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Expression and functional analysis of rat P23, a gut hormone-inducible isoform of trypsin, reveals its resistance to proteinaceous trypsin inhibitors

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Abstract

Rat P23 is an isoform of trypsin (ogens) synthesized by rat acinar cells. Expression of P23 is stimulated strongly by caerulein, an analogue of cholecystokinin (CCK). However, the physiological relevance of rat P23 in healthy and pathological conditions such as caerulein-induced pancreatitis is largely unknown. Using recombinant P23 trypsinogen and reconstitution analysis of zymogen autoactivation, unique inhibitor-resistance characteristics of P23 were elucidated. P23 cDNA was expressed in *Escherichia coli* periplasm, yielding recombinant P23 trypsinogen. Autoactivation of zymogen granule contents from caerulein-induced rat pancreas was also studied. Activation kinetics of P23 by enterokinase was similar to those of rat anionic trypsinogen, which is a major isoform of trypsinogen. Interestingly, rat pancreatic secretory trypsin inhibitor (PSTI), which protects against deleterious activation of trypsinogens in zymogen granules, failed to inhibit P23 trypsin even with four-fold molar excess, at which concentration it effectively inhibited rat anionic trypsin to almost 100%. P23 trypsin also showed marked resistance to proteinaceous trypsin inhibitors such as soybean trypsin inhibitor and aprotinin. P23 trypsin activated by enterokinase dramatically accelerated the cascade of autoactivation of anionic trypsinogen even in the presence of PSTI. Taken together with a previous observation that P23 is specifically upregulated 14-fold by 24-h caerulein infusion, these results suggest that elevated levels of P23 should be taken into consideration in the mechanism of trypsinogens within the pancreas in pathological conditions.

Keywords: Trypsin; Gut; Rat P23

1. Introduction

P23 was found originally in rat exocrine pancreas by two-dimensional isoelectric focusing and SDS-PAGE to be an unidentified protein with Mr=23,000 and an isoelectric point (p*I*) of 6.2 [1]. P23 is expressed only in trace amounts (<0.4% of total secretory proteins) in the unstimulated pancreas while caerulein, an analog of the physiological pancreatic secretagogue cholecystokinin (CCK), causes an acute 14-fold induction of P23 protein synthesis, so that it comprises up to 2% of total secretory proteins [1]. By this criterion, P23 is the most strongly regulated protein of the rat exocrine pancreas. Molecular cloning using a specific antibody against P23 revealed that rat P23 cDNA encodes a novel isoform of trypsinogen, whose similarities in amino acid sequence to two anionic (trypsinogen 1 and 2) and one basic (trypsinogen 3) isoform previously identified in the rat pancreas are 67%, 68% and 72%, respectively [2]. P23 contains all the major structural features of other trypsins [2].

Thus far, the physiological role and hormonal regulation of P23 are relatively unknown. Here, we describe the experimental expression and functional analysis of rat P23 and its unique inhibitor resistance.

2. Materials and methods

2.1. Materials

Rat anionic trypsinogen and pancreatic secretory trypsin inhibitor (PSTI) were purified from pure rat pancreatic juice collected from rats under anesthesia as described in Ref. [3]. Purified bovine trypsin (Type III) was purchased from Sigma. Enterokinase was purchased from NEB. A synthetic trypsin substrate, benzoyl-arginine-*p*-nitroanilide (BApNA) and an expression inducer, isopropyl-beta-D(−)-thiogalactopyrano-

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side (IPTG) were purchased from Wako Pure Chemicals. Protein concentration was determined using Bio-Rad protein assay reagent. Recombinant enterokinase and reagents for DNA manipulation were purchased from NEB. FOY-305 was provided from Ono Pharmaceutical Co., Ltd.

2.2. Construction of a P23 expression vector in Escherichia coli periplasm

Two oligonucleotide primers were used for polymerase chain reaction (PCR). The sense primer 5′-GATGACAAG-CTTGTTGGAGGCTACA-3′, corresponding to amino acid residues 21–29 of P23, was designed to contain *Hin*dIII restriction sites. M13–20 primer was utilized as an antisense primer. PCR was performed using P23 cDNA cloned into pBluescript [2] as a template, with Taq/Pfu polymerases. Samples were amplified for 25 cycles under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C. A DNA fragment of approximately 750 bp was treated with *Hin*dIII, isolated by electrophoresis on a 0.7% agarose gel, and recovered with a Gene Clean kit (Bio 101). The fragment was subcloned into the prokaryotic expression vector pFlag-1 (IBI) at its *Hin*dIII site as described previously [4]. The DNA sequence was determined to confirm insertion and integrity of the P23 sequence. Restriction enzyme analysis was also performed to determine the orientation of the inserts. Both constructs with normal and reversed (as a control) orientations relative to the promoter in pFlag-1 were selected for the following expression study.

2.3. Expression of P23 protein in E. coli periplasm

E. coli JM109 strains were transformed by pFlag carrying P23 or reversed P23 inserts using a standard calcium chloride method. Transformants were selected on plates containing ampicillin (50 μg/ml). Plasmid DNA was purified from these transformants and restriction patterns were checked with several restriction enzymes for DNA integrity. Transformants were preincubated at 37 °C with vigorous shaking in LB media with ampicillin to achieve log phase growth, at which point IPTG was added (0.05 mM). Periplasmic expression and purification were performed as described in Ref. [4]. Briefly, cells were harvested by centrifugation after 3 h incubation with IPTG in the presence of 5 mM reduced glutathione. Cells were washed with a Tris–HCl buffer, pH 7.5 containing 1 mM EDTA, and then treated with lysozyme (0.1 mg/ml) in the same buffer at 4 °C for 30 min. Periplasmic proteins were recovered in the supernatant after centrifugation at 10,000×*g* for 20 min. Expressed P23 was purified further by two cycles of sedimentation with ammonium sulfate and by HPLC with a TSK3000 gel filtration column. Elution was monitored by measuring absorbance at 280 nm and performing SDS-PAGE. Fractions with a molecular weight of 23 kDa and trypsin activity were pooled and stored at −20 °C until use. A control transformant with a Flag-1 vector carrying a reversed P23 insert was treated in the same manner and the periplasmic fraction was mock-purified with ammonium sulfate and by HPLC and then used as a control solution for enzyme assay.

2.4. Enzyme assay

The recombinant P23, reversed P23 fraction or purified anionic trypsinogen (100 μg/ml) was activated with enterokinase (1 μg/ml) at 37 °C for 40 min, and then BApNA (2 mM) was added. The mixtures were incubated at 37 °C. At given time points, a portion of each reaction was collected and the reaction stopped by addition of acetic acid (20%). *p*-Nitroanilines liberated by trypsin activity were measured with a spectrophotometer at 410 nm. Dependency of the reactions on enterokinase activation was studied by performing the same reaction in the absence of enterokinase and comparing the results.

2.5. Effects of trypsin inhibitors on the recombinant P23 and native anionic trypsins

Recombinant P23 or purified anionic trypsinogen was activated with enterokinase as described above, and then various proteinaceous or synthetic trypsin inhibitors were added at various inhibitor/enzyme ratios. The reaction mixtures were incubated at 22 °C for 15 min to allow inhibitor/enzyme interaction, and then BApNA was added. Reactions were carried out and stopped as described earlier. Inhibitor-resistant trypsin activity was expressed as a percentage of the activity in control reactions without inhibitors.

2.6. In vitro studies of acceleration of autoactivation by rat P23 trypsin

Highly purified rat anionic trypsinogen (90 μg/ml) was incubated with recombinant P23 trypsinogen (10 μg/ml) for various periods of time in the presence or absence of enterokinase (0.1 μg/ml) and/or purified rat PSTI (2 μg/ml) at 37 °C. Experiments conducted without addition of P23 trypsinogen were controlled by addition of an equivalent amount of anionic trypsinogen (10 μg/ml), in order to maintain a total of 100 μg/ml trypsinogen in the reaction mixtures. The amount of P23 trypsinogen to be added was estimated to be similar to those found in rat pancreatic secretions after 24-h stimulation by caerulein (about one-tenth of total anionic trypsinogens) [1]. The amount of PSTI to be added was made to be similar to physiological conditions, which effectively inhibits about 5% of total trypsin activity in pancreatic secretions [5]. At given time points, portions of the samples were collected and assayed with BApNA for activated trypsin activity. Trypsin-like activity of enterokinase toward BApNA was negligible.

3. Results

3.1. Expression of P23 trypsinogen as a secretory protein in E. coli periplasm

E. coli transformed with pFlag-1 carrying P23 cDNA produced a protein with a molecular mass corresponding to a trypsinogen (23 kDa) in the periplasm, representing about 10% of all periplasmic proteins upon IPTG induction (Fig. 1, lane 1 vs. 2). As a control, *E. coli* with pFlag-1 carrying reverse-oriented P23 cDNA did not express any significant protein at the corresponding molecular weight with or without IPTG (Fig. 1, lanes 3 and 4). Western blotting using polyclonal anti-dog trypsinogen antibody was weakly positive, confirming that the 23-kDa band in lane 2 is a trypsinogen-related molecule (data not shown). A large portion (>90%) of the expressed P23 protein remained in insoluble forms in the bacteria even after lysis with 1% Triton X-100. SDS-PAGE with beta-mercaptoethanol showed that inclusion bodies contained a protein with a molecular mass of about 25 kDa, suggesting that the soluble form of P23 was translocated to the periplasm and an Omp A signal peptide (with a predicted size of 2046 Da for 21 amino acids) was released, resulting in formation of a proenzyme (P23 trypsinogen) form in the periplasm. The apparent low efficiency of translocation may be due to saturation of the *E. coli* translation machinery with a high level of exogenous protein expression. The expressed P23 trypsinogen in the periplasm was further purified by two rounds of ammonium sulfate precipitation and HPLC gel filtration, producing a homogenous sample to be used for enzymatic assays.

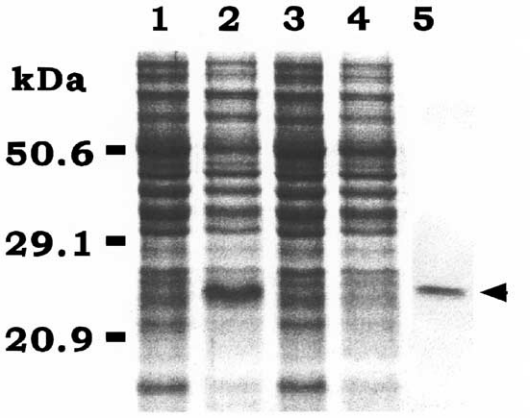

Fig. 1. Expression of recombinant P23 trypsinogen in *E. coli* periplasm. *E. coli* transformed with pflag-1 carrying P23 cDNA (lanes 1 and 2) or reverse-oriented p23 cDNA (lanes 3 and 4) were incubated in LB media with (lanes 2 and 4) or without (lanes 1 and 3) IPTG. Cells were harvested and the periplasmic fractions were subjected to 10% SDS-PAGE. Apparent molecular weight shown by prestained molecular markers are indicated as kDa at far left. P23 trypsinogen purified by ammonium sulfate precipitation/HPLC gel filtration is indicated in lane 5 with an arrow head.

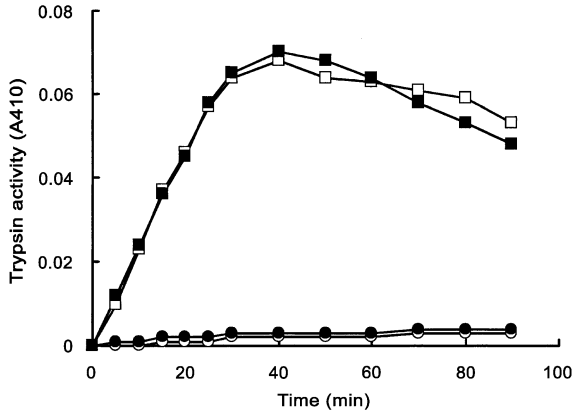

Fig. 2. Enterokinase-dependent activation of rat anionic and p23 trypsinogens. Recombinant rat P23 trypsinogen (closed symbols) or purified rat anionic trypsinogen (open symbols) (100 μg/ml) were incubated in the presence (square) or absence (circle) of enterokinase (1 μg/ml). Samples were taken at given incubation times, mixed with BApNA (2 mM) as a substrate and incubated again at 37 °C for 15 min. *p*-Nitroanilides liberated by trypsin cleavage were measured with a spectrophotometer (Beckman DU-650) at 410 nm. Trypsin activity was expressed as the rate of A410 increase per minute.

3.2. Enteropeptidase-dependent activation of P23 trypsinogen

Expressed P23 trypsinogen showed enteropeptidase-dependent activation, with a time-dependent profile similar to that of purified rat anionic trypsinogen (Fig. 2). A control preparation similarly purified from *E. coli* periplasm transformed with the reversed P23 cDNA did not show any significant trypsin activity either in the presence or absence of enteropeptidase, indicating efficient separation of recombinant P23 from endogenous *E. coli* proteases by the purification (data not shown).

3.3. Inhibition of P23 trypsin with various trypsin inhibitors

The effect of various trypsin inhibitors on P23 trypsin was compared with their effects on rat anionic trypsin and bovine trypsin. Increasing concentrations of inhibitors were incubated with 100 μg/ml of each trypsin, and then the remaining resistant trypsin activities were measured. Rat anionic and bovine trypsins were completely inhibited by equimolar (100 μg/ml) soybean trypsin inhibitor (SBTI), whereas P23 was inhibited by 20–30% with the same concentration of SBTI (Fig. 3A). Similar results were obtained when P23 was mixed with rat pancreatic secretory trypsin inhibitor (rPSTI), a physiological inhibitor present together with trypsinogens in the zymogen granules, possibly to prevent premature trypsin activation. In the presence of a four-fold molar excess of rPSTI (100 μg/ml), control trypsins were inhibited completely, whereas more than 95% of the original P23 activity remained (Fig. 3B). A very similar profile was obtained when these trypsins were challenged by bovine pancreatic trypsin inhibitor (BPTI),

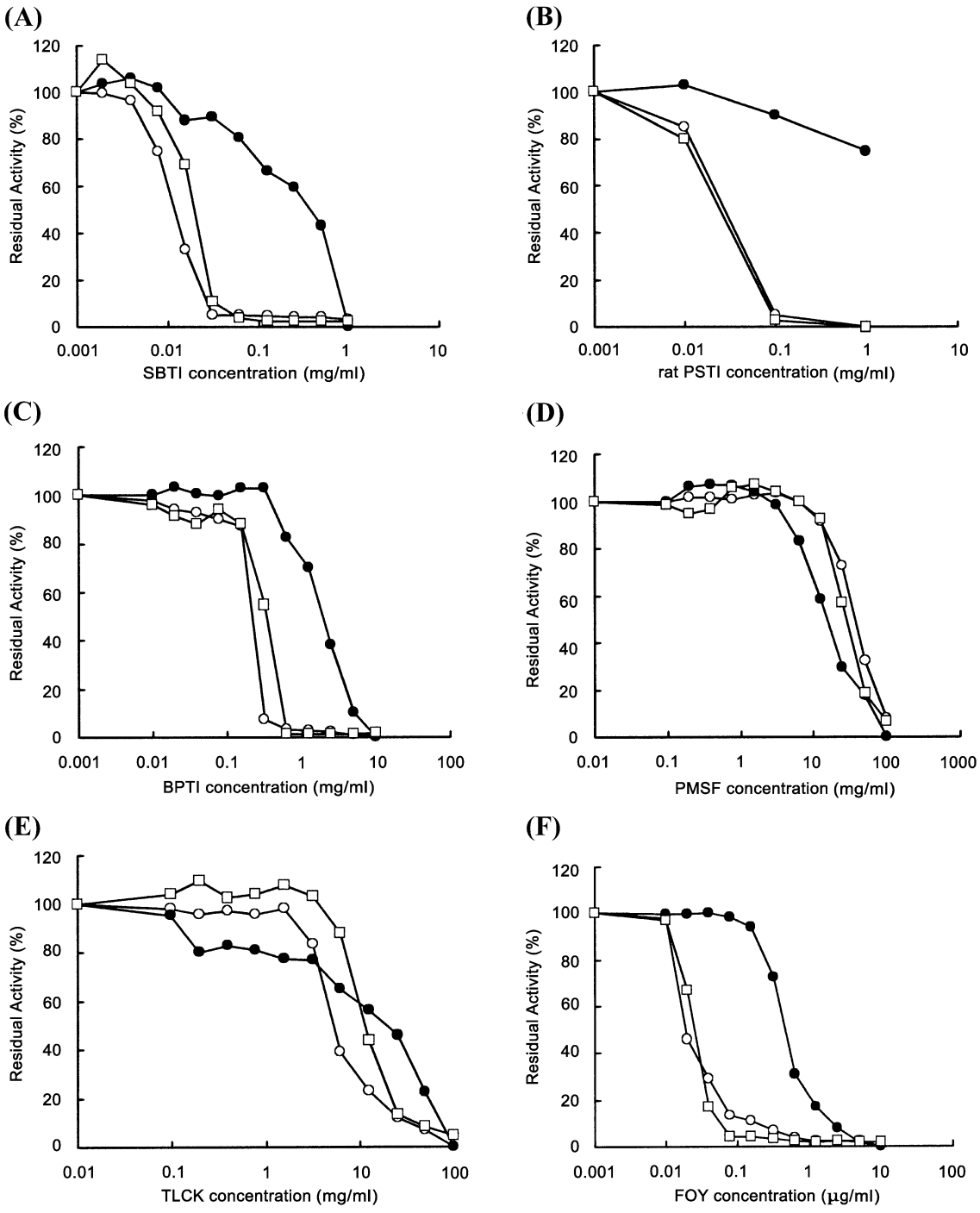

Fig. 3. Inhibition profiles of rat p23 trypsin, rat anionic trypsin and bovine trypsin with various trypsin inhibitors. One hundred microgram per milliliter each of recombinant p23 trypsin (closed circle), purified rat anionic trypsin (open circle), or bovine trypsin (open square) was activated with enterokinase (1 μg/ml) as described and then incubated with one of the given trypsin inhibitors at various concentrations as indicated at 22 °C for 15 min. Residual trypsin activity was assayed using BApNA and expressed as a percentage of total activity without the inhibitor. (A) Soybean trypsin inhibitor (SBTI), (B) rat pancreatic secretory trypsin inhibitor (rPSTI), (C) bovine pancreatic trypsin inhibitor (BPTI/aprotinin), (D) phenylmethylsulfonylfluoride (PMSF), (E) *p*-toluenesulfonyl-l-lysine chloromethyl ketone (TLCK), (F) FOY-305 (*n*,*n*-dimethylcarbamoylmethyl 4-(4-guanidinobenzoyloxy) phenylacetate methane sulfate). Data are expressed as the mean of three experiments, all of which varied less than 5%.

also known as aprotinin or Trasylol, a strong, widely used trypsin inhibitor in both experimental and clinical settings (Fig. 3C). However, P23 was sensitive to synthetic serine protease inhibitors such as PMSF (Fig. 3D) and TLCK (Fig. 3E) at concentrations comparable to those used to inhibit rat anionic trypsin and bovine trypsin, confirming that P23 is a member of the serine protease family. When FOY-305, a low molecular weight inhibitor, was used, rat P23 trypsin

was more resistant than rat anionic and bovine trypsins (Fig. 3F), but was almost completely inhibited at higher FOY-305 concentrations.

3.4. In vitro studies of autoactivation acceleration by rat P23 trypsin

Based on the observation that P23 trypsin shows resistance to PSTI, which is thought to protect against trypsinogen autoactivation, an experiment to assess the ability of increased levels inhibitor-resistant P23 trypsinogen to induce autoactivation was performed in vitro.

When purified anionic trypsinogen was incubated with or without purified recombinant P23 trypsinogen (at 9:1 molar ratio) at 37 °C up to 16 h, trypsin autoactivation was not detected in either sample (Fig. 5). This finding indicates that purified trypsinogens are stable and not activated spontaneously under such conditions. When a small amount of enterokinase, an artificial initiator of activation, was added, anionic trypsinogens, with or without P23 trypsinogen, were all activated very quickly. When similar experiments were performed in the presence of purified PSTI, a physiological inhibitor of trypsin autoactivation, anionic trypsinogen was only slowly activated by enterokinase, indicating that PSTI attenuates the initial rate of trypsin autoactivation. On the other hand, when P23 trypsinogen was present in the reaction mixture, trypsin activation was accelerated dramatically, suggesting that P23 trypsinogen was activated by enterokinase to P23 trypsin, a PSTI-resistant enzyme that could promote the cascade of trypsin autoactivation free from PSTI control. The autoactivation promoted by the activated P23 trypsin was observed to overcome PSTI inhibitory effects on the other trypsinogens after an initial period of time. In conclusion, these results indicate that P23 trypsinogen is stable and does not promote trypsin autoactivation by itself but requires activation by an independent initiator (enterokinase, present in this experimental case but absent in the exocrine pancreas).

4. Discussion

Based on observations presented here, rat P23 trypsin (ogen) appears to be a unique isoform of trypsin, which is resistant to proteinaceous trypsin inhibitors such as PSTI and SBTI. A similar isoform of trypsin has been described in human pancreatic secretions and named mesotrypsin, due to its p*I* of 5.95, which is ‘meso’, or in the middle, between other acidic and basic trypsins [6,7]. Human mesotrypsin is a minor species of pancreatic digestive enzyme and shows resistances to naturally occurring trypsin inhibitors similar to those exhibited in P23. Structural and functional analyses suggest that rat P23 trypsinogen is a functional homologue of human mesotrypsinogen. However, the degree to which human mesotrypsinogen is regulated by CCK or its analogues should be investigated further. Structural comparison of rat P23 and human mesotrypsinogen revealed several unique characteristics. Upon comparison of rat P23 or human mesotrypsin to other known trypsin isoforms, it is found that the catalytic triad of His-63, Asp-107 and Ser-201 (numbers are based on the rat P23 sequence), as well as an obligatory Asp at position 194, are all conserved.

However, at amino acid 198, close to the active center of the enzyme, an Asp residue is found in P23, rather than an Asn, which is conserved throughout all known rat and human trypsins (Fig. 4). This substitution may change the

	195										201
Rat P23 trypsinogen	-Gly-	Gly-	Lys-	Asp-	Ser-	Cys-	Asp-	Gly-	Asp-	Ser-	Gly-
Rat Ta1	-	-	-	-	-	-	Asn	-	-	-	-
Rat Ta2	-	-	-	-	-	-	Asn	-	-	-	-
Rat Tc	-	-	-	-	-	-	Asn	-	-	-	-
Human trypsin 1	-	-	-	-	-	-	Asn	-	-	-	-
Human trypsin 2	-	-	-	-	-	-	Asn	-	-	-	-
Human mesotrypsin	-	-	-	-	-	-	Asn	Arg	-	-	-

Fig. 4. Comparison of amino acid sequences of various trypsin active sites. Number corresponding to amino acid residues in rat p23 trypsinogen are indicated at the top. Residue 195 is an obligatory Asp that determines substrate specificity toward basic amino acids and is conserved in all rat and human trypsins. Residue 200 is the active serine in the triad and is also conserved in all rat and human trypsins. Note the unique Asp-198, instead of Asn, in rat p23 and the unique Arg-199 in human mesotrypsinogen, which may be responsible for their altered interactions with trypsin inhibitors. Ta, anionic trypsin (two different anionic trypsins have been described in rats); Tc, cationic trypsin.

electrical and steric environment in the active center. A similar, in terms of site, but different, in terms of amino acid charge, substitution has been found in human mesotrypsin, which has an Arg instead of the conserved Gly in position 199.

Based upon an X-ray crystallographic structure of a complex between rat trypsin and BPTI [8], the Asp→Asn mutation at position 198 was modeled using the software Quanta (Molecular Simulations). The Van der Waals radii of Asp-198 in rat P23 and Arg-17 in BPTI were found to overlap for all possible rotations (data not shown). This suggests that the two residues cannot co-exist in the complex as it is currently structured, and would disrupt the binding between the enzyme and inhibitor. Further analysis of the change of the microscopic molecular environment in the active center of P23 and mesotrypsin could explain why they are not inhibited by large proteinaceous trypsin inhibitors such as PSTI, BPTI and SBTI, but can be blocked using small synthetic trypsin inhibitors such as PMSF and TLCK.

The fact that P23, a hormone-inducible trypsin isoform, exhibits marked inhibitor resistance implies that it may perform several physiologically relevant functions. If levels of rat P23 trypsin are increased by a specific stimulus such as CCK, caerulein or by pathogenic conditions, the inhibitor-resistant trypsin may cause potentially deleterious effects upon autoactivation within pancreatic tissues, especially in the setting of pancreatitis. Caerulein-induced pancreatitis is a widely acknowledged model as an experimental pancreatitis [9]. Supramaximal doses of CCK, caerulein or cholinergic agonists have been found to induce pancreatic damage manifested by edema, increased serum levels of pancreatic enzymes, inflammation and necrosis in rats [10], mice [11] and even healthy human [12]. Although molecular mechanisms that initiate or apply the form of pancreatitis are largely unknown, it has been pointed out that premature activation of pancreatic zymogens within pancreatic tissues is a key step for progression of the pathological consequence [9]. When P23 is turned out to be unique PSTI-resistant trypsin, one can speculate that activated P23 trypsin is able to further cleave trypsinogens to trypsins in the presence of PSTI or other endogenous trypsin inhibitors, and thus promote the activation cascade. Rat PSTI mRNA expression has been shown to be upregulated by CCK [13], it only occurs by one- to two-fold and is much less responsible than the expression of P23 induced by CCK analogue. Parallel regulation of anionic trypsinogen and PSTI was also observed under various dietary conditions in rats [14]. However, the ratio between the rates of P23 and PSTI expression might be much higher (P23≫PSTI) in the setting of caerulein–infusion, resulting in insufficient amounts of PSTI to attenuate the cascade of trypsin autoactivation promoted by activated P23. In vitro studies shown in Fig. 5 demonstrate the possibilities that imbalance between P23 and PSTI cause the promotion of trypsinogen activation cascade.

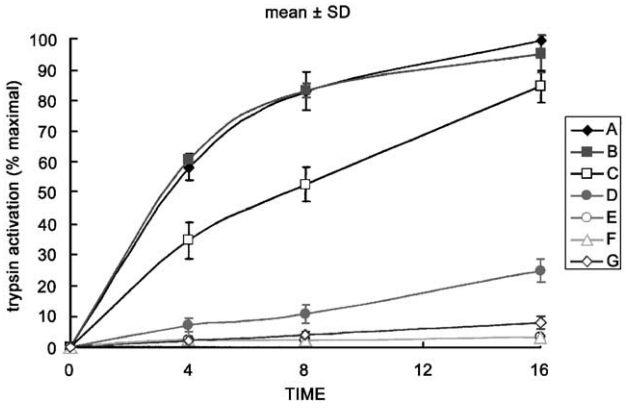

Fig. 5. In vitro studies of autoactivation acceleration by rat P23 trypsin. Highly purified rat anionic trypsinogen (Ta) (90 μg/ml) was incubated with recombinant P23 trypsinogen (10 μg/ml) in the presence or absence of enterokinase (0.1 μg/ml) and/or purified rat PSTI (2 μg/ml) at 37 °C: (A), (C) and (E). The experiments lacking P23 trypsinogen were compensated by addition of an equivalent amount of anionic trypsinogen (10 μg/ml) in order to maintain a total of 100 μg/ml trypsinogen in every reaction mixture: (B), (D) and (F). Increment of P23 trypsinogen (10 μg/ml) alone was also measured in the presence of enterokinase (0.1 μg/ml) and purified rat PSTI (2 μg/ml): (G). At given time points, portions of the samples were collected and assayed with BApNA for activated trypsin activity. (A) Ta+enterokinase+P23; (B) Ta+enterokinase; (C) Ta+enterokinase+PSTI+P23; (D) Ta+enterokinase+PSTI; (E) Ta+P23; (F) Ta alone; (G) P23+enterokinase+PSTI. Data are expressed as percentages of maximal activation and reported as the means of four experiments ± 1 S.D.

In conclusion, rat P23 is found to be a unique PSTI-resistant trypsin. Taken together with the fact that P23 is upregulated 14-fold after 24-h caerulein infusion, these results suggest that rat P23 plays an important role in the progression of the experimental animal model of caerulein-induced pancreatitis. An in vitro autoactivation assay system and recombinant P23 will be useful tools for further investigation of the mechanism underlying its pathogenesis.

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