Mesotrypsin is an unusual human trypsin isoform with inhibitor resistance and the ability to degrade trypsin inhibitors. Degradation of the protective serine protease inhibitor Kazal type 1 (SPINK1) by mesotrypsin in the pancreas may contribute to the pathogenesis of pancreatitis. Here we tested the hypothesis that the regulatory digestive protease chymotrypsin C (CTRC) mitigates the harmful effects of mesotrypsin by cleaving the autolysis loop. As human trypsins are post-translationally sulfated in the autolysis loop, we also assessed the effect of this modification. We found that mesotrypsin cleaved in the autolysis loop by CTRC exhibited catalytic impairment on short peptides due to a 10-fold increase in $K_{\text{m}}$, it digested $\beta$-casein poorly and bound soybean trypsin inhibitor with 10-fold decreased affinity. Importantly, CTRC-cleaved mesotrypsin degraded SPINK1 with markedly reduced efficiency. Sulfation increased mesotrypsin activity but accelerated CTRC-mediated cleavage of the autolysis loop and did not protect against the detrimental effect of CTRC cleavage. The observations indicate that CTRC-mediated cleavage of the autolysis loop in mesotrypsin decreases protease activity and thereby protects the pancreas against unwanted SPINK1 degradation. The findings expand the role of CTRC as a key defense mechanism against pancreatitis through regulation of intrapancreatic trypsin activity.

The human pancreas secretes three trypsinogen isoforms, commonly known as cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen (1). Although all three are acidic in character, the names reflect their relative isoelectric points. The three human trypsinogens are encoded by the PRSS1 (serine protease 1), PRSS2, and PRSS3 genes, respectively (2). Cationic and anionic trypsinogen constitute 90–95% of the total trypsinogen content in pancreatic juice, whereas mesotrypsinogen is a minor isoform (3–6). Trypsinogens are discharged to the duodenum where they become activated to trypsin by their specific activator, the serine protease enteropeptidase. Trypsinogens have the unique ability of undergoing autoactivation, a molecular reaction during which trypsin activates trypsinogen in a self-amplifying manner. Cationic and anionic trypsinogens autoactivate readily, whereas mesotrypsinogen cannot autoactivate (6–8). Premature activation of trypsinogen to trypsin inside the pancreas causes pancreatitis and mutated forms of cationic trypsinogen are often found in patients with hereditary pancreatitis (9).

Although mesotrypsinogen cannot autoactivate, it can be activated by cationic and anionic trypsin in the pancreas during premature trypsinogen activation (7, 8). Active mesotrypsin is resistant to trypsin inhibitors such as pancreatic SPINK1 (serine protease inhibitor Kazal type 1) and it can readily degrade or inactivate the protease inhibitors (6, 7, 10–17). Consequently, during pathological intrapancreatic trypsin activation, mesotrypsin can contribute to pancreatitis by reducing protective SPINK1 levels. Our earlier observations indicated that mesotrypsin is cleaved by the regulatory digestive protease chymotrypsin C (CTRC) in the autolysis loop (18). Because cleavage of the autolysis loop in homologous serine proteases such as thrombin and factor Xa was shown to cause catalytic changes (19–20), we hypothesized that mesotrypsin function might be regulated in a similar manner. Therefore, in the present study, we assessed the functional properties of mesotrypsin cleaved in the autolysis loop by CTRC. Because human trypsins are post-translationally sulfated in the autolysis loop on Tyr-154 (21–24), we also studied the effect of this modification on mesotrypsin activity and its regulation by CTRC.

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The abbreviations used are: PRSS1, serine protease 1, human cationic trypsinogen; PRSS2, serine protease 2, human anionic trypsinogen; PRSS3, serine protease 3, human mesotrypsinogen; CTRC, chymotrypsin C; Hu3, human mesotrypsinogen or mesotrypsin; SBTI, soybean trypsin inhibitor; SPINK1, serine protease inhibitor Kazal type 1.
Chymotrypsin C inactivates mesotrypsin

Results

Cleavage of the autolysis loop in mesotrypsinogen by CTRC

When mesotrypsinogen was incubated with 5 nM CTRC in the absence of calcium, multiple bands migrating in the 10–20-kDa range on reducing SDS-PAGE were generated (Fig. 1A). N-terminal protein sequencing by Edman degradation revealed that CTRC cleaved mesotrypsinogen after the conserved Leu-81 in the calcium-binding loop, and after Phe-150 and Leu-148 in the autolysis loop (Fig. 1B). Cleavage at Phe-150 was preferred over Leu-148 by about 2-fold. The autolysis loop was cleaved more rapidly than the Leu-81 site resulting in a relatively stable two-chain mesotrypsinogen intermediate. This species was then slowly digested further at Leu-81. The role of the Leu-81 CTRC-cleavage site in facilitating degradation was previously characterized in cationic and anionic trypsinogen (18, 25–27). CTRC cleavage after Leu-148 was also observed in anionic trypsinogen, but not in cationic trypsinogen, which contains Ala at this position (27). However, anionic trypsinogen is rapidly degraded by CTRC without the stable autolysis loop-cleaved intermediate observed here for mesotrypsinogen (27). The Phe-150–cleavage site is absent in cationic and anionic trypsinogen, suggesting a positive evolutionary selection for the CTRC-mediated cleavage of the autolysis loop in mesotrypsinogen. Although not shown, millimolar concentrations of calcium reduced the rate of CTRC-mediated cleavages at all sites.

CTRC also cleaved mesotrypsinogen after the conserved Phe-18 in the activation peptide but the resulting small mobility shift was not apparent on the reducing SDS-PAGE used. The functional significance of the Phe-18 CTRC-cleavage site has been extensively analyzed in other trypsinogens (see “Discussion”) and we did not investigate it further in the present study.

Cleavage of the autolysis loop in L81A-mesotrypsinogen by CTRC

To study the CTRC-mediated cleavage of the autolysis loop without interference from cleavage at Leu-81, we generated the L81A mesotrypsinogen mutant. Human trypsinogens are sulfated on Tyr-154, which forms part of the S2’ substrate-binding subsite in trypsin (Fig. 1B) (21–24). Because of its proximity to the CTRC-cleavage sites in the autolysis loop, sulfation may alter CTRC cleavage rates and may affect catalytic properties of the CTRC-cleaved mesotrypsinogen. Therefore, in the following experiments we studied both nonsulfated and sulfated forms of L81A-mesotrypsinogen and L81A-mesotrypsin.

First, we analyzed CTRC-mediated cleavage of L81A-mesotrypsinogen in the absence and presence of 1 and 10 mM calcium. CTRC (5 nM) rapidly cleaved the L81A mutant in the autolysis loop and generated two closely migrating bands (Fig. 2A). N-terminal protein sequencing confirmed that the upper band corresponded to the N-terminal fragment of mesotrypsinogen, whereas the lower band contained the C-terminal products of cleavages at Phe-150 and Leu-148. As observed with WT mesotrypsinogen, CTRC preferentially cleaved L81A-mesotrypsinogen at Phe-150 versus at Leu-148, yielding a 2:1 product ratio. Sulfated L81A-mesotrypsinogen was cleaved more rapidly by CTRC than its nonsulfated counterpart. Increasing calcium concentrations protected against cleavage (Fig. 2B). Next, we studied the functional consequences of the autolysis loop cleavage by CTRC.

Catalytic properties of L81A-mesotrypsin cleaved in the autolysis loop by CTRC

The autolysis loop is located near the substrate-binding site of mesotrypsin and helps to shape the prime-side–binding subsites (Fig. 1B). Thus, cleavage of the autolysis loop by CTRC may alter the function of mesotrypsin. To test this notion, we prepared CTRC-cleaved L81A-mesotrypsin by cleaving the autolysis loop to completion in L81A-mesotrypsinogen and then activating the cleaved form by enteropeptidase. When the
time course of enteropeptidase-mediated activation was followed, nonsulfated and sulfated L81A-mesotrypsinogen developed high trypsin activity, whereas activity of the CTRC-cleaved forms was markedly reduced (Fig. 3).

Next, we measured kinetic parameters of the various L81A-mesotrypsin forms (nonsulfated, sulfated, nonsulfated CTRC-cleaved, sulfated CTRC-cleaved) using the Suc-Ala-Ala-Pro-Lys-p-nitroanilide, Suc-Ala-Ala-Pro-Arg-p-nitroanilide, and N-benzyloxycarbonyl-Gly-Pro-Arg-p-nitroanilide peptide substrates (Table 1). Sulfation of mesotrypsin caused small decreases both in the $K_m$ (2.3–3.3–fold) and the $k_{cat}$ (1.5–2–fold) and a small increase in the specificity constant (1.3–1.9–fold). CTRC-cleaved mesotrypsinogen increased $K_m$ values by about an order of magnitude, whereas $k_{cat}$ values were relatively unaffected. As a result, the specificity constant ($k_{cat}/K_m$) of cleaved L81A-mesotrypsin, both nonsulfated and sulfated, decreased by about 10-fold relative to the intact proteases.

To examine catalytic activity on a larger protein substrate, we digested bovine $\beta$-casein with cleaved and uncleaved L81A-mesotrypsin, using both nonsulfated and sulfated forms (Fig. 4). L81A-mesotrypsin rapidly digested the protein substrate in 60 min and sulfation increased the rate by about 5-fold. Importantly, CTRC-cleaved L81A-mesotrypsin variants degraded casein at markedly slower rates. Thus, cleavage by CTRC
Chymotrypsin C inactivates mesotrypsin

Figure 3. Activation of CTRC-cleaved L81A-mesotrypsinogen with enteropeptidase. Nonsulfated and sulfated (SO₄) L81A-mesotrypsinogen (Hu₃) were incubated at 2 μM concentration with 50 nM CTRC in 0.1 M Tris (pH 8.0) and 0.05% Tween 20 at 37 °C for 1 h in a 150-μl final volume. Activation of CTRC-cleaved L81A-mesotrypsinogen was then initiated by the addition of 60 ng/ml of recombinant human enteropeptidase and 1 mM calcium chloride. At the indicated time points, mesotrypsin activity was measured with 0.3 mM Suc-Ala-Ala-Pro-Lys-4-Nitroanilide substrate, as described under “Experimental procedures.” Data points represent the average of three experiments ± S.D.

redacted activity of nonsulfated L81A-mesotrypsin by 8-fold and that of sulfated L81A-mesotrypsin by 30-fold.

Inhibitor binding of L81A-mesotrypsin cleaved in the autolysis loop by CTRC

Trypsin inhibitors such as SPINK1 and soybean trypsin inhibitor (SBTI) poorly inhibit mesotrypsin (6, 7, 10–17). To examine the effect of CTRC-mediated cleavage of the autolysis loop on inhibitor binding, we measured binding of SBTI to sulfated L81A-mesotrypsin. We determined Michaelis-Menten parameters with a short peptide substrate in the presence of increasing inhibitor concentrations. As expected from a purely competitive inhibitor, the presence of SBTI increased the 𝐾ₘ, whereas the 𝑘ₑ₄₅ remained essentially unchanged (Table 2). The 𝐾ₘ values were plotted as a function of SBTI concentration and apparent 𝐾ₘ values were calculated as shown and described in the legend to Fig. 5. SBTI inhibited sulfated L81A-mesotrypsin with a 𝐾ₘ value of 0.9 μM. In contrast, SBTI inhibited the CTRC-cleaved L81A-mesotrypsin form with a 𝐾ₘ value of 8.6 μM, which indicates about an order of magnitude weaker binding, in agreement with the similarly higher 𝐾ₘ values observed on short peptide substrates (Table 1).

Inhibitor digestion by L81A-mesotrypsin cleaved in the autolysis loop by CTRC

In the next experiments, we examined whether weakened inhibitor binding would result in reduced digestion of trypsin inhibitors. Mesotrypsin cleaves the reactive site-peptide bond of SBTI in the inhibitory loop resulting in an ~50–50% equilibrium of cleaved and uncleaved SBTI forms (7). We studied the digestion of SBTI by intact and CTRC-cleaved L81A-mesotrypsin (nonsulfated and sulfated) using reducing SDS-PAGE (Fig. 6). Sulfated L81A-mesotrypsin cleaved SBTI at least 2-fold faster than nonsulfated L81A-mesotrypsin, as judged by the more rapid attainment of the equilibrium. Importantly, when cleaved in the autolysis loop by CTRC, both nonsulfated and sulfated L81A-mesotrypsin proved to be essentially inactive in SBTI digestion and generated only very faint cleavage products.

Finally, we incubated human SPINK1 with intact and cleaved L81A-mesotrypsin, using both nonsulfated and sulfated forms (Fig. 7). SPINK1 is digested by mesotrypsin at multiple sites resulting in complete degradation, which is easier to follow by the loss of inhibitory activity rather than SDS-PAGE (7). We found that intact L81A-mesotrypsin degraded SPINK1 within the 60-min time course studied and the sulfated species was slightly more active than the nonsulfated form. In contrast, CTRC-cleaved L81A-mesotrypsin showed negligible inhibitor-degrading activity (sulfated form) or none at all (nonsulfated form).

Discussion

Mesotrypsin is a unique human digestive protease, which seems to have evolved for the digestion of dietary trypsin inhibitors. A large body of work supports this contention (6, 7, 10–17). Mesotrypsin is poorly inhibited by proteinaceous trypsin inhibitors due to the presence of the bulky and charged Arg-198 in the S2’ subsite, which interferes with inhibitor binding. This steric clash and the resultant binding defect seems to be a prerequisite to inhibitor digestion, which takes place at the reactive site-peptide bond in most cases. In addition to Arg-198, mesotrypsin evolved a number of additional amino acid residues that facilitate inhibitor digestion (17). Because of its ability to degrade trypsin inhibitors, mesotrypsin can inactivate SPINK1, the inhibitor produced by pancreatic acinar cells that is responsible for protecting the pancreas from premature, pathological trypsin activation (1, 6, 7). Decreased SPINK1 levels result in pancreatitis, as indicated by the association of loss-of-function SPINK1 mutations and chronic pancreatitis (9). Because the regulatory digestive protease CTRC protects the pancreas against pathological trypsin activation (9), we speculated that CTRC cleavage might influence mesotrypsin function in a manner that mitigates SPINK1 degradation.

The regulatory effects of CTRC on the major human trypsinogen isoforms have been previously characterized (reviewed in Ref. 9). In cationic trypsinogen, CTRC cleaves after Leu-81 in the calcium-binding loop (18, 25, 26). Subsequent trypsin-mediated cleavage at Arg-122 results in inactivation and eventual degradation of trypsinogen. CTRC also cleaves the activation peptide of cationic trypsinogen at Phe-18 and removes the N-terminal tripeptide (25, 28). This cleavage results in faster autoactivation due to the release of an inhibitory interaction between Asp-218 in cationic trypsin and the tetra-Asp residues in the trypsinogen activation peptide (28). As a result of these two regulatory cleavages, the initial rate of autoactivation of cationic trypsinogen is accelerated but final trypsin levels become markedly suppressed (25). Thus, the important outcome is reduced trypsin activity and protection against pancreatitis. Loss-of-function mutations in CTRC impair the efficiency of this protective mechanism and result in elevated risk of chronic pancreatitis (29). PRSS1 mutations associated with hereditary pancreatitis block or decrease CTRC-dependent trypsinogen degradation or increase CTRC-mediated N-terminal processing and consequent stimulation of autoactivation (9, 25). In any event, mutated cationic trypsinogen activates to higher trypsin levels, which increases the risk for intrapancreatic trypsin activation and pancreatitis. The autoactivation of
anionic trypsinogen is controlled by CTRC much more tightly with an additional cleavage observed at Leu-148 in the autolysis loop (27). Furthermore, anionic trypsinogen lacks the Cys-139–Cys-206 disulfide bridge, which renders it more susceptible to degradation. As a result, CTRC-mediated suppression of trypsin activity is more efficient, which explains why mutations in this isof orm are not found in hereditary pancreatitis. Importantly, N-terminal processing of anionic trypsinogen by CTRC does not cause accelerated autoactivation. Instead, a slight inhibition is observed (27, 28) because anionic trypsin lacks Asp-218 and contains Tyr-218, as most mammalian trypsins.

We found that CTRC-mediated cleavages at Phe-18 and Leu-81 were also conserved in mesotrypsinogen and these sites likely play similar roles as in other trypsinogens. However, in contrast to cationic and anionic trypsinogens, mesotrypsinogen was also cleaved in the autolysis loop at Phe-150 and to a lesser degree at Leu-148. Importantly, this CTRC-cleaved product seemed to be relatively stable and did not suffer rapid degradation. Therefore, we were intrigued by the possible functional consequences of the autolysis loop cleavage in mesotrypsin. Cleavage of the autolysis loop in the homologous serine proteases thrombin and factor Xa were shown to alter catalytic activity and result in some degree of functional impairment (19, 20). We speculated that mesotrypsin might be regulated by CTRC in a similar manner, and the cleavage of the autolysis loop might prevent SPINK1 degradation. To test this notion, we studied the L81A-mesotrypsin variant, which was selectively cleaved in the autolysis loop by CTRC. We found that CTRC-cleaved L81A-mesotrypsin remained functional, however, its catalytic activity was reduced by at least an order of magnitude. This effect was due to a large increase in the $K_m$. Binding of the trypsin inhibitor SBTI was decreased by 10-fold and digestion of SBTI and SPINK1 by CTRC-cleaved L81A-mesotrypsin was also diminished. Thus, CTRC-mediated cleavage of the autolysis loop in mesotrypsin results in loss of protease activity, decreased inhibitor binding, and impaired inhibitor degradation. In all likelihood this occurs due to altered interactions between the substrate and the prime-side substrate-binding subsites in mesotrypsin (see Fig. 1B).

Trypsinogens in primates undergo post-translational sulfation on Tyr-154 (21–24). Other mammalian trypsinogens are not sulfated and the physiological significance of this modification in humans is unclear. Sulfation slightly increases autoactivation of cationic trypsinogen and it steers the S2’ subsite selectivity of cationic and anionic trypsin toward basic amino acids (22–24). A common African variant in anionic trypsinogen abolishes sulfation without any known disease association (24). Because of the proximity of Tyr-154 to the CTRC cleavage sites in the autolysis loop, we studied the effect of CTRC cleavage on sulfated L81A-mesotrypsin as well. We found that sulfation slightly accelerated the CTRC-mediated cleavage of the autol-

### Table 1

**Enzyme kinetic parameters of intact (uncleaved) and CTRC-cleaved L81A-mesotrypsin**

Measurements were performed as described under “Experimental procedures.” AAPK-pNA, Suc-Ala-Ala-Pro-Lys-p-nitroanilide; AAPR-pNA, Suc-Ala-Ala-Pro-Arg-p-nitroanilide; GPR-pNA, N-benzoyloxycarbonyl-Gly-Pro-Arg-p-nitroanilide. Mean values ± S.D. are shown ($n = 3$).

|                      | AAPK-pNA | AAPR-pNA | GPR-pNA |
|----------------------|----------|----------|---------|
|                      | Uncleaved | Cleaved  | Uncleaved | Cleaved  | Uncleaved | Cleaved  |
| Non-sulfated         |          |          |          |          |          |         |
| $k_{cat}$ (s$^{-1}$) | 240 ± 3  | 240 ± 4  | 223 ± 2  | 191 ± 13 | 154 ± 2  | 131 ± 2  |
| $K_m$ (μM)           | 191 ± 9  | 3223 ± 183 | 493 ± 2.3 | 1346 ± 213 | 36.1 ± 1.9 | 377 ± 16 |
| $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | 1.26 | 0.07 | 4.52 | 0.14 | 0.42 | 0.35 |
| Sulfated             |          |          |          |          |          |         |
| $k_{cat}$ (s$^{-1}$) | 156 ± 2  | 176 ± 2  | 112 ± 1  | 119 ± 1  | 88.5 ± 1.3 | 136 ± 2  |
| $K_m$ (μM)           | 84.2 ± 4.1 | 888 ± 42 | 190 ± 1.0 | 185 ± 4  | 11.1 ± 0.7 | 93.3 ± 5.4 |
| $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | 1.85 | 0.20 | 5.89 | 0.64 | 5.40 | 1.46 |

*Chymotrypsin C inactivates mesotrypsin*

![Figure 4. Degradation of casein by CTRC-cleaved L81A-mesotrypsin.](image-url)
Chymotrypsin C inactivates mesotrypsin

Table 2
Enzyme kinetic parameters of intact (uncleaved) and CTRC-cleaved sulfated L81A-mesotrypsin in the presence of SBTI

| Condition         | SBTI (µM) | 0.5 µM | 1 µM | 1.5 µM | 2 µM | 5 µM |
|-------------------|-----------|--------|------|--------|------|------|
| Uncleaved         | No SBTI   | 112 ± 1| 114 ± 1| 111 ± 1| 108 ± 1| 117 ± 2|
|                   | 0.5 µM    | 122 ± 1| 37.5 ± 0.9 | 46.7 ± 0.9 | 52.8 ± 1.9 | 119 ± 6|
| CTRC-cleaved      | No SBTI   | 190 ± 1| 31.3 ± 1.8 | 46.7 ± 0.9 | 52.8 ± 1.9 | 119 ± 6|
|                   | 0.5 µM    | 119 ± 1| 279 ± 7 | 348 ± 11 | 373 ± 12 | 598 ± 22|
| CTRC-cleaved      | 2.5 µM    | 124 ± 1| 119 ± 1 | 125 ± 1 | 119 ± 1 | 112 ± 2|
|                   | 5 µM      | 254 ± 8| 279 ± 7 | 348 ± 11 | 373 ± 12 | 598 ± 22|
|                   | 7.5 µM    | 185 ± 4| 279 ± 7 | 348 ± 11 | 373 ± 12 | 598 ± 22|
|                   | 10 µM     | 185 ± 4| 279 ± 7 | 348 ± 11 | 373 ± 12 | 598 ± 22|
|                   | 15 µM     | 185 ± 4| 279 ± 7 | 348 ± 11 | 373 ± 12 | 598 ± 22|

Nomenclature

Amino acid residues in human mesotrypsinogen (Hu3) were numbered beginning with the first methionine of the primary translation product according to the recommendations of the Human Genome Variation Society.

Expression plasmids and mutagenesis

The coding DNA for human mesotrypsinogen (Hu3) including the secretory signal peptide was cloned into the pcDNA3.1(−) expression plasmid using the Xhol and BamHI restriction sites. The pTrapT7-Hu3 bacterial expression plasmid for mesotrypsinogen and all other expression plasmids were described previously (7, 18, 24, 25, 33, 34). Mutation L81A was introduced with overlap-extension PCR mutagenesis and ligated into the pTrapT7-Hu3 and pcDNA3.1(−)-Hu3 plasmids.

Expression and purification of human trypsinogens

Nonsulfated human trypsinogens were expressed in Escherichia coli BL21(DE3), refolded in vitro, and purified by cation affinity chromatography, as described before (30, 31). Sulfated mesotrypsinogen was expressed in HEK 293T cells grown in T75 flasks and co-transfected with 8 µg of pcDNA3.1(−)-Hu3 and 2 µg of pcDNA3.1(−)-TPST2 plasmid DNA (24). The TPST2 gene codes for the Golgi-resident enzyme tyrosylprotein sulfotransferase 2. Sulfated trypsinogen was purified from 400 ml of conditioned medium by cation chromatography. The concentration of mesotrypsinogen preparations was calculated from their UV absorbance at 280 nm using an extinction coefficient of 41,535 M⁻¹ cm⁻¹. Cationic trypsinogen was activated with enteropeptidase and cationic trypsin concentrations were determined by active site titration with ecotin.

Transfection of HEK 293T cells

Cells were grown to 80–90% confluence, as described (32). Polyethyleneimine transfection reagent stock was prepared by the dilution of 45 µg of branched polyethyleneimine (catalogue number 408727, Sigma-Aldrich) with 80 ml of distilled water, and the pH was adjusted to 7.0 with hydrochloric acid (35). Transfection mixture was prepared by mixing 1 ml of Opti-MEM (Thermo Fisher Scientific) reduced serum medium with 10 µg of pcDNA3.1(−) plasmid DNA and 60 µl of polyethylenimine stock. The mixture was incubated for 20 min at 23 °C and then added to the T75 tissue culture flask containing 5 ml of Dulbecco’s modified Eagle’s medium with appropriate supplements. After 15 h incubation at 37 °C, the cells were rinsed with Opti-MEM, and 20 ml of Opti-MEM was added to the flask and incubated for 48 h. The conditioned medium was harvested and

Figure 5. Inhibition of CTRC-cleaved L81A-mesotrypsin by SBTI. Michaelis-Menten parameters of intact (uncleaved) and CTRC-cleaved sulfated L81A-mesotrypsin (Hu3-SO4 L81A, 2 nM) were determined with the Suc-Ala-Ala-Pro-Arg-p-nitroanilide substrate in the absence and presence of increasing SBTI concentrations at 23 °C, as described under “Experimental procedures.” Results are given in Table 2. The Km values were plotted as a function of SBTI concentration and competitive inhibitory constants (Ks) were calculated by dividing the y axis intercept with the slope of the linear fits. This value corresponds to the negative of the x axis intercept. Data points represent the average of three measurements. Errors were omitted for clarity.

ysis loop in mesotrypsinogen. Sulfated mesotrypsin degraded bovine β-casein at a 5-fold higher rate than the nonsulfated enzyme, whereas digestion of trypsin inhibitors was increased to a lesser extent. Importantly, however, CTRC-mediated cleavage of the autolysis loop had strong inhibitory effects both on the nonsulfated and sulfated L81A-mesotrypsin forms.

In summary, in the present study we demonstrated that CTRC-mediated cleavage of the autolysis loop in mesotrypsin decreases its activity and results in diminished SPINK1 degradation. Prevention of SPINK1 loss due to mesotrypsin-mediated degradation can mitigate intrapancreatic trypsin activation, the earliest event in the pathogenesis of pancreatitis. This novel mechanism expands the protective role of CTRC in the pancreas.

Experimental procedures

Materials

EcoTin was produced, purified, and immobilized in our laboratory as reported previously (30, 31). SBTI was purchased from Sigma and further purified by MonoQ chromatography. Human CTRC and SPINK1 containing C-terminal His10 tags were expressed in HEK 293T cells and purified by nickel-affinity chromatography according to our published protocols (26, 32). Human enteropeptidase was purchased from Bio-Techne R&D Systems and activated with human cationic trypsin.
20 ml of fresh Opti-MEM was added and collected again after 48 h incubation.

**Preparation of CTRC-cleaved L81A-mesotrypsin**

L81A-mesotrypsinogen (2 μM) was incubated with 50 nM CTRC in 0.1 M Tris-HCl (pH 8.0) for 1 h at 37 °C. CTRC-cleaved L81A-mesotrypsinogen was then supplemented with 1 mM calcium chloride and activated with 300 ng/ml of human enteropeptidase for 30 min at 37 °C. Note that in the experiment in Fig. 3 we used lower enteropeptidase concentrations (60 ng/ml) so that the time course of activation could be appreciated.

**Mesotrypsin activity assay**

Mesotrypsin activity was measured with 0.3 mM Suc-Ala-Ala-Pro-Lys-<sub>p</sub>-nitroanilide (Bachem) trypsin substrate in 100 μl of assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM calcium chloride, and 0.05% Tween 20). At the indicated time points, 2.5-μl aliquots were mixed with 92.5 μl of assay buffer and the reaction was started by adding 5 μl of the 6 mM substrate solution. Substrate cleavage was monitored for 1 min at 405 nm in a plate reader. The velocity of the enzymatic reactions was determined from the initial linear portion of the curves.

**Enzyme kinetic measurements**

Michaelis-Menten kinetic parameters of mesotrypsin variants were determined with chromogenic substrates Suc-Ala-Ala-Pro-Lys-<sub>p</sub>-nitroanilide, Suc-Ala-Ala-Pro-Arg-<sub>p</sub>-nitroanilide, and N-benzyloxycarbonyl-Gly-Pro-Arg-<sub>p</sub>-nitroanilide (Bachem). Substrate concentrations were varied between 5 and 16,200 μM in a final volume of 200 μl of 0.1 M Tris-HCl (pH 8.0), 1 mM calcium chloride, and 0.05% Tween 20. Reactions were initiated by the addition of 2 nM mesotrypsin. \( K_m \) and \( k_{cat} \) values were calculated from hyperbolic fits to plots of velocity versus substrate concentration.

**Gel electrophoresis and densitometry**

Samples from cleavage reactions were precipitated with 10% TCA, incubated on ice for 5 min, and centrifuged for 10 min at 20,000 × g. Precipitated samples were resuspended in 2× SDS sample buffer (Bio-Rad) and electrophoresed on reducing polyacrylamide gels. Gel bands were visualized by Western blotting and imaging with a ChemiDoc XRS+ imaging system (Bio-Rad). Band intensities were quantified and plotted using ImageJ (version 1.42) software.
Chymotrypsin C inactivates mesotrypsin

![Graph showing the degradation of human SPINK1 by CTRC-cleaved L81A-mesotrypsin](image)

Figure 7. Degradation of human SPINK1 by CTRC-cleaved L81A-mesotrypsin. The SPINK1 trypsin inhibitor was incubated at 0.5 μM concentration with 200 nM of the indicated L81A-mesotrypsin (Hu3) variants (nonsulfated, sulfated (SO4), nonsulfated CTRC-cleaved, sulfated CTRC-cleaved) in 0.1M Tris-HCl (pH 8.0), 1 mM calcium chloride, and 0.05% Tween 20. After 3 min incubation at 23 °C, the activity of cationic trypsin was measured by adding 5 μl of 5 nM cationic trypsin in 0.1M Tris-HCl (pH 8.0), 1 mM calcium chloride, and 0.05% Tween 20. From the results, Loss of SPINK1 inhibitory activity was expressed as the percent gain in the activity of cationic trypsin relative to its expected full activity. Data points represent the average of three experiments ± S.D.

16,000 × g. The supernatants were aspirated and the protein pellets were dissolved in 15 μl of reducing Laemmli buffer containing 0.1 M DTT and heat denatured for 5 min at 95 °C. The final volume. At the indicated times, 5

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**Chymotrypsin C inactivates mesotrypsin**

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