Identification of one- and two-chain forms of trypsinogen 1 produced by a human gastric adenocarcinoma cell line

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It has previously been reported that two kinds of human gastric adenocarcinoma cell lines (STKM-1 and MKN28) secrete a trypsin-like enzyme. In this study, four molecular forms of the enzyme (26, 25, 24 and 23 kDa on non-reducing SDS/PAGE) were purified from the serum-free conditioned medium of STKM-1 cells. Analysis of N-terminal amino acid sequences showed that the 26 kDa protein was a two-chain form of trypsinogen 1 which had been produced by proteolytic cleavage of the Arg¹⁰⁷-Val¹⁰⁸ bond of trypsinogen 1, and the 24 kDa protein was the one-

chain form of trypsinogen 1. The 25 and 23 kDa proteins were the activated forms of the two-chain and one-chain trypsinogen 1 respectively. Isoelectric focusing gave pI values of 6.3 and 6.6 for the 26 kDa two-chain form and the 24 kDa one-chain form of trypsinogen 1 respectively. Comparison of the proteolytic activities indicated that the one-chain trypsin 1 had amidolytic activity about four times higher than that of the two-chain enzyme.

INTRODUCTION

A number of proteinases and their natural inhibitors secreted into the extracellular matrix are involved in a variety of biological processes such as tumour invasion and metastasis, tissue reconstruction, angiogenesis and cell migration. Many reports have suggested that a family of metalloproteinases called matrix metalloproteinases play a major role in matrix degradation by tumour cells [1,2]. Plasminogen activator is a serine proteinase that is often secreted by tumour cells [3–5]. It is believed to contribute to matrix degradation of tumour cells by activating plasminogen to plasmin. However, little is known about the role of other serine proteinases in the malignant growth of tumour cells.

To clarify the molecular mechanism of tumour invasion and metastasis, we have characterized various matrix metalloproteinases [6–8] and their natural inhibitors [9,10] secreted by cancer cells. During the course of these studies, we found that two human malignant gastric carcinoma cell lines secrete multiple serine proteinases. These enzymes were purified and shown to be identical with or related to trypsin, plasmin and tissue kallikrein [11]. These matrix serine proteinases, which can hydrolyse cell-adhesion proteins in the extracellular matrix such as fibronectin and laminin, are probably involved in matrix degradation of tumour cells.

Trypsin genes constitute a multiple gene family. Emi et al. [12] and Kang et al. [13] have isolated cDNA clones encoding three human trypsinogen isoenzymes (trypsinogens 1, 2 and 3) from a human pancreatic cDNA library. Human trypsinogens 1 and 2 have been purified and characterized from pancreatic juice and tissue [14,15]. Koivunen et al. [16,17] purified two trypsinogen isoenzymes named tumour-associated trypsinogen-1 (TAT-1) and TAT-2 from cyst fluid of human ovarian carcinoma. TAT-1 and TAT-2 are very similar to human pancreatic trypsinogen 1 and 2 respectively in structure, but there are significant differences in substrate specificity and susceptibility to proteinase inhibitors [16].

In the present study, we purified four forms of trypsin-related proteins from the conditioned medium of human gastric carcinoma cell line STKM-1, and identified them as one- and two-chain forms of trypsinogen 1 and their activated forms.

MATERIALS AND METHODS

Cell and culture conditions

The human gastric cancer cell line STKM-1 was a gift from Dr. S. Yanoma, Kanagawa Cancer Centre, Kanagawa, Japan. This cell line was maintained in autoclavable RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10 % fetal calf serum (Hyclone, Logan, UT, U.S.A.) [11]. Plastic culture dishes were gifts from Sumibe Medical (Tokyo, Japan), and plastic roller bottles were purchased from Becton Dickinson (Oxnard, CA, U.S.A.).

Purification of trypsin-like enzymes

Trypsin-like enzymes were purified from serum-free conditioned medium of STKM-1 cells by a series of procedures including molecular-sieve chromatography, immunoaffinity chromatography on an anti-trypsin antibody-conjugated Sepharose column, and two runs of reversed-phase h.p.l.c. (rp-h.p.l.c.), essentially as described previously [11]. The protein yield was 224 mg in conditioned medium (3 litres), 32 mg after molecular-sieve chromatography, 60 µg after immunoaffinity chromatography and 15 µg (10 µg of 24 kDa trypsinogen 1 and 5 µg of 26 kDa trypsinogen 1) after rp-h.p.l.c.

Electrophoretic analyses

SDS/PAGE, zymography and isoelectric focusing of the purified enzymes were carried out as described previously [10,11].

Assay of proteinase activities

Enzyme preparations were assayed for amidolytic activity on five

Abbreviations used: rp-h.p.l.c., reversed-phase h.p.l.c.; PVDF, poly(vinylidene difluoride); TAT, tumour-associated trypsinogen; Bz, benzyl; Boc, t-butoxycarbonyl; Mec, 7-amino-4-methylcoumarin.

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synthetic peptides. Purified proenzymes (10 nM) were activated by incubation with bovine enterokinase (1:1, w/w) in 100 μ l of 20 mM Tris/HCl (pH 7.5) at 25 °C for 30 min, and then mixed with 1 μ l of the individual substrates (0.1 mM) in 20 mM Tris/HCl (pH 7.5). 7-Amino-4-methylcoumarin liberated from the substrates during incubation was determined fluorimetrically with excitation at 380 nm and emission at 460 nm using a Cyto Fluor 2350 fluorimeter (Millipore, Bedford, MA, U.S.A.).

N-Terminal analysis

The N-terminal amino acid sequences of purified trypsin-like proteins were analysed with an Applied Biosystems model 473A protein sequencer by the courtesy of Applied Biosystems Laboratory (Tokyo, Japan).

Determination of protein concentration

Protein concentration was determined by the dye method with a Bio-Rad protein assay kit (Richmond, CA, U.S.A.), with bovine IgG as standard.

Reagents

Bovine intestine enterokinase was purchased from Sigma (St. Louis, MO, U.S.A.); lysyl endopeptidase was from Wako Pure Chemical Industries (Osaka, Japan); five synthetic substrates for trypsin were obtained from the Peptide Institute (Osaka, Japan); Immobilon poly(vinylidene difluoride) membrane (PVDF) was from Millipore.

RESULTS

Purification of four forms of trypsin-like enzyme

Trypsin-like proteins were purified from the serum-free conditioned medium of STKM-1 cells as described in the Materials and methods section. The proteins purified by immunoaffinity chromatography showed four bands of apparent molecular size 26, 25, 24 and 23 kDa on SDS/PAGE under non-reducing conditions (Figure 1). The 25 and 23 kDa proteins showed much higher gelatinolytic activity than the others on gelatin zymography. These proteins were separated into two peaks by rp-h.p.l.c. on a C4 column (Figure 2). When analysed by non-reducing SDS/PAGE, peak 1 showed a major band at 24 kDa and a minor band at 23 kDa, and peak 2 showed a major band at 26 kDa and a minor band at 25 kDa. On reducing SDS/PAGE, the apparent molecular size of the 24 kDa protein was shifted to 28 kDa, whereas the 26 kDa protein split into two peptides of apparent molecular size 13 and 9 kDa, indicating that the 26 kDa protein consisted of two peptide chains linked by disulphide bonds (results not shown).

The isoelectric points of the purified 24 and 26 kDa proteins were 6.6 and 6.3 respectively on thin-layer polyacrylamide-gel isoelectric focusing (results not shown).

Structural analysis

In order to identify the 24 and 26 kDa proteins, their N-terminal amino acid sequences were analysed with an automated protein sequencer after electrophoretic blotting of the proteins from an SDS/polyacrylamide gel on to a PVDF membrane. When the 24 kDa protein, separated by non-reducing SDS/PAGE, was analysed, a single N-terminal sequence up to the 20th amino acid residue was determined (Figure 3). This sequence was consistent with the N-terminal sequence of human pancreatic trypsinogen 1. Sequencing of the 26 kDa protein yielded two amino acids in

each cycle. The sequences obtained contained the N-terminal sequence of the 24 kDa protein. By subtracting this sequence, the other N-terminal sequence of the 26 kDa protein was found to be the internal sequence of trypsinogen 1 from residue 108 to residue 127. These results suggest that the purified 26 kDa protein was a two-chain form of trypsinogen 1, which had been produced by proteolytic cleavage of the Arg¹⁰⁷-Val¹⁰⁸ bond of trypsinogen 1.

The N-terminal amino acid sequences of the 23 and 25 kDa proteins, which were coeluted as minor proteins with the 24 and 26 kDa proteins on rp-h.p.l.c. respectively, were also analysed as described above (Figure 3). The sequence of the 23 kDa protein corresponded to that of human pancreatic trypsinogen 1 from residue 9 to residue 28. The 25 kDa protein gave two different sequences: one was the same as that of the 23 kDa protein, and the other corresponded to residues 108–127 of trypsinogen 1. These results indicate that the 23 and 25 kDa proteins were the activated forms of the 24 and 26 kDa trypsinogens respectively.

To confirm further that the purified proteins were human trypsinogen 1 and its derivatives, the purified 24 kDa protein and authentic human pancreatic trypsin 1 were digested by lysyl endopeptidase and subjected to peptide mapping by rp-h.p.l.c.

Figure 1 SDS/PAGE and zymography of trypsin-like proteins purified by immunoaffinity chromatography

(1) SDS/PAGE on a 14% gel under non-reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. (2) Zymography on a gelatin-containing gel. Molecular masses (kDa) are indicated on the right. Other experimental conditions are given in the text.

Figure 2 Separation of two major trypsin-like proteins by repeated rp-h.p.l.c. on SynCropack RP-4 column

Trypsin-like proteins purified by three types of chromatography (Figure 1) were applied to the second rp-h.p.l.c. column as described in the text. Inset, SDS/PAGE profiles of peak 1 (lane 1) and peak 2 (lane 2). Arrowheads indicate the 24 and 23 kDa proteins in lane 1 and the 26 and 25 kDa proteins in lane 2.

26 kDa	(1)APFDDDDKIV ¹¹ GGYNXEENXV
	(2) ¹⁰⁸ VSXIXLPXAP ¹¹⁸ PAXGXKXLIX
25 kDa	(1)IVGGYNXEEN ¹⁹ SVPYQVSLNS
	(2) ¹⁰⁸ VSTISLPTAP ¹¹⁸ PATGXKXLIS
24 kDa	(1)APFDDDDKIV ¹¹ GGYNXEEXSV
23 kDa	(1)IVGGYNXEEN ¹⁹ SVPYQVSLNS

Figure 3 N-Terminal amino acid sequences of purified 23, 24, 25 and 26 kDa trypsin-like proteins

X denotes unidentified amino acids. The 26 and 25 kDa proteins had two different sequences, one of which appeared to be identical with those of the 24 and 23 kDa proteins respectively. The second sequences were determined by subtraction. The numbers indicate the corresponding residues in human trypsinogen 1.

Figure 4 rp-h.p.l.c. of lysyl endopeptidase-digested peptides from human trypsin 1 (a) and purified 24 kDa protein (b)

The proteins were carboxamidomethylated by iodoacetamide and then digested for 3 h by a 1:200 (w/w) amount of lysyl endopeptidase in 2 M urea at 33 °C. A Cosmosil 5C18 h.p.l.c. column was previously equilibrated with 0.05% trifluoroacetic acid. Peptides bound to the column were eluted with a linear gradient of 0–80% (v/v) acetonitrile in 60 min at a flow rate of 0.5 ml/min. The peptides indicated by arrows were collected and analysed for N-terminal amino acid sequences (see Figure 5).

(Figure 4). Some of the resultant peptides were further analysed for N-terminal amino acid sequences. Most of the digested peptides of the 24 kDa protein were eluted at the same positions

Figure 5 Structure of human trypsinogen 1 and its cleavage sites giving rise to two-chain and active forms

The large arrow indicates the site that is cleaved to produce the two-chain form; the small arrow indicates the site cleaved to produce activation. The amino acid sequences of peptides A–E in Figure 4 are indicated by bars. The molecular masses of the heavy chain (Val¹⁰⁸–Ser²³²) and the light chain (Ala¹–Arg¹⁰⁷) of the two-chain trypsinogen are calculated to be 12 943 and 12 093 Da respectively.

Table 1 Activities of the two purified forms of trypsin 1 on synthetic substrates

The purified 24 and 26 kDa proteins (10 nM) were activated by enterokinase, and then incubated in reaction mixtures containing the indicated synthetic substrates at 25 °C for 15 min. Other experimental conditions are described in the Materials and methods section. The 26 kDa protein is the two-chain form of trypsinogen 1 and the 24 kDa protein is the one-chain form of trypsinogen 1. The values in parentheses relative activities.

Substrate	k_{cat} (s ⁻¹)	
	26 kDa	24 kDa
Boc-Phe-Ser-Arg-Mec	7.5 (27)	28.0 (100)
Boc-Leu-Thr-Arg-Mec	3.1 (11)	11.9 (42)
Boc-Gln-Ala-Arg-Mec	2.5 (9)	12.9 (43)
Boc-Val-Pro-Arg-Mec	2.1 (7)	9.5 (34)
Bz-Arg-Mec	0 (0)	0 (0)

as those of trypsin 1, except for peptides D and E, which were absent from trypsin 1. Peptide D corresponded to the propeptide of human trypsinogen 1 (residues 1–8), and a partially determined sequence of peptide E corresponded to an internal sequence (residues 9–18) (Figure 5). The apparent absence of peptide E from authentic trypsin may be due to incomplete digestion or incomplete chromatographic separation. The sequences of three other peptides (A, B and C) corresponded to internal sequences of human trypsinogen 1 (residues 78–87, 209–216 and 226–232 respectively). These results confirm that the 24 kDa protein is identical with human pancreatic trypsinogen 1. The structure of human trypsinogen 1 and the cleavage sites that give rise to two-chain and activated forms are illustrated in Figure 5.

Activity of the two forms of trypsin 1

The activities of the two forms of trypsin 1 against five synthetic peptide derivatives are summarized in Table 1. For the assay, the 26 kDa two-chain and 24 kDa one-chain forms of trypsinogen

1 were previously activated by enterokinase, the natural activator of trypsinogen. Both enzymes hydrolysed Boc-Phe-Ser-Arg-NH-Mec most effectively, Boc-Leu-Thr-Arg-NH-Mec, Boc-Gln-Ala-Arg-NH-Mec and Boc-Val-Pro-Arg-NH-Mec moderately, and Bz-Arg-NH-Mec not at all. For each substrate except for Bz-Arg-NH-Mec, the single-chain trypsin 1 had about four times higher activity than the two-chain form. Authentic human pancreatic trypsin 1 exhibited essentially the same substrate specificity as the 26 and 24 kDa enzymes (results not shown).

DISCUSSION

In the present study, one- and two-chain forms of trypsinogen 1 and their activated forms were purified and identified from the serum-free conditioned medium of the human gastric carcinoma cell line STKM-1. The two-chain forms of trypsinogen 1 and trypsin 1 had been cleaved at the internal Arg¹⁰⁷-Val¹⁰⁸ bond.

Trypsinogen is synthesized in the pancreas, secreted into the duodenum and converted into the active single-chain form β -trypsin by enterokinase or by autolysis [18,19]. This activation is attained by proteolytic removal of the N-terminal eight amino acids of trypsinogen. It is also known that purified bovine β -trypsin is converted into a two-chain form called α -trypsin by autolytic cleavage at the Lys¹³¹-Ser¹³² bond, and α -trypsin is further converted into an inactive three-chain form called pseudotrypsin by autolytic cleavage at the Lys¹⁷⁶-Asp¹⁷⁷ bond [18,19]. Maroux et al. [20] reported that incubation of purified bovine trypsin produced a two-chain form on autolytic cleavage at the internal Arg¹⁰⁵-Val¹⁰⁶ bond, which had a similar activity to single-chain trypsin. This conversion was prevented by the presence of 5 mM Ca²⁺. The two-chain trypsin found in this study seems to correspond to neither α -trypsin nor pseudotrypsin but to the two-chain trypsin reported by Maroux et al. [20]. Unlike their two-chain trypsin, however, our two-chain trypsin had about one-quarter of the activity of the single-chain trypsin.

The proportions of the 26 and 24 kDa forms in conditioned medium varied among subclones of STKM-1 cells, but it was hardly affected by the purification procedure (results not shown). In some preparations of conditioned medium, the 26 kDa two-chain trypsinogen 1 was detected almost exclusively. These facts suggest that the conversion of trypsinogen 1 into its two-chain form probably occurs in the culture or during concentration of the conditioned medium rather than during the purification procedure itself. These cells may have an efficient system for converting single-chain trypsinogen 1 into its two-chain form. This conversion may be catalysed by not only trypsin 1 but also plasmin and tissue kallikrein [11]. The conversion of trypsinogen 1 into its two-chain form appears to prevent excess expression of trypsin activity.

Koivunen et al. [21] detected two trypsinogen-like proteins

(TAT-1, -2) in conditioned media of three human cancer cell lines (colon carcinoma, fibrosarcoma and erythroleukaemia) by immunoblotting analysis. We have recently detected trypsin 1-like activity in many human cancer cell lines besides the two gastric carcinoma cell lines, including bladder cell carcinoma, schwannoma, glioblastoma and ovarian carcinoma (N. Koshikawa, H. Yasumitsu, Y. Nagashima, M. Umeda and K. Miyazaki, unpublished work). Labombardi et al. [22] also observed a membrane-bound trypsin-like proteinase in Walker 256 carcinosarcoma cells. These results suggest that trypsin 1 and related enzymes may be expressed in many types of cancer cell and play an important role in extracellular matrix degradation by these cells.

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