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Purification and Identification of a Novel and Four Known Serine Proteinase Inhibitors Secreted by Human Glioblastoma Cells[1]

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Our previous studies have shown that some human cancer cell lines produce pancreatic trypsinogen, plasminogen, and tissue-type kallikrein. To understand the regulatory mechanism of these proteinases, serine proteinase inhibitors secreted by human glioblastoma cell line T98G were analyzed by gelatin reverse zymography with trypsin. The serum-free conditioned medium of T98G cells showed more than ten trypsin inhibitor bands ranging from 16 to 150 kDa in the reverse zymography. Major trypsin inhibitors were purified by trypsin-affinity chromatography. Analysis of their N-terminal amino acid sequences demonstrated that the purified inhibitors were identical to the secreted forms of amyloid protein precursors (APPs), tissue factor pathway inhibitor (TFPI), placental protein 5 (PP5)/TFPI-2, and secretory leukocyte proteinase inhibitor (SLPI). In addition, a novel 25-kDa trypsin-binding protein, tentatively named p25TI, was identified. p25TI showed weak inhibitory activity against trypsin in reverse zymography as compared with the other inhibitors. The secretion of multiple forms of serine proteinase inhibitors by human cancer cells raises the possibility that they might be involved in the abnormal growth of cancer cells.

Key words: cancer, glioblastoma, reverse zymography, serine proteinase inhibitor, trypsin inhibitor.

Malignant growth of tumor cells is supported by many secreted factors such as growth factors, cell attachment factors, proteinases, and their inhibitors. Among them, proteinases contribute to an essential event in extracellular matrix (ECM) proteolysis, which is required for tumor invasion (reviewed in Refs. *1-3*). Many recent studies have shown that a family of metalloproteinases, so-called "matrix metalloproteinases (MMPs)," plays a critical role in the ECM degradation by invasive cancer cells. MMPs have a high degree of specificity for ECM proteins, such as collagens, elastin, fibronectin, and laminin (*1-3*). The activities of MMPs are regulated by three kinds of tissue inhibitors of metalloproteinases (TIMPs) (*1-3*).

It is also known that many kinds of cancer cells produce two types of plasminogen activators (PAs), tissue-type (t-PA), and urokinase-type (u-PA) (*4*). These PAs convert plasminogen to plasmin, and the activated plasmin is believed to degrade ECM proteins and to activate the latent precursors of various MMPs. Moreover, u-PA may directly contribute to the ECM degradation, because it is able to hydrolyze gelatin (*5*). We previously found that two human gastric carcinoma cell lines secreted three kinds of ECM-degrading serine proteinases, pancreatic trypsinogen 1, plasminogen, and tissue-type kallikrein, but they hardly secreted MMPs (*6, 7*). Trypsinogen is also produced by human ovarian carcinoma cell lines (*5, 8*) and other cancer cell lines (*9*). We recently found that trypsinogen mRNA and protein are expressed in most human ovarian cancer tissues (*8*) and human gastric cancer tissues (to be published). Trypsin has higher activity than plasmin in ECM degradation and pro-MMP activation (*6, 10*). Therefore, it seems likely that trypsin plays an important role in tumor invasion and some other pathological processes. It is also expected that the activities of trypsin and other matrix serine proteinases are regulated by natural inhibitors *in vivo*. However, little is known about the species of secreted serine proteinase inhibitors, other than two plasminogen activator inhibitors (PAIs). To find the natural regulators of the ECM-degrading enzymes, we have been analyzing the inhibitors secreted by tumor cells using reverse zymography on gelatin-containing gels (*11-13*). Recently we found that human glioblastoma cell line T98G secreted a serine proteinase inhibitor called placental protein 5 (PP5), and we cloned its cDNA, showing that PP5 is identical with a very recently reported Kunitz-type serine proteinase inhibitor, tissue factor pathway inhibitor-2 (TFPI-2) (*14*).

In the present study, serine proteinase inhibitors secret-

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Abbreviations: APP, amyloid protein precursor; CBB, Coomassie Brilliant Blue R-250; ECM, extracellular matrix; KPI, Kunitz-type proteinase inhibitor; PP5, placental protein 5; SLPI, secretory leukocyte proteinase inhibitor; TFA, trifluoroacetic acid; TFPI, tissue factor pathway inhibitor.

ed by T98G cells were purified and their partial amino acid sequences were determined. This analysis demonstrated that the tumor cells secreted at least five kinds of serine proteinase inhibitors including a novel inhibitor of 25 kDa.

MATERIALS AND METHODS

Preparation of Serum-Free Conditioned Medium of T98G Cells—The human glioblastoma cell line T98G (JCRB 9041), which was provided by the Japanese Cancer Research Resource Bank, was grown to confluence in 850-cm² roller bottles containing 200 ml of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (GIBCO; Grand Island, NY, USA), DME/F12, supplemented with 10% fetal calf serum (FCS). The cells were then rinsed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (BSS) and culture was continued in 200 ml of serum-free DME/F12. The serum-free conditioned medium was harvested three times/week and clarified by sequential centrifugation at 1,500 rpm for 15 min and at 9,500 rpm for 30 min. Proteins in the pooled conditioned medium (about 4 liters) were precipitated by ammonium sulfate at 80% saturation. The resultant protein precipitate was collected by centrifugation, dissolved in 40 ml of 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 0.005% Brij-35, and dialyzed against the same buffer.

Purification of Trypsin Inhibitors—The concentrated serum-free conditioned medium of T98G cells was subjected to molecular-sieve chromatography on a Cellulofine GCL-2000m column (Chisso, Tokyo), previously equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 0.005% Brij-35, as described before (*6*). The resultant fractions containing trypsin inhibitors were pooled, and applied to a trypsin-Sepharose column (10×50 mm), which had been prepared by mixing bovine trypsin (Mochida Pharmaceuticals, Tokyo) with CNBr-Sepharose CL-4B (Pharmacia LKB; Uppsala, Sweden) and then equilibrated with the same buffer as above. The loaded column was washed with 5 bed volumes of 20 mM Tris-HCl (pH 7.5) containing 1.0 M NaCl and then with 2 bed volumes of 20 mM Tris-HCl (pH 7.5), and eluted with 5 bed volumes of 0.05% trifluoroacetic acid (TFA), at a flow rate of 10 ml/h. The eluted trypsin inhibitors were finally purified by reverse-phase HPLC on a SynChropack RP-4 column (0.41×25 cm) (SynChrom, Lafayette, IN, USA) under the conditions described before (*6*).

Reverse Zymography for Trypsin Inhibitors—Unless otherwise noted, sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE) was carried out on 10% or 12.5% polyacrylamide slab gels (90×90×0.75 mm) under reducing or nonreducing conditions, as reported before (*6*). Proteins on the gels were stained with Coomassie Brilliant Blue R-250 (CBB). Reverse zymography for trypsin inhibitors was carried out by modifying the reverse zymography for gelatinase inhibitors described before (*11, 12*). Samples were subjected to SDS-PAGE on polyacrylamide gels containing 0.1% (w/v) SDS and 1 mg/ml gelatin (Difco; Detroit, MI, USA) under non-reducing conditions and then renatured in 50 mM Tris-HCl (pH 7.5) containing 2.5% (v/v) Triton X-100 and 0.1 M NaCl. The renatured gels were incubated at 37°C for 18 h in 12 ml of a reaction mixture consisting of 50 mM Tris-HCl (pH 7.5) and 10 ng/ml bovine trypsin (Mochida Pharmaceuticals) as the indicator enzyme. The resultant gels were stained with CBB. Trypsin inhibitors separated on the gels formed CBB-stained gelatin bands by blocking the gelatinolytic activity of trypsin. The trypsin concentration was critical for the optimal detection of trypsin inhibitors.

Protein Sequencing—The N-terminal amino acid sequences of trypsin inhibitors were analyzed with an Applied Biosystems model 477A protein sequencer. In most cases, sample proteins were separated by reducing SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The blotted protein bands were cut out and applied to the protein sequencer.

Determination of Protein Concentration—Protein concentration was determined by the dye binding method with a Bio-Rad protein assay kit (Richmond, CA, USA), using bovine IgG as the standard.

RESULTS

Reverse Zymography of Conditioned Medium—Reverse zymography for detection of serine proteinase inhibitors has been reported by Hanspal *et al.* (*13*). In order to improve the sensitivity of this method, we used a minimum concentration (10 ng/ml) of trypsin and a prolonged incubation time (18 h). A minimum amount of 0.5 ng/lane of bovine aprotinin was clearly detected by the improved reverse zymography.

Using the highly sensitive reverse zymography with trypsin, trypsin inhibitors secreted by various human cancer cell lines have been analyzed. Most of the cancer cell lines showed multiple inhibitor bands on the reverse zymogram (to be published). In particular, the conditioned medium of human glioblastoma cell line T98G showed a number of inhibitor bands ranging from 16 to 150 kDa in approximate molecular mass under non-reducing conditions (Fig. 1). Two intense bands of 25-30 and 100 kDa and at least 10 minor bands are seen in the reverse zymogram.

To identify the trypsin inhibitors secreted by T98G cells, the conditioned medium was fractionated by molecular-sieve chromatography (Fig. 2, A-C). The resultant fractions were divided into three pools on the basis of the kinds of inhibitors (Fig. 2C). Pool 1 (fraction no. 50-66) contained a major inhibitor of 100 kDa and minor ones of 120, 90, 55,

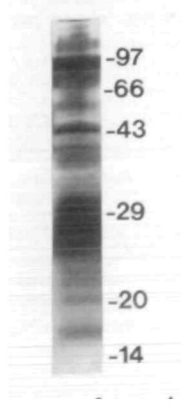

Fig. 1. **Reverse zymogram of trypsin inhibitors present in conditioned medium of T98G cells.** The serum-free conditioned medium of T98G cells was concentrated 30-fold by ammonium sulfate precipitation, and a 15-μl aliquot was subjected to reverse zymography. Ordinate, molecular mass in kDa.

and 45 kDa. Pool 2 (fraction no. 67-76) contained a major inhibitor of 35 kDa and two minor ones of 29 and 24 kDa, and pool 3 (fraction no 77-86) contained two major inhibitors of 24 and 26 kDa and minor ones of 21 and 19 kDa. An additional minor inhibitor of 16 kDa was also detected in the reverse zymography of pool 3 on a higher concentration (12.5%) of polyacrylamide gel.

Purification of Trypsin Inhibitors—We previously purified gelatinase A inhibitors of 100 and 90 kDa from conditioned medium of human bladder carcinoma cell line EJ-1 and identified them as secreted forms of amyloid β protein precursor (APP) (*11*); they have a Kunitz-type trypsin inhibitor (KPI) domain and are often called protease nexin II. When pool 1 obtained from the molecular-sieve chromatography of the T98G conditioned medium was applied to an anion-exchange column (Shodex QA-824; Showa Denko, Tokyo), the two trypsin inhibitors of 100 and 90 kDa were tightly bound to the column and eluted forming a single protein peak at the same NaCl concentration as in the case of the secreted APPs (*11*). The 100- and 90-kDa inhibitors were reactive to a monoclonal antibody against human secreted APP in Western blotting analysis (data not shown). When the N-terminal amino acid sequences of the 100- and 90-kDa inhibitors were analyzed with an automated protein sequencer, both N-terminal amino acid sequences determined were identical to that of the mature

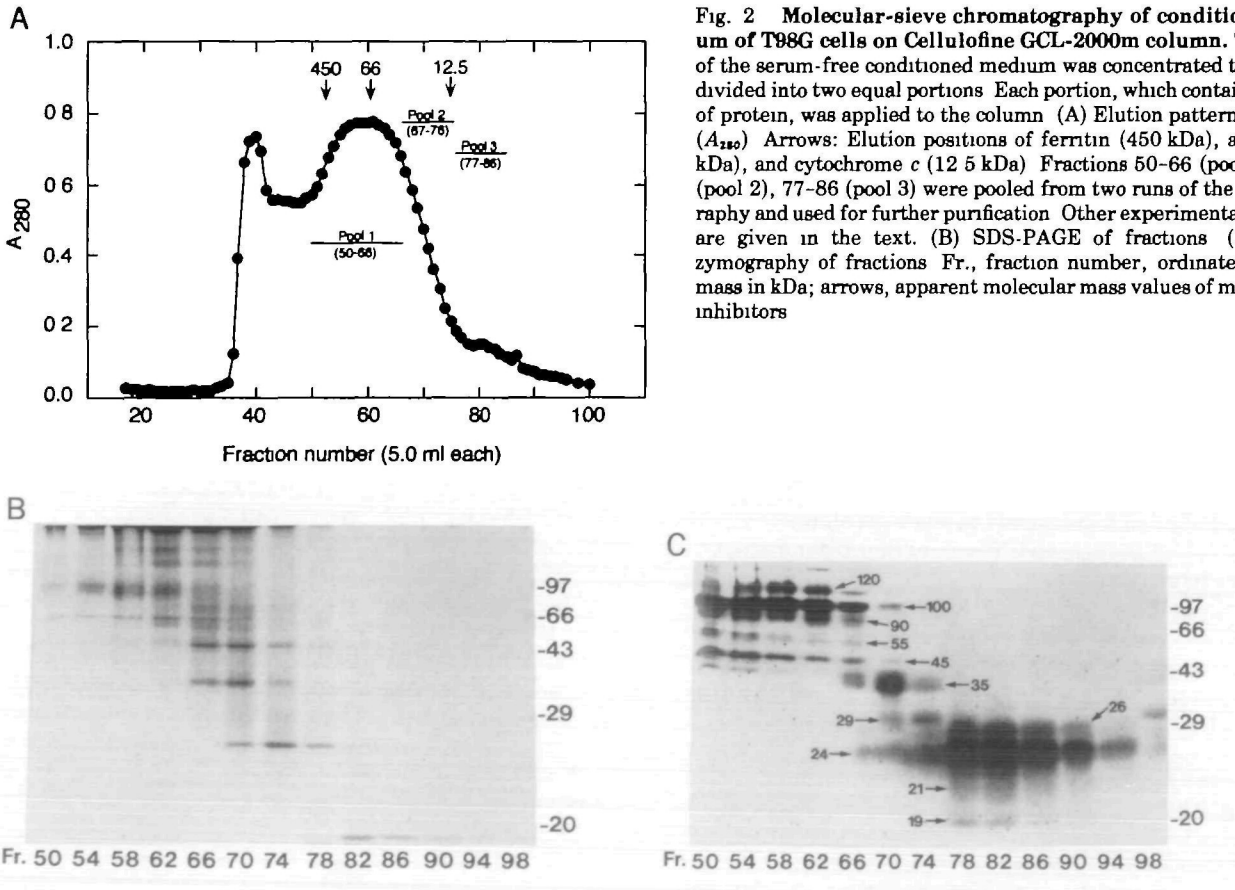

Fig. 2 **Molecular-sieve chromatography of conditioned medium of T98G cells on Cellulofine GCL-2000m column.** Three liters of the serum-free conditioned medium was concentrated to 30 ml and divided into two equal portions Each portion, which contained 120 mg of protein, was applied to the column (A) Elution pattern of proteins (A_{280}) Arrows: Elution positions of ferritin (450 kDa), albumin (66 kDa), and cytochrome *c* (12 5 kDa) Fractions 50-66 (pool 1), 67-76 (pool 2), 77-86 (pool 3) were pooled from two runs of the chromatography and used for further purification Other experimental conditions are given in the text. (B) SDS-PAGE of fractions (C) Reverse zymography of fractions Fr., fraction number, ordinate, molecular mass in kDa; arrows, apparent molecular mass values of major trypsin inhibitors

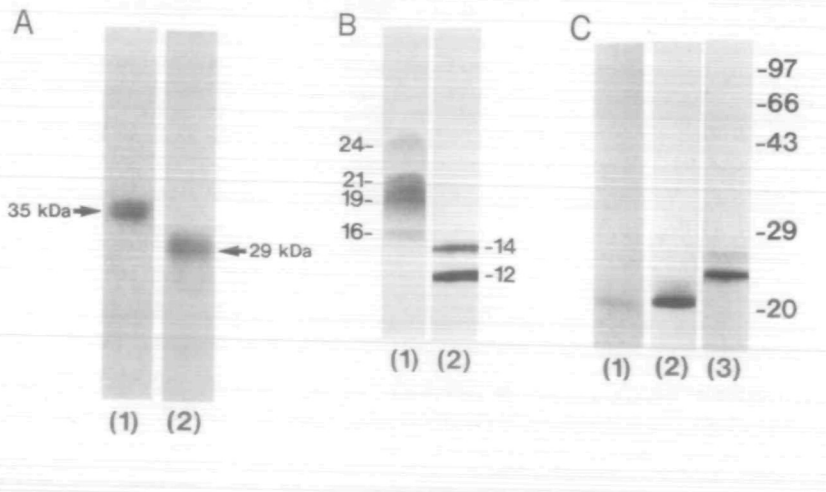

Fig 3. **SDS-PAGE of purified trypsin inhibitors.** Separated proteins were stained with CBB. Ordinate, molecular mass in kDa. (A) 35-kDa (lane 1) and 29-kDa (lane 2) trypsin inhibitors purified from pool 2 SDS-PAGE was carried out under nonreducing conditions. (B) A major trypsin inhibitor fraction obtained from pool 3. Lane 1, non-reduced; lane 2, reduced. (C) A 25-kDa trypsin-binding protein purified by the second trypsin-affinity chromatography of pool 3 and subsequent reverse-phase HPLC. Lane 1, reverse zymography; lane 2, non-reducing SDS-PAGE; lane 3, reducing SDS-PAGE.

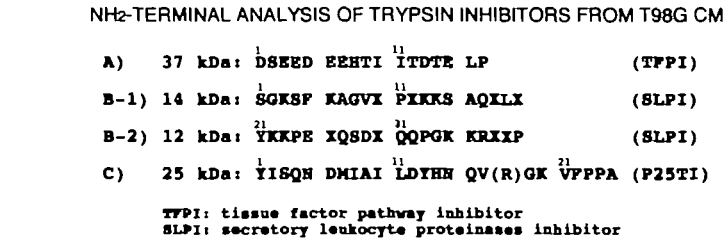

Fig. 4. **N-terminal amino acid sequences of purified trypsin inhibitors.** Major inhibitors obtained by reverse-phase HPLC were separated by reducing SDS-PAGE, and the resultant reduced protein bands were subjected to N-terminal amino acid sequencing. (A) A 37-kDa protein from pool 2, which corresponds to the non-reduced 35-kDa inhibitor (Fig. 3A, lane 1). (B) 14-kDa and 12-kDa proteins (Fig. 3B, lane 2) obtained by reducing SDS-PAGE of the mixture of 24-, 21-, 20-, and 16-kDa inhibitors from pool 3. (C) A 25-kDa trypsin-binding protein (Fig. 3C, lane 2) obtained by reducing SDS-PAGE of the non-reduced 20-kDa inhibitor from pool 3. X denotes an unidentified amino acid.

APP (data not shown). Thus, the 100- and 90-kDa inhibitors corresponded to the secreted form of either APP770 or APP751 (*11*). Although the 120-kDa inhibitor was not identified in this study because of its poor recovery in the anion-exchange chromatography, it appeared to be the highly glycosylated form of APP (*15*).

Pool 2 obtained from the molecular-sieve chromatography, which contained 35- and 29-kDa inhibitors, was applied to a trypsin-Sepharose affinity column, and bound proteins were eluted with 0.05% TFA (pH 2.5). When the inhibitor fractions were subjected to reverse-phase HPLC, the 35- and 29-kDa inhibitors were separately eluted at about 42% and about 37% acetonitrile, respectively (Fig. 3A). The N-terminal amino acid sequence of the 35-kDa inhibitor, the molecular mass of which increased to 37 kDa on reducing SDS-PAGE, was identical to that of the mature protein of tissue factor pathway inhibitor (TFPI) (Fig. 4A). The 29-kDa trypsin inhibitor was identified as placental protein 5 (PP5) as reported previously (*14*).

Pool 3, which contained heterogeneous inhibitor proteins between 16 and 29 kDa, was also subjected to the trypsin affinity chromatography. Bound proteins were eluted with TFA from the column and then subjected to reverse-phase HPLC as described above. The main fractions from the reverse-phase HPLC showed four broad protein bands of 24, 21, 19, and 16 kDa on non-reducing SDS-PAGE, but showed two bands of 14 and 12 kDa on reducing SDS-PAGE (Fig. 3B). When each band on the non-reducing gel was cut out and subjected to reducing SDS-PAGE, the 24-kDa inhibitor was shifted to 14 kDa, but the three others were shifted to 12 kDa (data not shown). The N-terminal sequences of the 14- and 12-kDa proteins were identical to the sequences of human secretory leukocyte proteinase inhibitor (SLPI) from residue 1 to residue 20, and from residue 21 to residue 40, respectively (Fig. 4B). This indicated that the heterogeneous inhibitor bands between 16 and 24 kDa observed in Figs. 1 and 2C can be mostly attributed to SLPI.

To isolate trypsin inhibitors having relatively low affinity to trypsin, the flow-through fraction of pool 3 from the trypsin affinity column was repeatedly applied to the same column. Bound proteins were eluted with TFA, and further purified by reverse-phase HPLC. The main fractions obtained from the reverse-phase HPLC showed an apparently single band of 20 kDa and one of 25 kDa in non-reducing and reducing SDS-PAGE, respectively (Fig. 3C, lanes 2 and 3). The 20-kDa band showed a relatively weak trypsin-inhibitory activity on reverse zymography (Fig. 3A, lane 1). When the 25 N-terminal amino acid residues of the 25-kDa inhibitor were determined and the GenBank/EMBL/NBRF data bases were searched, no homologous proteins were found, indicating that it is a novel protein (Fig. 4C).

DISCUSSION

In this study, we have demonstrated the presence of multiple forms of serine proteinase inhibitors in the serum-free conditioned medium of human glioblastoma T98G cells using reverse zymography, and identified secreted APPs, TFPI, PP5/TFPI-2, SLPI, and a novel 25-kDa inhibitor (p25TI).

APPs are precursor proteins of amyloid β protein, which is known to form the core of senile plaques in Alzheimer's disease. There are three major forms of APP, APP770, APP751, and APP695, from which either amyloid β protein or a secreted form of the APPs is produced by the action of different proteinases (*16*). The secreted forms of APP770 and APP751, which are also known as protease nexin II, have a KPI domain and inhibit the activities of trypsin, chymotrypsin, plasmin, coagulation factor XIa, and other serine proteinases (*17-19*). We recently purified secreted APPs from conditioned medium of a human bladder carcinoma cell line and showed that APPs have inhibitory activity against gelatinase A, an important member of the matrix metalloproteinases (*11*). There are some other reports showing the secretion of APPs by cultured cancer cell lines (*15, 19, 20*).

In contrast to APPs, production of TFPI, PP5/TFPI-2, and SLPI by cancer cells has rarely been reported. TFPI, which has three tandem KPI domains, is known to be an important inhibitor of the blood coagulation system (*21, 22*). It inhibits the activities of the coagulation factors Xa and VIIa and other serine proteinases. PP5 was originally found as one of the major placental proteins having a serine proteinase-inhibitory activity by Bützow *et al.* (*23*). It is found in various body fluids such as maternal serum at advanced pregnancy, ovarian follicular fluid and seminal plasma (*24, 25*). We (*14*) and Sprecher *et al.* (*26*) independently cloned its cDNA and showed that it has a TFPI-like structure. We also showed that its mRNA is expressed in various tissues, such as placenta, pancreas, heart, liver, and kidney and also in some human ovarian cancer cell lines (*14*). Like TFPI, PP5/TFPI-2 is likely to function as a coagulation inhibitor, because it is also expressed in vascular endothelial cells (*25, 26*). However, it is also possible that the two TFPIs may play roles in the regulation of some cellular functions.

SLPI, a potent inhibitor of leukocyte elastase, cathepsin G, chymotrypsin, and trypsin, is found in body fluids of parotid, bronchus, cervix, and testis (*27*). SLPI is thought to play a protective role against the proteolytic degradation of these tissues. Recombinant human SLPI has been subjected to clinical trial for diseases involving excessive proteolytic activities, such as cystic fibrosis and genetic emphysema (*28*). Although the reported molecular mass of SLPI is 14 kDa, our purified SLPI showed multiple bands

between 16 and 24 kDa on non-reducing SDS-PAGE. It seems likely that SLPI exists as a dimer linked with a disulfide bond(s) in the conditioned medium of T98G cells. In addition, the apparent heterogeneity of SLPI seems to be due to the difference in the sugar moiety and/or limited hydrolysis by trypsin in the purification step.

In addition to the four known serine proteinase inhibitors, we found a novel trypsin-binding protein of 25 kDa, p25TI. Because p25TI was purified in a very small amount, it was not well characterized in this study. p25TI showed weak trypsin-inhibitory activity in reverse zymography. In a preliminary assay in solution, it significantly inhibited the activity of trypsin but not that of thrombin (data not shown). Its cDNA cloning and subsequent production of the recombinant protein are essential to examine its serine proteinase inhibitor activities and other biological activities in more detail.

We have obtained data indicating that the inhibitors identified in this study are produced by many other human cancer cell lines, including cervical carcinomas, lung adenocarcinomas, bladder carcinomas, and oral squamous carcinomas (to be published). In addition, we have observed that normal human fibroblasts secrete lesser amounts of APPs compared to their SV40-transformed cell lines. Other serine proteinase inhibitors such as α1-antitrypsin, pancreatic secretory trypsin inhibitor, and plasminogen activator inhibitors have also been reported to be secreted by some cancer cell lines (*29-31*). Therefore, most cancer cell lines including T98G cells appear to secrete excess amounts of serine proteinase inhibitors compared to their target proteinases. These inhibitors seem to protect the producer cells from excess proteolysis by matrix serine proteinases such as trypsin, plasmin and tissue-type kallikrein.

It seems important to note that proteinase inhibitors exert some biological activities other than proteinase inhibition. Secreted APPs have been reported to show growth stimulation and cell adhesion activities towards some cultured cells (*32, 33*). Pancreatic secretory trypsin inhibitor stimulates the growth or DNA synthesis of endothelial cells (*31*) and fibroblasts (*34*). A similar growth-modulating activity has been reported with urinary trypsin inhibitor (*35*) and tissue inhibitors of metalloproteinases (TIMPs) (*36*). Furthermore, some serine proteinase inhibitors have been reported to show chemotactic activity (*37*) and neurite-promoting activity (*38*). It has generally been accepted that matrix proteinases promote invasive growth of cancer cells, while their natural inhibitors exert suppressive effects on malignant growth. However, the secretion of multiple forms of serine proteinase inhibitors by cancer cells, as well as their multifunctional characters, give rise to another possibility, that serine proteinase inhibitors might be involved in the abnormal growth of cancer cells. To test this possibility, we are currently investigating the effect of transfection of cDNAs for some serine proteinase inhibitors into human cancer cell lines on their cellular function.

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