Human Mesotrypsin Is a Unique Digestive Protease Specialized for the Degradation of Trypsin Inhibitors

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Mesotrypsin is an enigmatic minor human trypsin isoform, which has been recognized for its peculiar resistance to natural trypsin inhibitors such as soybean trypsin inhibitor (SBTI) or human pancreatic secretory trypsin inhibitor (SPINK1). In search of a biological function, two conflicting theories proposed that due to its inhibitor-resistant activity mesotrypsin could prematurely activate or degrade pancreatic zymogens and thus play a pathogenetic or protective role in human pancreatitis. In the present study we ruled out both theories by demonstrating that mesotrypsin was grossly defective not only in inhibitor binding, but also in the activation or degradation of pancreatic zymogens. We found that the restricted ability of mesotrypsin to bind inhibitors or to hydrolyze protein substrates was solely due to a single evolutionary mutation, which changed the serine-protease signature glycine 198 residue to arginine. Remarkably, the same mutation endowed mesotrypsin with a novel and unique function: mesotrypsin rapidly hydrolyzed the reactive-site peptide bond of the Kunitz-type trypsin inhibitor SBTI, and irreversibly degraded the Kazal-type temporary inhibitor SPINK1. The observations suggest that the biological function of human mesotrypsin is digestive degradation of trypsin inhibitors. This mechanism can facilitate the digestion of foods rich in natural trypsin inhibitors. Furthermore, the findings raise the possibility that inappropriate activation of mesotrypsinogen in the pancreas might cause or promote the development of human pancreatitis. In this regard, it is noteworthy that the well known pathological trypsinogen activator cathepsin B exhibited a preference for the activation of mesotrypsinogen of all three human trypsinogen isoforms, suggesting a biochemical mechanism for mesotrypsinogen activation in pancreatic acinar cells.

The human pancreas secretes three isoforms of trypsinogen, which are encoded by the PRSS (protease, serine) genes PRSS1 (OMIM 276000), PRSS2 (OMIM 601564), and PRSS3. On the basis of their electrophoretic mobility, they are commonly referred to as cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen. The two major isoforms, cationic and anionic trypsinogen, constitute the bulk of secreted trypsinogen, whereas levels of mesotrypsinogen were reported between 3 and 10% of total trypsinogen content in normal pancreatic juice (see Table I in Ref. 1; Fig. 8 in Ref. 2; and Table I in Ref. 3). Rinderknecht et al. (4) first discovered mesotrypsin in 1978 as a new inhibitor-resistant protease found in human pancreatic tissue and fluid, and a systematic characterization was published in 1984 (2). A cDNA coding for mesotrypsinogen was cloned from human pancreas in 1997 (5), and the crystal structure of mesotrypsin complexed with benzanidazole was solved in 2002 (6). An alternatively spliced form of mesotrypsinogen in which the signal peptide is replaced with a novel sequence encoded by an alternative exon 1 is expressed in the human brain (7). Although usually referred to as “brain trypsinogen,” there is no evidence for the activation of this novel chimeric molecule, which might have a function unrelated to proteolytic activity (8).

The most intriguing property of mesotrypsin is its resistance to polypeptide trypsin inhibitors (see Table V in Ref. 2), such as the Kunitz-type soybean trypsin inhibitor (SBTI)1 or the Kazal-type pancreatic secretory trypsin inhibitor (SPINK1, serine protease inhibitor, Kazal type 1, OMIM 167790) (2, 5, 6). Analysis of the recent crystal structure of mesotrypsin provided compelling evidence that the presence of an arginine residue in place of the highly conserved Gly198 (Gly193 in the chymotrypsin numbering system) is responsible for the peculiar inhibitor resistance of mesotrypsin (6). Arg198 occupies the S2′ subsite and its long side-chain sterically clashes with protein inhibitors and possibly substrates. Furthermore, the charge of the guanidino group contributes to the strong clustering of positive charges around the primary specificity pocket of mesotrypsin. However, no direct experimental evidence has ever been presented for the proposed role of Arg198.

Despite the high resolution crystal structure, the biological function of mesotrypsin has remained mysterious. In two clearly 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 (2, 5). It was also suggested that mesotrypsin might have been “abandoned by the process of evolution,” and has no important role in digestion or pancreatic physiology (2, 8). In the present study we identified a unique and specific role for mesotrypsin in the degradation of trypsin inhibitors. Furthermore, we found that this distinctive enzymatic activity was endowed by the evolutionary selection of Arg198. Finally, we showed that the lysosomal cysteine protease cathepsin B activated mesotrypsinogen at a higher rate, relative to the activation of human cationic and anionic trypsinogens. The observations not only indicate a physiological role for mesotrypsin, but also suggest

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1 The abbreviations used are: SBTI, soybean trypsin inhibitor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
that premature activation of mesotrypsinogen could contribute to the pathogenesis of human pancreatitis by reducing the protective levels of SPINK1.

EXPERIMENTAL PROCEDURES

Materials—N-CBZ-Gly-Pro-Arg-p-nitroanilide was purchased from Sigma. Suc-Ala-Ala-Pro-Phe-p-nitroanilide was from Bachem (King of Prussia, PA), ultrapure bovine enterokinase was from Biozyme Laboratories (San Diego, CA), and reagent grade bovine serum albumin was from Biocell Laboratories (Rancho Dominguez, CA). Bovine chymotrypsinogen A, TLLC-treated bovine chymotrypsin and TPCK-treated bovine trypsin was obtained from Worthington Biochemical Corp. (Lakewood, NJ). The concentration of chymotrypsinogen used for the periplasmic space where it acquires its native fold. In the SM138/pTrap expression systems. In the SM138/pTrap expression systems, mesotrypsinogen exhibited an —3-fold higher turnover number ($k_{\text{cat}}$) with a comparable $K_m$ value relative to cationic or anionic trypsin (Table I).

Inhibitor Resistance of Mesotrypsin Is Caused by Arg198—On the basis of sequence alignments (5) and a crystal structure (6), it was proposed that human cationic trypsinogen (11, 12). The pTrap-T7 plasmid harboring the mesotrypsinogen gene was transformed into the E. coli Rosetta(DE3) strain (Novagen), which is a BL21(DE3) derivative strain carrying a chromosomal copy of T7 RNA polymerase under the control of the lacZ promoter. 50-ml cultures were grown in Luria-Bertani medium with 100 $\mu$g/ml ampicillin and 54 $\mu$g/ml chloramphenicol to an OD$_{600}$ nm of 0.5, induced with 1 mM isopropyl-1-thio-B-galactopyranoside, and grown for an additional 3 h. Cells were harvested by centrifugation and inclusion bodies were isolated by sonication and centrifugation. In vitro refolding of mesotrypsinogen from the inclusion bodies was accomplished as described previously (11, 12, 16). Finally, refoldedzymogen was purified on an active site titration with p-nitrophenyl-p'-guanidinobenzoate (Sigma) as described in (9). Soybean (Glycine max) trypsin inhibitor (Kunitz type) was from Fluka (93619), and was further purified on an affinity column containing immobilized S200A mutant human cationic trypsin.

Human SPINK1 was expressed in Saccharomyces cerevisiae and purified on the S200A affinity column. Inhibitor concentrations were determined by titration with bovine trypsin. Human pro-elastase II (ELA2A) was expressed in Escherichia coli and purified by eqn affinity chromatography. Details of expression for SPINK1 and ELA2A will be described elsewhere. Eotin, human cationic trypsinogen, and human anionic trypsinogen were expressed and purified as described previously (10–13). Human recombinant cathepsin B was a generous gift from Paul M. Steed (Research Department, Novartis Pharmaceuticals, Summit, NJ). Before use, cathepsin B was activated with 1 mM dithiothreitol (final concentration) for 30 min on ice.

Expression and Purification of Mesotrypsinogen—The gene encoding mesotrypsinogen was PCR-amplified from the IMAGE clone 2659811 (GenBank accession AW125865), purchased from Invitrogen (Carlsbad, CA), and reagent grade bovine serum albumin was purchased from Sigma. Suc-Ala-Ala-Pro-Phe-p-nitroanilide was from Bachem (King of Prussia, PA). Bovine chymotrypsinogen A, TLUC-treated bovine chymotrypsin and TPCK-treated bovine trypsin was obtained from Worthington Biochemical Corp. (Lakewood, NJ). The concentration of chymotrypsinogen used for the periplasmic space where it acquires its native fold. In the SM138/pTrap expression systems. In the SM138/pTrap expression systems, mesotrypsinogen exhibited an —3-fold higher turnover number ($k_{\text{cat}}$) with a comparable $K_m$ value relative to cationic or anionic trypsin (Table I).

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tively converted to trypsin, indicating that the degradation was mediated by trypsin and not enterokinase (not shown). Activation in the presence of high Ca\(^{2+}\)/HCl concentrations (50 mM) at room temperature (22°C) increased the trypsin yield, and eventually pure and active R198G-mesotrypsin preparation could be obtained by separating the degradation products on a benzamidine affinity column. Catalytic parameters of R198G-mesotrypsin on N-CBZ-Gly-Pro-Arg-p-nitroanilide were essentially identical to those of cationic or anionic trypsin (Table I). Strikingly, R198G-mesotrypsin fully regained its sensitivity to protein trypsin inhibitors, and formed tight inhibitory complexes with SBTI or SPINK1 (Fig. 2). As expected, wild-type mesotrypsin was resistant to these inhibitors. The experiments confirmed that the unique inhibitor resistance of mesotrypsin was the result of a single evolutionary amino acid change, which replaced the small conserved Gly198 residue with a bulky Arg. Notably, in addition to rendering mesotrypsin resistant to inhibitors, the evolutionary selection of the potentially trypsin-sensitive Arg198 side chain also stabilized mesotrypsin(ogen) against autocatalytic degradation. This apparent paradox is resolved if we assume that Arg198 blocks access to the mesotrypsin active site not only for protein inhibitors, but also for protein substrates, and thus renders mesotrypsin relatively inactive toward its own trypsin-sensitive sites. This notion will be further explored by the subsequent experiments in this report.

**Mesotrypsin Cannot Activate Pancreatic Zymogens**—Next,
we tested the hypothesis that because of its inhibitor resistance mesotrypsin can activate pancreatic zymogens unopposed by SPINK1, and this mechanism might play a role in the development of human pancreatitis (2, 5). This theory was already contradicted by sporadic observations indicating that mesotrypsin was defective in activating bovine chymotrypsinogen (2) or human cationic and anionic trypsinogen (16). As shown in Fig. 3, these previous findings were fully confirmed by our experiments in which mesotrypsin was used to activate human anionic and cationic trypsinogen. Both zymogens autoactivated spontaneously to trypsin as a function of time, and addition of cationic trypsin, anionic trypsin or R198G-mesotrypsin markedly enhanced this process. In contrast, inclusion of mesotrypsin had no appreciable effect on trypsinogen activation, and mesotrypsinogen itself exhibited no autoactivation either.

Wild-type mesotrypsin did not activate bovine chymotrypsinogen A, whereas R198G-mesotrypsin was essentially as active as cationic or anionic trypsin. When rates of chymotrypsinogen activation were compared quantitatively, mesotrypsin proved to be 500–1000-fold less efficient than cationic trypsin (not shown). Finally, the activation experiments were extended to human pro-elastase 2 (ELA2A), which was efficiently activated by human cationic trypsin, and even better by R198G mesotrypsin mutant. Bovine chymotrypsinogen A and human pro-elastase 2 were activated with 32 and 75 nM trypsin, respectively. Although not shown, in other experiments mesotrypsin at 125 nM concentration activated ~5% of bovine chymotrypsinogen in 90 min, but had no measurable activating effect on human pro-elastase 2. Protease activities were determined as described under “Experimental Procedures,” and expressed as percent of maximal activity.
tide bond is the most sensitive trypsinolytic site in cationic K23Q-trypsinogen. We demonstrated previously that this peptide bond can be fully repaired by the restoration of Gly198. For these experiments we used activation-resistant mutant SPINK1-resistant mesotrypsin, which eliminates trypsinogen, and thus limits further escalation of the activation cascade (2, 5). To test this model, we examined the activity of mesotrypsin on the degradation of human cationic and anionic trypsinogen.

Impaired toward protein substrates gained clear experimental support. In terms of cleaving the activation peptide bonds of pancreatic zymogens, mesotrypsin appears to be 2–3 orders of magnitude less active than the major trypsin isoforms, and this defect can be fully repaired by the restoration of Gly198.

**Role of Mesotrypsin in Trypsinogen Degradation**—Previous work by Rinderknecht et al. (2) indicated that mesotrypsin can degrade bovine trypsinogen and possibly other pancreatic zymogens. This observation spawned the theory, that inappropriate trypsinogen activation in the pancreas is curbed not only by SPINK1, which mops up active trypsin, but also by the SPINK1-resistant mesotrypsin, which eliminates trypsinogen, and thus limits further escalation of the activation cascade (2, 5). To test this model, we examined the activity of mesotrypsin on the degradation of human cationic and anionic trypsinogen.

For these experiments we used activation-resistant mutant trypsinogen, in which Lys-23 in the activation site was replaced with Gln (K23Q). K23Q-trypsinogen is an ideal model substrate to study trypsinogen degradation without interference from trypsinogen activation (13, 18). Fig. 4 shows that in the absence of Ca\(^{2+}\) (in 1 mM EDTA, pH 8.0, 37 °C) mesotrypsin slowly cleaved the Arg\(^{122}\)–Val\(^{123}\) peptide bond in cationic K23Q-trypsinogen. We demonstrated previously that this peptide bond is the most sensitive trypsinolytic site in cationic trypsinogen (18). In contrast to a widely held belief, cleavage at this site does not result in any further degradation or inactivation, but yields a double-chain trypsin(ogen) species, which is functionally equivalent to its single-chain parent enzyme. Furthermore, cleavage never proceeds to completion, but due to trypsin-mediated re-synthesis of the Arg\(^{122}\)–Val\(^{123}\) peptide bond an equilibrium is established between the single-chain and the double-chain species. Mesotrypsin-mediated digestion of the Arg\(^{122}\)–Val\(^{123}\) peptide bond in 1 mM EDTA resulted in the expected hydrolysis equilibrium with ~18% single chain and 82% double chain trypsinogen present (Fig. 4, A and C). Addition of 1 mM Ca\(^{2+}\) significantly decreased the rate of cleavage, and at 240 min only 20% double chain trypsinogen was observed (Fig. 4, B and C). This was not a true equilibrium yet, because previous studies indicated that in the presence of Ca\(^{2+}\) the equilibrium mixture should contain 40% double chain and 60% single chain trypsinogen (18). The results clearly demonstrate that mesotrypsin is capable of cleaving the Arg\(^{122}\)–Val\(^{123}\) peptide bond. However, comparison of mesotrypsin (Fig. 4) and cationic trypsin (cf. Fig. 4 in Ref. 18) in their ability to cleave cationic K23Q-trypsinogen revealed that mesotrypsin was at least 500-fold less active. Other than cleaving at Arg\(^{122}\)–Val\(^{123}\) mesotrypsin did not degrade single chain or double chain cationic K23Q-trypsinogen to any extent.

In contrast to cationic K23Q-trypsinogen, digestion of the proteolytically less stable anionic K23Q-trypsinogen by mesotrypsin resulted in complete zymogen degradation, with a t\(_{1/2}\) of ~50 min (Fig. 5, A and C). Under similar conditions (pH 8.0, 1 mM EDTA, 37 °C, 1:10 trypsin-to-zymogen ratio), cationic trypsin degraded anionic K23Q-trypsinogen 22-fold more rapidly, with a half-life of 2.25 min (cf. Fig. 3 in Ref. 13). Addition of 1 mM Ca\(^{2+}\) almost completely stabilized anionic K23Q-trypsinogen and only ~25% mesotrypsin-mediated degradation was detected over the 240-min time course studied (Fig. 5, B and C). Taken together, the results demonstrate that the two major human trypsinogen isoforms are poor substrates for mesotrypsin, and dispute a protective role for mesotrypsin-mediated zymogen degradation in pancreatic physiology. Furthermore, the slow but measurable degradation of anionic trypsinogen by mesotrypsin also indicates that the loss of affinity toward protein substrates is not always several orders of magnitude, and a “specific” mesotrypsin substrate might exist, which can avoid the guarding side chain of Arg\(^{198}\).

**Mesotrypsin Is Weakly Inhibited by SPINK1 or SBTI**—We hypothesized that although mesotrypsin degrades human anionic trypsinogen very slowly, this process might still be significant under conditions when all other trypsins are inhibited by SPINK1 and only mesotrypsin is active. To demonstrate the feasibility of such a scenario, we digested anionic K23Q-trypsinogen with mesotrypsin in the presence of SPINK1. To our surprise, SPINK1 inhibited mesotrypsin activity even in submicromolar concentrations, and no degradation of anionic trypsinogen was detectable (not shown). These data seemed to contradict the observation in Fig. 2 that SPINK1 does not bind to mesotrypsin. However, the experimental setup used in Fig. 2 only tests for tight binding inhibition, which withstands dissociation during the assay. Relatively weak inhibitory complexes would quickly dissociate when the sample was diluted into the substrate-containing assay buffer, and no inhibition would be apparent. Thus, the assay used in Fig. 2 is not appropriate for testing weak inhibition. To characterize the relatively weaker inhibition of mesotrypsin by SPINK1, catalytic parameters (K\(_{m}\), k\(_{cat}\)) of mesotrypsin were measured on N-CBZ-Gly-Pro-Arg-p-nitroanilide in the presence of increasing concentrations of inhibitor. From [inhibitor] versus K\(_{m}\) plots a K\(_{i}\) value of 1.5 μM was estimated (not shown). Similar experiments with SBTI...
yielded a $K_i$ value of 0.42 μM (not shown), which was identical to the $K_i$ value determined by Katona et al. (6) using progress curve analysis. Interestingly, while SBTI acted in a purely competitive fashion, SPINK1 in the higher concentration range also exhibited non-competitive inhibition of mesotrypsin, for reasons that are not readily apparent. In any event, the results clearly indicate that mesotrypsin retained low but significant affinity toward trypsin inhibitors, and challenge the notion that mesotrypsin can act uncontrolled in the presence of SPINK1.

Mesotrypsin Rapidly Cleaves the Reactive Site Peptide Bond of SBTI—Seminal work from the Laskowski laboratory demonstrated that in the complexes of proteases and canonical protease inhibitors, the reactive site peptide bond of the inhibitor gets slowly cleaved, resulting in an equilibrium mixture of double chain “modified” inhibitor and single-chain “virgin” inhibitor (Ref. 19 and references therein). We hypothesized that due to the low but still significant affinity, mesotrypsin recognizes protein trypsin inhibitors as substrates and may rapidly hydrolyze their reactive site peptide bonds. A precedent for this notion was found by Estell and Laskowski (20, 21), who demonstrated that trypsin-1 from the starfish Dermasterias imbricata (leather star) cleaved the reactive sites of SBTI and bovine pancreatic trypsin inhibitor at an increased rate. In the experiment shown in Fig. 6A, we incubated 500 nM SBTI with 10 nM mesotrypsin (final concentrations) and at indicated times aliquots were taken and the virgin (intact) SBTI concentration was determined using bovine trypsin (see “Experimental Procedures” for a description of the assay). Remarkably, a rapid decrease in the concentration of virgin SBTI was observed, and the reaction reached a plateau in about 20 min, with ∼40% of the original virgin inhibitor remaining. Analysis of the digests reactions on reducing SDS-PAGE revealed that mesotrypsin cleaved SBTI at a single site (Fig. 6B), resulting in the appearance of two new bands on the gels. N-terminal sequencing of the major fragment yielded a sequence of Ile-Arg-Phe-Ile42-Ala, confirming that the cleaved peptide bond was the Arg68-Ile69 reactive site of SBTI (numbering starts with Met1). Densitometric quantitation of the virgin SBTI band indicated that ∼40–44% virgin (intact) and 56–60% modified (cleaved) inhibitor was present in the equilibrium mixture, which was in good agreement with the results of the functional assay in Fig. 6A. At the same pH but under somewhat different assay conditions (15 mM KCl, 21 °C) the Laskowski laboratory reported a hydrolysis equilibrium containing 78% modified SBTI after digestion with Dermasterias imbricata trypsin-1 or bovine trypsin (20). In control experiments 50 nM cationic or anionic trypsin was incubated with 500 nM SBTI (final concentrations), and after the initial 50 nM decrease due to complex formation between trypsin and SBTI, no further change in the concentration of free virgin SBTI was detectable for 70 h.

Mesotrypsin Degrades Human SPINK1—In contrast to SBTI, which forms stable complexes with trypsin, SPINK1 is a so-called “temporary inhibitor,” because trypsin-SPINK1 complexes irreversibly dissociate over time (22). First, reversible digestion of the Lys41–Ile42 reactive site peptide bond occurs, which is followed by the irreversibly inactivating cleavage of

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**Fig. 5.** Degradation of human anionic trypsinogen (PRSS2) by mesotrypsin (PRSS3). Anionic K23Q-trypsinogen (2 μM final concentration) was digested with mesotrypsin (200 nM final concentration) at 37 °C in 0.1 M Tris-HCl (pH 8.0) containing 1 mM EDTA (panel A) or in 1 mM Ca$^{2+}$ (panel B). Samples were precipitated with trichloroacetic acid (10% final concentration) at the indicated times and analyzed by SDS-PAGE and Coomassie Blue staining. Panel C, densitometric quantitation of the intact anionic K23Q-trypsinogen band.

**Fig. 6.** Hydrolysis of the reactive site of SBTI by mesotrypsin. Panel A, SBTI (500 nM) was incubated with 10 nM mesotrypsin (final concentration) at 37 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl$_2$, and 1 mg/ml bovine serum albumin. Cleavage of the reactive site was followed by measuring the decrease in the association rate of modified SBTI with bovine trypsin, as described under “Experimental Procedures.” Panel B, alternatively, samples were precipitated with 10% trichloroacetic acid (final concentration) and analyzed by reducing SDS-PAGE and Coomassie Blue staining. The asterisk indicates the band subjected to N-terminal protein sequencing.
the Arg<sup>67</sup>–Gln<sup>68</sup> bond (23–25). In addition, peptide bonds Arg<sup>28</sup>–Glu<sup>29</sup>, Arg<sup>65</sup>–Lys<sup>66</sup>, and Lys<sup>75</sup>–Ser<sup>76</sup> are also subject to tryptic attack (23–25). In our experiments, temporary inhibition followed a relatively rapid time course (t<sub>1/2</sub>, 2–4 h) when human cationic or anionic trypsin was in some excess to SPINK1, however, at lower trypsin-to-SPINK1 ratios the reaction proceeded dramatically slower. Thus, when cationic or anionic trypsin (50 nM, final concentration) was reacted with SPINK1 (500 nM, final concentration) at a 1:10 ratio, no trypsin activity was detectable up to 90 h, and free SPINK1 levels did not change measurably either (not shown). In sharp contrast, incubation of 50 nM mesotrypsin with 500 nM SPINK1 (final concentrations) resulted in a much more rapid decrease of active SPINK1 concentration (Fig. 7B), which eventually resulted in complete elimination of SPINK1 activity. To confirm this notion, a sample of the digestion mixture taken at 40 min was applied to a ProSorb PVDF cartridge (Applied Biosystems) and subjected to Edman degradation. In addition to the native N terminus, two major

![Fig. 7. Degradation of human SPINK1 by mesotrypsin. Panel A, SPINK1 (500 nM) was incubated with 50 nM mesotrypsin, 500 nM bovine chymotrypsin, or 500 nM human elastase 2 (final concentrations) at 37 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl<sub>2</sub>, and 1 mg/ml bovine serum albumin. Residual inhibitory activity was measured with bovine trypsin, as described under “Experimental Procedures.” Panel B, SPINK1 (15 μM) was incubated with 0.75 μM mesotrypsin (final concentrations) at 37 °C in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl<sub>2</sub>. Samples were precipitated with 20% trichloroacetic acid (final concentration) at indicated times; separated on 16% Tricine-SDS gels under reducing conditions and visualized by Coomassie Blue staining. Note that SPINK1 stains very poorly, and the relative intensities of the mesotrypsin and SPINK1 bands do not reflect the actual ratio of their concentrations in the reaction mixtures. Panel C, sites of mesotrypsin cleavage in SPINK1, as determined from N-terminal sequencing of a sample of the digestion mixture at 40 min in B.](http://www.jbc.org/)

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new N termini were identified, which indicated cleavages at peptide bonds Lys\(^{41}\)–Ile\(^{42}\) (the reactive site) and Lys\(^{75}\)–Ser\(^{76}\). Two additional N termini were found in smaller yield, which revealed cleavages at Arg\(^{28}\)–Glu\(^{29}\) and Arg\(^{67}\)–Gln\(^{68}\). Although we could not establish the exact order of cleavages from these data, the results clearly confirmed that one of the major targets of mesotrypsin was the reactive site of SPINK1, and the other tryptic cleavages corresponded to those previously described. Therefore, we conclude that mesotrypsin-mediated SPINK1 degradation followed the established mechanism of “temporary inhibition,” but at a markedly higher rate.

**Cathepsin B Is a Potential Pathological Activator of Mesotrypsinogen**—The observations described above directly suggest the idea that premature activation of mesotrypsinogen in the pancreas can degrade protective SPINK1 and eventually cause pancreatitis. Since we found that mesotrypsin cannot autoactivate, we were left with the dilemma of identifying a possible activating enzyme for mesotrypsinogen in the pancreas. In this regard, the two major trypsin isoforms appeared to be good candidates. However, when mesotrypsinogen was activated with cationic trypsin, anionic trypsin, or enterokinase under physiologically optimal conditions (37 °C, pH 8.0, 1 mM Ca\(^{2+}\)), human trypsin generated less than 20% of the potentially maximal mesotrypsin activity in 2 h (Fig. 8A). Gel analysis indicated that the mesotrypsinogen band was slowly converted to trypsin, and after 2 h approximately half of the mesotrypsinogen remained unactivated. In addition, a considerable fraction of mesotrypsinogen was degraded, despite the presence of 1 mM Ca\(^{2+}\) (not shown). In the absence of Ca\(^{2+}\), mesotrypsinogen was highly susceptible to degradation by cationic or anionic trypsin, and half-lives of 2.3 min and 0.5 min were measured, respectively. The \(t_{1/2}\) value of degradation by cationic trypsin was almost identical to the previously determined half-life of anionic K23Q-trypsinogen in the presence of cationic trypsin (2.25 min, see Fig. 3 in Ref. 13). Thus, mesotrypsinogen seems to be similar in its proteolytic stability to anionic trypsinogen, while cationic trypsinogen is at least 20-fold more stable (cf. Fig. 3 in Ref. 13). In conclusion, the results indicate that cationic and anionic trypsin are more likely to play a role in mesotrypsinogen degradation than activation.

**FIG. 8. Activation of mesotrypsinogen by human trypsins and cathepsin B.** Panel A, mesotrypsinogen (2 μM) was activated with human cationic trypsin (PRSS1), anionic trypsin (PRSS2), mesotrypsin (PRSS3) or enterokinase (200 ng/ml) at 37 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM Ca\(^{2+}\), and 2 mg/ml bovine serum albumin. Panel B, human cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2), and mesotrypsinogen (PRSS3) were activated at 2 μM concentration with human cathepsin B (90 μg/ml) at 37 °C in 0.1 M sodium acetate buffer (pH 4.0) in the presence of 1 mM dithiothreitol, 2 mg/ml bovine serum albumin, 1 mM K-EDTA, and 300 μM benzamidine. Aliquots (2.5 μl) 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 maximal activity determined by enterokinase activation at pH 8.0, in 20 mM CaCl\(_2\) and 2 mg/ml bovine serum albumin.
The lysosomal cysteine protease cathepsin B was shown to play a critical role in various experimental pancreatitis models as the intracellular catalyst of pathological trypsinogen activation (Refs. 26, 27, and references therein). Cathepsin B is also found in the secretory pathway of the human pancreas, where it is co-localized with trypsinogen (27). In vitro, cathepsin B is a potent activator of cationic trypsinogen, with a pH optimum of 4.0 (16, 27, 28). When activation of the three human trypsinogen isoforms was compared at pH 4.0, an interesting picture emerged (Fig. 8B). Rapid activation of mesotrypsinogen was apparent, which was followed by slow degradation. Compared with cationic and anionic trypsinogen, the initial rate of mesotrypsinogen activation was −2-fold and 6-fold higher, respectively. Therefore, under certain conditions, cathepsin B can rather selectively activate mesotrypsinogen, and potentially initiate the mesotrypsin-mediated degradation of SPINK1.

**DISCUSSION**

The experiments presented in this study provide three important observations: (1) Mesotrypsin can rapidly hydrolyze the reactive-site peptide bond of Kunitz-type trypsin inhibitors and degrade Kazal-type temporary inhibitors (2). 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 (3). Cathepsin B can preferentially activate mesotrypsinogen of the three human trypsinogen isoforms, under certain conditions. These observations clearly define a physiological purpose for mesotrypsin and also suggest a potential pathological role, in which prematurely activated mesotrypsin can degrade protective SPINK1 and cause pancreatitis.

The results strongly argue that human mesotrypsin plays a unique and highly specialized role in the degradation of trypsin inhibitors. 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 isofrom. Such a specialized function can also explain the relatively low levels of mesotrypsin secretion, in contrast to the largely nonspecific digestive enzymes. Conceivably, mesotrypsin exerts its effect in concert with other digestive proteases, which might attack the partially digested inhibitors. 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 Ref. 19). We hypothesized that mesotrypsin might be also responsible for the elimination of endogenous SPINK1 secreted to the duodenum, however, model experiments indicated that at physiological concentrations SPINK1 was bound by cationic or anionic trypsin and the presence of mesotrypsin did not facilitate the irreversible dissociation of SPINK1 (data not shown). Thus, elimination of unwanted SPINK1 seems to occur through the “temporary inhibition” mechanism by major trypsin isoforms. From the experimental data it appears that mesotrypsin-mediated degradation of inhibitors becomes significant at high inhibitor concentrations, while in the absence of inhibitors mesotrypsin is probably degraded by the true digestive trypsins.

Undoubtedly, the most exciting implication of the observations presented here is that inappropriate activation of human mesotrypsinogen in the pancreas might degrade protective SPINK1 levels and cause pancreatitis. The role of SPINK1 in protecting against pancreatitis is clearly supported by the association of SPINK1 mutations with certain forms of chronic pancreatitis (e.g. Refs. 29–31). Should mesotrypsinogen get converted to mesotrypsin prematurely in the pancreas, the ensuing degradation of SPINK1 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. Remarkably, in our in vitro experiments cathepsin B robustly activated mesotrypsinogen, with a measurable preference over cationic or anionic trypsinogen. 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.

Is mesotrypsin unique to humans? Rinderknecht et al. (2) reported that no inhibitor-resistant trypsin activity was found in pancreatic extracts from dog, cow, pig, rat, mouse, or hamster or in pancreatic juice from dog or hamster. Nonetheless, examination of the numerous rat trypsinogen genes found in databanks revealed that trypsinogen V contains a bulky tyrosine residue at position 198. While there is no evidence that functional protein is expressed from the rat trypsinogen V mRNA, this protein would be expected to exhibit mesotrypsin-like inhibitor-resistance and inhibitor-degrading properties. Interestingly, the corresponding mouse gene is probably a pseudogene with a 9 amino acid deletion in the N-terminal half of trypsinogen. No other sequenced trypsinogen gene carries the mesotrypsin signature mutation. Trypsin-1 from *Dermasterias imbricata* (20, 21) is clearly a mesotrypsin-like enzyme, however, the protein or DNA sequence has not been determined for this trypsin yet. With the ongoing sequencing of several vertebrate genomes, the discovery of new mesotrypsin orthologs can be expected in the near future. Finally, evolutionary mutations in trypsin at positions other than 198 can also result in mesotrypsin-like properties. In this regard, rat trypsinogen IV was described as partially inhibitor resistant, presumably due to the presence of a negatively charged aspartic acid, which replaced the conserved neutral glutamine residue found at position 197 (mesotrypsinogen numbering) in other mammalian trypsinogens (32).

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Human Mesotrypsin Is a Unique Digestive Protease Specialized for the Degradation of Trypsin Inhibitors
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