Purification and identification of a binding protein for pancreatic secretory trypsin inhibitor: a novel role of the inhibitor as an anti-granzyme A

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Pancreatic secretory trypsin inhibitor (PSTI) is a potent trypsin inhibitor that is mainly found in pancreatic juice. PSTI has been shown to bind specifically to a protein, distinct from trypsin, on the surface of dispersed cells obtained from tissues such as small intestine. In the present study, we affinity-purified the binding protein from the 2 % (w/v) Triton X-100-soluble fraction of dispersed rat small-intestinal cells using recombinant rat PSTI. Partial N-terminal sequencing of the purified protein gave a sequence that was identical with the sequence of mouse granzyme A (GzmA), a tryptase produced in cytotoxic lymphocytes. We confirmed the formation of an affinity-cross-linked complex between ^{125}I-labelled PSTI and recombinant rat GzmA (rGzmA). *In situ* hybridization and immunostaining revealed the existence of GzmA-expressing intraepithelial lymphocytes in the rat small intestine. We concluded that the PSTI-binding protein isolated from the dispersed cells is GzmA that is produced in the lymphocytes of the tissue. The rGzmA hydrolysed the *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT), and the BLT hydrolysis was inhibited by PSTI. Sulphated glycosaminoglycans, such as fucoidan or heparin, showed almost no effect on the inhibition of rGzmA by PSTI, whereas they decreased the inhibition by antithrombin III. In the present paper, we propose a novel role of PSTI as a GzmA inhibitor.

Key words: affinity purification, glycosaminoglycan, granzyme A, lymphoid tissue, pancreatic secretory trypsin inhibitor.

INTRODUCTION

Pancreatic secretory trypsin inhibitor (PSTI), initially found by Kazal et al. [1], is mainly produced in the acinar cells of the pancreas and is secreted into pancreatic juice. PSTI is a small protein with a molecular mass of about 6.5 kDa [2]. Its role has been postulated to be the prevention of inadvertent proteolysis in the pancreas or pancreatic duct caused by premature activation of trypsinogen [2]. The inhibitory specificity is very narrow, with strong inhibitory capacity against pancreatic trypsin, but with very little or no inhibitory capacity against various proteases with trypsin-like specificity [3]. Considerable amounts of PSTI have also been detected in a variety of extrapancreatic tissues [4]. In addition, Ogawa and co-workers [5,6] have found elevated serum immunoreactive PSTI content in patients with severe inflammation and tissue destruction, where almost no trypsin activity in blood has been detected. These previous results suggest that PSTI has other important functions outside the pancreas.

We previously found a specific binding protein for PSTI on the surface of dispersed cells obtained from small intestine, colon, liver, stomach and kidney [7]. Autoradiography of the affinity-cross-linked complex of the ^{125}I-labelled PSTI and the binding protein indicated a molecular mass of about 33 kDa and 53 kDa after SDS/PAGE (12 % gels), under reducing and non-reducing conditions respectively, suggesting that the binding molecule is distinct from trypsin. However, the chemical structure, the production site and the role of the specific binding remain to be investigated.

The purpose of the present study was to identify the binding protein for PSTI. We affinity-purified the binding protein with a recombinant PSTI from small intestine, where the binding site is the most abundant [7]. Partial N-terminal sequencing of the purified protein revealed that it is identical with granzyme A (GzmA), a lymphocyte tryptase [8]. The present study documents a potential role of PSTI as the tryptase inhibitor.

EXPERIMENTAL

Materials

Male Wistar rats were purchased from Japan Shizuoka Laboratory Center (Hamamatsu, Japan). Trypsin (type III, from bovine pancreas), γ-globulin, soybean trypsin inhibitor (type IS), *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) [9], 5,5′-dithiobis-(2-nitrobenzoic acid) and fucoidan (from *Fucus vesiculosus*) were purchased from Sigma (St. Louis, MO, U.S.A.). Antithrombin III (ATIII), chondroitin sulphate A, and heparin were purchased from Wako Pure Chemicals Co. (Tokyo, Japan). An anti-GzmA polyclonal antibody (D-15) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Ni^{2+}-charged resin was purchased from CytoSignal Research

Abbreviations used: ATIII, antithrombin III; BLT, *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester; BS_3, bis(sulphosuccinimidyl) suberate; CCK, cholecystokinin; CTL, cytotoxic T-lymphocyte; GdmCl, guanidinium chloride; GzmA, granzyme A; GzmB, granzyme B; IL, interleukin; NK cells, natural killer cells; PSTI, pancreatic secretory trypsin inhibitor; rGzmA, rat GzmA; rhPSTI, recombinant human PSTI; rrPSTI–His, recombinant rat PSTI with C-terminal polyhistidine tag.

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The nucleotide sequence data of the rat granzyme A are available in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB082125.

Products Co. (Irvine, CA, U.S.A.). Recombinant human PSTI (rhPSTI) was a gift from Mochida Pharmaceutical Co. (Tokyo, Japan). $Na^{125}I$ and [α-^{35}S]dATP were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.). Synthetic oligonucleotides were purchased from Espec Oligo Service Inc. (Ibaraki, Japan). KOD Dash DNA polymerase for PCR amplification, AMV reverse transcriptase XL and DNA ligase were purchased from Toyobo (Osaka, Japan). Pre-stained protein markers (broad range) and restriction endonucleases were purchased from New England BioLabs Inc. (Beverly, MA, U.S.A.). All other reagents used were of analytical grade.

Preparation of a 2 % (w/v) Triton X-100-soluble fraction from dispersed small-intestine cells and detection of affinity-cross-linked complex between ^{125}I-labelled PSTI and its binding protein

The preparation of dispersed rat small-intestinal mucosal cells, purification of PSTI from rat pancreatic juice and ^{125}I-labelling of the purified PSTI (^{125}I-labelled PSTI) were as described in [7]. The dispersed cells were suspended in 2 % (w/v) Triton X-100 solution (10^7 cells/ml) and incubated for 1 h at 4 °C. After incubation, the mixture was centrifuged at 10 000 ***g*** for 20 min at 4 °C, and 10 μl of the resultant supernatant [2 % (w/v) Triton X-100-soluble fraction] was incubated for 1 h with ^{125}I-labelled PSTI (10 ng) in 100 μl of a binding buffer containing 25 mM Hepes, pH 8.0, 500 mM NaCl and 0.1 % (w/v) Triton X-100, at 37 °C in the presence or absence of a 500-fold excess amount of unlabelled native or recombinant PSTI. Then, after the addition of 100 μl of 0.2 % (w/v) γ-globulin and 200 μl of 25 % (w/v) poly(ethylene glycol) solution, the reaction mixture was incubated for 20 min on ice, followed by centrifugation at 10 000 ***g*** for 10 min at 4 °C. Pellets were resuspended in 30 μl of binding buffer in which 0.4 mM bis(sulphosuccinimidyl) suberate (BS_3) (Pierce Chemical Co., Rockford, IL) had been dissolved, and were incubated at room temperature (22 °C) for 30 min. The suspension was mixed with 30 μl of 2 × SDS sample buffer [1 × buffer: 0.05 M Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.005 % (w/v) Bromophenol Blue], with or without dithiothreitol at a final concentration of 12 mM, boiled for 3 min, and then subjected to SDS/PAGE (12 % gels). The resulting gel was vacuum-dried and exposed to X-ray film (Fuji Film Co., Tokyo, Japan) at –70 °C for 24 h.

Production of recombinant PSTI

Recombinant rat PSTI with a polyhistidine tag at the C-terminus (rrPSTI–His) was produced using an EasySelect™ *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, U.S.A.). Using a previously cloned full-length cDNA as template [10], a DNA fragment containing the sequence of mature rat PSTI (Gly^1–Cys^{61}) was amplified by PCR with sense and anti-sense primers, 5′-GGGTAACCCTCCAGCT-3′ and 5′-GTCGACACCGCAAGTCCCTCTCCT-3′ respectively. The PCR product was ligated into *Sma*I-linearized pT7Blue-2 vector (Novagen Inc., Madison, WI, U.S.A.). The construct was double-digested with *Bal*I and *Sal*I, and the 285 bp restriction fragment was ligated into *Pml*I- and *Sal*I-double-digested pPICZαC, a *Pichia pastoris* vector for secreted expression. The pPICZαC-rrPSTI–His vector was introduced into *P. pastoris* strain KM71H by electroporation using an Electro Cell Manipulator 600 (BTX Inc., San Diego, CA, U.S.A.). A transformant that was resistant to Zeocin™ (0.5 mg/ml) was selected and cultured according to the manufacturer's instructions. The recombinant protein that was secreted into the culture medium was purified using Ni^{2+}-charged resin, and was treated with recombinant enterokinase (Novagen Inc.). The concentration and trypsin-inhibitory activity of the rrPSTI–His were determined as in [7].

Affinity purification and detection of the binding protein

The 2 % (w/v) Triton X-100-soluble fraction from dispersed small-intestinal cells was concentrated by ultrafiltration using Ultrafree-15 (Millipore Co., Bedford, MA, U.S.A.) and subjected to gel filtration on a PD-10 column (Amersham Biosciences), equilibrated with a buffer [25 mM Hepes, pH 8.0, 500 mM NaCl, 10 mM imidazole and 0.1 % (w/v) Triton X-100]. The sample was diluted with the equilibration buffer so that the final protein concentration was approx. 10 mg/ml, and then soybean trypsin inhibitor was added at a final concentration of 0.1 mg/ml. Starting material (10 ml) was incubated for 16 h at room temperature with 0.5 ml of Ni^{2+}-charged resin to which approx. 1 mg of the rrPSTI–His had bound. After incubation, the resin was washed and then boiled for 3 min with 0.5 ml of 2 × SDS sample buffer with no reducing reagent. The eluate was subjected to gel filtration on a NAP-5 column (Amersham Biosciences), equilibrated with 0.01 % (w/v) Triton X-100 solution, and concentrated by ultrafiltration.

The purified protein was analysed by Western ligand blotting. After SDS/PAGE (12 % gels) under reducing or non-reducing conditions, proteins were transferred on to PVDF membrane (Fluorotrans®; Nihon Genetics Inc., Tokyo, Japan), and the membrane was washed twice for 5 min at room temperature with PBS containing 0.1 % (w/v) Tween 20 (PBST). The membrane was then incubated successively with PBST containing 6 M guanidinium chloride (GdmCl), PBST containing 3 M GdmCl, PBST containing 1.5 M GdmCl, PBST containing 0.75 M GdmCl, PBST containing 0.38 M GdmCl and PBST containing 0.19 M GdmCl, each for 3 min at room temperature, and finally washed twice with PBST for 10 min. The membrane was sequentially blocked for 16 h at 4 °C in PBST containing 1 % (w/v) BSA, and was incubated for 2 h at 37 °C with 1 × 10^5 c.p.m./ml of ^{125}I-labelled PSTI in PBST with 1 % (v/v) BSA. BS_3 was then added to a final concentration of 0.4 mM, followed by incubation for 1 h at room temperature. The membrane was washed three times with PBST for 10 min at room temperature, air-dried, and autoradiographed at –70 °C for 24 h with an intensifying screen.

Determination of amino acid sequence for the binding protein

After separation of the affinity-purified protein by SDS/PAGE (12 % gels) under non-reducing conditions, the gel fragment containing the protein of interest (45–50 kDa) was excised, slurried using a pestle in a microcentrifuge tube containing 300 μl of 0.1 % (w/v) Triton X-100 solution and incubated for 4 h at 4 °C. After incubation, the mixture (500 μl) was placed on Attoprep MF (ATTO Inc., Tokyo, Japan) and centrifuged at 10 000 ***g*** for 10 min at 4 °C. The solution that passed through the filter was concentrated by ultrafiltration and was subsequently subjected to SDS/PAGE (12 % gels) under reducing conditions. Following SDS/PAGE, the proteins were transferred on to a PVDF membrane and lightly stained with Coomassie Brilliant Blue. A portion of the membrane at which a protein band (28 kDa) was detected was excised and subjected to N-terminal sequence with an automated protein sequencer (Procise 494 cLC; Applied Biosystems, Tokyo, Japan).

cDNA cloning and recombinant protein production of rat GzmA

The DNA fragment encoding the binding protein (GzmA) was amplified by PCR using small-intestinal cDNAs synthesized as template and a set of degenerate primers, 5′-GGIGGIGAYA-CIGTIGTICCIC-3′, corresponding to the determined sequence (Gly^3–His^{10}), and 5′-CTNCCAGARTCNCCRTTRCA-3′, corresponding to Cys^{178}–Ser^{184} of mouse GzmA [11]. A PCR fragment of about 550 bp was obtained. This was subcloned into pBluescript® II SK(–) vector (Stratagene, La Jolla, CA, U.S.A.) and the DNA sequence was determined. Using the PCR frag ment as a probe, a rat jejunal cDNA library (Stratagene) was screened by plaque hybridization to obtain full-length cDNAs. We amplified a DNA fragment containing the sequence of the active form of rat GzmA (Ile^1–Val^{233}) by PCR with primers 5′-ATCATT-GGAGGAGACACAG-3′ and 5′-GGTCGACAGCGCCCTTC-GC-3′. The PCR product was subcloned into *Sma*I-linearized pT7Blue-2 vector to generate pT7Blue-GzmA. A DNA portion of 5 bp (AGCCC), which interrupts the direct junction of the enterokinase recognition site (Asp-Asp-Asp-Asp-Lys) and the N-terminus of the active GzmA (Ile^1), was then removed as-follows. Using pT7Blue-GzmA as a template, a PCR product (3796 bp) was amplified with the primers 5′-ATCATTGG-AGGAGACACAG-3′ and 5′-CTTGTCGTCGTCATCGC-3′, and was then self-ligated. The construct (pT7BlueDDDDK-I-GzmA) was double-digested with *Bal*I and *Sal*I, and the restriction fragment (709 bp) was ligated into *Pml*I- and *Sal*I-double-digested pPICZαC to generate pPICZαC-GzmA. The procedures for production and purification of the recombinant rat GzmA (rGzmA) using *P. pastoris* were essentially the same as those of rrPSTI–His. The purified rGzmA was activated by the recombinant enterokinase and then repurified using Ni^{2+}-charged resin to remove the enterokinase. The protein concentration of activated rGzmA was titrated by the method of Beresford et al. [12].

Binding and inhibitory activity of PSTI towards rGzmA

Approx. 10 ng of the purified and enterokinase-activated rGzmA (active rGzmA) was incubated with ^{125}I-labelled PSTI (10 ng) in 100 μl of binding buffer, and the affinity-cross-linked complex was detected by autoradiography.

The inhibitory activity of PSTI towards the active rGzmA was determined as follows. Aliquots of the active rGzmA (approx. 5 ng) were incubated with 500 μM (or 250 μM) BLT and 500 μM 5,5′-dithiobis-(2-nitrobenzoic acid), in a buffer [20 mM Hepes, pH 8.0, 145 mM NaCl and 0.1 % (w/v) Triton X-100] in the presence or absence of the purified rat PSTI at 22 °C in a final volume of 200 μl. The colour development was measured at 405 nm using a microplate reader (Multiskan MS; Labsystems Co., Helsinki, Finland). The non-enzymic rate of absorbance increase was subtracted from the enzyme-catalysed rate. For comparison, the inhibitory activity of ATIII towards the rGzmA and that of PSTI towards the bovine trypsin (approx. 5 ng used) were determined under the same conditions. Three separate experiments were performed in triplicate. The inhibitory activities of ATIII and PSTI were also determined in the presence of chondroitin sulphate A (1 mg/ml), fucoidan (50 μg/ml) or heparin (250 μg/ml), under the same conditions.

In situ hybridization and immunostaining

Two non-overlapping antisense oligonucleotides, which are complementary to nucleotide sequences 52–96 and 781–825 of the rat GzmA cDNA, were used as probes for *in situ* hybridization (the nucleotide numbering starts from the adenine base of the translation initiation codon). These oligonucleotides were labelled with [α-^{35}S]dATP, using terminal deoxyribonucleotidyl transferase (Invitrogen) at a specific radioactivity of 0.5×10^9 d.p.m./μg of DNA. The preparation of frozen sections of normal rat small intestine, conditions for prehybridization, hybridization and washing were as described in [13]. The sections were dipped in Kodak NTB2 nuclear track emulsion and exposed for one month.

The procedure for immunostaining was described previously in [14]. Briefly, frozen sections from rat small intestine, 10 μm in thickness, were incubated with an anti-GzmA goat polyclonal antibody (1:1000 dilution) in PBST containing 5 % (v/v) goat serum for 6 h at room temperature. After several washes with PBST, sections were incubated with a peroxidase-labelled rabbit anti-goat IgG at a dilution of 1:160 for 60 min. After washing the slides with PBS, the colour was developed using a Histofine™ immunostaining kit (Nichirei Co., Tokyo, Japan).

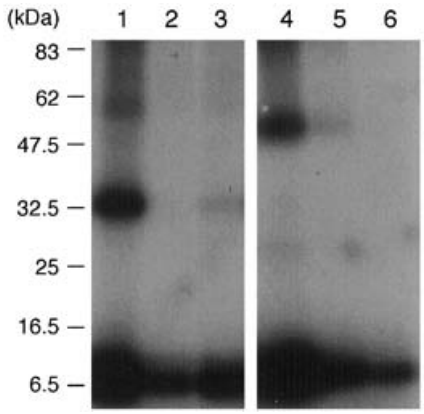

Figure 1 Autoradiography of the affinity-cross-linked complex of the ^{125}I-labelled PSTI and the binding protein in 2 % (w/v) Triton X-100-soluble fraction of dispersed rat small-intestinal cells

The 2 % (w/v) Triton X-100-soluble fraction (10 μl) from dispersed small-intestinal cells of rats was incubated with 2×10^6 c.p.m. of ^{125}I-labelled PSTI (10 ng) in 100 μl buffer at 37 °C for 1 h in the presence or absence of a 500-fold excess amount of unlabelled PSTI or rrPSTI–His. The complex was precipitated with γ-globulin and poly(ethylene glycol), cross-linked with BS_3, and subjected to SDS/PAGE (12 % gels) followed by autoradiography. Lane 1, without unlabelled PSTI or rrPSTI–His, but with dithiothreitol (DTT); lane 2, with unlabelled PSTI and DTT; lane 3, with unlabelled rrPSTI–His and DTT; lane 4, without unlabelled PSTI or rrPSTI–His and without DTT; lane 5, with unlabelled PSTI, but without DTT; lane 6, with unlabelled rrPSTI–His, but without DTT. The molecular masses of the protein standards are indicated.

RESULTS

Purification and N-terminal sequencing of the binding protein for PSTI

The binding protein from dispersed rat small-intestinal cells was first solubilized with detergent. When ^{125}I-labelled PSTI was incubated with the 2 % (w/v) Triton X-100-soluble fraction from the dispersed cells, 33 kDa and 53 kDa bands were detected by SDS/PAGE under reducing and non-reducing conditions respectively (Figure 1, lanes 1 and 4). These bands were not detected in the presence of a large excess of non-labelled purified PSTI (Figure 1, lanes 2 and 5) or non-labelled rrPSTI–His (Figure 1, lanes 3 and 6). The size of the signals obtained and the inhibition by the non-labelled PSTI were in agreement with previous results using intact dispersed cells [7]. The results

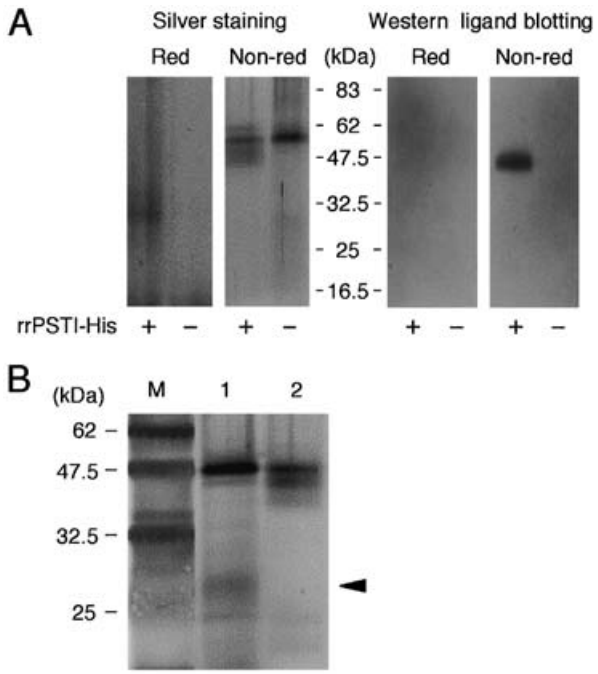

Figure 2 Detection of the purified PSTI-binding protein

(**A**) Detection of the purified protein by silver staining and Western ligand blotting. The 2% (w/v) Triton X-100-soluble fraction of dispersed small-intestinal cells was incubated with Ni^{2+}-charged resin with (+) or without (–) rrPSTI–His. The protein(s) bound were eluted and subjected to SDS/PAGE (12% gels) under reducing (Red) or non-reducing (Non-red) conditions, followed by silver staining or Western ligand blotting as described in the Experimental section. The molecular masses of the protein standards are indicated. (**B**) Silver staining profile of the purified protein by two-step SDS/PAGE. The protein(s) that bound to the Ni^{2+}-charged resin with (lane 1) or without (lane 2) rrPSTI–His were separated once by SDS/PAGE (12% gels) under non-reducing conditions. Then the portion of gel containing the protein of interest (approx. 45 kDa) was excised, and the eluted protein(s) were re-subjected to SDS/PAGE (12% gels) under reducing conditions. The position of the protein (28 kDa), in which the N-terminal sequence was determined, is indicated by an arrowhead on the right. Lane M shows the protein size marker, and the molecular masses are indicated in kDa.

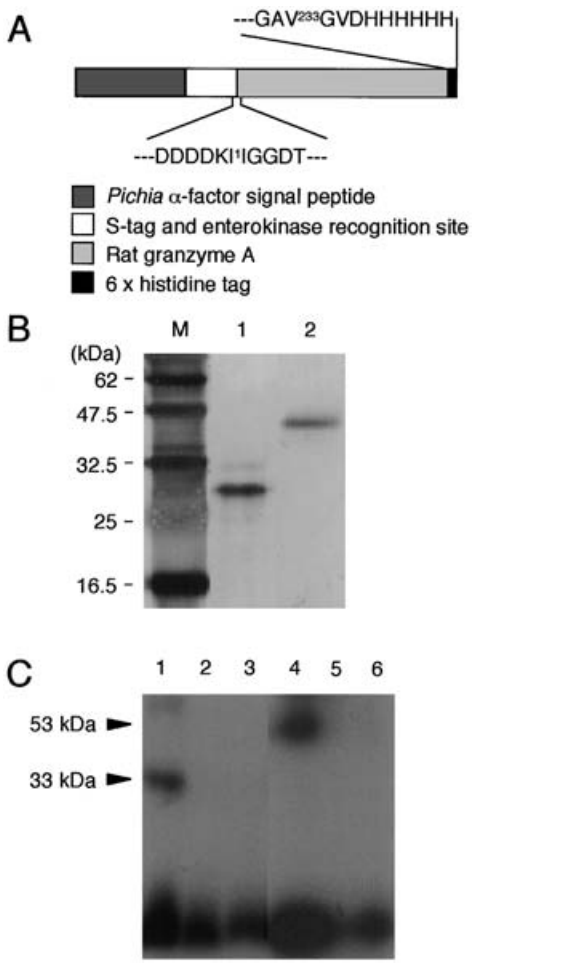

Figure 3 Production and characterization of recombinant rGzmA and its binding to PSTI

(**A**) *P. pastoris* expression construct for rGzmA. Amino acids are shown by their single-letter codes. The N- and C-terminal amino acid residues of the rat GzmA (Ile^1 and Val^{233} respectively) are indicated. (**B**) SDS/PAGE of purified and active rGzmA. After the enterokinase cleavage, the rGzmA was subjected to SDS/PAGE (12% gels) under reducing (lane 1) and non-reducing (lane 2) conditions followed by silver staining. Lane M shows the protein size markers with the molecular masses indicated. (**C**) Autoradiography of the affinity-cross-linked complex of the ^{125}I-labelled PSTI and the active rGzmA. Lane 1, without unlabelled PSTI or rhPSTI, but with dithiothreitol (DTT); lane 2, with unlabelled PSTI and DTT; lane 3, with unlabelled rhPSTI and DTT; lane 4, without unlabelled PSTI or rhPSTI and without DTT; lane 5, with unlabelled PSTI, but without DTT; lane 6, with unlabelled rhPSTI, but without DTT. The position of the affinity-cross-linked complex is indicated by the arrowhead.

showed that the treatment of cells with 2% (w/v) Triton X-100 solubilized the binding protein and that rrPSTI–His is capable of binding to the protein.

The 2% (w/v) Triton X-100-soluble fraction from dispersed small-intestinal cells was incubated with Ni^{2+}-charged resin, to which rrPSTI–His bound. Bound proteins were subjected to SDS/PAGE. Silver staining revealed major protein bands of 28 kDa and 45 kDa, under reducing and non-reducing conditions respectively, which were not detected when the 2% (w/v) Triton X-100-soluble fraction was incubated with resin alone (Figure 2A). Under non-reducing conditions, Western ligand blotting with ^{125}I-labelled PSTI as a probe clearly revealed a signal of 45 kDa that was not visible when the fraction was incubated with resin alone. Under reducing conditions, there were no signals, probably because the binding protein was irreversibly denatured (Figure 2A).

Because the N-terminal sequence of the binding protein could not be determined following a single SDS/PAGE step, owing to contaminating proteins, the protein was purified further. The proteins were first separated by SDS/PAGE under non-reducing conditions, and then proteins of about 45 kDa were eluted from the gel and resubjected to SDS/PAGE under reducing conditions, giving a major band at 28 kDa (Figure 2B). Sequencing of its N-terminal gave the sequence Ile-Ile-Gly-Gly-Asp-Thr-Val-Val-Pro-His-Ser-Arg, which matches the N-terminal sequence of mouse GzmA perfectly.

Production of recombinant GzmA and its specific binding to PSTI

We isolated a full-length cDNA encoding rat GzmA and produced a recombinant protein. The clone contained an open reading frame of 261 amino acids; 26 amino acid residues of signal peptide, two charged amino acid residues of propeptide, and 233 amino acid residues of active GzmA. The predicted protein sequence showed high sequence similarity with mouse and human GzmA (80.8% and 74.7% respectively).

rGzmA was produced using *P. pastoris* (Figure 3A). The rGzmA produced in the present study was designed to be activated *in vitro* by enterokinase. The active rGzmA gave signals of

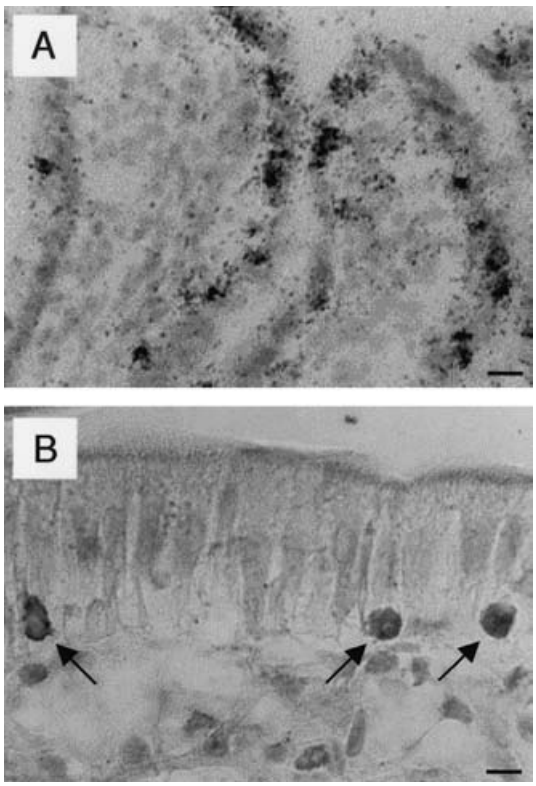

Figure 4 Localization of GzmA on the normal adult rat small intestine by *in situ* hybridization and immunostaining

(**A**) *In situ* hybridization with an antisense oligonucleotide specific to the rat GzmA mRNA (52–96). Positive signals for GzmA mRNA are mainly present on the epithelial layer. The signals were less evident in the lamina propria. Scale bar, 100 μm. (**B**) Immunostaining with an anti-GzmA antibody. Intra-epithelial lymphocytes stained by the antibody are indicated by arrows. Scale bar, 10 μm.

about 28 kDa and about 45 kDa on SDS/PAGE under reducing and non-reducing conditions respectively (Figure 3B), indicating that the rGzmA forms a disulphide-linked homodimer as native GzmA does [8,15]. We were able to detect an affinity-cross-linked complex of ^{125}I-labelled PSTI and active rGzmA that was not detectable in the presence of an excess amount of non-labelled rat PSTI or rhPSTI (Figure 3C).

Localization of GzmA in the rat small intestine

We localized GzmA in the rat small intestine. *In situ* hybridization with an antisense oligonucleotide corresponding to nucleotides 52–96 of GzmA mRNA revealed that the positive signals were mainly localized in the epithelial layer (Figure 4A). We obtained a similar result using another antisense oligonucleotide corresponding to nucleotides 781–825 of the mRNA (results not shown). The pattern of the signal distribution indicated that GzmA mRNA was selectively expressed in cells scattered within the epithelium. When the tissue sections were immunostained with an anti-GzmA antibody, immunoreactivity was shown in the intra-epithelial lymphocytes, but not in the epithelial lining cells (Figure 4B).

Inhibitory property of PSTI against rGzmA

BLT, a synthetic substrate used for the determination of GzmA activity [9], was hydrolysed by the active rGzmA, but

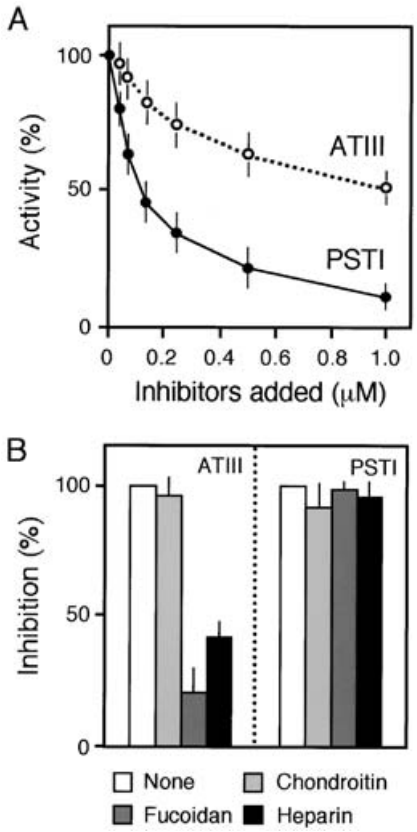

Figure 5 Inhibitory property of PSTI towards rGzmA

(**A**) Inhibition of rGzmA by PSTI. The active rGzmA (0.5 nM) was incubated with BLT (250 μM) in Hepes buffer as described in the Experimental section. The change in absorbance at 405 nm at 22 °C was measured in the presence of the PSTI purified from rat pancreatic juice at the concentrations indicated. For comparison, the inhibition of rGzmA by ATIII was determined under the same conditions. The BLT esterase activity of rGzmA in the absence of each inhibitor was taken as 100 %. Results are means $\pm$ S.D. of three separate experiments performed in triplicate. (**B**) The effects of sulphated glycosaminoglycans on the inhibition of rGzmA by ATIII and PSTI. The inhibition of rGzmA by human ATIII (1 μM) or purified rat PSTI (0.1 μM), concentrations at which each inhibitor shows about 50 % inhibition in the absence of glycosaminoglycans, was determined in the presence of chondroitin sulphate A (Chondroitin) (1 mg/ml), fucoidan (50 μg/ml) or heparin (250 μg/ml). The inhibition by each inhibitor in the absence of glycosaminoglycans was taken as 100 %. Results are means $\pm$ S.D. of three separate experiments performed in triplicate.

not by purified rGzmA before enterokinase cleavage. BLT hydrolysis by active rGzmA was inhibited by concomitant incubation with purified rat PSTI or human ATIII (Figure 5A). The dissociation constants, K_i, calculated according to Dixon [16], were 34 ± 7 nM and 3281 ± 388 nM respectively. For comparison, the inhibitory activity of PSTI towards trypsin was determined under the same experimental conditions. The concentrations of PSTI required for 50 % inhibition of rGzmA and trypsin were 116 ± 24 nM and 10 ± 3 nM respectively.

The effects of sulphated glycosaminoglycans on the inhibition of rGzmA by ATIII or PSTI were examined. Inclusion of chondroitin sulphate A had no significant effect on the action of the inhibitors (Figure 5B). Fucoidan and heparin had almost no effect on the inhibition by PSTI, whereas they decreased the inhibition by ATIII (Figure 5B).

DISCUSSION

In the present study, we purified the binding protein for PSTI from rat small intestine and showed that it is identical with GzmA. We

previously found that the PSTI-binding protein was also present in stomach, colon, liver and kidney of rats [7]. The binding protein in these tissues may also be GzmA, because the biochemical characteristics and the size of the affinity-cross-linked complex were in agreement with those detected in the small intestine. In fact, GzmA-expressing lymphocytes have been found in a variety of human tissues, including stomach, colon and liver [17]. We also found the inhibitory action of PSTI *in vitro* towards the proteolytic activity of rGzmA, although the inhibitory capacity is likely to be lower than that against trypsin. In an earlier study, PSTI inhibited the proteolytic activity of acrosin, a serine protease in acrosomes [18]; the significance of the inhibition has remained unknown. Nevertheless, to our knowledge, the present study is the first report showing that PSTI inhibits GzmA.

It is known that GzmA, together with perforin and other granzymes, is packaged in cytoplasmic granules of the cytotoxic T-lymphocytes (CTLs) or natural killer (NK) cells that are found in lymphoid tissues and peripheral blood [8,17]. In the small intestine, the enzyme is known to be expressed in cytolytic lymphocytes within the epithelial layer, termed intra-epithelial lymphocytes [19,20]. GzmA and granzyme B (GzmB) enter viral-infected cells or growing tumours via pores formed by perforin in the target-cell membrane and have an important role as mediators of granule-mediated apoptosis [21]. Studies using GzmA- or GzmB- single-knockout mice have indicated that GzmB is the major CTL effector molecule for the induction of apoptosis, with GzmA having only a minor part [22]. These granzymes are also released into blood [23]. At the present time, GzmA has been shown to mediate extracellular functions such as conversion of pro-interleukin (IL)-1β into its active form [24], production of IL-6 and IL-8 by fibroblasts and/or epithelial cells (possibly via activation of protease-activated receptors on the cell surface) [25], and degradation of several extracellular matrix proteins [8]. It has been reported that the serum immunoreactive PSTI is elevated in patients with severe inflammation and tissue destruction [5,6]. Although it was hypothesized that PSTI was elevated so as to inhibit trypsin that is released into blood, immunoreactive trypsin was hardly detected in the blood of the patients, owing to the rapid masking of the molecule by the large amounts of circulating antiproteases such as α1-antitrypsin [5]. In contrast with trypsin, the proteolytic activity of GzmA has been demonstrated in the blood of patients with activated CTLs and NK cells [23]. Therefore, it can be speculated that one of the roles for extrapancreatic or blood PSTI is the control of the extracellular activity of GzmA. This notion is supported by the fact that the K_i value for the PSTI–GzmA complex determined by Dixon plot [16] (34 ± 7 nM) is similar to the concentrations of PSTI occurring in the serum of patients with acute pancreatitis (15–500 nM) [5].

In the present study, we showed that inhibition of rGzmA by PSTI occurs in the presence of sulphated glycosaminoglycans. GzmA was detected as a free form and as a complex with proteoglycans containing chondroitin sulphate A [26] and, in the plasma of patients with activated CTLs and NK cells, the free, but not the complexed, form appeared to be rapidly inactivated by circulating anti-proteases such as ATIII (approx. 65 kDa) [27], a candidate physiological inhibitor for GzmA [15]. In addition, Vettel et al. [28] reported charge-dependent binding of purified GzmA to the basement membranes of murine kidney. The binding was not observed in the presence of fucoidan, a sulphated glycosaminoglycan from algae that binds strongly to GzmA, and it was proposed that the binding of GzmA to the basement membrane containing proteoglycans protects the enzyme from inhibition by macromolecule inhibitors. In the present study, however, chondroitin sulphate A alone failed to reduce the inhibitory action of ATIII towards rGzmA, suggesting that binding of the sugar chains to the enzyme is insufficient for the protective effect of the proteoglycans. On the contrary, fucoidan or heparin decreased the inhibition by ATIII. A decrease in the inhibitory action of ATIII by heparin (1 mg/ml) was also shown when purified human GzmA was used [15]. These findings indicate that sulphated glycosaminoglycans, such as fucoidan or heparin, mimic the *in vivo* effect of the proteoglycans. The absence of any effect of fucoidan or heparin on PSTI activity (Figure 5B) can be, at least in part, attributed to the smaller size of the inhibitor (6.5 kDa), because a synthesized smaller inhibitor was also shown to inhibit GzmA activity, including enzyme bound to basement membrane [28].

It remains uncertain whether the cell-surface localization of GzmA occurs in intact tissues. We found that GzmA could be extracted when the dispersed rat small-intestinal cells were incubated in the presence of fucoidan at the concentration of 1 mg/ml (S. Tsuzuki, H. Hirayasu and T. Fushiki, unpublished work), suggesting the binding of the enzyme to negatively charged molecules on the cell surface. If it occurs *in vivo*, this might be a mechanism by which the enzyme is protected from inhibition by anti-proteases. At present, however, we cannot conclude this, because no apparent immunoreactivity for GzmA has been demonstrated on the surface of epithelial cells (or lymphocytes themselves) in the rat small intestine (Figure 4B). We have reported that intraduodenal infusion of a rat PSTI (designated as monitor peptide) stimulates the release of cholecystokinin (CCK) into blood from rat small intestine and have suggested that the action on CCK release is mediated by an unknown receptor on the luminal surface of CCK-producing cells [29,30]. It is, however, unlikely that GzmA serves as the receptor for monitor peptide/PSTI to mediate the hormone release, because the enzyme is not thought to reach the luminal surface of small-intestinal epithelial cells, including CCK-producing cells, after release from the intraepithelial lymphocytes in the intact tissue.

In the present study, we found that the binding protein for PSTI is identical with GzmA and that PSTI inhibited the proteolytic activity of rGzmA. The inhibition of GzmA activity by PSTI is strong enough to occur *in vivo* and it may occur even if GzmA is complexed with proteoglycans. The present results provide new insight regarding the role of PSTI outside the pancreas.

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