Chymotrypsin C (Caldecrin) Stimulates Autoactivation of Human Cationic Trypsinogen*

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Zsófia Nemoda and Miklós Sahin-Tóth

From the Department of Molecular and Cell Biology, Boston University, Goldman School of Dental Medicine, Boston, Massachusetts 02118

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Trypsin-mediated trypsinogen activation (autoactivation) facilitates digestive zymogen activation in the duodenum but may precipitate pancreatitis if it occurs prematurely in the pancreas. Autoactivation of human cationic trypsinogen is inhibited by a repulsive electrostatic interaction between the unique Asp218 on the surface of cationic trypsin and the conserved tetra-aspartate (Asp19–22) motif in the trypsinogen activation peptide (Nemoda, Z., and Sahin-Tóth, M. (2005) J. Biol. Chem. 280, 29645–29652). Here we describe that this interaction is regulated by chymotrypsin C (caldecrin), which can specifically cleave the Phe19–Asp19 peptide bond in the trypsinogen activation peptide and remove the N-terminal tripeptide. In contrast, chymotrypsin B, elastase 2A, or elastase 3A (proteinase E) are ineffective. Autoactivation of N-terminally truncated cationic trypsinogen is stimulated ~3-fold, and this effect is dependent on the presence of Asp218. Because chymotrypsinogen C is activated by trypsin, and chymotrypsin C stimulates trypsinogen activation, these reactions establish a positive feedback mechanism in the digestive enzyme cascade of humans. Furthermore, inappropriate activation of chymotrypsinogen C in the pancreas may contribute to the development of pancreatitis. Consistent with this notion, the pancreatitis-associated mutation A16V in cationic trypsinogen increases the rate of chymotrypsin C-mediated processing of the activation peptide 4-fold and causes accelerated trypsinogen activation in vitro.

The digestive enzyme cascade is a tightly regulated activation process of pancreatic zymogens in the duodenum. First, trypsinogen is activated to trypsin by enteropeptidase (enterokinase), and in turn, trypsin activates all other protease zymogens. Trypsin also activates trypsinogen, in a proteolytic reaction termed autoactivation, which is thought to have a physiological role in facilitating zymogen activation in the duodenum. However, the unique ability of trypsinogen to autoactivate renders this zymogen a potentially harmful disease-causing agent, since inappropriate autoactivation within the pancreas might initiate an autodigestive process and result in pancreatitis. The human pancreas produces three isoforms of trypsinogen encoded by separate genes, the PRSS1 (pro tease, serine 1), PRSS2, and PRSS3 genes (for a recent review, see Ref. 1 and references therein). On the basis of their relative isolectric points and electrophoretic mobility, the iso(pro)enzymes are commonly referred to as cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2), and mesotrypsinogen (PRSS3). PRSS1 (~60–70%) and PRSS2 (~30–40%) account for the majority of trypsinogens in the pancreatic juice, whereas PRSS3 is secreted in relatively low amounts. Furthermore, this minor isofrom exhibits defective inhibitor binding, and it cannot autoactivate or activate other protease zymogens (2).

The causative role of trypsinogen in pancreatitis is supported by the identification of mutations in the PRSS1 gene of patients with hereditary pancreatitis (3–7). In contrast, genetic variants of PRSS2 or PRSS3 have not been described in association with chronic pancreatitis (8). Three PRSS1 mutations, namely R122H (~70%), N29I (~25%), and A16V (~4%), have been found with relatively high frequency in multiple families, whereas 18 additional genetic variants have been identified only in very few patients (for an up-to-date list, see the pancreatitis mutation data base on the World Wide Web at www.uni-leipzig.de/pancreasmutation). Biochemical analyses of the frequently found R122H and N29I mutations as well as a subset of rare mutations (D19A, D22G, K23R, N29T) indicated that the common phenotypic change in the pancreatitis-associated mutants is an increased propensity for autoactivation (9–13). However, the third most frequently detected mutation, A16V, which alters the N-terminal amino acid of trypsinogen, has appeared to be an exception so far, because in a recent study, the A16V mutant failed to exhibit increased autoactivation (14).

Recently, we demonstrated that the conserved tetra-aspartate sequence (Asp19–22) in the trypsinogen activation peptide plays an essential role in suppressing autoactivation of human cationic trypsinogen (15). Two inhibitory interactions were identified that employ the activation peptide: one between Asp19 and the conserved hydrophobic S2 subsite on trypsin and another between Asp22 and the unique Asp218, which forms part of the S3 subsite on trypsin. Together, these interactions can suppress the rate of autoactivation by more than 2 orders of magnitude. Interestingly, the inhibitory mechanism of autoactivation due to the Asp19–Asp218 interaction appears to be specific for human cationic trypsinogen, since Asp218 is rarely found in vertebrate trypsinogens, which mostly contain a Tyr residue at the corresponding position. Here we report that not only is this inhibitory interaction unique to humans, but it is also regulated by a novel mechanism, which involves proteolytic processing of the activation peptide by chymotrypsin C. Autoactivation of the N-terminally truncated cationic trypsinogen increases significantly, which can facilitate physiological zymogen activation in the duodenum, and may play a role in the precipitation of pancreatitis in carriers of the A16V cationic trypsinogen mutation.

EXPERIMENTAL PROCEDURES

Nomenclature—The genetic abbreviations PRSS1 (pro tease, serine 1) and PRSS2 (pro tease, serine 2) are used to denote human cationic trypsinogen and anionic trypsinogen, respectively. Similarly, chymotrypsinogens B and C are abbreviated as CTRB and CTRC, and proelastase 2A and proelastase 3A are indicated as ELA2A and ELA3A. Note that chymotrypsin C is also known in the literature as caldecrin. Amino acid residues in the trypsinogen sequences are numbered according to their position in the native preproenzyme, starting with Met1. The first amino acid of the mature cationic trypsinogen is Ala16. The term “autoactiva-
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Trypsinogen is a trypsin precursor that is proteolytically activated by trypsin. The activation process involves both autocatalytic and zymogen-mediated cleavages at specific peptide bond sites. In this study, the authors focus on the autoactivation of chymotrypsinogen C (CTR-C), a human trypsinogen, and its inhibition by human pancreatic juice trypsinogen autoactivation (TTP).

**Materials and Methods:**
- **Expression Systems:** Recombinant human cationic trypsinogen (HRCT) was expressed in the Rosetta(DE3) strain of Escherichia coli, and the CTR-C fusion construct was expressed in the LG-3 strain. Recombinant HRCTs were expressed as intein fusions in the expression system that was designed to produce recombinant trypsinogens with homogenous, intact N-terminal sequences.
- **Purification and Activity Assays:** Purified CTR-C was refolded in vitro and its intrinsic catalytic activity and chymotrypsin C-mediated activation were measured. The autoactivation rates of CTR-C were compared between recombinant and authentic N-terminus preparations.
- **Data Analysis:** The autoactivation rates were analyzed using the Michaelis-Menten equation, and the effect of N-terminal processing on the autoactivation was determined.

**Results:**
- The autoactivation of recombinant CTR-C was accelerated in the LG-3 strain compared to the Rosetta(DE3) strain, indicating a difference in the efficiency of N-terminal processing.
- The autoactivation of recombinant CTR-C was inhibited by authentic N-terminus preparations, suggesting that the N-terminal sequence affects the autoactivation process.
- The authors also investigated the effect of the N-terminal sequence on the intrinsic catalytic activity of trypsinogen, finding that the N-terminal sequence influences the activity.

**Discussion:**
- The results provide insights into the mechanism of trypsinogen autoactivation and the role of the N-terminal sequence in this process.
- The findings have implications for understanding the activation of trypsinogen in vivo and for the development of therapeutic interventions targeting trypsinogen activation.

**Conclusion:**
- The study highlights the importance of the N-terminal sequence in the autoactivation of trypsinogen and demonstrates the potential for targeting this process in future therapeutic strategies.

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**References:**
- The authors reference previous studies that provide the foundation for their work, including the significance of the N-terminal sequence in trypsinogen activation.

**Corresponding Author:**
- Dr. John Doe, University of X, Y Department, 123 Research Road, City, State, Country, Phone: +1 1234567890, E-mail: john.doe@uox.edu

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2 The abbreviation used is: HEK, human embryonic kidney.
76,025 M$^{-1}$ cm$^{-1}$, CTRB, 47,605 M$^{-1}$ cm$^{-1}$ and CTRC, 64,565 M$^{-1}$ cm$^{-1}$. In mixed zymogen preparations purified from pancreatic juice, protein concentrations were estimated using the extinction coefficient of the predominant protease zymogen.

Enzymatic Assays—Trypsin activity was measured with the synthetic chromogenic substrate, N-benzoylcarbonyl-Gly-Pro-Arg-p-nitroanilide (0.14 mM final concentration). Chymotrypsin activity was assessed by N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (0.15 mM final concentration). One-min time courses of p-nitroaniline release were followed at 405 nm in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl$_2$, at room temperature using a Spectramax Plus 384 microplate reader (Molecular Devices).

Autoactivation assays and calculation of initial rates using progress curve analysis with KINSIM and FITSIM computer programs (21, 22) were described in Ref. 15. Autoactivation reactions contained 2 μM trypsinogen and were initiated by the addition of 10 nM trypsin (final concentrations). The initiating trypsin was always prepared from the corresponding trypsinogen preparation.

The rate of N-terminal processing of trypsinogens by CTRC, CTRB, ELA3A, and ELA2A was measured using SDS-PAGE. Zymogens were activated in 0.1 M Tris-HCl (pH 8.0) and 10 mM CaCl$_2$ with 10 nM trypsinogen (final concentration). Trypsinogens (2 μM concentration) were incubated with the indicated concentrations of active proteases in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl$_2$, and 1 mM benzamidine. Aliquots (100 μl) were withdrawn from the activation mixtures, and trypsinogens was precipitated with trichloroacetic acid (10% final concentration). The precipitate was recovered by centrifugation, dissolved in Laemmli sample buffer, and heat-denatured at 95 °C for 5 min in the absence of a reducing agent. Electrophoretic separation was performed on 13% SDS-PAGE minigels in standard Tris-glycine buffer, and gels were stained with Brilliant Blue R.

RESULTS

An N-Terminally Processed Cationic Trypsinogen Variant in Pancreatic Juice Is Associated with Increased Autoactivation—We have isolated cationic and anionic trypsinogens from three deidentified human pancreatic juice samples (PS7, PS13, and PS19). Our aim was to compare properties of autoactivation of native human trypsinogens to those of recombinant trypsinogens produced in E. coli. Unexpectedly, autoactivation of cationic trypsinogen isolated from PS19 was significantly increased relative to cationic trypsinogen from PS7, PS13, or from E. coli, which were comparable. Fig. 1A demonstrates time courses of autoactivation at pH 8.0, and Fig. 1B shows the pH dependence of the rate of autoactivation between pH 4.0 and 8.0. The increased autoactivation of cationic trypsinogen from PS19 was detectable between pH 6.0 and 8.0 and showed a maximum at pH 7.0. In contrast, anionic trypsinogen isolated from PS19 autoactivated slower than anionic trypsinogen from PS7, PS13, or E. coli; however, the difference was not significant (see Fig. 1C for time courses at pH 8.0 and Fig. 1D for pH dependence).

SDS-PAGE analysis of native and recombinant trypsinogens under reducing and nonreducing conditions revealed that native trypsinogens purified from PS19 are heterogeneous and exhibit two bands (Fig. 2). The doublet was best appreciated under nonreducing conditions in the 13% polyacrylamide gel used. The two cationic trypsinogen bands were transferred to polyvinylidene difluoride membrane and subjected to Edman degradation, which yielded two distinct N-terminal sequences (Fig. 2). One of the sequences corresponded to the N terminus of intact, mature cationic trypsinogen starting with Ala$^{16}$, whereas the other sequence showed a truncated N terminus, with the Ala$^{16}$-Pro$^{17}$-Phe$^{18}$ tripeptide missing. N-terminal sequencing of the anionic trypsinogen doublet revealed the same proteolytic modification. Interestingly, the shortened N-terminal sequence was also observed by Guy et al. (23), when the N-terminal sequences of native human trypsinogens were first determined (23). Clearly, a so far unidentified proteolytic activity in pancreatic juice cleaves off the N-terminal tripeptide of human trypsinogens. Remarkably, N-terminal processing stimulates autoactivation of cationic trypsinogen, whereas anionic trypsinogen remains largely unaffected.

Chymotrypsin C Activity Is Responsible for Stimulation of Autoactivation of Human Cationic Trypsinogen—The cleavage after Phe$^{18}$ suggested that a chymotrypsin-like enzyme was responsible for the observed
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FIGURE 3. MonoQ anion exchange chromatography of pancreatic juice samples PS19, PS7, and PS13. After injection of the 4-ml sample volume, the column was developed with a linear gradient of 0–0.5 M NaCl in 20 mM Tris-HCl (pH 8.0) at a flow rate of 1 ml/min. The elution profile of proteins was followed by UV absorbance at 280 nm, indicated by the black dashed line. Chymotrypsin activity was determined using the synthetic chromogenic substrate N-succinyl-Ala-Ala-Pro-Val-p-nitroanilide before (solid orange line) and after (solid red line) activation with trypsin, as described under “Experimental Procedures.” The three peaks with chymotrypsin-like activity were designated I, II, and III. There was no measurable chymotrypsin activity in the PS7 and PS13 fractions before activation with trypsin. Trypsin activity was assayed by N-benzoylargininyl-Gly-Pro-Arg-p-nitroanilide hydrolysis after activation by enteropeptidase (solid blue line). There was no detectable trypsin activity before enteropeptidase activation. The enzyme activities are indicated as absorbance change at 405 nm in 1 min (mOD/min).

N-terminal processing of human trypsinogens. Therefore, we set out to identify this enzyme in human pancreatic juice. We based our experimental approach on the reasonable assumption that the unknown enzyme would cleave the synthetic chromogenic chymotrypsin substrate N-succinyl-Ala-Ala-Pro-Val-p-nitroanilide, which is analogous to the N terminus of human trypsinogens in the P1-P2-P3 positions. Furthermore, it seemed likely that the unknown enzyme was present or activated in PS19, but not in PS7 and PS13, and thus comparative analysis of the three juices would be informative. The juice samples were loaded onto a MonoQ anion exchange column and eluted with a NaCl gradient (0–0.5 M). The flow-through and fractions 18 and 19 from PS19 (see orange trace in Fig. 3A). After activation with trypsin, three distinct chymotrypsin activity peaks emerged in all three juice samples, corresponding to the flow-through (peak I) and to fractions 14 and 15 (peak II) and fractions 18 and 19 (peak III). No spontaneous trypsin activity was observed in any of the chromatography fractions from the three juice samples. After activation with enteropeptidase, two trypsin activity peaks appeared, which represented cationic trypsinogen (fractions 24–26) and anionic trypsinogen (fractions 27–29).

The presence of chymotrypsin activity in peak III of PS19 even without activation with trypsin, suggested that the chymotrypsin-like enzyme in peak III was responsible for the N-terminal processing of human trypsinogens. To investigate this notion, we used affinity chromatography to purify the putative chymotrypsinogen from peaks I–III. The purified proteins were electrophoresed on 13% minigels, transferred to polyvinylidene difluoride membrane, and subjected to N-terminal protein sequencing. Peak II contained a single prominent band, which was identified as chymotrypsinogen B. Peak III exhibited three bands: an intense upper band corresponding to chymotrypsinogen C, a faint middle band that contained proelastase 3A, and a relatively weak lower band, which was chymotrypsinogen B. Finally, peak I purified from the MonoQ flow-through also contained all three bands, with chymotrypsinogen B as the predominant component (Fig. 4A). The chymotrypsinogen C band in peak III exhibited a fuzzy appearance on gels, suggesting that the protein might be glycosylated. Indeed, treatment with peptide-N-glycosidase F (New England Biolabs) resulted in a mobility shift on gels, whereas the mobility of chymotrypsinogen B was unaffected (not shown). The addition of trypsin also caused small but detectable mobility shifts for all proteins, indicating that the zymogens were activated by trypsin (not shown). Activity assays confirmed that trypsin-mediated activation resulted in the development of chymotrypsin activity in all three ecotin-affinity purified MonoQ peaks.

To test the effect of the purified chymotrypsins on trypsinogen function, autoactivation of recombinant cationic trypsinogen was measured in the presence of proteins from peaks I–III. Clearly, peak III stimulated autoactivation to a significant extent, whereas peak I was only marginally active, and peak II was devoid of stimulatory activity (Fig. 4B).

Efforts to purify chymotrypsinogen C completely free from the two small contaminating proteins (i.e. chymotrypsinogen B and proelastase 3A) present in peak III were unsuccessful; therefore, we expressed chymotrypsinogen C and proelastase 3A recombinantly in E. coli. To rule out that human elastase 2A, which may also cleave phenylalanyl peptide bonds, can process the N terminus of trypsinogens, recombinant proelastase 2A was also made. Fig. 4C demonstrates that recombinant human chymotrypsin C markedly stimulated autoactivation of cationic trypsinogen, whereas ELA3A and ELA2A had no stimulatory activity. Maximal chymotrypsin C-induced stimulation of the rate of autoactivation was ~3-fold at pH 8.0.

Taken together, the results clearly establish that human chymotrypsin C specifically stimulates autoactivation of human cationic trypsinogen, whereas chymotrypsin B, elastase 3A, and elastase 2A are ineffective.

Chymotrypsin C Excises the N-terminal Tripeptide of Human Trypsinogens—To confirm that stimulation of autoactivation by chymotrypsin C is mediated through N-terminal processing of cationic trypsinogen, the effect of purified peaks II and III as well as recombinant proteases was analyzed on SDS-polyacrylamide gels under nonreducing conditions, which clearly resolved the trypsinogen, N-terminally truncated trypsinogen, and trypsin bands. For these experiments, three different cationic trypsinogen preparations were used: native trypsinogen from pancreatic juice and recombinant trypsinogens expressed in the aminopeptidase P-deficient E. coli LG-3 strain or in HEK 293T cells (see “Experimental Procedures”). Trypsinogen produced in E. coli LG-3 or in...
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FIGURE 4. Effect of native and recombinant pancreatic proteases with chymotrypsin or elastase activity on the autoactivation of cationic trypsinogen. Exonin affinity chromatography was carried out to purify the proteasezymogens from the three MonoQ peaks with chymotrypsin activity (see Fig. 3). Peak I was from the flow-through fractions 2–6, peak II contained fractions 13–16, and peak III was pooled from fractions 18–20. In the experiments presented here,zymogens purified from PS13 were used. Identical results were obtained withzymogens purified from juice sample PS7 (not shown). Recombinant cationic trypsinogen, chymotrypsinogen C, proelastase 2A and proelastase 3A were expressed in E. coli Rosetta(DE3) and purified with exonin affinity chromatography. A, SDS-PAGE analysis of the ecotin affinity-purified peaks I, II, and III from pancreatic juice. Results of N-terminal sequencing are indicated. The N-terminal sequences correspond to chymotrypsinogen C (upper band), proelastase 3A (middle band), and chymotrypsinogen B (lower band). The Cys residues in the sequences were inferred from their expected positions. B, trypsin-mediated trypsinogen activation was carried out as described in the legend to Fig. 1A in the absence or presence of ~20 nM purified peak fractions (final concentrations). Protein concentrations were estimated based on the UV absorbance at 280 nm, using the theoretical extinction coefficient of the predominantzymogen, as described under “Experimental Procedures.” Because activation reactions contained 10 nM initial trypsin concentration, peak fractions were added directly aszymogens. Identical results were obtained when fractions were first preactivated with 10 nM trypsin (not shown). The rates of autoactivation calculated from progress curve analysis were as follows: control (open circles), 1.7 nM/min; peak I (solid diamonds), 2 nM/min; peak II (solid triangles), 1.7 nM/min; peak III (inverted solid triangles), 3.4 nM/min. C, the effect of purified recombinant pancreatic proteases on autoactivation of cationic trypsinogen. Initial concentrations of reactants were 2 μM cationic trypsinogen, 10 nM cationic trypsin, and 40 nM CTRC, ELA3A, or ELA2A, added as purifiedzymogens. Autoactivation was measured as described in the legend to Fig. 1A; the calculated initial rates were the following: control (open circles), 1.4 nM/min; CTRC (solid circles), 4.5 nM/min; ELA2A (solid triangles), 1.5 nM/min; ELA3A (solid inverted triangles), 1.4 nM/min.

FIGURE 5. N-terminal processing of cationic trypsinogen. Proteolytic removal of the N-terminal tripeptide from cationic trypsinogen preparations (2 μM final concentration) was measured in the presence of 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl2, 1 mM benzamidine, and the indicated activeproteases. Benzamidine was included in the reaction to prevent autoactivation of trypsinogens. Nonreducing SDS-PAGE was used to separate the two trypsinogen forms on 13% polyacrylamide gels, which adequately resolved the intact trypsinogen, the N-terminally processed trypsinogen, and the trypsin bands, as indicated. A, N-terminal processing by chymotrypsin C from peak III. Trypsinogens purified from pancreatic juice (native) or expressed recombinantly in human embryonic kidney 293T cells (HEK) or in E. coli LG-3 (LG) were incubated with chymotrypsin C from peak III at ~40 nM final concentration. B, lack of N-terminal processing of cationic trypsinogen (from E. coli LG-3) by chymotrypsin B from peak II (40 nM final concentration). C, N-terminal processing of cationic trypsinogen by recombinant pancreatic proteases. Recombinant trypsinogen expressed in E. coli LG-3 (LG) was used at 2 μM concentration. CTRC was at 30 nM concentration, ELA3A was at 100 nM, and ELA2A was at 100 nM. See “Experimental Procedures” for details. Chymotrypsinogen B and C from peaks II and III, respectively, were purified from juice sample PS13 for the experiments presented here. Identical results were obtained whenzymogens purified from juice sample PS7 were used (not shown).

HEK cells accurately mimics the intact N terminus of the native proenzyme (Ala16-Pro17-Phe18–). As shown in Fig. 5A, native chymotrypsin C from peak III proteolysed the N terminus of cationic trypsinogen, and the rate of processing was comparable for native and recombinant cationic trypsinogens from HEK cells or E. coli LG-3. Similarly, recombinant chymotrypsin C also efficiently removed the N-terminal tripeptide from cationic trypsinogen B isolated from peak II (Fig. 5B) or recombinant elastase 3A and elastase 2A (Fig. 5C) were completely devoid of processing activity.

Although not shown, we also observed that cationic trypsinogen produced in E. coli Rosetta(DE3) was processed by chymotrypsin C at a markedly increased rate (~20-fold). The enhanced processing is probably due to the extra N-terminal methionine residue (Met-Ala16-Pro17-Phe18–) present in the bulk of trypsinogen expressed in E. coli Rosetta(DE3). This unexpected observation offered the unique opportunity to use this type of recombinant trypsinogen preparation in experiments when rapid and complete processing was desirable (e.g. to examine the functional consequences of N-terminal processing).

N-terminal Processing of Cationic Trypsinogen by Chymotrypsin C Relieves the Inhibitory Effect of Asp218 on Autoactivation—The findings described above establish a novel regulatory mechanism through which autoactivation of cationic trypsinogen is controlled by chymotrypsin C. Furthermore, the experiments presented thus far raise the exciting question of why N-terminal trimming of the trypsinogen activation peptide...
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FIGURE 6. Effect of Asp<sup>218</sup> on the chymotrypsin C-mediated stimulation of trypsinogen autoactivation. Trypsin-mediated trypsinogen activation was carried out as described in the legend to Fig. 1A in the absence of chymotrypsin C (+CTR). A, recombinant wild-type anionic trypsinogen mutant. Autoactivation rates were 0.9 nM/min (control) and 2.3 nM/min (+CTR). B, recombinant wild-type cationic trypsinogen. Autoactivation rates were 1.6 nM/min (control) and 4.3 nM/min (+CTR). C, recombinant wild-type anionic trypsinogen mutant. Autoactivation rates were 17.4 nM/min (control) and 18.1 nM/min (+CTR). Although data are not shown, cationic trypsinogen mutant D218S was also tested, and no stimulation of autoactivation by chymotrypsin C was observed. Autoactivation rates of the D218S mutant were 12.1 and 13.3 nM/min, in the absence and presence of CTRC, respectively. D, recombinant wild-type anionic trypsinogen. Autoactivation rates were 4.2 nM/min (control) and 3.6 nM/min (+CTR). E, D218Y anionic trypsinogen mutant. Autoactivation rates were 0.9 nM/min (control) and 2.3 nM/min (+CTR).

increases autoactivation in a manner that is specific for cationic trypsinogen. In this context, we recently identified a unique inhibitory interaction in human cationic trypsinogen, which involves an electrostatic repulsion between Asp<sup>218</sup> on trypsin and Asp<sup>21</sup> within the tetra-aspartate motif (Asp<sup>19–22</sup>) of the trypsinogen activation peptide (15). Asp<sup>218</sup> is not present in the large majority of vertebrate trypsins, and typically a Tyr residue occupies the same position. Human anionic trypsinogen also contains a Tyr at position 218, and, consequently, the electrostatic inhibitory mechanism is not operational in this isoform. The differential effect of the chymotrypsin C-mediated N-terminal processing on the two human trypsins suggested that disruption of the Asp<sup>218</sup>-Asp<sup>21</sup> interaction might be the mechanism underlying autoactivation stimulation. To test this notion, cationic trypsinogen mutant D218Y was treated with chymotrypsin C, and autoactivation was measured. Fig. 6, A and B, show that mutation of Asp<sup>218</sup> to Tyr (D218Y) abolished the stimulatory effect of chymotrypsin C. Although not shown, we also tested the D218S mutant, which contains a different uncharged residue (Ser) at position 218, and, again, no stimulation was observed. In the converse experiment, Asp<sup>218</sup> was introduced into human anionic trypsinogen (mutant Y218D). Incubation of wild-type anionic trypsinogen with chymotrypsin C had no effect on autoactivation (Fig. 6C), whereas autoactivation of the Y218D mutant was notably stimulated (Fig. 6D). In conclusion, the observations provide compelling evidence that chymotrypsin C stimulates autoactivation of human cationic trypsinogen through alleviation of the Asp<sup>218</sup>-mediated inhibition.

The Pancreatitis-associated Mutation A16V Increases N-terminal Processing of Cationic Trypsinogen—The observations presented above raise the possibility that chymotrypsin C-mediated stimulation of autoactivation might play a role in the pathogenesis of human pancreatitis, where premature trypsinogen activation is believed to be an early, initiating event. To obtain support for this theory, we have tested the effect of the three cationic trypsinogen mutations that are most frequently found in human hereditary pancreatitis. Previous studies have demonstrated that mutations N29I and R122H increase autoactivation of human cationic trypsinogen (9–11); however, mutation A16V has no such effect (14). In the experiments presented in Fig. 7, chymotrypsin C-mediated N-terminal processing of wild-type cationic trypsinogen and mutant A16V was followed on SDS-PAGE under nonreducing conditions. Remarkably, chymotrypsin C processed the A16V mutant 4-fold more rapidly than wild-type cationic trypsinogen. Recombinant trypsinogens expressed either in E. coli or HEK cells were tested, with identical results. Analysis of mutation N29I revealed a small but reproducible (1.2–1.4-fold) stimulation in N-terminal processing, whereas mutation R122H had no effect (not shown). The results strongly suggest that stimulation of autoactivation of cationic trypsinogen by chymotrypsin C plays a role in chronic pancreatitis associated with the A16V mutation.

DISCUSSION

In this study, we demonstrated that proteolytic removal of the N-terminal tripeptide from human cationic trypsinogen by chymotrypsin C results in marked stimulation of autoactivation. The chymotrypsin C-processed cationic trypsinogen becomes a better substrate for trypsin due to the disruption of the inhibitory interaction between Asp<sup>218</sup> on cationic trypsin and Asp<sup>21</sup> within the conserved tetra-Asp motif of the trypsinogen activation peptide. Presumably, N-terminal truncation of cationic trypsinogen by chymotrypsin C results in a conformational change within the remainder of the activation peptide, which repositions Asp<sup>21</sup> and thereby mitigates the Asp<sup>218</sup>-Asp<sup>21</sup> electrostatic repulsion. Previous work demonstrated that complete abolition of the Asp<sup>218</sup>-Asp<sup>21</sup> interaction by replacing Asp<sup>218</sup> with Tyr resulted in an ~11-fold increase in autoactivation (15). Therefore, the 3-fold increase in autoactivation upon chymotrypsin C treatment indicates that the loss of the N-terminal tripeptide only partly relieves the Asp<sup>218</sup>-Asp<sup>21</sup> dependent inhibition. Chymotrypsin C also cleaves off the N-terminal tripeptide from human anionic trypsinogen, but the proteolytic processing has
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no significant effect on the autoactivation of this trypsinogen isoform, which contains a Tyr in place of Asp. However, if an Asp residue is artificially introduced into anionic trypsinogen in place of Tyr, autoactivation of the resulting mutant becomes stimulated by chymotrypsin C.

Chymotrypsin C was first isolated from pig pancreas as an anionic chymotrypsin that exhibited protease activity distinct from the cationic bovine or porcine chymotrypsin A or the anionic bovine chymotrypsin B. Thus, chymotrypsin C displayed a broad specificity toward tryrosyl, phenylalanyl, methionyl, tryptophanyl, leucyl, glutaminyl, and asparaginyl peptide bonds but, characteristically, hydrolyzed leucyl peptide bonds in synthetic and natural substrates with significantly higher activity than chymotrypsin A or B. In the bovine pancreas, chymotrypsinogen C was found in a binary complex with procarboxypeptidase A or in ternary complex with procarboxypeptidase A and proprotease E (Refs. 26 and 27 and references therein). The crystal structure of the ternary complex has been determined (28, 29). Chymotrypsin C and chymotrypsin B (43%) have an active site that is distinctly different from that of chymotrypsin A or the anionic bovine chymotrypsin.

Chymotrypsin C stimulates autoactivation of human cationic trypsinogen establishes a novel positive feedback mechanism, which facilitates activation of human trypsinogens in the digestive enzyme cascade of humans. Activation of secreted pancreatic trypsinogens in the duodenum is initially catalyzed by enteropeptidase. The active trypsin can in turn activate trypsinogen (autoactivation) and other pancreatic protease zymogens, including chymotrypsinogen C. Active chymotrypsin C can process the N terminus of still unactivated trypsinogens. It remains unclear why only in this particular sample of human cationic trypsinogen using recombinant trypsinogens produced in the E. coli LG-3 strain as self-splicing intein-trypsinogen fusions. In contrast to the previously characterized N29I and R122H variants, mutation A16V had no stimulatory effect on autoactivation, and even a slight inhibition was observed (14). The present study offers a plausible mechanistic explanation for the pathogenic action of the A16V mutation, which is fundamentally similar to the effect of the other mutations and yet differs substantially enough to account for the different clinical phenotype. Thus, mutation A16V stimulates autoactivation of cationic trypsinogen but does it indirectly by means of chymotrypsin C. Consequently, some degree of premature trypsinogen activation followed by activation of chymotrypsin C must occur before the detrimental effect the A16V mutation is manifested. This stands in contrast to N29I or R122H and a handful of other mutations, which directly increase autoactivation of cationic trypsinogen and lead to the development of pancreatitis with a higher probability.

From the three juice samples studied here, only one (PS19; see Fig. 3) contained spontaneous chymotrypsin C activity and N-terminally processed trypsinogens. It remains unclear why only in this particular sample was chymotrypsinogen C prematurely activated. No clinical information was available with the identified juice samples obtained for this study. To address this question, characterization of chymotrypsin C activity and trypsinogen processing in juice samples from patients with well-defined pancreatic pathologies will be necessary.

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REFERENCES

1. Chen, J. M., and Ferec, C. (2003) in Nature Encyclopedia of the Human Genome (Cooper D. N., ed) pp. 645–650, Macmillan, London.
2. Sahin-Tóth, M. (2005) Protein Pept. Lett. 12, 457–464
3. Whitcomb, D. C., Gorry, M. C., Preston, R. A., Furey, W., Sossenheimer, M. J., Ulrich, C. D., Martin, S. P., Gates, L. K., Jr, Amann, S. T., Toskes, P. P., Liddle, R., McGrath, K., Uomo, G., Post, J. C., and Ehrlich, G. D. (1996) Nat. Genet. 14, 141–145
4. Applebaum-Shapiro, S. E., Finch, R., Pfützer, R. H., Hepp, L. A., Gates, L. A., Amann, S., Martin, S., Ulrich, C. D., and Whitcomb, D. C. (2001) Pancreatology 1, 439–443
5. Reim, V., Witt, H., Bauer, N., Bedeker, H., Rosendahl, J., Teich, N., and Missner, J. (2003) TOP1, 146–154
6. Howes, N., Lerch, M. M., Greenhall, W., Stocken, D. D., Ellis, I., Simon, P., Truninger, K., Ammann, R., Cavallini, G., Charnley, R. M., Uomo, G., Delhaye, M., Spick, J., Drumm, B., Jansen, J., Mountford, R., Whitcomb, D. C., Neoptolemos, J. P., and...
European Registry of Hereditary Pancreatitis and Pancreatic Cancer (EUROPAC) (2004) Clin. Gastroenterol. Hepatol. 2, 252–261
7. Otsuki, M., Nishimori, I., Hayakawa, T., Hirota, M., Ogawa, M., Shimosegawa, T., and Research Committee on Intractable Disease of the Pancreas (2004) Pancreas 28, 200–206
8. Chen, J. M., Audrezet, M. P., Mercier, B., Quere, L., and Ferec, C. (1999) Scand. J. Gastroenterol. 34, 831–832
9. Sahin-Tóth, M. (2000) J. Biol. Chem. 275, 22750–22755
10. Sahin-Tóth, M., and Toth, M. (2000) Biochem. Biophys. Res. Commun. 278, 286–289
11. Szilágyi, L., Kénesi, E., Katona, G., Kaslik, G., Juhász, G., and Gráf, L. (2001) J. Biol. Chem. 276, 24574–24580
12. Teichert, N., Ockenga, J., Hoffmeister, A., Manns, M., Mössner, J., and Keim, V. (2000) Gastroenterology 119, 461–465
13. Chen, J. M., Kukor, Z., Le Marechal, C., Tóth, M., Tsakiris, L., Raguenes, O., Ferec, C., and Sahin-Tóth, M. (2003) Protein Expr. Purif. 10.1016/j.pep.2006.01.023
14. Nemoda, Z., and Sahin-Tóth, M. (2005) J. Biol. Chem. 280, 29645–29652
15. Pasternak, A., Liu, X., Lin, T. Y., and Hedstrom, L. (1998) Biochemistry 37, 16201–16210
16. Keim, V., Jovanna, I. L., Oreille, B., Verdier, J. M., Busing, M., Hopt, U., and Dagorn, J. C. (1992) Gastroenterology 103, 248–254
17. Lengyel, Z., Pál, G., and Sahin-Tóth, M. (1998) Protein Expression Purif. 12, 291–294
18. Kukor, Z., Tóth, M., and Sahin-Tóth, M. (2003) Eur. J. Biochem. 270, 2047–2058
19. Szepessy, E., and Sahin-Tóth, M. (2006) Pancreatology 6, 117–122
20. Barshop, B. A., Wrenn, R. F., and Frieden, C. (1983) Anal. Biochem. 130, 134–145
21. Zimmerle, C. T., and Frieden, C. (1989) Biochem. J. 258, 383–387
22. Guy, O., Lombardo, D., Bartelt, D. C., Amic, J., and Figarella, C. (1978) Biochemistry 17, 1669–1675
23. Folk, J. E., and Schirmer, E. W. (1965) J. Biol. Chem. 240, 181–192
24. Folk, J. E., and Cole, P. W. (1965) J. Biol. Chem. 240, 193–197
25. Peanasky, R. J., Gratecos, D., Baratti, J., and Roverey, M. (1969) Biochim. Biophys. Acta. 181, 82–92
26. Keil-Dlouha, V., Puisis, A., Marie, A., and Keil, B. (1972) Biochim. Biophys. Acta 276, 531–535
27. Gomis-Rüth, F. X., Gómez, M., Bode, W., Huber, R., and Avilés, F. X. (1995) EMBO J. 14, 4387–4394
28. Gomis-Rüth, F. X., Gómez-Ortiz, M., Vendrell, J., Ventura, S., Bode, W., Huber, R., and Avilés, F. X. (1997) J. Mol. Biol. 269, 861–880
29. Tomomura, A., Fukushige, T., Noda, T., Noikura, T., and Saheki, T. (1992) FEBS Lett. 301, 277–281
30. Tomomura, A., Fukushige, T., Tomomura, M., Noikura, T., Nishii, Y., and Saheki, T. (1993) FEBS Lett. 335, 213–216
31. Tomomura, A., Tomomura, M., Fukushige, T., Akiyama, M., Kubota, N., Kumaki, K., Nishii, Y., Noikura, T., and Saheki, T. (1995) J. Biol. Chem. 270, 30315–30321
32. Tomomura, A., Akiyama, M., Itoh, H., Yoshino, I., Tomomura, M., Nishii, Y., Noikura, T., and Saheki, T. (1996) FEBS Lett. 386, 26–28
33. Tomomura, A., Yamada, H., Fujimoto, K., Inaba, A., and Katoh, S. (2001) FEBS Lett. 508, 454–458
34. Witt, H., Luck, W., and Becker, M. (1999) Gastroenterology 117, 7–10
35. Pfützer, R. H., and Whitcomb, D. C. (1999) Gastroenterology 117, 1507–1508
36. Chen, J. M., Raguenes, O., Ferec, C., Deprez, P. H., Verellen-Dumoulin, C., and Andruilli, A. (1999) Gastroenterology 117, 1508–1509
37. Teichert, N., Bauer, N., Müssner, J., and Keim, V. (2002) Am. J. Gastroenterol. 97, 341–346