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Proteolytic Activation of Tissue-Type Plasminogen Activator by the Culture Media of Mouse Cancer Cells

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ABSTRACT—Human single-chain tissue-type plasminogen activator (tPA) was activated by the culture media of mouse B16 melanoma and Lewis lung carcinoma cells. This activation was due to the proteolytic conversion of single-chain tPA to two-chain tPA. Typical serine proteinase inhibitors, such as diisopropylfluorophosphate and aprotinin, strongly inhibited the proteolytic activation, suggesting that the enzyme responsible for this activation is a serine proteinase. Through a process of gel filtration, the molecular weight of the putative tPA-activating enzyme was estimated to be approximately 35 kDa. Expression of the tPA mRNA was demonstrated by Northern blot analysis both for the melanoma and carcinoma cells. Zymographic experiments showed that the culture medium of the melanoma cells contained active two-chain tPA. These results indicate that a common serine proteinase may be involved in the proteolytic activation of single-chain tPA in these cancer cells.

INTRODUCTION

Tissue-type plasminogen activator (tPA) is a serine proteinase which converts plasminogen into plasmin through its proteolytic activity. tPA is widely distributed in tissues and organs (Barrett and McDonald, 1980; Wilson *et al.*, 1980), and is believed to be primarily responsible for removal of fibrin from the vascular tree (Collen and Lijnen, 1991). The enzyme is thought to play a role in a variety of biological events, such as ovulation (Beers *et al.*, 1975; Tsafiriri *et al.*, 1989), embryo implantation (Strickland *et al.*, 1976), embryogenesis (Menoud *et al.*, 1989), cell invasion (Ossowski and Reich, 1983; Sappino *et al.*, 1989), and brain function (Qian *et al.*, 1993; Seeds *et al.*, 1995; Tsirka *et al.*, 1995). It has been established that tPA is synthesized by endothelial cells and secreted into the blood stream by certain stimuli (Aoki, 1974; Rijken *et al.*, 1980). In addition, various cell lines are known to produce the activator (Rickles *et al.*, 1988; Rijken and Collen, 1981; Wallèn *et al.*, 1983).

The tPA preparation purified from the culture medium of human melanoma cell lines (Bowes) generally contains two molecular forms of the enzyme: one consists of a single polypeptide chain with Mr = 64000 and the other consists of two polypeptide chains of about equal size (Mr = 32000 and 30000) (Rifkin *et al.*, 1974; Wallèn *et al.*, 1983). However, melanoma tPA can be purified in the one-chain form, provided that cell culturing and purification are performed in the presence of the serine proteinase inhibitor aprotinin (Andreassen

et al., 1984; Ichinose *et al.*, 1984; Wallèn *et al.*, 1983). These facts suggest that a serine proteinase(s), which is either secreted into the medium or present on the cell surface, is involved in the conversion of a single-chain form to the two-chain enzyme. Ichinose *et al.* (1984) stated that tissue kallikrein or a tissue kallikrein-like proteinase is responsible for the formation of the two-chain form of tPA. But the nature of the enzyme remains unclear.

In the present study, we found that the culture media of mouse melanoma and lung cancer cells contained a detectable activity capable of activating tPA. Both cell lines secreted an aprotinin-sensitive, serine proteinase which is able to produce the two-chain form of tPA. The secreted proteinases showed basically the same properties including their apparent molecular sizes. These results indicate that a common serine proteinase is involved in the proteolytic activation of precursor tPA in both cells.

MATERIALS AND METHODS

Cells and reagents

Mouse B16 melanoma cells (RCB 0557) and Lewis lung carcinoma cells (RCB 0558) were obtained from RIKEN Cell Bank (Tsukuba, Japan). Dulbecco's modified Eagle medium (DMEM), L-glutamine, penicillin-streptomycin, and lactalbumin hydrolysate were purchased from GIBCO (Gland Island, NY, USA). Fetal bovine serum was obtained from Hyclone (Logan, UT, USA). Peptide 4-methylcoumaryl-7-amide (MCA) substrates, leupeptin, antipain, and E-64 were purchased from the Peptide Institute (Osaka, Japan). Diisopropylfluorophosphate (DFP), phenylmethanesulfonyl fluoride (PMSF), N^α-p-tosyl-L-lysine chloromethyl ketone (TLCK), N^α-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N-ethylmaleimide (NEM), aprotinin, benzamidin and soybean trypsin inhibitor (SBTI)

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were obtained from Sigma (St. Louis, MO, USA). Human single-chain tPA (sctPA), two-chain tPA (tPA) and goat anti-human melanoma tPA antibody were purchased from Biopool AB (Umeå, Sweden). Human plasminogen was obtained from Chromogenic (Mölnådal, Sweden). Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore Corp (Bedford, MA, USA). An ECL Western blot detection kit was purchased from Amersham (Buckinghamshire, England). A Diol-300 HPLC column was obtained from Shimadzu (Kyoto, Japan). Sources of other chemicals were described previously (Ohnishi *et al.*, 1997).

Cell culture

Mouse B16 melanoma cell line and Lewis lung carcinoma cell line were grown in 20 ml of DMEM containing fetal bovine serum (10%), L-glutamine (0.3 mg/ml), penicillin (100 u/ml), and streptomycin (0.1 mg/ml). When the cells became confluent, they were washed twice with phosphate-buffered saline (PBS) and cultured further in the medium containing lactoalbumin hydrolysate (1.3 mg/ml) but lacking fetal bovine serum. After 2 days, the medium and cells were separately collected.

Enzyme activity assay

Human single-chain tPA was incubated with samples in 100 μ l in 0.1 M Tris-HCl buffer (pH 8.0) at 37°C. After 60 min of incubation, 50 μ g of aprotinin, a specific inhibitor for the present enzyme but not for tPA, were added to the reaction mixture. The mixture was then adjusted to a 495 μ l by adding 0.1 M Tris-HCl buffer (pH 8.0). The enzyme activity of activated tPA in the mixture was determined with MCA substrate according to the method of Barrett (1980) with slight modification. The assay was carried out at 37°C for 30 min in 500 μ l in a reaction comprising 0.1 M Tris-HCl buffer (pH 8.0) and 0.1 mM Boc-Gln-Gly-Arg-MCA. The reaction was terminated by the addition of 2.5 ml of 0.1 M sodium acetate buffer (pH 4.3) containing 0.1 M monochloroacetic acid. The amount of 7-amino-4-methylcoumarin (AMC) released was measured with a spectrofluorophotometer (RF-5000 model, Shimadzu, Kyoto, Japan) at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Enzyme activity was expressed as the amount of AMC released at 37°C per min.

Preparation of cellular membrane and soluble fractions, and culture media

In most cases, culture media were concentrated by ultrafiltration using a Centriprep 30, Centricon 30, or Microcon 30 (Amicon, Beverly, MA, USA).

Cells collected from culture dishes using PBS containing 1 mM EDTA were centrifuged at 1000 \times g for 5 min at 4°C. The precipitated cells were suspended in a small volume of PBS containing 0.1 mM EDTA and homogenized with a Polytron homogenizer. The homogenate was centrifuged at 1000 \times g for 10 min at 4°C, and the resulting supernatant was further centrifuged at 50000 \times g for 15 min at 4°C. The supernatant thus obtained was referred to as "cytosol fraction". The precipitate was again suspended in a small volume of PBS and centrifuged at 50000 \times g for 15 min at 4°C. The resulting precipitate was resuspended in PBS and used as "membrane fraction".

Western blotting

Samples of human sctPA incubated with the culture medium or cellular fractions of cancer cells were separated by SDS-PAGE (Laemmli, 1970) under reducing conditions and transferred to PVDF membrane (Towbin *et al.*, 1979). The blotted membrane was incubated with goat anti-human tPA monoclonal antibody at 1:2000 dilution and subsequently with the second antibody (Ohnishi *et al.*, 1997). Immunoreactive signals were detected using an ECL Western blot detection kit (Amersham, Buckinghamshire, England) according to the protocol provided by the manufacturer. Under the conditions, the 32 kDa polypeptide chain of tPA is strongly detected by the antibody, but the 30 kDa polypeptide chain is hardly visible.

Gel filtration

The culture medium was treated with ammonium sulfate at 60% saturation. The precipitated material was collected by centrifuging at 10000 \times g for 10 min at 4°C, and dissolved in a small volume of cold distilled water. Aliquots (50 μ l) of the sample thus concentrated were applied to a Diol-300 gel filtration column previously equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 1 ml/min using a Gilson HPLC apparatus (Middleton, WI, USA). Standard molecular weight marker proteins used were thyroglobulin (669 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa).

Zymographic detection of tPA

Plasminogen/casein zymography was performed according to the method of Tomooka *et al.* (1992) with slight modification. Briefly, samples were electrophoresed on 10% SDS-PAGE gels containing casein (6.4 mg/mini-gel) and human plasminogen (120 μ g/mini-gel) under nonreducing conditions. After electrophoresis, gels were washed twice in 2.5% Triton X-100 for 30 min to remove SDS, and then incubated with shaking in 0.1 M glycine-NaOH (pH 8.3) for 18 hr at 37°C. The gels were stained with 0.25% Coomassie brilliant blue R-250 to visualize zones of lysis. Samples were also run on the gels with casein only, to show that the zones of lysis were attributable to plasminogen activator.

RNA isolation and Northern blot analysis

Total cellular RNAs of Lewis lung carcinoma and B16 melanoma cells were isolated from confluent monolayers using the guanidine isothiocyanate-cesium chloride method (Chirgwin *et al.*, 1979). Poly(A)⁺ RNAs were selected by oligo(dT)-cellulose column chromatography.

A mouse tPA cDNA fragment was amplified by polymerase chain reaction from a Lewis lung carcinoma poly(A)⁺ RNA. A sense primer of 5'-ACTTACCAACAGCACCAGTC-3' corresponding to nucleotides 229-248 and an antisense primer of 5'-AAGATGATTGGGGGGG-AACC-3' corresponding to nucleotides 1172-1191 of mouse tPA (Rickles *et al.*, 1988) were used. The corresponding 963 bp products were subcloned into the *EcoRV* site of pBluescript (+) plasmid vector (Stratagene, La Jolla, CA, USA) and the cloned nucleotide sequence was confirmed using an Applied Biosystems model 373 DNA Sequencer (Foster City, CA, USA).

For Northern analysis 2 μ g poly(A)⁺ RNAs were subjected to electrophoresis on a 1.5% agarose formaldehyde gel and then transferred to a Nytran-plus membrane (Schleicher and Schuell, Dassel, Germany). The blot was hybridized with a ³²P-labelled 963 bp mouse tPA cDNA fragment (described above) at 42°C in 50% formamide, 5 \times Denhardt's solution, 5 \times SSPE, 1% SDS, and 100 μ g/ml salmon sperm DNA, and it was washed with increasing stringency, with a final wash of 0.1 \times SSC/0.1% SDS at 50°C. The same blot was then stripped and rehybridized with a ³²P-labelled 2.0 kb human β -actin cDNA to serve as a loading control (Ponte *et al.*, 1984).

RESULTS AND DISCUSSION

The culture media of mouse B16 melanoma and Lewis lung carcinoma cells contained detectable tPA-activating activity. We noticed the enzyme activity increased by several times when assayed with the concentrated media. Figure 1A shows a difference in the enzyme activity of the carcinoma cell culture medium before and after concentration by ultrafiltration. We also found that dialysis of the culture medium against 50 mM Tris-HCl (pH 7.5) brought about detectable increase in the activating activity. These observations suggest the presence of an inhibitor(s) in the medium. Indeed,

tPA-activating activity of the concentrated medium was remarkably suppressed when the filtrate obtained using ultrafiltration was included in the reaction. Furthermore, a comparable inhibition was achieved by the addition of fresh DMEM (Fig. 1B). Figure 1C demonstrates the inhibition of proteolytic conversion of sctPA (Mr = 64,000) to two-chain tPA (Mr = 32,000 and 30,000) by DMEM. Essentially the same results were obtained in the experiments with the melanoma cell cul-

ture medium. From these results, it is clear that an ingredient(s) of this medium has an inhibitory effect on the tPA-activating enzyme. Therefore, the culture media concentrated approximately 50-fold by ultrafiltration were used for subsequent experiments to minimize the effect of DMEM on enzyme activity.

Figure 2 shows the distribution of tPA-activating activity in the media and cellular fractions of the cultured cells. The

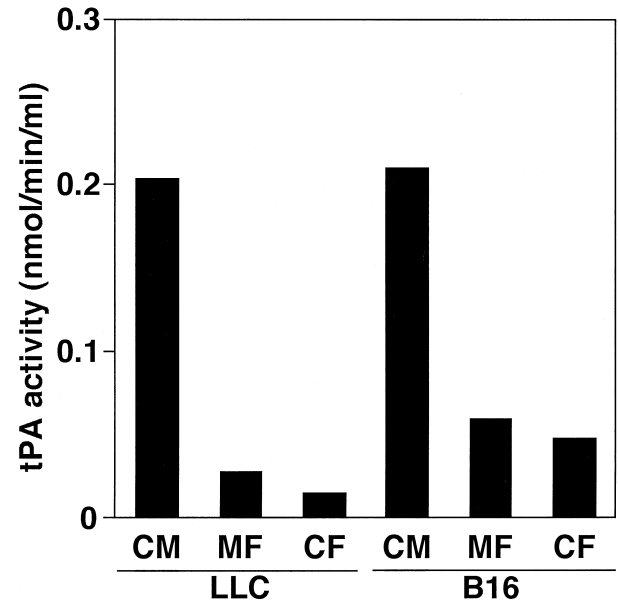
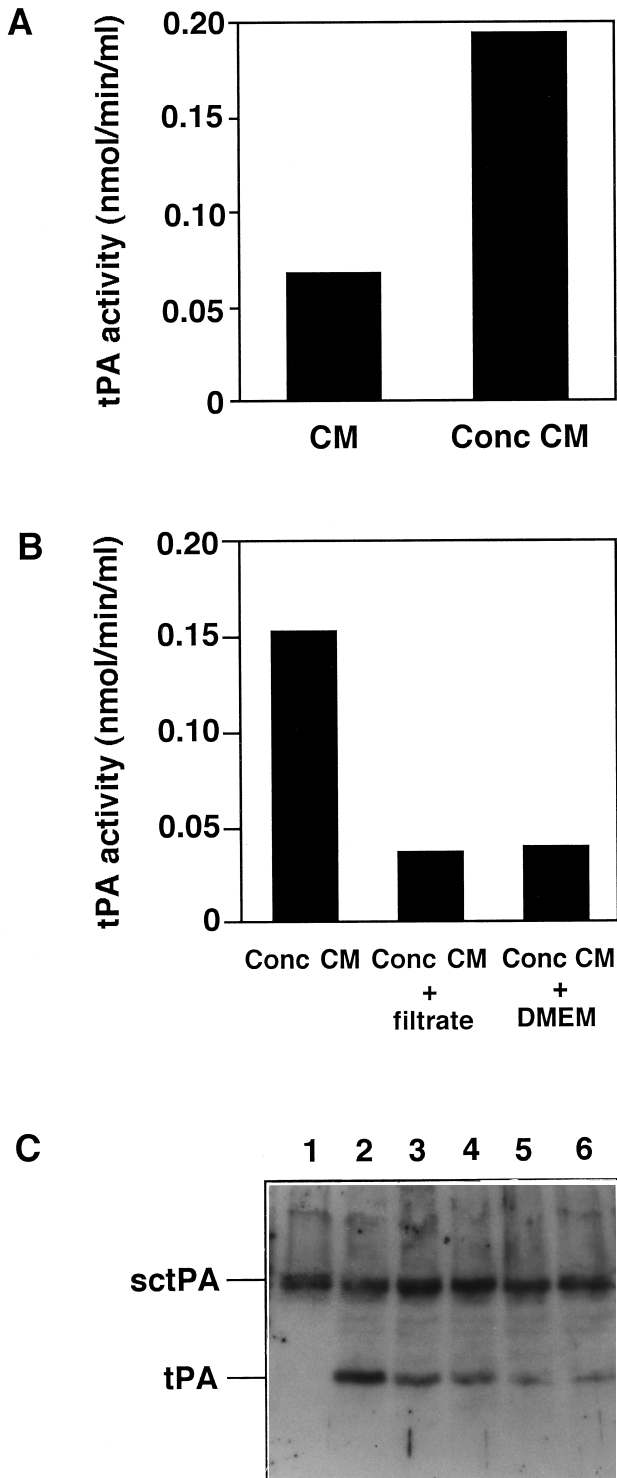


Fig. 2. Distribution of tPA-activating enzyme activity in culture cells. sctPA (200 ng) was incubated at 37°C for 8 hr with 6 µg of the concentrated culture media (CM), cellular membrane fraction (MF), or cytosol fraction (CF), and the activated tPA activity was then assayed using Boc-Gln-Gly-Arg-MCA. Average values of two determinations are shown.

Fig. 1. Effect of DMEM on tPA activation by mouse Lewis lung carcinoma cell culture medium. **(A)** Human sctPA (0.2 µg) was incubated in 0.1 M Tris-HCl (pH 8.0) at 37°C for 60 min in 100 µl with 8 µg protein of untreated conditioned culture medium (CM) or the 50-fold-concentrated medium (Conc CM). The activated tPA activity was selectively measured using Boc-Gln-Gly-Arg-MCA. Neither sctPA nor the culture medium exhibited a significant activity toward the synthetic substrate. Average values of two determinations are shown. **(B)** sctPA (0.2 µg) was incubated in 0.1 M Tris-HCl (pH 8.0) at 37°C in 100 µl with 40 µg of the concentrated culture medium alone (Conc CM) or in the presence of the filtrate (20 µl) derived from ultrafiltration (Conc CM + filtrate) or 20 µl of fresh DMEM (Conc CM + DMEM). After incubation for 60 min, activated tPA activity was measured. Average values of two determinations are shown. **(C)** The concentrated culture medium (7.8 µg protein) was preincubated in 0.1 M Tris-HCl (pH 8.0) in 20 µl with varying amounts (0 - 8 µl) of fresh DMEM for 10 min. sctPA (33 ng) was then added to each reaction and the mixtures were further incubated at 37°C for 60 min. Proteolytic conversion of sctPA to two-chain tPA (tPA) in the mixtures was monitored by Western blot analysis. Amounts of DMEM used in the preincubation were 0 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), and 8 µl (lane 6). Lane 1 represents sctPA alone. Of the two polypeptide chains (32 kDa and 30 kDa) that comprise tPA, the 32 kDa chain is only visible in this experiment.

conditioned media had the highest specific activity in both cells. The enzyme activities recovered from the media constituted 93% and 86% of the total activity in Lewis lung carcinoma and B16 melanoma cells, respectively. The results suggest that the enzyme is an extracellular proteinase secreted from the cells. Indeed, we observed that in both cell cultures the tPA-activating activity in the medium increased linearly up to 36 hr of culture and then leveled off. Based on this observation, culture medium was routinely collected at 48 hr of cell culture. The conditioned media of both Lewis lung carcinoma and B16 melanoma cells showed tPA-activating activity at a comparable level. In an experiment, the medium conditioned with Lewis lung carcinoma cells for 48 hr was found to contain the enzyme activity of 7880 nmol/min/dish, while that with B16 melanoma cells was 6320 nmol/min/dish.

A progressive activation of sctPA was observed with an increasing amount of the enzyme samples (Fig. 3A). Western blot analysis of the reaction mixtures indicated that the activation underlies the production of two-chain tPA from its single-chain precursor molecule (Fig. 3B). The time-dependent activation of sctPA with a fixed amount of enzyme sample is shown in Fig. 4A. Prolonged incubation resulted in no decline of activated tPA activity, suggesting that both enzymes activate tPA by cleaving a specific peptide bond at the presumed activation site with no further hydrolysis leading to its degradation. Consistent with this idea, the two-chain tPA once produced in the reaction remained stable even after a 6 hr-incubation period (Fig. 4B). As described above, tPA activation profiles were slightly different between the culture media from B16 melanoma and Lewis lung carcinoma cells. At present, it is difficult to determine whether or not the difference is significant, because the contents of the activating enzyme as well as the inhibitor substance in the enzyme samples are unknown.

Effects of various proteinase inhibitors on the enzyme activity were examined (Fig. 5). Aprotinin, DFP, and leupeptin strongly inhibited the conversion of sctPA to two-chain tPA, while other inhibitors were without effect. These results are consistent with the classification of the two culture medium tPA-activating enzymes as serine proteinases.

The concentrated media of B16 melanoma and Lewis lung carcinoma cells were separately analyzed using a Diol-300 HPLC column. Both media gave a single activity peak in the same position, eluting between ovalbumin and chymotrypsinogen. The estimated molecular weight of the enzymes was approximately 35000 (Fig. 6). The results of gel filtration experiments also indicate that the media obtained from both cell cultures contained only one species of tPA-activating proteinase. However, we cannot rule out completely at this time the possibility that several tPA-activating enzymes with a similar molecular size are present in the culture media. Also, we examined enzyme activity toward synthetic MCA substrates, such as butyloxycarbonyl(Boc)-Gln-Arg-Arg-MCA, Boc-Gln-Gly-Arg-MCA, and Pro-Phe-Arg-MCA, with the fractions containing tPA-activating activity derived from the gel filtration column. These substrates are widely used to screen proteinases with a trypsin-like substrate specificity. Since proteolytic acti-

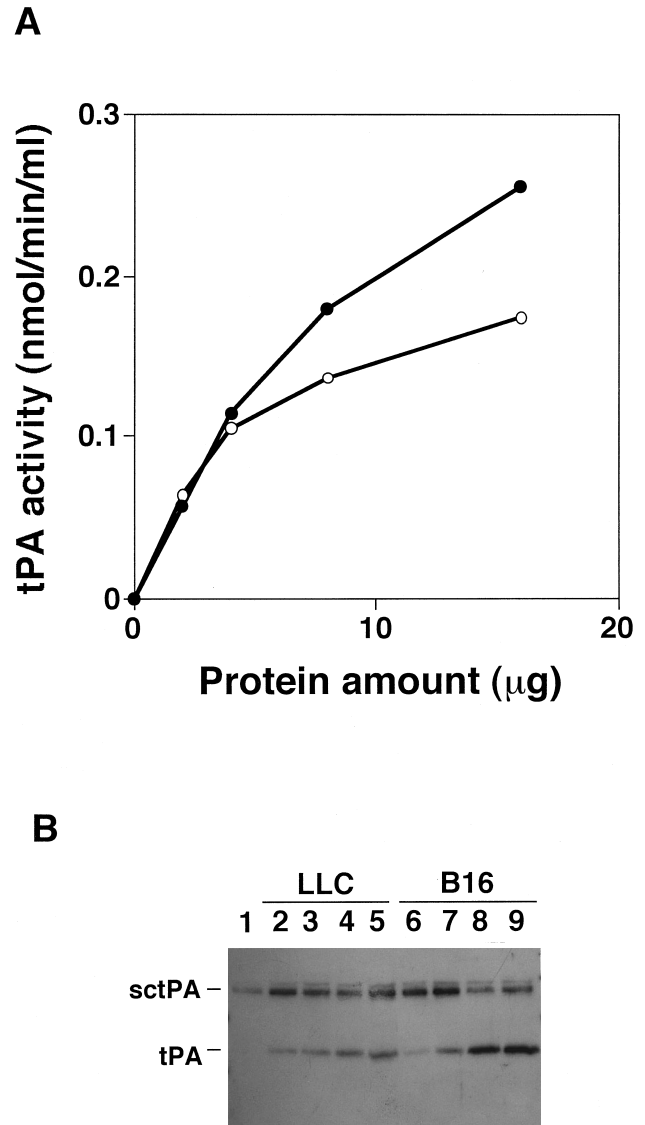


Fig. 3. Dose-dependent activation of sctPA by the culture media. **(A)** sctPA (200 ng) was incubated in 0.1 M Tris-HCl (pH 8.0) at 37°C for 60 min in 100 µl with an increasing amount of the concentrated culture media of the melanoma (○) and carcinoma cells (●). Eighty µl of the incubation mixtures were assayed for the activated tPA activity using Boc-Gln-Gly-Arg-MCA. Average values of two determinations are shown. **(B)** Remaining samples (20 µl) of the incubation mixtures in **(A)** were subjected to reducing SDS-PAGE/Western blot analysis for assessing the proteolytic conversion of sctPA to tPA. sctPA alone (lane 1) or sctPA incubated with 2 µg (lanes 2 and 6), 4 µg (lanes 3 and 7), 8 µg (lanes 4 and 8) and 16 µg (lanes 5 and 9) of the culture media were applied.

vation of tPA is known to involve the hydrolysis of an internal peptide bond Arg²⁷⁵-Ile²⁷⁶ (in human sequence) resulting in a two-chain structure held together by disulfide bonds, the synthetic substrates would have been effectively hydrolyzed by the current tPA-activating proteinase. Contrary to expectations, the enzyme fraction did not exhibit any detectable activity toward the substrates. These results indicate that the putative tPA-activating enzyme is highly specific for the protein

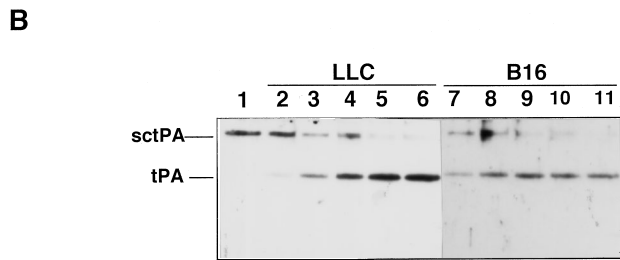
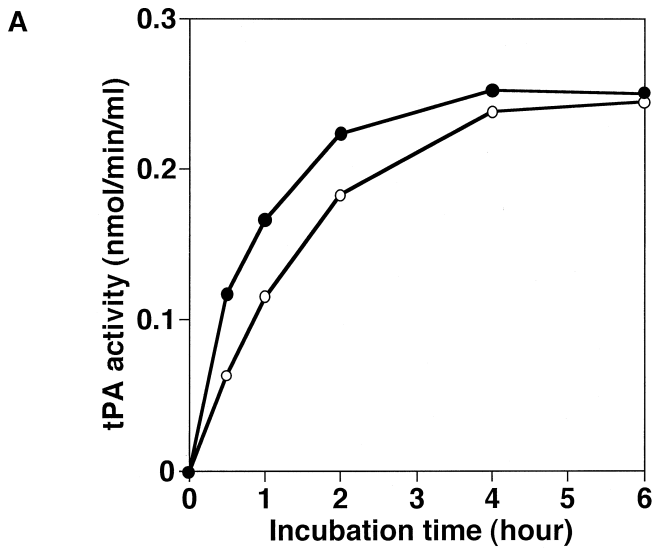


Fig. 4. Time-course of sctPA activation by the culture media. (A) sctPA (3.2 μg) was separately incubated in 0.1 M Tris-HCl (pH 8.0) in 1600 μl with the concentrated culture media (128 μg) of the melanoma cells (○) or carcinoma cells (●). Eighty μl aliquots of the incubation mixtures were taken at the indicated times for enzyme activity assay of the activated tPA. Average values of two determinations are shown. (B) Remaining 20 μl aliquots of the incubation mixtures in (A) were taken at the indicated times, and subjected to reducing SDS-PAGE/Western blot analysis for assessing the proteolytic conversion of sctPA to tPA. sctPA incubated with the media for 0 (lane 1), 0.5 (lanes 2 and 7), 1 (lanes 3 and 8), 2 (lanes 4 and 9), 4 (lanes 5 and 10), and 6 hr (lanes 6 and 11) were applied.

substrate sctPA.

To determine whether tPA is expressed in the Lewis lung carcinoma cells and B16 melanoma cells, we carried out Northern blot analyses using isolated poly(A)⁺ RNAs and a 963-bp probe. As shown in Fig. 7A, a single transcript with a size of 2.8 kb was detected in both RNAs. The mRNA size was exactly the same as that reported for mouse F9 teratocarcinoma cells (Ricklees *et al.*, 1988). We further carried out a zymographic analysis to detect tPA in the culture media. Since the analysis was conducted under nonreducing conditions, active tPA, which consists of two polypeptides chains ($M_r = 32,000$ and $30,000$) associated covalently, was expected to appear in a position corresponding to about $M_r = 60,000$. Indeed, the analysis of the culture medium of B16 melanoma cells revealed a band representing active tPA (Fig. 7B). How-

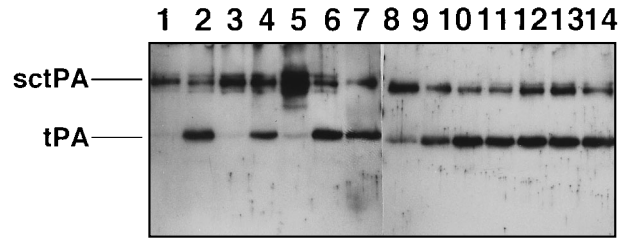


Fig. 5. Effects of proteinase inhibitors on the tPA-activating enzyme of B16 melanoma cell culture medium. The concentrated culture medium (8 μg protein) was preincubated at 37°C for 10 min in 19 μl in 0.1 M Tris-HCl (pH 8.0) with various inhibitors. To each mixture was added 0.2 μg tPA in 1 μl . After incubation for 4 hr at 37°C, the reaction mixtures were subjected to reducing SDS-PAGE/Western blot analysis for assessing the proteolytic conversion of sctPA to tPA. Lane 1, sctPA only; lane 2, without inhibitor; lane 3, aprotinin (0.4 mg/ml); lane 4, SBTI (0.8 mg/ml); lane 5, DFP (4 mM); lane 6, benzamide (4 mM); lane 7, PMSF (2 mM); lane 8, leupeptin (0.2 mM); lane 9, antipain (0.2 mM); lane 10, TLCK (0.4 mM); lane 11, TPCK (0.4 mM); lane 12, E-64 (0.2 mM); lane 13, NEM (4 mM), and lane 14, EDTA (4 mM). The medium from Lewis lung carcinoma cells gave the same inhibitor profile.

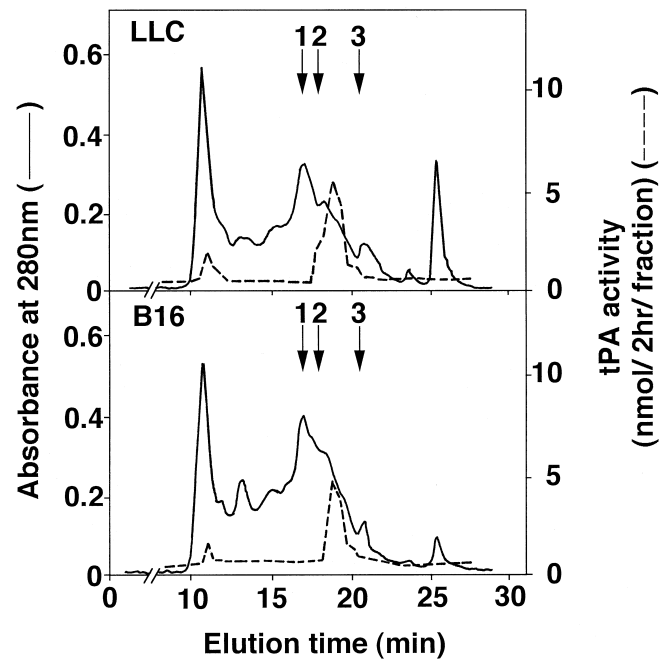


Fig. 6. Gel filtration analysis of tPA-activating enzyme. The culture media of the carcinoma cells (LLC) and melanoma cells (B16) were treated with ammonium sulfate at 60% saturation, and the precipitates were collected and dissolved in a small volume of cold distilled water. Fifty μl of the sample (200 μg protein) were applied to a Diol-300 gel filtration column. The column was equilibrated and eluted with 0.1 M Tris-HCl (pH 8.0) containing 0.15 M NaCl. Fractions of 500 μl were collected and aliquots of 50 μl were incubated at 37°C for 2 hr in 100 μl in 0.1 M Tris-HCl (pH 8.0) with 0.2 μg sctPA. The activated tPA activity was assayed using Boc-Gln-Gly-Arg-MCA. Elution positions of bovine serum albumin (1), ovalubumin (2), and chymotrypsinogen (3) are shown.

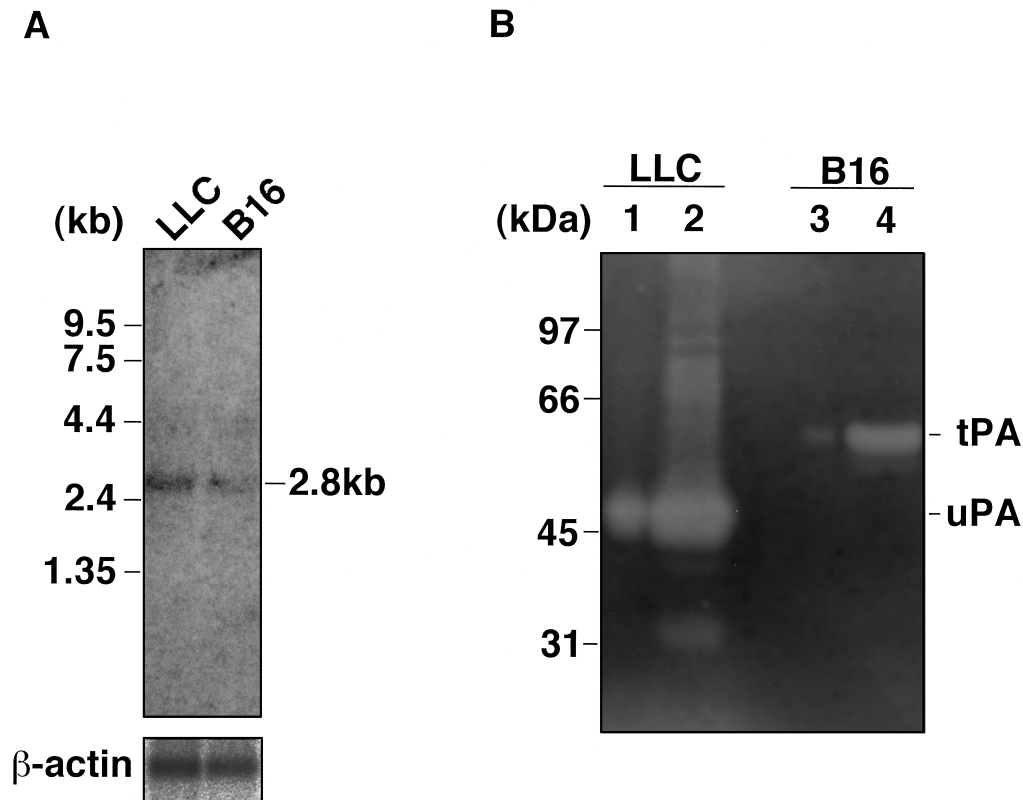


Fig. 7. Detection of the tPA mRNA and activity. **(A)** Two μ g poly(A)⁺ RNA isolated from the carcinoma (LLC) and melanoma cells (B16) were electrophoresed, blotted, and hybridized with ³²P-labelled 963 bp fragment of mouse tPA cDNA. The β -actin mRNA was also detected using a ³²P-labelled 2.0 kb human β -actin cDNA. **(B)** The concentrated culture media of the carcinoma (LLC) and melanoma cells (B16) were subjected to zymographic analysis using a 10% SDS-PAGE gel containing casein and plasminogen under the nonreducing conditions. Sample amounts loaded were 4 μ g (lanes 1 and 3) and 40 μ g (lanes 2 and 4). Positions of molecular weight markers (left) and of tPA and uPA (right) are shown.

ever, no tPA activity was seen with the lung carcinoma cell culture medium. In contrast, the activity of urokinase-type plasminogen activator (uPA) was detected in the medium of the carcinoma cells but not melanoma cells. It is unclear at present why the tPA activity cannot be detected in the carcinoma cell culture medium in spite of the fact that the cells express its mRNA. Clearly, further studies are necessary to solve this problem. Western blot analysis of the media using tPA antibody failed to visualize specifically tPA. This was probably due to reduced sensitivity of the antibody to mouse tPA because the antibody used in this study was produced against human, but not mouse, tPA. Since anti-mouse tPA antibody was not available in our laboratory, immunological demonstration of the presence of tPA in the media was not successful. In short, these results clearly show that both B16 melanoma and Lewis lung carcinoma cells express the tPA mRNA and that the culture medium of the melanoma cells contains tPA in active form.

This study showed that two different mouse tumor cell lines secrete a serine proteinase capable of activating proteolytically tPA. The present data suggest that the enzymes secreted from these cells are identical. Judging from its behavior to various proteinase inhibitors, the enzyme resembles the tPA-activating enzyme previously detected in the medium of human melanoma cell culture (Ichinose *et al.*, 1984). In

addition, our finding that the mouse proteinase has a molecular weight of about 35,000 is consistent with the idea that it is a tissue kallikrein-like enzyme. However, further studies are necessary to determine whether this assumption is valid.

tPA has been studied extensively because of its importance in many biological events. In contrast, reports of studies on enzymes involved in proteolytic activation of tPA have been limited. A main reason for this is the fact that sctPA itself has considerable activity toward plasminogen if co-factor fibrin is present. Therefore, tPA activation by proteolysis is generally thought to be less important. However, the present observation that tPA-producing cells simultaneously secrete its activating enzyme tempts us to speculate that proteolytic activation of tPA might be a more important biological event than we thought. Although a variety of proteinases are reported to activate tPA proteolytically *in vitro* (Ichinose *et al.*, 1984), their *in vivo* roles as a tPA activator remain unclear. We tentatively assume that the 35kDa serine proteinase identified in this study is a physiological activator of the sctPA zymogen at least in mouse B16 melanoma and Lewis lung carcinoma cells. A future study of particular interest would examine whether the expression of this proteinase is closely associated with that of tPA in normal tPA-producing cells of animal tissues.

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