matrix proteins (21) and a decrease in proteolytic activity due to production of PAI-1 (26) are essential features for the support of soft agar growth of nontumorigenic cells, TGF $\beta$  might regulate anchorage-independent growth by regulating the proteolytic activity of these cells (see 37). TGF $\beta$  is, however, inhibitory for A549 and several other cell types in substratum-attached cultures, and other mechanisms must therefore be involved in monolayer growth inhibition.

It is not yet established how different malignant cells respond to TGF $\beta$  in the production of PAI-1. It has recently been shown that protease-nexin I and anti-u-PA antibodies decrease tumor cell-induced degradation of pericellular matrices (4, see reference 31). TGF $\beta$ -induced extracellular matrix-associated PAI-1 is expected to have a similar effect (28); thus TGF $\beta$  might regulate pericellular proteolysis through its action on PAI-1. A positive correlation between optimal proteolytic activity and anchorage-independent growth could be an essential part of the action of TGF $\beta$ . TGF $\beta$  might attain this goal by changing the balance between PAs and PAIs. An alteration in such a response might indicate that the cells have changed in their responsiveness to TGF $\beta$  (see 37). Identification of novel inhibitory and stimulatory signals that operate between proteolytic enzymes and growth factors may



reveal new regulatory routes of invasive and malignant growth.

We thank Ms. Mary Aakre and Ms. Jana Wolfshohl, M.Sc., for technical assistance, and Ms. Rebecca Koransky and Ms. Aira Halonen for secretarial work. F. Blasi is grateful to Drs. A. Roberts, R. Assoian, and M. Sporn for their support in the early phases of this study.

This study was supported by grant CA42572, awarded by the National Cancer Institute, DHHS. Work carried out in Naples was supported by Consiglio Nazionale delle Ricerche, PF Oncologia and PF Ingegneria Genetica.

Received for publication 11 March 1987, and in revised form 21 September 1987.

## References

1. Andreasen, P. A., T. H. Christensen, J.-Y. Huang, L. S. Wilson, and K. Danø. 1986. Hormonal regulation of extracellular plasminogen activators and Mr 54,000 plasminogen activator inhibitor in human neoplastic cells lines, studied with monoclonal antibodies. *Mol. Cell. Endocrinol.* 45: 137-147.
2. Andreasen, P. A., A. Riccio, K. G. Welinder, R. Douglas, R. Sartorio, L. S. Nielsen, C. Oppenheimer, F. Blasi, and K. Danø. 1986. Plasminogen activator type-1: reactive center and amino terminal heterogeneity determined by protein and cDNA sequencing. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 209:213-218.
3. Åstedt, B., I. Lecander, T. Brodin, A. Lundblad, and K. Low. 1985. Purification of a specific placental plasminogen activator inhibitor by monoclonal antibody and its complex formation with plasminogen activator. *Thromb. Haemostasis.* 53:122-125.
4. Bergman, B. L., R. W. Scott, A. Bajpai, S. Watts, and J. F. Baker. 1986. Inhibition of tumor-cell-mediated extracellular matrix destruction by a fibroblast proteinase inhibitor, protease nexin I. *Proc. Natl. Acad. Sci. USA.* 83:996-1000.
5. Blasi, F., J. D. Vassalli, and K. Danø. 1987. Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors. *J. Cell Biol.* 104:801-804.
6. Cajot, J.-F., E. O. K. Kruihof, W.-D. Schleuning, B. Sordat, and F. Bachmann. 1986. Plasminogen activators, plasminogen activator inhibitors and procoagulant analyzed in twenty human tumor cell lines. *Int. J. Cancer.* 38: 719-727.
7. Cheifetz, S., B. Like, and J. Massagué. 1986. Cellular distribution of type I and type II receptors for transforming growth factor- $\beta$ . *J. Biol. Chem.* 261:9972-9978.
8. Crutchley, D. J., L. B. Conanan, and J. R. Maynard. 1981. Human fibroblasts produce inhibitor directed against plasminogen activator when treated with glucocorticoids. *Ann. NY Acad. Sci.* 370:609-616.
9. Danø, K., P. A. Andreasen, J. Grøndahl-Hansen, P. Kristensen, L. S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139-266.
10. DeLarco, J. E., and G. J. Todaro. 1978. Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA.* 75:4001-4005.
11. Derynck, R., J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn, and D. V. Goeddel. 1985. Human transforming growth factor- $\beta$  cDNA sequence and expression in tumor cell lines. *Nature (Lond.)* 316:701-705.
12. Eaton, D. L., and J. B. Baker. 1983. Phorbol esters and mitogens stimulate human fibroblast secretion of plasmin-activatable plasminogen activator and protease nexin, an antiactivator/antiplasmin. *J. Cell Biol.* 97:323-328.
13. Erikson, L. A., D. A. Lawrence, and D. J. Loskutoff. 1984. Reverse fibrin autoradiography: a method to detect and partially characterize protease inhibitors after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* 137:454-463.
14. Ferraiuolo, R., M. P. Stoppelli, P. Verde, S. Bullock, P. Lazzaro, F. Blasi, and C. Pietropaolo. 1984. Synthesis and induction by tetradecanoyl phorbol acetate of urokinase in cultured human kidney carcinoma cells. *J. Cell Physiol.* 121:368-374.
15. Frolík, C. A., L. M. Wakefield, D. M. Smith, and M. B. Sporn. 1984. Characterization of a membrane receptor for transforming growth factor- $\beta$  in normal rat kidney cells. *J. Cell Biochem.* 259:10995-11000.
16. Goustin, A. S., E. B. Leof, G. D. Shipley, and H. L. Moses. 1986. Growth factors and cancer. *Cancer Res.* 46:1015-1029.
17. Granelli-Piperno, A., and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. *J. Exp. Med.* 146:223-234.
18. Grimaldi, G., P. P. DiFiore, E. L. Kajtaniak, J. Falco, and F. Blasi. 1986. Modulation of urokinase plasminogen activator gene expression during the transition from the quiescent to the proliferative state in normal mouse cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:855-861.
19. Gross, J. L., M. N. Krupp, D. B. Rifkin, and M. D. Lane. 1983. Down-regulation of epidermal growth factor receptor correlates with plasminogen activator activity in human A431 epidermoid carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 80:2276-2280.
20. Hodgson, G. P., P. K. Elder, T. Ono, D. N. Foster, and M. J. Getz. 1983. Structure and expression of mouse VL30 genes. *Mol. Cell. Biol.* 3:2221-2231.
21. Ignatz, R. A., and J. Massagué. 1986. Transforming growth factor- $\beta$  stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. Biol. Chem.* 261:4337-4335.
22. Keski-Oja, J., and A. Vaheri. 1982. The cellular target for the plasminogen activator, urokinase, in human fibroblasts: 66,000 dalton protein. *Biochim. Biophys. Acta.* 720:141-146.
23. Keski-Oja, J., E. B. Leof, R. M. Lyons, R. J. Coffey, and H. L. Moses. 1987. Transforming growth factors and the regulation of neoplastic cell growth. *J. Cell Biochem.* 33:95-107.
24. Keski-Oja, J., R. Raghov, M. Sawdey, D. J. Loskutoff, A. E. Postlethwaite, A. H. Kang, and H. L. Moses. 1988. Regulation of mRNAs for type-1 plasminogen activator inhibitor, fibronectin and type I procollagen by transforming growth factor- $\beta$ : divergent responses in lung fibroblasts and carcinoma cells. *J. Biol. Chem.* In press.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
26. Laiho, M., O. Saksela, P. A. Andreasen, and J. Keski-Oja. 1986. Enhanced secretion and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor- $\beta$ . *J. Cell Biol.* 103:2403-2410.
27. Laiho, M., O. Saksela, and J. Keski-Oja. 1986. Transforming growth factor- $\beta$  alters plasminogen activator activity in human skin fibroblasts. *Exp. Cell Res.* 164:399-407.
28. Laiho, M., O. Saksela and J. Keski-Oja. 1987. Transforming growth factor- $\beta$  induction of type-1 plasminogen activator inhibitor: pericellular deposition and sensitivity to exogenous urokinase. *J. Biol. Chem.* In press.
29. Lee, L. S., and I. B. Weinstein. 1979. Epidermal growth factor, like phorbol esters, induces plasminogen activator in HeLa cells. *Nature (Lond.)* 274:696-697.
30. Leof, E. B., J. A. Proper, A. S. Goustin, G. D. Shipley, P. E. DiCorleto, and H. L. Moses. 1986. Induction of c-sis mRNA and platelet-derived growth factor-like activity by transforming growth factor, type- $\beta$ : a proposed model for indirect mitogenesis involving autocrine activity. *Proc. Natl. Acad. Sci. USA.* 83:2453-2457.
31. Low, D. A., J. B. Baker, W. C. Koonce, and D. D. Cunningham. 1981. Released protease-nexin regulates cellular binding, internalization, and degradation of serine proteases. *Proc. Natl. Acad. Sci. USA.* 78:2340-2344.
32. Lund, L. R., A. Riccio, P. A. Andreasen, L. S. Nielsen, P. Kristensen, M. Laiho, O. Saksela, F. Blasi, and K. Danø. 1987. Transforming growth factor- $\beta$  is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1281-1286.
33. Massagué, J. 1985. Subunit structure of a high-affinity receptor for type- $\beta$  transforming growth factor: evidence for a disulfide-linked glycosylated receptor complex. *J. Biol. Chem.* 260:7059-7066.
34. Moses, H. L., E. B. Branum, J. A. Proper, and R. A. Robinson. 1981. Transforming growth factor production by chemically transformed cells. *Cancer Res.* 41:2842-2848.
35. Moses, H. L., R. F. Tucker, E. B. Leof, R. J. Coffey, Jr., J. Halper, and G. D. Shipley. 1985. Type- $\beta$  transforming growth factor is a growth stimulator and a growth inhibitor. *Cancer Cells (Cold Spring Harbor)* 3:65-71.
36. Nagamine, Y., M. Sudol, and E. Reich. 1983. Hormonal regulation of plasminogen activator mRNA production in porcine kidney cells. *Cell.* 32: 1181-1190.
37. Newman, M. J., E. Lane, M. A. Nugent, and E. Racker. 1986. Induction of anchorage-independent growth by epidermal growth factor and altered sensitivity to type- $\beta$  transforming growth factor in partially transformed rat kidney cells. *Cancer Res.* 46:5842-5850.
38. Nielsen, L. S., P. A. Andreasen, J. Grøndahl-Hansen, L. Skriver, and K. Danø. 1986. Plasminogen activators catalyze conversion of inhibitor from fibrosarcoma cells to an inactive form with a lower apparent molecular mass. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 196:269-273.
39. Odenakker, G., H. Ashino-Fuse, J. Van Damme, A. Billiau, and P. De Somer. 1983. Effects of 12-O-tetradecanoylphorbol-13-acetate on the production of mRNAs for human tissue-type plasminogen activator. *Eur. J. Biochem.* 131:481-487.
40. Quigley, J. P. 1979. Phorbol ester-induced morphological changes in transformed chick fibroblasts: evidence for direct catalytic involvement of plasminogen activator. *Cell.* 17:131-141.
41. Reich, E. 1980. Activation of plasminogen. A general mechanism for producing localized extracellular proteolysis. In *Molecular Basis of Biological Degradative Processes*. R. D. Berlin, L. Herrman, I. H. Lepow, and J. M. Tanzer, editors. Academic Press, Inc., New York. 155-169.
42. Rifkin, D. B. 1978. Plasminogen activator synthesis by cultured human embryonic lung cells: characterization of the suppressive effect of corticosteroids. *J. Cell Physiol.* 97:421-428.



43. Roberts, A. B., M. A. Anzano, L. C. Lamb, J. M. Smith, and M. B. Sporn. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc. Natl. Acad. Sci. USA.* 78:5339-5343.
44. Roberts, A. B., M. A. Anzano, L. M. Wakefield, N. S. Roche, D. F. Stern, and M. B. Sporn. 1985. Type- $\beta$  transforming growth factor: a bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA.* 82:119-123.
45. Saksela, O. 1985. Radial caseinolysis in agarose: a simple method for detection of plasminogen activator activity in the presence of inhibitory substances and serum. *Anal. Biochem.* 111:276-282.
46. Saksela, O. 1985. Plasminogen activation and regulation of pericellular proteolysis. *Biochim. Biophys. Acta.* 823:35-65.
47. Saksela, O., M. Laiho, and J. Keski-Oja. 1985. Regulation of plasminogen activator activity in human fibroblastic cells by fibrosarcoma cell-derived factors. *Cancer Res.* 45:3214-3219.
48. Scott, T. W. and J. B. Baker. 1983. Purification of human protease nexin. *J. Biol. Chem.* 258:10439-10444.
49. Scott, T. W., B. L. Bergman, A. Bajpai, R. T. Hersh, H. Rodriguez, B. N. Jones, C. Barreda, S. Watts, and J. B. Baker. 1985. Protease nexin. Properties and a modified purification procedure. *J. Biol. Chem.* 260:7029-7034.
50. Stoppelli, M. P., A. Corti, A. Soffientini, G. Cassani, F. Blasi, and R. K. Assoian. 1985. Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor of U937 monocytes. *Proc. Natl. Acad. Sci. USA.* 82:4939-4943.
51. Stoppelli, M. P., C. Tacchetti, M. V. Cubellis, A. Corti, V. J. Hearing, G. Cassani, E. Appella, and F. Blasi. 1986. Autocrine saturation of pro-urokinase receptors on human A431 cells. *Cell.* 45:675-684.
52. Stoppelli, M. P., P. Verde, G. Grimaldi, E. K. Locatelli, and F. Blasi. 1986. Increase in urokinase plasminogen activator mRNA synthesis in human carcinoma cells is a primary effect of the potent tumor-promoter phorbol myristate acetate. *J. Cell Biol.* 102:1235-1241.
53. Tucker, R. F., E. L. Branum, G. D. Shipley, R. J. Ryan, and H. L. Moses. 1984. Specific binding to cultured cells of [<sup>125</sup>I]-labeled type- $\beta$  transforming growth factor from human platelets. *Proc. Natl. Acad. Sci. USA.* 81:6757-6761.
54. Tucker, R. F., G. D. Shipley, H. L. Moses, and R. W. Holley. 1984. Growth inhibitor from BSC-1 cells closely related to platelet type- $\beta$  transforming growth factor. *Science (Wash.).* 226:705-707.
55. van Mourik, J. A., D. A. Lawrence, and D. J. Loskutoff. 1984. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. *J. Biol. Chem.* 259:14914-14921.
56. Vassalli, J. D., D. Baccino, and D. Belin. 1985. A cellular binding site for the M<sub>r</sub> 55,000 form of the human plasminogen activator, urokinase. *J. Cell Biol.* 100:86-92.
57. Verde, P., M. P. Stoppelli, P. Galeffi, P. P. Di Nocera, and F. Blasi. 1984. Identification and primary sequence of an unspliced human urokinase poly-(A<sup>+</sup>) RNA. *Proc. Natl. Acad. Sci. USA.* 81:4727-4731.
58. Wilson, E. L., and E. Reich. 1979. Modulation of plasminogen activator synthesis in chick embryo fibroblasts by cyclic nucleotides and phorbol myristate acetate. *Cancer Res.* 39:1566-1579.