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CHYMOTRYPSIN C (CALDECRIN) STIMULATES AUTOACTIVATION OF HUMAN CATONIC TRYPSINOGEN

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Abstract

Trypsin-mediated trypsinogen activation (autoactivation) facilitates digestive zymogen activation in the duodenum, but may precipitate pancreatitis if occurs prematurely in the pancreas. Autoactivation of human cationic trypsinogen is inhibited by a repulsive electrostatic interaction between the unique Asp^{218} on the surface of cationic trypsin and the conserved tetra-aspartate ($Asp^{19–22}$) motif in the trypsinogen activation peptide (Nemoda Z, Sahin-Tóth M. *J Biol Chem* 2005; 280:29645–52). Here we describe that this interaction is regulated by chymotrypsin C (caldecrin), which can specifically cleave the Phe^{18}-Asp^{19} 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 approximately 3-fold and this effect is dependent on the presence of Asp^{218}. Because chymotrypsinogen C is activated by trypsin, and chymotrypsin C stimulates trypsinogen activation, these reactions establish a positive feed-back 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, as 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* (protease, serine, 1), *PRSS2* and *PRSS3* genes (for a recent review see [1] and references therein). On the basis of their relative isoelectric 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 isoform 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

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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 database at the www.uni-leipzig.de/pancreasmutation website). 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 ($Asp^{19–22}$) 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 Asp^{22} and the conserved hydrophobic S2 subsite on trypsin; and another between Asp^{21} and the unique Asp^{218}, 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 owing to the Asp^{21}-Asp^{218} interaction appears to be specific for human cationic trypsinogen, as Asp^{218} 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 (protease, serine, 1) and PRSS2 (protease, 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 pro-elastase 2A and pro-elastase 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 pre-proenzyme, starting with Met^{1}. The first amino acid of the mature cationic trypsinogen is Ala^{16}. The term "autoactivation" is used to describe trypsin-mediated trypsinogen activation. The intrinsic catalytic activity of trypsinogen is approximately 10^{8}–fold lower than the activity of trypsin, therefore, its impact on trypsinogen activation is negligible in our experiments [16].

Human pancreatic juice samples

De-identified samples of pure human pancreatic juice were received in lyophilized form, as a kind gift from Dr. Niels Teich (University of Leipzig, Gemany). The juice was collected from patients who underwent pancreas transplantation with bladder drainage of exocrine pancreatic secretions. To prevent zymogen activation, Trasylol (aprotinin) was added at the time of collection. See reference [17] for details.

Recombinant trypsinogen preparations

Because the routinely used recombinant trypsinogen preparations expressed in *E. coli* Rosetta (DE3) cells contain abnormal N termini (see below), in this study recombinant trypsinogens were also made in the *E. coli* LG-3 strain and human embryonic kidney (HEK) 293T cells,

which produce trypsinogens with homogenous, authentic N terminal sequences. In the experiments, which analyze rates of chymotrypsin C-mediated N-terminal processing of cationic trypsinogen, recombinant preparations from LG-3 or HEK cells were used exclusively. On the other hand, we observed that N-terminal processing of trypsinogen preparations from *E. coli* Rosetta(DE3) cells is markedly accelerated. Therefore, these trypsinogen preparations proved to be valuable in experiments, which examined the *effect* of N-terminal processing. The source of the recombinant preparations used in the different experiments is clearly indicated in the figure legends.

Purification of native protease zymogens from pancreatic juice

Fifty mg lyophilized pancreatic juice powder was solubilized with 1 mL 10 mM HCl, and insoluble material was removed by centrifugation. The supernatant was diluted with 3 mL water and loaded onto a MonoQ anion-exchange column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 8.0). Proteins were eluted with a 0–0.5 M NaCl gradient at 1 mL/min flow rate and 1 mL fractions were collected. Aliquots of fractions were subjected to trypsin- and chymotrypsin-activity assays before and after activation with enteropeptidase or trypsin, respectively. Briefly, 10 μL fraction was mixed with 40 μL assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$) and 150 μL *N*-CBZ-Gly-Pro-Arg-p-nitroanilide trypsin substrate or *N*-Suc-Ala-Ala-Pro-Phe-p-nitroanilide chymotrypsin substrate, dissolved in assay buffer, was added to a final concentration of 0.14 mM and 0.15 mM, respectively. After recording the absorbance change at 405 nm for 1 min in a microplate reader, 0.032 nM recombinant human enteropeptidase (R & D Systems, Minneapolis) or 10 nM cationic trypsin was added (final concentrations). After 1 min (enteropeptidase) or 5 min (trypsin) incubation, the rate of p-nitroaniline release was measured again for 1 min. Zymogens were further purified from high-activity fractions by ecotin-affinity chromatography, as reported previously [18].

Expression of recombinant protease zymogens in the *E. coli* Rosetta (DE3) strain

The pTrap-T7 expression plasmids harboring the PRSS1, PRSS2 and ELA2A genes were described earlier [9,10,19,20]. Note that in this expression plasmid, the sequence encoding the secretory signal peptide is deleted and a codon for an initiator methionine is placed before the mature zymogen sequence. The PRSS1 D218Y, D218S and the PRSS2 Y218D mutants were constructed previously [15]. The cDNA for chymotrypsinogen C (CTRC) was PCR-amplified from IMAGE clone #5221216 (GenBank accession BI832476; this corresponds to the reported HC1 variant with Arg80 instead of Trp80) with the sense primer 5′-GCC TGT GCC ATG GCT TGT GGG GTG CCC AGC TTC CCG CCC AAC-3′ and antisense primer 5′-CCC AGC GTC GAC TCA CAG CTG CAT TTT CTC GTT GAT CC-3′. The sense primer introduces a Met-Ala sequence in place of the secretory signal peptide of CTRC. The cDNA for pro-elastase 3A (ELA3A) was PCR-amplified from IMAGE clone #3950453 (GenBank accession BC007028) using the sense primer 5′-GCC GTT GCC ATG GCT GGC TAT GGC CCA CCT TCC TCT CAC TCT TCC-3′ and the antisense primer 5′-TTG GTT GTC GAC TTA GTG GCT TGC TAT GGT CTC CTC AAT CC-3′. The sense primer replaces the secretory signal peptide of ELA3A with a Met-Ala-Gly sequence and the antisense primer changes the amber stop codon (TAG) to ochre (TAA). The CTRC and ELA3A PCR products were digested with *Nco* I and *Sal* I restriction enzymes and cloned under the control of the T7 promoter in the pTrap-T7 expression plasmid.

Recombinant protease zymogens were expressed in the *E. coli* Rosetta(DE3) strain as cytoplasmic inclusion bodies. Small scale expression and *in* vitro refolding of the zymogens was carried out as reported previously [9,10], except that 6 M guanidine-HCl was used to solubilize the pro-elastases and chymotrypsinogen C from inclusion bodies.

Expression of recombinant human cationic trypsinogen with an authentic N terminus in the *E. coli* LG-3 strain

Trypsinogens expressed from the pTrapT7 plasmid in *E. coli* Rosetta(DE3) contain a mixture of abnormal N termini, which consists of ~70 % Met-Ala^{16}-Pro^{17}-Phe^{18}- and ~30 % Pro^{17}-Phe^{18}- sequences. Recently, we developed an *E. coli* expression system that was designed to produce recombinant trypsinogens with a homogenous, intact N terminal sequence of Ala^{16}-Pro^{17}-Phe^{18}-. The method involves the use of a self-splicing mini-intein fused in frame with the N terminus of human cationic trypsinogen and a newly engineered *E. coli* strain deficient in aminopeptidase P, designated *E. coli* LG-3. Details of the construction of the intein-trypsinogen fusion construct and the LG-3 strain are described elsewhere [14]. Wild-type cationic trypsinogen and mutants A16V, N29I and R122H were expressed as intein fusions in the LG-3 strain. After C-terminal self-cleavage of the intein, trypsinogens with the native N terminus (Ala^{16}-Pro^{17}-Phe^{18}-) accumulated in inclusion bodies. *In vitro* refolding and purification of trypsinogens was then carried out according to the protocol described previously [9,10,18].

Expression of human cationic trypsinogen in human embryonic kidney (HEK) 293T cells

The PRSS1 cDNA was PCR-amplified from IMAGE clone #6217518 (GenBank accession CA778152) and cloned into the pcDNA3.1(-) plasmid using the *Xho* I and *Bam*H I restriction sites. Mutation A16V was generated by site-directed mutagenesis. HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10 % fetal bovine serum (FBS), 1 % penicillin and 4 mM L-glutamine. Cells were grown in the same medium but without penicillin before transfection in 75 cm^2 flasks to 95 % confluence. Transfections were carried out in Opti-MEM I reduced-serum medium with 2 mM L-glutamine (Invitrogen) using 32 μg plasmid DNA and 80 μL Lipofectamine 2000™ reagent (Invitrogen). After 5 hours, the medium was supplemented with DMEM and FBS to a 10 % final concentration. After 24 hours, cells were washed with Opti-MEM containing 2 mM L-glutamine and 1 mM benzamidine; and covered with 20 mL of the same medium. Conditioned medium was then harvested and replenished with fresh medium every other day for 10–12 days. Collected media were pooled (100–120 mL) and trypsinogens were purified by ecotin-affinity chromatography [18].

Protein concentrations

Concentrations of the purified zymogen solutions were calculated from their ultraviolet absorbance at 280 nm, using the following theoretical extinction coefficients. PRSS1, 36,160 M^{-1} cm^{-1}; PRSS2, 37,440 M^{-1} cm^{-1}; ELA2A, 73,505 M^{-1} cm^{-1}; ELA3A, 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*-CBZ-Gly-Pro-Arg-*p*-nitroanilide (0.14 mM final concentration). Chymotrypsin activity was assessed by *N*-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (0.15 mM final concentration). One-minute 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 [15]. Autoactivation reactions contained 2 μM trypsinogen and were initiated by 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 trypsin (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 trypsinogen 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 minutes in the absence of a reducing agent. Electrophoretic separation was performed on 13 % SDS-PAGE mini gels 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 de-identified 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 non-reducing conditions revealed that native trypsinogens purified from PS19 are heterogeneous and exhibit two bands (Fig 2). The doublet was best appreciated under non-reducing conditions in the 13 % polyacrylamide gel used. The two cationic trypsinogen bands were transferred to PVDF 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. (1978), 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 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*-Suc-Ala-Ala-Pro-Phe-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

eluted fractions were collected and analyzed for the presence of chymotrypsin activity before and after incubation with low concentrations of trypsin, which was added to activate pro-enzymes. Trypsin activity was also measured in each fraction before and after activation of trypsinogens with enteropeptidase. Fig 3 demonstrates the elution (ultraviolet absorbance at 280 nm) and enzyme activity profiles for all three juice samples. Before activation with trypsin, chymotrypsin activity was negligible in the PS7 and PS13 fractions, but was clearly detectable in the flow-through and fractions 18–19 from PS19 (see orange colored 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 14–15 (peak II) and fractions 18–19 (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 (fraction 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, ecotin-affinity chromatography was used to purify the putative chymotrypsinogens from peaks I, II, and III. The purified proteins were electrophoresed on 13 % minigels, transferred to PVDF 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 pro-elastase 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 (PGNase F, New England Biolabs) resulted in a mobility shift on gels, whereas the mobility of chymotrypsinogen B was unaffected (not shown). 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, II and 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 pro-elastase 3A) present in peak III were unsuccessful; therefore, we expressed chymotrypsinogen C and pro-elastase 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 pro-elastase 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 circa 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 non-reducing 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 human embryonic kidney (HEK) 293T cells (see *Experimental Procedures* for details). Trypsinogen produced in *E. coli* LG-3 or in HEK cells accurately mimics the intact N terminus of the native pro-enzyme (Ala^{16}-Pro^{17}-Phe^{18}-). As shown in Fig 5A, native chymotrypsin C from peak III proteolyzed 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, confirming the identity of the active component in peak III (Fig 5C). In sharp contrast, chymotrypsin 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-Ala^{16}-Pro^{17}-Phe^{18}-) 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 Asp^{218} 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 why N-terminal trimming of the trypsinogen activation peptide 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^{218} on trypsin and Asp^{21} within the tetra-asparatate motif ($Asp^{19–22}$) of the trypsinogen activation peptide [15]. Asp^{218} 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 trypsinogens suggested that disruption of the Asp^{218}-Asp^{21} 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 6A and B show that mutation of Asp^{218} 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^{218} 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^{218}-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 non-reducing conditions. Remarkably, chymotrypsin C processed the A16V mutant 4-fold more rapidly than wild-type cationic trypsinogen. Recombinant trypsinogens expressed either in *E. coli* LG-3 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, while 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^{218} on cationic trypsin and Asp^{21} 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 re-positions Asp^{21} and thereby mitigates the Asp^{21}-Asp^{218} electrostatic repulsion. Previous work demonstrated that complete abolition of the Asp^{21} - Asp^{218} interaction by replacing Asp^{218} with Tyr resulted in an approximately 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^{218}-dependent inhibition. Chymotrypsin C also cleaves off the N-terminal tripeptide from human anionic trypsinogen, but the proteolytic processing has no significant effect on the autoactivation of this trypsinogen isoform, which contains a Tyr in place of Asp^{218}. However, if an Asp residue is artificially introduced into anionic trypsinogen in place of Tyr^{218}, 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 [24]. Thus, chymotrypsin C displayed a broad specificity toward tyrosyl, 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 [24,25]. In the bovine pancreas, chymotrypsinogen C was found in binary complex with procarboxypeptidase A or in ternary complex with procarboxypeptidase A and pro-proteinase E [26,27 and references therein]. The crystal structure of the ternary complex has been determined [28,29]. Chymotrypsin C is almost certainly the same protein as caldecrin, a serum-calcium decreasing protein isolated from porcine and rat pancreas and later cloned from rat and human pancreas [30-34]. Porcine, rat and human caldecrins have chymotrypsin-like activity after activation with trypsin and exhibit a very high sequence identity to bovine chymotrypsin C (75–81 %), suggesting that these proteins represent orthologs of the same digestive enzyme. The protease activity and the effect on serum calcium appear to be distinct and unrelated functions, although

both require activation of the zymogen by trypsin. The gene for human chymotrypsin C (*CTRC* gene) is located on chromosome 1, in the vicinity of the pro-elastase 2A and 2B (*ELA2A* and *ELA2B*) genes. Indeed, the extent of homology between human chymotrypsin C and elastase 2A is higher (64 %) than between chymotrypsin C and chymotrypsin B (43 %).

The discovery that chymotrypsin C stimulates autoactivation of human cationic trypsinogen establishes a novel positive feed-back 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, and thereby enhance autoactivation of the most abundant cationic isoform. As a result, overall conversion of trypsinogen to trypsin is accelerated and the digestive protease capacity in the chyme may be increased. Chymotrypsin C appears to be universally expressed in the pancreas of various species, however, the stimulatory effect described here is probably human-specific due to the conspicuous absence of Asp^{218} in vertebrate trypsinogens. In addition, the N-terminal Ala^{16}-Pro^{17}-Phe^{18}- sequence is also unique to human trypsinogens and other N termini may not be good substrates for chymotrypsin C.

The observation that the pancreatitis-associated mutation A16V markedly increases N-terminal processing of cationic trypsinogen by chymotrypsin C provides compelling evidence that the chymotrypsin C-mediated stimulation of autoactivation plays a role in the pathogenesis of human pancreatitis. Inappropriate intrapancreatic trypsinogen activation is an early event in pancreatitis and cationic trypsinogen mutations that *in vitro* accelerate autoactivation are frequently associated with hereditary pancreatitis. The A16V mutation was first reported in 1999, and almost 10 % of pediatric patients with chronic pancreatitis were shown to be heterozygous carriers [35]. In follow-up studies the mutation was also identified in adult patients with chronic pancreatitis and to date approximately 25 patients (pediatric and adult) have been documented worldwide [6,36–38]. Interestingly, in contrast to the more frequent N29I and R122H mutations, which show autosomal dominant inheritance, the A16V mutation was more often found in sporadic cases with no family history. This suggests that this mutation might have a somewhat different mechanism of action than N29I or R122H. Since the A16V mutation affects the first amino acid of the activation peptide, which forms part of the signal peptide cleavage site, it was proposed that the mutation might cause defective intracellular transport of cationic trypsinogen. However, such a mechanism seems unlikely, because the signal-peptide cleavage reaction is typically not influenced by the amino-acid distal to the cleavage site and valine is frequently found in this position in other secretory proteins, including human mesotrypsinogen. Furthermore, we measured the rate of trypsinogen secretion from transiently transfected HEK 293T cells and found no difference between wild-type and A16V mutant cationic trypsinogens (not shown), indicating that signal-peptide cleavage is not affected by the mutation. Recently, we have characterized the effect of mutation A16V on autoactivation of cationic trypsinogen using recombinant trypsinogens produced in the aminopeptidase P-deficient *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 chymotrypsinogen 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 de-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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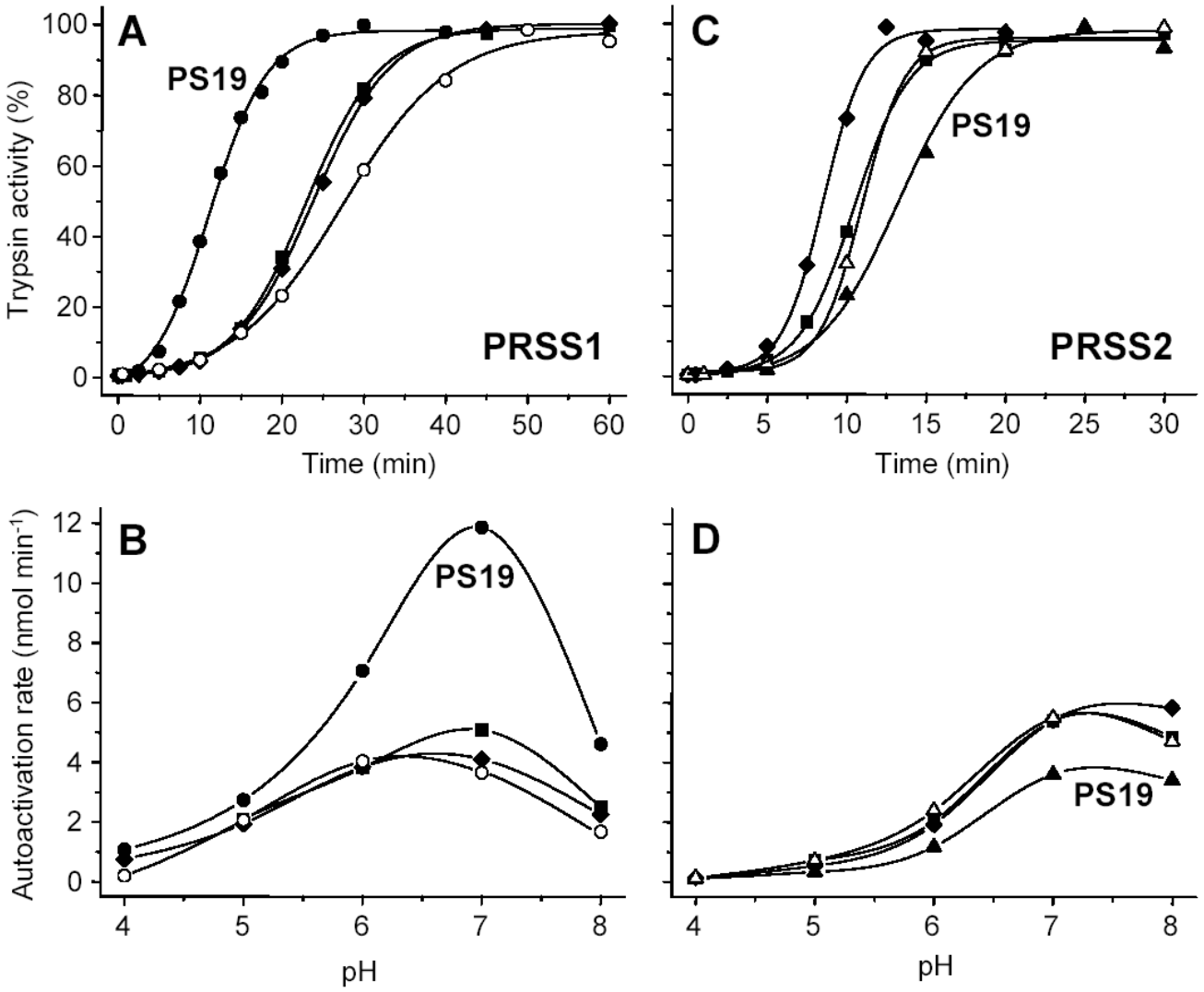

Figure 1.

Autoactivation of native cationic (PRSS1) and anionic (PRSS2) trypsinogens from human pancreatic juice. Trypsin-mediated trypsinogen activation was measured at 37 °C, using 2 μM trypsinogen and 10 nM trypsin initial concentrations. The buffers used were Na-acetate, (pH 4.0 and 5.0); Na-MES (pH 6.0); Na-HEPES (pH 7.0) and Tris-HCl (pH 8.0). Solid symbols represent native trypsinogens purified from pancreatic juice samples PS7 (diamonds), PS13 (squares) and PS19 (circles for PRSS1 and triangles for PRSS2). For comparison, recombinant trypsinogens expressed in *E. coli* Rosetta(DE3) are also shown (open circles for PRSS1 and open triangles for PRSS2). **A.** Time courses of PRSS1 autoactivation measured in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 2 mg/mL bovine serum albumin. Trypsin activity was expressed as percent of potential maximal activity, which was determined by activation with human enteropeptidase. **B.** pH dependence of PRSS1 autoactivation. Initial rates were calculated from time-courses of autoactivation using progress curve analysis, as described in [15]. **C.** Time-courses of autoactivation of PRSS2 measured in 0.1 M Tris-HCl (pH 8.0) and 10 mM $CaCl_2$. **D.** pH dependence of autoactivation of PRSS2.

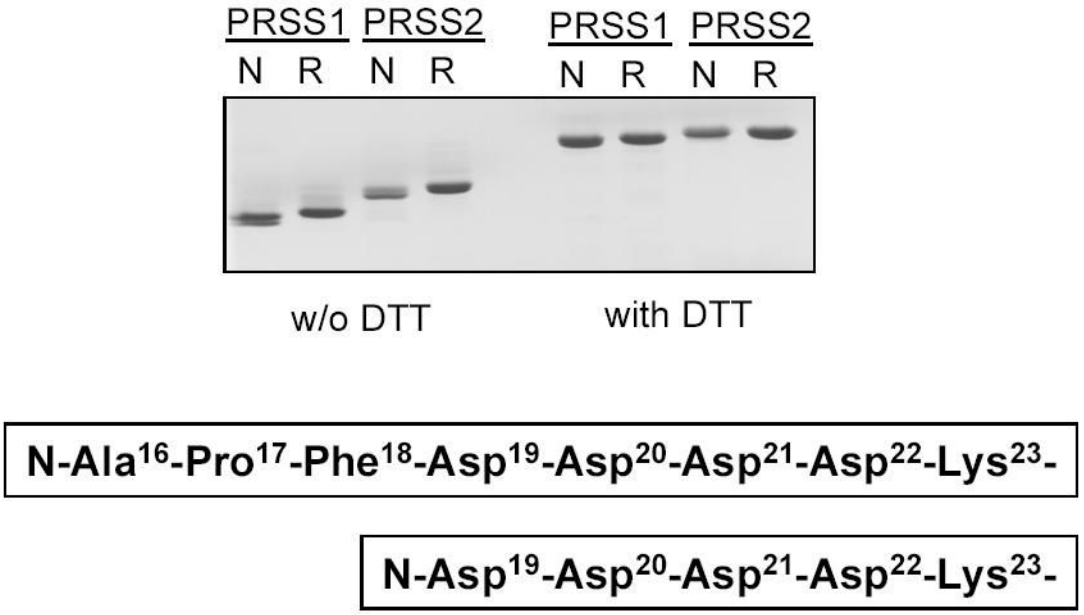

Figure 2.

N-terminal heterogeneity of trypsinogens purified from pancreatic juice PS19. Approximately 5 μg native (N) or recombinant (R) trypsinogens were heat-denatured at 95 °C for 5 min in Laemmli sample buffer either in the presence or absence of 100 mM dithiotreithol (DTT) and electrophoresed on 13 % SDS-polyacrylamide mini-gels. Gels were stained with Brilliant Blue R. Native trypsinogens exhibiting double bands were transferred to PVDF membrane and subjected to N-terminal sequencing. In addition to the native, intact sequence, a new truncated form was detected, with the N-terminal Ala^{16}-Pro^{17}-Phe^{18} tripeptide deleted.

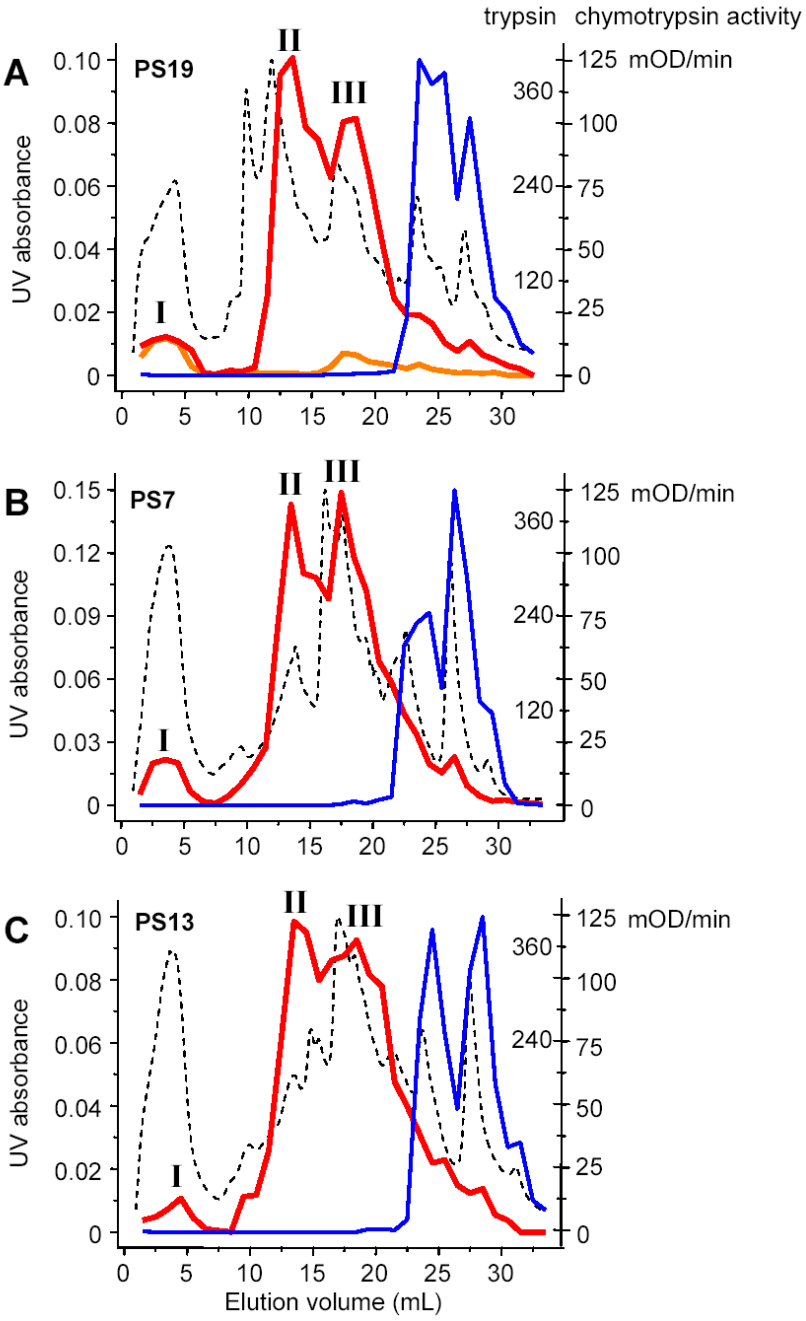

Figure 3.

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*-Suc-Ala-Ala-Pro-Phe-p-nitroanilide before (solid orange line) and after (solid red line) activation with trypsin, as described in *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*-CBZ-

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).

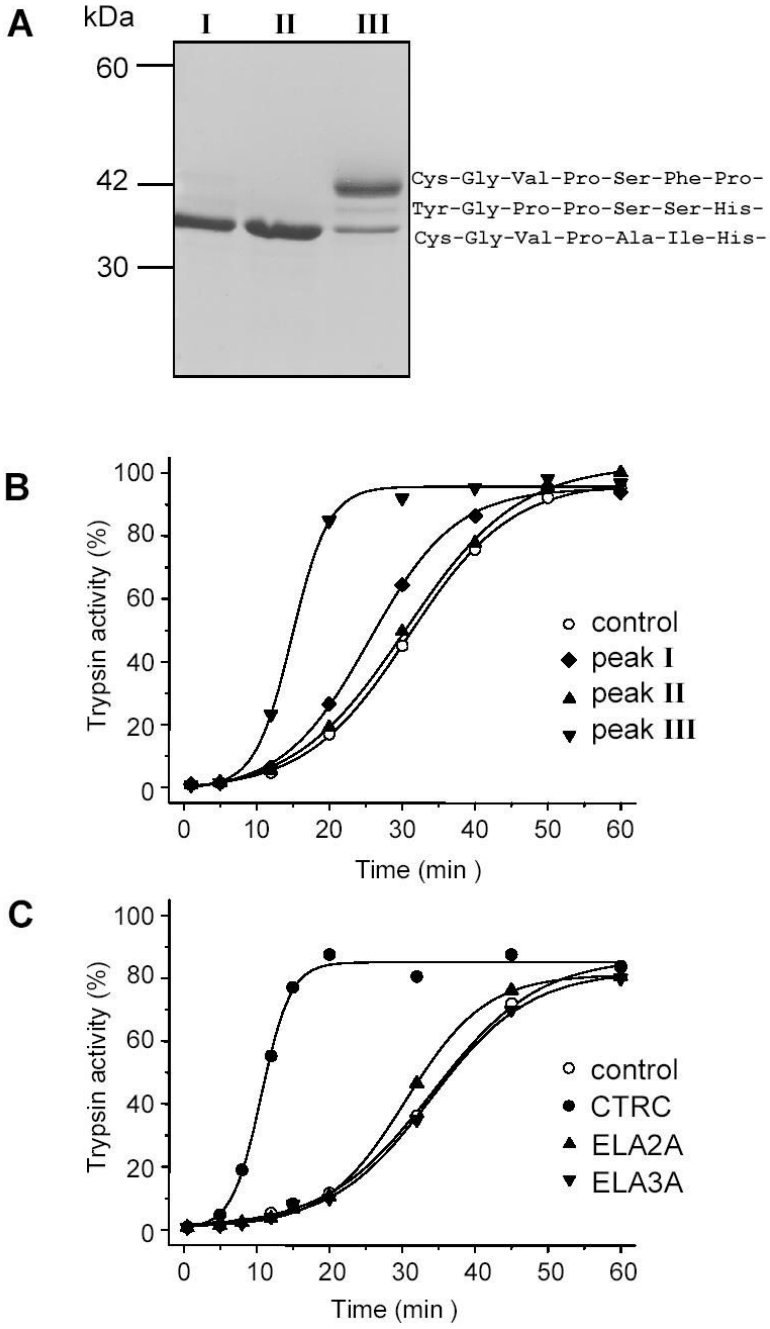

Figure 4.

Effect of native and recombinant pancreatic proteases with chymotrypsin or elastase activity on the autoactivation of cationic trypsinogen. Ecotin affinity chromatography was carried out to purify the protease zymogens 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 with zymogens purified from juice sample PS7 (not shown). Recombinant cationic trypsinogen, chymotrypsinogen C, pro-elastase 2A and pro-elastase 3A were expressed in *E. coli* Rosetta(DE3) and purified with ecotin-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), pro-elastase 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 Figure 1A in the absence or presence of approximately 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 predominant zymogen, as described in *Experimental Procedures*. Because activation reactions contained 10 nM initial trypsin concentration, peak fractions were added directly as zymogens. Identical results were obtained when fractions were first pre-activated 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 chymotrypsin C (CTRC), elastase 3A (ELA3A) or elastase 2A (ELA2A), added as purified zymogens. Autoactivation was measured as described in 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.

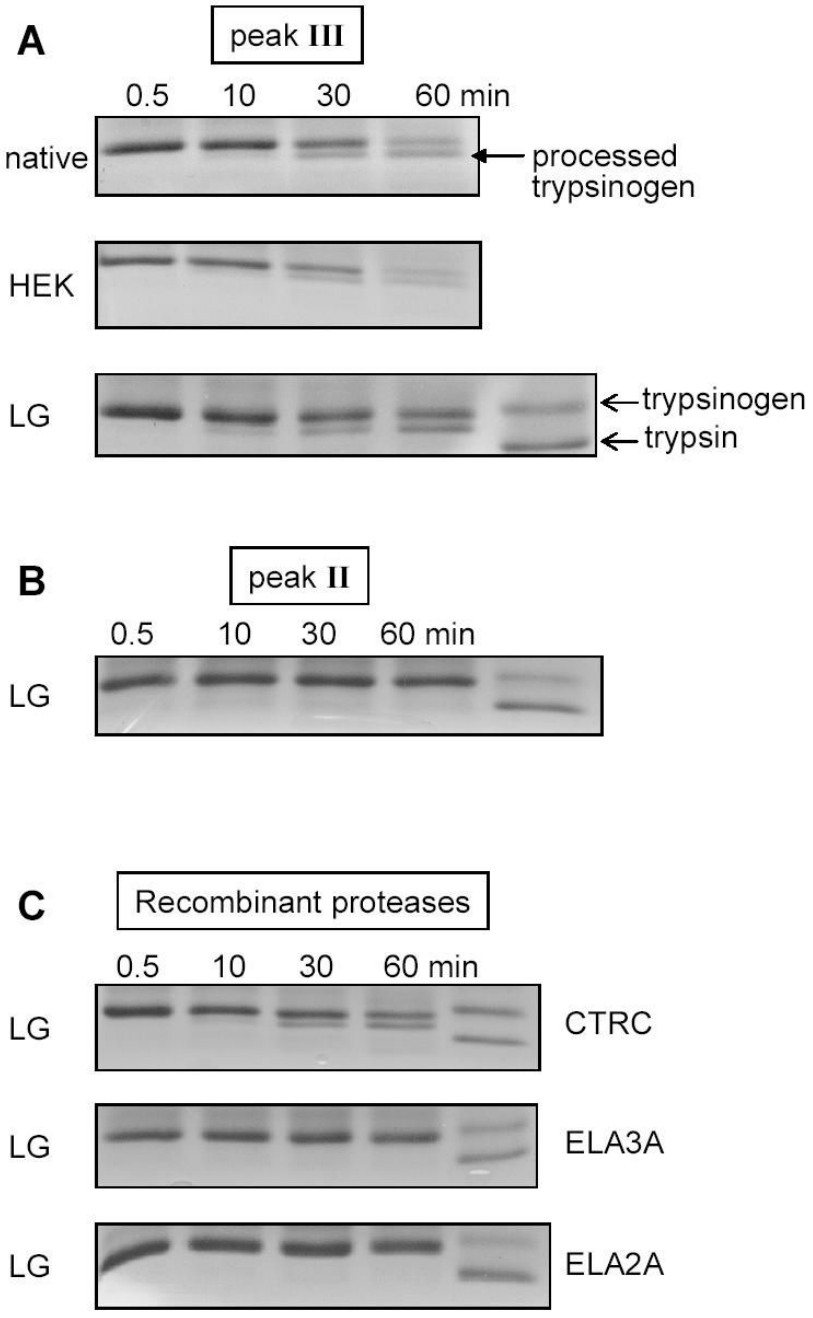

Figure 5.

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 $CaCl_2$, 1 mM benzamidine and the indicated active proteases. Benzamidine was included in the reaction to prevent autoactivation of trypsinogens. Non-reducing 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, chymotrypsin C (30 nM concentration); ELA3A, elastase 3A (100 nM concentration); ELA2A, elastase 2A (100 nM concentration). 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 when zymogens purified from juice sample PS7 were used (not shown).

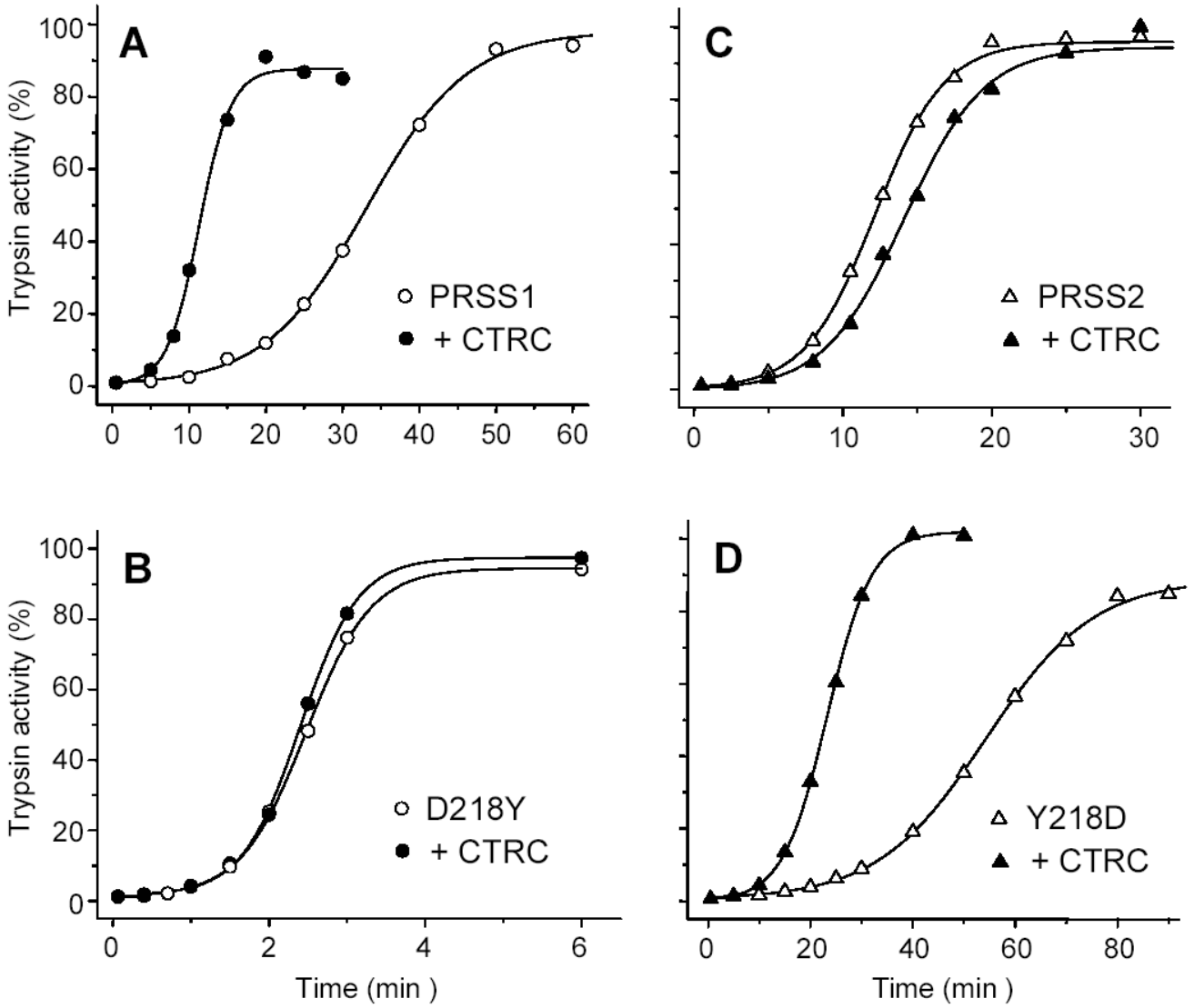

Figure 6.

Effect of Asp^{218} on the chymotrypsin C-mediated stimulation of trypsinogen autoactivation. Trypsin-mediated trypsinogen activation was carried out as described in Fig 1A in the absence (open symbols) or presence (solid symbols) of recombinant chymotrypsin C (+ CTRC) at 40 nM concentration. Recombinant trypsinogens were expressed in *E. coli* Rosetta(DE3). These preparations exhibit enhanced rates of N-terminal processing by chymotrypsin C, which ensured complete processing even for the rapidly autoactivating D218Y mutant. **A.** Recombinant wild-type cationic trypsinogen. Autoactivation rates were 1.6 nM/min (control) and 4.3 nM/min (+ CTRC). **B.** D218Y cationic trypsinogen mutant. Autoactivation rates were 17.4 nM/min (control) and 18.1 nM/min (+ CTRC). 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 nM/min and 13.3 nM/min, in the absence and presence of CTRC, respectively. **C.** Recombinant wild-type anionic trypsinogen. Autoactivation rates were 4.2 nM/min (control) and 3.6 nM/min (+ CTRC). **D.** Y218D anionic trypsinogen mutant. Autoactivation rates were 0.9 nM/min (control) and 2.3 nM/min (+ CTRC).

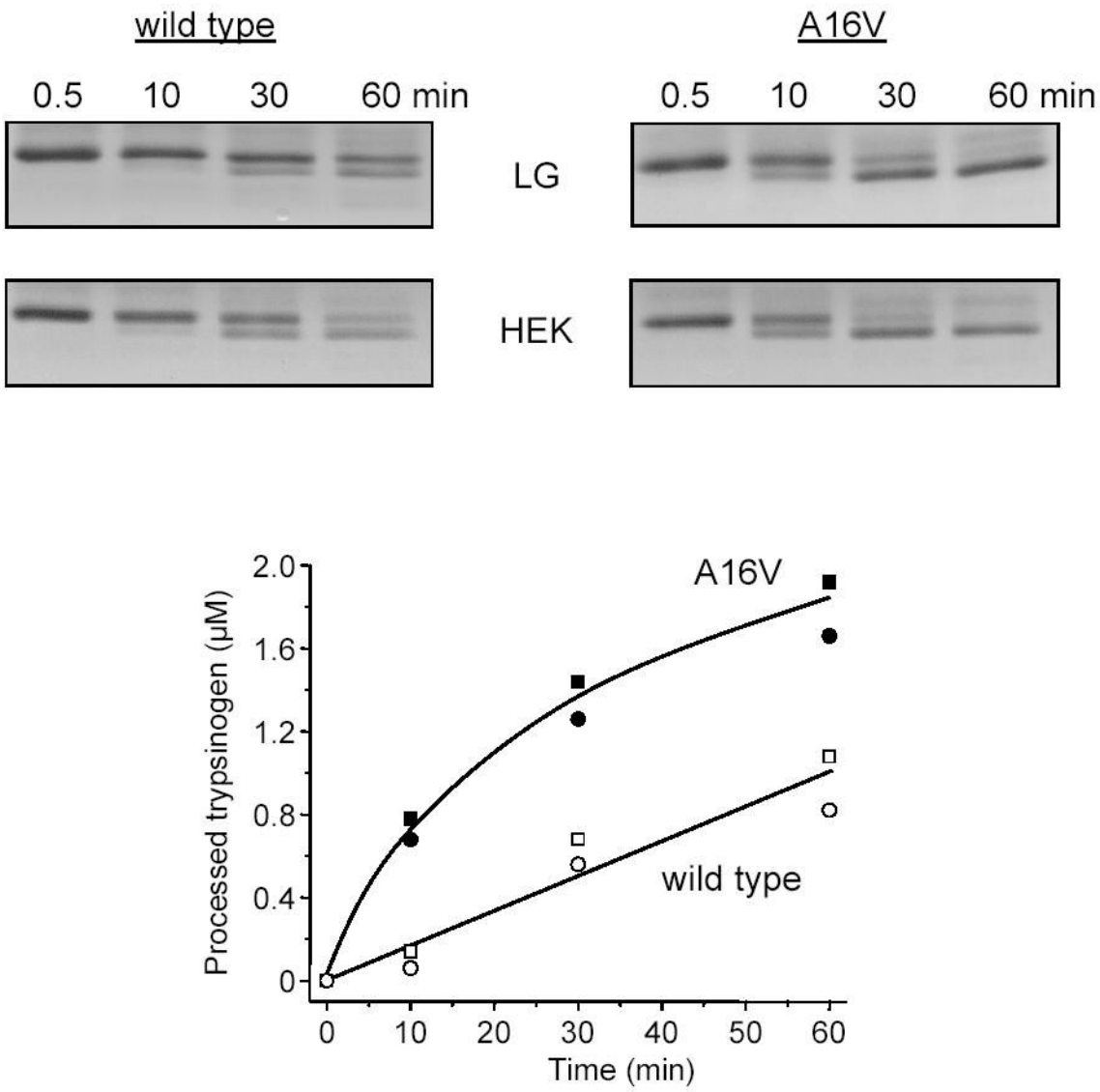

Figure 7.

Effect of the pancreatitis-associated A16V mutation on the N-terminal processing of cationic trypsinogen by chymotrypsin C. Recombinant wild-type (open symbols) and A16V mutant (solid symbols) cationic trypsinogen (2 μM) was incubated with 30 nM recombinant chymotrypsin C (final concentrations) and at the indicated time points samples were precipitated with trichloroacetic acid and analyzed by SDS-PAGE under non-reducing conditions. The graph illustrates the densitometric analysis of the gels shown. Experimental details are given in the legend to Fig 5. Recombinant trypsinogen preparations from two independent sources were tested; from *E. coli* LG-3 strain (circles) and from human embryonic kidney (HEK) 293T cells (squares).