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HUMAN MESOTRYPSIN DEFIES NATURAL TRYPSIN INHIBITORS. FROM PASSIVE RESISTANCE TO ACTIVE DESTRUCTION.

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Abstract

More than twenty years ago Rinderknecht et al. identified a minor trypsin isoform resistant to natural trypsin inhibitors in the human pancreatic juice. At the same time, Estell and Laskowski found that an inhibitor-resistant trypsin from the pyloric caeca of the starfish *Dermasterias imbricata* rapidly hydrolyzed the reactive-site peptide bonds of trypsin inhibitors. A connection between these two seminal discoveries was made recently, when human mesotrypsin was shown to cleave the reactive-site peptide bond of the Kunitz-type soybean trypsin inhibitor and degrade the Kazal-type pancreatic secretory trypsin inhibitor. These observations indicate that proteases specialized for the degradation of protease inhibitors are ubiquitous in metazoa and prompt new investigations into their biological significance. Here we review the history and properties of human mesotrypsin and discuss its function in the digestive degradation of dietary trypsin inhibitors and possible pathophysiological role in pancreatitis.

Discovery

In 1978 Rinderknecht et al. identified mesotrypsin as a minor, inhibitor-resistant trypsin found in human pancreatic tissue and fluid [1]. The bulk of trypsinogen secreted by the human pancreas consists of two major isoforms, commonly referred to as cationic trypsinogen (pI = 6.2 – 6.4) and anionic trypsinogen (pI = 4.4 – 4.9), on the basis of their relative isoelectric points [2]. The catchy name “mesotrypsinogen” was coined [3] to reflect the intermediate pI (5.5 – 5.7) and electrophoretic mobility of “zymogen X” [4], the pro-enzyme of the novel inhibitor-resistant trypsin.

Genomics, transcriptomics, proteomics

The human trypsinogen genes are intercalated between the beta T cell receptor genes on chromosome 7 [5,6]. Cationic and anionic trypsinogen are encoded by the *PRSS* (protease, serine) genes *PRSS1* (OMIM #276000) and *PRSS2* (OMIM #601564), respectively. Interestingly, the *PRSS3* gene encoding mesotrypsinogen translocated from chromosome 7q34 to chromosome 9p11.2 [5,6]. As a result, the mesotrypsinogen gene acquired an alternative exon-1, located approximately 45 kilobases upstream of the original exon-1 (Fig 1). Note that the earlier used *PRSS4* gene designation for mesotrypsinogen with the alternative exon-1 is incorrect, because it would indicate that two separate genes exist for mesotrypsinogen [6]. The proper gene name is *PRSS3*, which can give rise to two different mRNAs generated through alternative splicing and/or alternative promoter use.

Numbering of amino-acid residues in the proteins discussed here is based on the primary translation product of the mRNA, and thus starts with Met1. The reactive sites of soybean trypsin inhibitor and human pancreatic secretory trypsin inhibitor correspond to peptide bonds Arg63-Ile64 and Lys18-Ile19 in the mature proteins, respectively. Arg198 in mesotrypsinogen is designated as Arg193 in the crystallographic numbering [12].

A cDNA coding for mesotrypsinogen was first cloned in 1990 [7], but the reported sequence (trypsinogen III) contained several mistakes and the correct mesotrypsinogen cDNA was described in 1997 [8]. An alternatively spliced cDNA of mesotrypsinogen in which exon-1 encoding the secretory signal-peptide is replaced by the alternative exon-1 was cloned from the human brain [9]. Because of nucleotide differences from the incorrectly sequenced trypsinogen III, the “brain trypsinogen” was named trypsinogen IV. This nomenclature remains acceptable, because mesotrypsinogen and trypsinogen IV are, in fact, different proteins. However, it is important to point out that there is no evidence for the secretion and/or activation of trypsinogen IV, which might even have a function unrelated to proteolytic activity [6]. Expression at the mRNA level of both mesotrypsinogen splice variants is also confirmed by a multitude of EST clones. It appears that the pancreas exclusively expresses the secretory form of mesotrypsinogen, while the brain and other extrapancreatic tissues express the alternatively spliced trypsinogen IV. The use of the alternative exon-1 is probably facilitated by the defective splicing site (GCGAGT) at the 5' end of intron-1 (Fig 1). Available evidence suggests that transcription of alternative exon-1 is initiated at multiple sites and translation of trypsinogen IV might be initiated from a non-conventional CTG codon [8, Z. Nemoda, and M. S.-T., unpublished observations].

At the protein level, the secretory form of mesotrypsinogen was isolated from the pancreas by gel electrophoresis and expression levels were reported between 3 and 10 % of the total trypsinogen content in normal pancreatic juice [2–4,10]. Elevated mesotrypsinogen levels were found in the pancreatic juice of chronic alcoholics [4,10] and decreased levels were reported in patients with chronic pancreatitis [4]. The presence of trypsinogen IV was demonstrated by immunostaining in PC-3 and SW480 epithelial cell lines and association with cytoplasmic vesicles was observed [11]. In agreement with the absence of a secretory signal peptide, no secretion of trypsinogen IV was detected from these cell lines. It remains unclear whether trypsinogen IV can acquire a trypsinogen-like fold inside the cell.

Crystal structure

The crystal structure of mesotrypsin complexed with benzamidine was solved in 2002 [12]. This study provided the first compelling evidence that the presence of an arginine residue in place of Gly198 (Gly193 in the chymotrypsinogen numbering system) is responsible for the peculiar inhibitor resistance of mesotrypsin. Gly198 is highly conserved among chymotrypsin-like proteases, including all known vertebrate trypsinogens. Arg198 in mesotrypsin occupies the S2' subsite and its long side-chain sterically clashes with protein inhibitors (see Figure 4 in ref [12]). Furthermore, the charge of the guanidino group may also affect inhibitor binding by contributing to the strong clustering of positive charges around the primary specificity pocket of mesotrypsin.

Inhibitor resistance

Arguably, the most intriguing property of mesotrypsin has been its characteristic resistance to naturally occurring polypeptide inhibitors. Mesotrypsin is readily inhibited by small-molecular weight trypsin-inhibitors; e.g. benzamidine, *p*-amino benzamidine, *N*^α-*p*-tosyl-L-lysine chloromethyl ketone; and leupeptin; or general serine-protease inhibitors, such as diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride [3,8,12]. In contrast, standard-mechanism protease inhibitors tested to date are unable to form a tight complex with mesotrypsin. These include bovine pancreatic trypsin inhibitor (Kunitz), human pancreatic secretory trypsin inhibitor (Kazal), soybean trypsin inhibitor (Kunitz) (Fig 2, left panel), lima bean inhibitor, canine submandibular gland inhibitor, chicken ovomucoid, Alzheimer precursor protein trypsin inhibitor domain, and ecotin [3,8,12,13]. Mesotrypsin is also resistant to the serum serpin alpha-1 antitrypsin [3]. The term “resistant” is misleading, because natural

trypsin inhibitors still exhibit significant affinity towards the enzyme, although the equilibrium dissociation constant (K_D) is 2–7 orders of magnitude increased (e.g. see Table 2. in [12]). The relatively weak inhibition is generally not apparent in laboratory experiments when mesotrypsin is pre-incubated with the inhibitor followed by dilution into high concentrations of a small turnover substrate. Under these conditions the loose inhibitory complexes rapidly dissociate and no inhibition is observed (Fig 2, left panel). To demonstrate and quantify the inhibitory effect of soybean trypsin inhibitor (Kunitz) on mesotrypsin, catalytic parameters (K_M , k_{cat}) of mesotrypsin were measured with the chromogenic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide in the presence of increasing concentrations of the inhibitor (Fig 2, right panel). By plotting the apparent K_M values against the inhibitor concentration, a K_i value of 0.42 μ M was estimated, which was identical to the K_i value determined by Katona et al. using progress curve analysis [12]. The results clearly indicate that mesotrypsin retained low but significant affinity toward trypsin inhibitors, and dispute the notion that mesotrypsin can act uncontrolled in the presence of inhibitors. The long misunderstood inhibitor resistance of mesotrypsin created a number of unrealistic models for the enzyme's biological function. In these models mesotrypsin was supposed to activate or degrade other zymogens in the pancreas or catalyze protein digestion in the gut; unaffected by the presence of trypsin inhibitors [3,8].

Substrate specificity

Mesotrypsin possesses true trypsin-like specificity and hydrolyzes peptide bonds after Arg and Lys residues. Commercial chromogenic or fluorogenic peptide substrates routinely used for trypsin activity measurements are typically cleaved by mesotrypsin with high catalytic efficiency. Mesotrypsin often hydrolyzes small peptide substrates with no P' subsite contacts at significantly higher rates than other trypsins [3,13]. Consequently, using turnover substrates for the determination of mesotrypsin concentration by comparing to a trypsin “standard” can be inaccurate.

In contrast to its high activity on peptide substrates, mesotrypsin cleaves protein substrates poorly. Intuitively, it seems straightforward that if Arg198 perturbs binding of polypeptide inhibitors to mesotrypsin, the same should apply to polypeptide substrates as well. However, the “normal” catalytic activity of mesotrypsin on laboratory trypsin substrates has shrouded its defective activity on natural substrates for decades, despite early observations that mesotrypsin cannot activate chymotrypsinogen [3]. More recent experiments unequivocally confirmed that mesotrypsin is a defective digestive trypsin, when compared to other pancreatic trypsins [13,14]. Interestingly, however, the extent of the “digestive defect” varies greatly among protein substrates tested. Pancreatic zymogens (chymotrypsinogen, trypsinogen, proelastase) are activated by mesotrypsin to their respective active enzymes at rates that are 500–1000-fold lower than activation by cationic or anionic trypsin [13,14]. Mesotrypsinogen does not exhibit any appreciable autoactivation either. Mesotrypsin-mediated cleavage of the Arg122-Val123 peptide bond in human cationic trypsinogen is also 500-fold slower when compared to cationic trypsin [13]. On the other hand, mesotrypsin degrades human anionic trypsinogen only 20-fold slower than human cationic trypsin [13]. Finally, we found that the rate of liberation of trichloroacetic acid-soluble peptides from casein is ca. 3-fold decreased relative to anionic or cationic trypsin (R. Szmola and M. S.-T., unpublished observations).

Role of Arg198

First, on the basis of sequence alignment with other trypsins, it has been suggested that Arg198 is the major determinant of the inhibitor resistance exhibited by mesotrypsin against proteinaceous trypsin inhibitors [8]. Subsequently, this notion gained support from the analysis of the mesotrypsin crystal structure (see above) [12]. Finally, direct experimental evidence for the role of Arg198 came from site-directed mutagenesis of Arg198 to Gly in mesotrypsin

[13]. The resulting R198G-mesotrypsin mutant became fully sensitive to soybean trypsin inhibitor (Kunitz) (Fig 2) and human pancreatic secretory trypsin inhibitor (Kazal). Furthermore, R198G-mesotrypsin activated chymotrypsinogen almost as well as cationic or anionic trypsin, indicating a complete restoration of the digestive capacity of mesotrypsin. In conclusion, the evolutionary selection of Arg198 is solely responsible for the unique inhibitor resistance and restricted substrate specificity of mesotrypsin [13].

Digestion of trypsin inhibitors

Mesotrypsin rapidly hydrolyzes the reactive-site peptide bond of soybean trypsin inhibitor (Kunitz)

As detailed in the accompanying articles of this issue, in complexes of proteases and standard-mechanism protease inhibitors, the reactive-site peptide bond of the inhibitor undergoes slow hydrolysis, resulting in an equilibrium mixture of double-chain “modified” inhibitor and single-chain “virgin” inhibitor [see also ref [15] and references therein]. Normally, this process is extremely slow and can take months to reach equilibrium. In 1980 Estell and Laskowski found that an inhibitor-resistant trypsin from the starfish *Dermasterias imbricata* (leather star) cleaved the reactive sites of soybean trypsin inhibitor (Kunitz) and bovine pancreatic trypsin inhibitor (Kunitz) at significantly increased rates [16,17]. We hypothesized that mesotrypsin might be a functional ortholog of the starfish trypsin, which recognizes protein trypsin inhibitors as substrates and rapidly hydrolyzes their reactive-site peptide bonds. In the experiment shown in Fig 3, mesotrypsin was incubated with a 50-fold molar excess of soybean trypsin inhibitor at 37 °C. At indicated times aliquots were withdrawn, mixed with bovine trypsin and incubated at room temperature for 1 min before the residual trypsin activity was measured. Because association of the modified (cleaved) soybean trypsin inhibitor to bovine trypsin is considerably slower than association of the virgin inhibitor, the inhibitory activity detected after 1 min incubation with bovine trypsin is a specific measure of the virgin soybean trypsin inhibitor concentration [18]. A rapid decrease in the concentration of virgin inhibitor was observed, and the reaction reached a plateau in about 20 min, with approximately 40 % of the original virgin inhibitor remaining, suggesting that the cleaved inhibitor might inhibit further conversion. SDS-PAGE analysis and N-terminal sequencing confirmed that mesotrypsin cleaved the Arg88-Ile89 reactive-site peptide bond of the inhibitor, yielding an equilibrium mixture of approximately 40–44 % virgin and 56–60 % modified inhibitor. At the same pH but under somewhat different assay conditions (0.5 M KCl, 21 °C) the Laskowski laboratory reported a hydrolysis equilibrium containing 78 % modified inhibitor after digestion with *Dermasterias imbricata* trypsin-1 or bovine trypsin [16].

Mesotrypsin degrades human pancreatic secretory trypsin inhibitor

The Kazal-type pancreatic secretory trypsin inhibitors also belong to the standard-mechanism trypsin inhibitors, but in contrast to soybean trypsin inhibitor (Kunitz), which forms stable complexes with trypsin, the secretory inhibitors are so-called “temporary inhibitors”, because they irreversibly dissociate from the protease over time [19]. Temporary inhibition follows a relatively rapid time-course ($t_{1/2}$ 2–4 hours) when trypsin is in some excess to the inhibitor, however, at lower trypsin-to-inhibitor ratios the reaction proceeds dramatically slower [19]. For example, when cationic or anionic trypsin was reacted with human pancreatic secretory inhibitor at a 1:10 ratio, no trypsin activity was detectable up to 90 hours, and free inhibitor levels did not change measurably either [13]. The mechanism of temporary inhibition involves a series of ordered trypsinolytic cleavages. First, reversible digestion of the Lys41-Ile42 reactive-site peptide bond must occur, which is followed by the irreversibly inactivating cleavage of the Arg67-Gln68 bond [20–22]. In addition, peptide bonds Arg28-Glu29, Arg65-Lys66 and Lys75-Ser76 are also subject to tryptic attack [20–22]. As shown in Fig 3, incubation of mesotrypsin with a 10–20-fold excess of pancreatic secretory trypsin inhibitor results in the

gradual elimination of the inhibitor. The two chains generated by cleavage of the reactive-site peptide bond are only transiently visible on the reducing SDS-tricine polyacrylamide gel, and eventually the inhibitor is digested to peptides too small to resolve. N-terminal sequencing of the peptides confirmed that mesotrypsin-mediated degradation of pancreatic secretory inhibitor follows the established mechanism of “temporary inhibition”, but at a markedly higher rate. It is interesting to compare how mesotrypsin and “normal” trypsins degrade pancreatic secretory trypsin inhibitors. Trypsin forms a tight complex with the inhibitor and the obligatory cleavage of the reactive-site bond is the slowest, rate-limiting step, which is then followed by rapid digestion of other inactivating sites. In contrast, mesotrypsin hydrolyzes the reactive-site bond at a high rate, but due to its “digestive defect”, the subsequent inactivating cleavages occur more slowly and thus determine the rate of the overall inhibitor inactivation.

Activation of mesotrypsinogen to mesotrypsin

The physiological activator of mesotrypsinogen is enterokinase (enteropeptidase), which is localized to the brush border membrane of enterocytes in the duodenum and activates all pancreatic trypsinogens secreted. In contrast to cationic and anionic trypsinogen, mesotrypsinogen cannot autoactivate [13]. Activation by anionic or cationic trypsin is also slow and inefficient, and results in the degradation of mesotrypsinogen to a significant extent [13]. In the absence of Ca^{2+} , cationic or anionic trypsin degrade mesotrypsinogen within minutes. When trypsinolytic degradation of the three pancreatic trypsinogens are compared, anionic trypsinogen and mesotrypsinogen show similar stability, while cationic trypsinogen is 10–20-fold more resistant [23,24]. Mesotrypsinogen is also activated by the lysosomal cysteine protease cathepsin B, which is responsible for trypsinogen activation in experimental pancreatitis [25,26]. The activation time-course is biphasic, a rapid initial activation is followed by a slower degradation (Fig 4). The initial rate of activation is at least 2-fold higher relative to cationic trypsinogen and 6-fold faster when compared to anionic trypsinogen.

Physiological role

Degradation of dietary trypsin inhibitors appears to be the only role of pancreatic mesotrypsin [13]. Obviously, such a function would be advantageous in the digestion of foods rich in naturally occurring trypsin inhibitors, and would provide a rationale for the evolution of this trypsin isoform. In most cases, cleavage of the reactive-site peptide bond already results in an apparent loss of inhibition, as the modified inhibitor associates with trypsin more slowly. Some inhibitors can be then further digested by mesotrypsin, while others might be degraded in concert with other digestive proteases. A known example is carboxypeptidase B, which can cleave off the newly generated C-terminal Lys or Arg residues after hydrolysis of the reactive-site peptide bonds and thus completely inactivate trypsin inhibitors (see also discussion in [16]). From the experimental data it appears that mesotrypsin-mediated degradation of inhibitors becomes significant at high inhibitor concentrations that exceed active trypsin levels in the duodenum. At lower concentrations, inhibitors will be complexed by the secretory trypsins. Similarly, elimination of endogenous pancreatic secretory trypsin inhibitor secreted to the duodenum probably occurs through the “temporary inhibition” mechanism by major trypsin isoforms and not by mesotrypsin-mediated degradation. Finally, because of its digestive defect, mesotrypsin cannot play a significant role in the degradation of dietary proteins, given the higher catalytic efficiencies and larger abundance of cationic and anionic trypsins.

Extrapancreatic mesotrypsin

A recent study suggested that extrapancreatic mesotrypsin might be a specific activator of protease activated receptors 2 and 4 on epithelial cells [11]. The authors based their conclusion on the identification of trypsinogen IV in epithelial cell lines and the demonstration that mesotrypsin activates protease-activated receptors. However, as discussed above, it is unclear

whether a secretory form of mesotrypsin is expressed in extrapancreatic tissues; and properties of the presumably intracellular “trypsinogen IV” are even more obscure. Furthermore, independent studies comparing activation of protease-activated receptors with the three human pancreatic trypsins demonstrated that cationic and anionic trypsins catalyze robust activation, while mesotrypsin is ineffective (Z. Grishina, W. Halangk, M. S.-T., G. Reiser - in preparation). Thus, this question remains controversial, awaiting further studies.

Paneth cells, the secretory epithelial cells of the small intestine, were also shown to express trypsinogen IV and anionic trypsinogen mRNA. The Paneth cell trypsins were proposed to be the processing enzymes for the antimicrobial peptide defensin-5 [27].

Biotechnological applications

The inhibitor-degrading enzyme of the starfish has been patented for the purpose of inhibitor degradation in food samples [28]. The patent claims “A method for overcoming trypsin inhibition by soybean trypsin inhibitor in soy based foodstuffs, which method comprises treating the soybean meal used in said foodstuff with a proteolytically effective amount of starfish trypsin 1 and with a proteolytically effective amount of supplementary proteolytic enzyme.” The supplementary enzyme was carboxypeptidase B. In another claim, the authors effectively propose artificial mesotrypsin supplementation: “A method of overcoming trypsin inhibition by soybean trypsin inhibitor in a subject animal, which method comprises administering orally to said animal a proteolytically effective amount of, or a composition containing an proteolytically effective amount of, starfish trypsin 1 in a dosage form resistant to the digestive conditions of the stomach.”

In addition to inhibitor degradation, the unique substrate specificity of mesotrypsin among the trypsin-like proteases can be exploited in proteomics, which relies heavily on the use of specific proteases coupled with mass spectrometric analysis.

Pathological role

The idea of mesotrypsin roaming the pancreas unopposed by trypsin inhibitors has fascinated scientists since its discovery. In two conflicting theories, it was proposed that premature activation of mesotrypsin in the pancreas might cause or protect against pancreatitis, as the inhibitor-resistant trypsin activity can freely activate or degrade other pancreatic zymogens [3,8]. Experimental data clearly indicate that mesotrypsin cannot activate pancreatic zymogens (trypsinogen, chymotrypsinogen, proelastase) and degradation of human trypsinogens by mesotrypsin is also defective [3,13,14]. Thus, mesotrypsin is unlikely to play a role in activation or degradation of pancreatic zymogens.

On the other hand, the observation that mesotrypsin digests trypsin inhibitors raises the possibility that mesotrypsin could contribute to the pathogenesis of human pancreatitis by reducing the protective levels of pancreatic secretory trypsin inhibitor. Natural mutations of this inhibitor are associated with chronic pancreatitis, indicating that protease inhibition is an important safeguard against pancreatitis [29–31]. Should mesotrypsinogen get converted to mesotrypsin prematurely in the pancreas, the ensuing degradation of the pancreatic inhibitor might represent an immediate risk factor for the development of pancreatitis. Such a model of disease onset would require a relatively specific activator for mesotrypsinogen in the pancreatic acinar cells. *In vitro* cathepsin B robustly activated mesotrypsinogen, with a measurable preference over cationic or anionic trypsinogen (Fig 4) [13]. Thus, the biochemical basis clearly exists for a putative mesotrypsin-induced pancreatitis model, and future research will confirm or rule out the medical significance of such a mechanism. In this regard, identification of pancreatitis-associated mutations that stabilize mesotrypsin or loss-of-function mutations that protect against pancreatitis could provide particularly strong evidence.

Extrapancreatic trypsinogen IV

Transgenic expression of human trypsinogen IV in the neurons of the mouse brain resulted in enhanced production of glial fibrillar acidic protein by astrocytes with no evidence of neurodegeneration [32]. The significance of this observation remains to be determined.

In addition to tumor-derived epithelial cell lines [11], trypsinogen IV mRNA was also identified in esophageal squamous cell carcinomas and gastric adenomas, and down-regulation via promoter hypermethylation was correlated with higher degree of tumor invasion [33].

Genetic variants

The Single Nucleotide Polymorphism database (dbSNP; www.ncbi.nlm.nih.gov/SNP) lists 4 genetic variants of the *PRSS3* gene that alter the coding region of the gene. With the exception of E32del, none of these variants have been confirmed or characterized by published studies.

The E32del variant, in which Glu32 is deleted, was first described as the “b-form” of trypsinogen IV cloned from human brain [9]. The brain cDNA used for cloning was heterozygous for this mutation and genomic sequencing of 2 additional individuals revealed the heterozygous presence of E32del in one of them. Chen et al. also found a heterozygous E32del carrier in 1 of 6 hereditary pancreatitis patients tested for mesotrypsinogen mutations [34]. Recently, we screened 97 German patients with chronic pancreatitis of alcoholic etiology and 109 healthy controls for the presence of the E32del variant and characterized the biochemical properties of E32del-mesotrypsinogen [35]. Higher allele frequency of the E32del variant was detected in the control population (25.7 % vs. 18.0 %), but the difference was not significant ($p=0.062$). Recombinant mesotrypsin-E32del exhibited normal catalytic activity, characteristic inhibitor-resistance and inability to activate pancreatic zymogens. Degradation of trypsin inhibitors was also unaffected by the E32del genotype. Interestingly, mesotrypsinogen-E32del was biochemically distinguishable from mesotrypsinogen by its faster activation with bovine enterokinase, while activation by human enterokinase, trypsin or cathepsin B was unchanged. The results classify mesotrypsinogen-E32del as a frequent polymorphic variant, which is not associated with chronic alcoholic pancreatitis.

Mesotrypsins in animals

At the time of its discovery, mesotrypsin seemed to be specific for humans, because Rinderknecht et al. reported that no inhibitor-resistant trypsin activity was found in pancreatic extracts from dog, cow, pig, rat, mouse and hamster or in pancreatic juice from dog and hamster [3]. With the ongoing sequencing of several vertebrate genomes, orthologs of mesotrypsin have been identified and more can be expected in the near future. Mesotrypsin-like enzymes in animals can be divided into structural orthologs, which carry the mesotrypsin signature mutation of Gly198 (chymotrypsinogen # Gly193), and functional orthologs, which exhibit inhibitor resistance.

As to structural orthologs, the recently sequenced chimpanzee genome contains an authentic mesotrypsinogen gene, which corresponds to the E32del variant. Both forms of the rat trypsinogen V mRNA code for a bulky tyrosine residue at position 198 [36] and there is new evidence that trypsinogen V is also expressed at the protein level [37]. Future studies will clarify whether or not rat trypsin V exhibits mesotrypsin-like inhibitor-resistance and inhibitor-degrading properties. The mouse ortholog of the rat trypsinogen V gene is a pseudogene (trypsinogen 3).

Among the functional orthologs of mesotrypsin, trypsin-1 from *Dermasterias imbricata* [16, 17,27] is a well-documented example, however, the amino-acid or cDNA sequence has not

been determined yet. Rat trypsinogen IV was described as partially inhibitor resistant, presumably due to the presence of the negatively charged aspartic acid 198 (corresponding to Gln197 in mesotrypsinogen or chymotrypsinogen #192), which replaced the conserved neutral glutamine residue found at this position in other mammalian trypsinogens [38]. This trypsinogen is hardly detectable in the normal pancreas, but cerulein stimulation results in significant upregulation [39]. Two potential orthologs (94 % identity at the amino-acid level) can be found in the mouse genome (trypsinogen 4 and 5), with no data on their expression or function. Finally, the expression of inhibitor-resistant trypsin isoforms has been implicated in the adaptation of lepidopteran insects to plant proteinase inhibitors, but the amino-acid determinants of the inhibitor resistance are unknown [40].

Summary

Mesotrypsin can rapidly hydrolyze the reactive-site peptide bond of the Kunitz-type soybean trypsin inhibitor and degrade the Kazal-type pancreatic secretory trypsin inhibitor. This unique activity is caused by the evolutionary selection of Arg198, which also rendered mesotrypsin resistant to protein inhibitors and limited its capacity to cleave protein substrates. These observations clearly define a physiological purpose for mesotrypsin in inhibitor degradation and also suggest a potential pathological role, in which prematurely activated mesotrypsin can degrade protective pancreatic secretory trypsin inhibitor and predispose to pancreatitis. Consistent with this notion, mesotrypsinogen is strongly activated by the pathological trypsinogen activator cathepsin B. Finally, mesotrypsin-like enzymes seem to be widespread in the animal kingdom, suggesting that protease-inhibitor degradation is a mechanism of general physiological significance.

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Figure 1. Organization of the *PRSS3* gene on chromosome 9p11.2. The GCGAGT sequence indicates the suboptimal splicing site, which probably facilitates alternative splicing of exon-1 and exon-1A. The location of the TATA-box containing promoter upstream of exon-1 is indicated. The promoter elements upstream of exon-1A have not been identified yet. Exons are not drawn to scale.

Figure 2.

Inhibition of human mesotrypsin by soybean trypsin inhibitor (Kunitz). **Left panel.** Tight-binding inhibition of cationic trypsin (PRSS1), anionic trypsin (PRSS2), mesotrypsin (PRSS3) and R198G-mesotrypsin [13]. Trypsins were incubated with the indicated concentrations of soybean trypsin inhibitor at 22 °C in 100 μL of 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 and 1.5 mg/mL bovine serum albumin for 10 min. Residual trypsin activity was then determined after addition of 100 μL N-CBZ-Gly-Pro-Arg-p-nitroanilide to 0.1 mM final concentration. Trypsin concentrations were 25 nM with the exception of wild-type mesotrypsin, which was used in 15 nM concentration. **Right panel.** Competitive inhibition of mesotrypsin. The K_M values for N-CBZ-Gly-Pro-Arg-p-nitroanilide were determined in the presence of the indicated concentrations of soybean trypsin inhibitor at 22 °C, in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 and 1.5 mg/mL bovine serum albumin. The mesotrypsin concentration was 1 nM. The K_M values were plotted against the inhibitor concentration and the inhibitory constant (K_i) was determined from the x-axis intercept of the linear fit.

Figure 3.

Digestion of trypsin inhibitors by mesotrypsin. Inhibitors and mesotrypsin were incubated at the indicated concentrations at 37 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and 1 mg/mL bovine serum albumin, and residual inhibitory activity was determined against bovine trypsin. Alternatively, samples were precipitated with trichloroacetic acid and analyzed by reducing SDS-PAGE and Coomassie-blue staining. **Left panel.** Hydrolysis of the reactive site of soybean trypsin inhibitor (Kunitz). **Right panel.** Degradation of human pancreatic secretory trypsin inhibitor (Kazal) by mesotrypsin. Note that pancreatic secretory trypsin inhibitor stains poorly with Coomassie blue, and the relative band intensities do not reflect the actual concentrations of mesotrypsin and inhibitor. Figure modified from [13].

Figure 4.

Activation of human trypsinogens by cathepsin B. Human cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2) and mesotrypsinogen (PRSS3) were activated at 2 μ M concentration with human cathepsin B (90 μ g/mL) at 37 $^{\circ}$ C in 0.1 M Na-acetate buffer (pH 4.0) in the presence of 1 mM dithiothreitol, 2 mg/mL bovine serum albumin, 1 mM K-EDTA, and 300 μ M benzamidine. Aliquots (2.5 μ L) were withdrawn at indicated times and trypsin activity was measured on the synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. Trypsin activity was expressed as percentage of potential maximal activity. Figure adapted from [13].

Table 1

Mesotrypsinogen gene variants that affect the coding region, as listed in the Single Nucleotide Polymorphism database.

SNP entry	Mutation
rs3831310	E32del
rs1048379	Y175C
rs1063273	T167S
rs855581	A131T