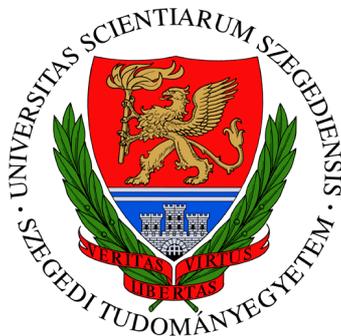


MUTATIONS OF THE TRYPSINOGEN ACTIVATION PEPTIDE IN HEREDITARY PANCREATITIS

Ph.D. Thesis

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Articles closely related to the subject of the thesis and cited in the thesis

- I. **Geisz A**, Hegyi P, Sahin-Tóth M. Robust autoactivation, chymotrypsin C independence and diminished secretion define a subset of hereditary pancreatitis associated cationic trypsinogen mutants. *FEBS J* (2013 Apr 18, Epub ahead of print), doi:10.1111/febs.12292 [IF: 3.79]
- II. Joergensen MT*, **Geisz A***, Brusgaard K, Schaffalitzky de Muckadell OB, Hegyi P, Gerdes A, Sahin-Tóth M. Intragenic duplication: a novel mutational mechanism in hereditary pancreatitis. *Pancreas* 40, 540–546 (2011). *Equal contributors with shared first authorship. [IF: 2.386]

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- III. Pallagi P, Venglovecz V, Rakonczay Z Jr, Borka K, Korompay A, Ózsvári B, Judák L, Sahin-Tóth M, **Geisz A**, Schnúr A, Maléth J, Takács T, Gray MA, Argent BE, Mayerle J, Lerch MM, Wittman T, Hegyi P. Trypsin reduces pancreatic ductal bicarbonate secretion by inhibiting CFTR Cl⁻ channels and luminal anion exchangers. *Gastroenterology* 141, 2228–2239.e6 (2011). [IF: 11.675]
- IV. Czepán M, Rakonczay Z Jr, Varró A, Steele I, Dimaline R, Lertkowitz N, Lonovics J, Schnúr A, Biczó G, **Geisz A**, Lázár G, Simonka Z, Venglovecz V, Wittmann T, Hegyi P. NHE1 activity contributes to migration and is necessary for proliferation of human gastric myofibroblasts. *Pflugers Arch* 463, 459–475 (2012). [IF: 4.463]

Article not related to the subject of the thesis

- V. Zádori D*, **Geisz A***, Vámos E, Vécsei L, Klivényi P. Valproate ameliorates the survival and the motor performance in a transgenic mouse model of Huntington's disease. *Pharmacol Biochem Behav* 94, 148–153 (2009). *Equal contributors with shared first authorship. [IF: 2.967]

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TABLE OF CONTENTS

1	INTRODUCTION.....	4
1.1	Trypsinogen mutations in hereditary chronic pancreatitis.....	4
1.2	The regulatory effect of chymotrypsin C.....	5
2	AIMS OF THE STUDY.....	6
3	EXPERIMENTAL PROCEDURES.....	7
3.1	Nomenclature.....	7
3.2	Patients.....	7
3.3	Genetic analyses.....	7
3.4	Plasmid construction and mutagenesis.....	7
3.5	Expression and purification of protease zymogens.....	8
3.6	Trypsinogen autoactivation.....	8
3.7	Gel electrophoresis and densitometry.....	8
3.8	Secretion measurements of human cationic trypsinogens.....	8
4	RESULTS.....	10
4.1	A novel intragenic duplication in the <i>PRSS1</i> gene.....	10
4.2	Activation characteristics of K23_I24insIDK mutant cationic trypsinogen.....	10
4.3	Autoactivation of hereditary pancreatitis associated activation peptide mutants in the absence and presence of chymotrypsin C.....	11
4.4	Chymotrypsin C mediated degradation: Cleavage of the Leu81-Glu82 peptide bond in the activation peptide mutants.....	11
4.5	Elevated autoactivation by chymotrypsin C: N-terminal processing of activation peptide mutants.....	12
4.6	Effect of the activation peptide mutations on calcium binding.....	12
4.7	Effect of calcium on autoactivation of the activation peptide mutants.....	12
4.8	Secretion of trypsinogen activation peptide mutants from transiently transfected HEK 293T cells.....	13
5	DISCUSSION.....	14
6	ACKNOWLEDGEMENTS.....	18

1 INTRODUCTION

1.1 Trypsinogen mutations in hereditary chronic pancreatitis

Hereditary chronic pancreatitis (HCP) is a very rare type of early onset chronic pancreatitis. It is defined as an autosomal dominant genetic disorder characterized by incomplete penetrance and variable expressivity. Heterozygous mutations in the serine protease 1 (*PRSSI*) gene have been identified as causative genetic changes in 25-80% of cases in different studies. The *PRSSI* gene encodes human cationic trypsinogen, the dominant digestive proenzyme in human pancreatic secretions. The discovery that the disease is associated with *PRSSI* mutations demonstrates that trypsinogen plays a central role in the pathogenesis of human pancreatitis. The biochemical mechanism behind the genetic factors involves increased ectopic activation of trypsin in the pancreas and failure of protective mechanisms responsible for trypsin inactivation.

Up to now, approximately 70% of the identified HCP cases carry the R122H mutation and about 20 % the N29I mutation. In the remaining 10% of the documented variants, there is an interesting subgroup composed by mutations affecting the activation peptide of human cationic trypsinogen. The trypsinogen activation peptide (TAP) is an eight amino acid long N-terminal sequence containing a strongly conserved tetra-Asp motif preceding the Lys23-Ile24 scissile peptide bond. The characteristic tetra-Asp sequence is presumed to serve as an enteropeptidase recognition motif. Previous data from our laboratory showed that in human cationic trypsinogen the negatively charged tetra-Asp sequence plays only a limited role in enteropeptidase recognition, but it is essential for suppression of autoactivation. It has also been demonstrated that millimolar concentrations of Ca^{2+} , which binds to the above mentioned tetra-Asp motif in the activation peptide and shields the negative charges, can increase trypsinogen activation. Thus, mutations that neutralize any of the Asp residues in the activation peptide, are expected to cause increased autoactivation of trypsinogen.

In addition to the relatively common A16V mutation, there were three other mutations in the activation peptide of human cationic trypsinogen found in association with hereditary pancreatitis namely D19A, D22G and K23R variants. All four TAP mutations were identified in the heterozygous state, and mutations D22G and K23R were found only in a single family each (see details at www.pancreasgenetics.org). Mutation

A16V exhibited variable penetrance and was also found in sporadic cases with no family history.

Although these activation peptide mutations are fairly rare, their functional characterization strongly contributed to the elucidation of the pathomechanism of genetically determined pancreatitis. Biochemical analyses of TAP mutants D19A, D22G and K23R revealed that the common phenotypic change is a markedly increased propensity for autoactivation. In fact, these mutations offered the first convincing evidence that increased autoactivation was a pathologically relevant mechanism in hereditary pancreatitis. However, even though TAP mutations stimulate autoactivation in a dramatic manner, these variants cause the same clinical phenotype as the most frequent R122H and N29I mutations. Mutations D19A and D22G eliminate Asp residues from the characteristic tetra-Asp motif and thereby mitigate its inhibitory effect. Mutation K23R changes the P1 Lys residue to Arg, which is preferred by trypsin owing to a favorable electrostatic interaction in the specificity pocket of the protease. Mutation A16V has no direct effect on autoactivation; however, it increases N-terminal processing of the activation peptide by chymotrypsin C (CTRC), which, in turn, leads to increased autoactivation of cationic trypsinogen.

1.2 The regulatory effect of chymotrypsin C

Functional characterization of HCP-associated *PRSSI* mutations revealed that there are at least two pathological pathways mediating increased pancreatitis risk. Mutations exert their effect via a so-called trypsin-dependent pathological pathway by directly increasing trypsinogen autoactivation or by altering the CTRC-dependent activation and/or degradation of cationic trypsinogen. Alternatively, *PRSSI* mutations can cause misfolding, intracellular retention and degradation with consequent endoplasmic reticulum stress.

The mechanism of action for the most frequently found mutations have been recently elucidated and involves increased resistance against CTRC-mediated degradation and/or increased sensitivity to CTRC-dependent stimulation of autoactivation. CTRC is a pancreatic serine protease which controls autoactivation of human cationic trypsinogen by selectively cleaving regulatory sites within the TAP and the calcium binding loop. The dominant effect of CTRC is trypsinogen degradation, which is triggered by cleavage of the Leu81-Glu82 peptide bond in the calcium binding loop and is facilitated by a trypsin-mediated autolytic cleavage of the Arg122-Val123 peptide bond. CTRC also degrades

active trypsin by the same mechanism but at a slower rate. It has been shown that HCP-associated mutations N29I, N29T, V39A, R122C and R122H decrease or block cleavages at these sites and thereby increase trypsin levels generated during autoactivation.

A secondary, less prominent effect of CTRC on autoactivation is mediated by cleavage of the activation peptide of cationic trypsinogen at the Phe18-Asp19 peptide bond. In human cationic trypsinogen, inhibition of autoactivation is dependent on Asp218, which participates in a repulsive electrostatic interaction with the tetra-Asp motif. CTRC cleavage at the Phe18-Asp19 peptide bond results in a shortened activation peptide, causing partial liberation of the inhibitory interaction with Asp218 and increased autoactivation. Earlier studies proved that pancreatitis-associated mutations A16V and, to a lesser extent, N29I increase N-terminal processing of the activation peptide by CTRC and thereby stimulate trypsinogen autoactivation.

2 AIMS OF THE STUDY

In a hereditary pancreatitis family from Denmark, we identified a novel intragenic duplication of 9 nucleotides in exon 2 of the *PRSSI* gene (c.63_71dup) which at the amino-acid level resulted in the insertion of three amino acids within the activation peptide of cationic trypsinogen (K23_I24insIDK). Our aim was to **characterize the effect of the novel K23_I24insIDK *PRSSI* alteration on the function of human cationic trypsinogen (I).**

In light of the recently discovered CTRC-dependent unifying pathomechanism for HCP, **this study was further aimed at clarifying the role of CTRC in the mechanism of action of the activation peptide mutations (II).**

3 EXPERIMENTAL PROCEDURES

3.1 Nomenclature

Nucleotide numbering reflects the coding DNA numbering with +1 corresponding to the A of the ATG translation initiation codon in *PRSSI*. Amino-acid residues were numbered starting with the initiator methionine of the primary translation product, in accordance with the recommendations of the Human Genome Variation Society.

3.2 Patients

Our study was approved by the Scientific Ethics Committee of the Odense University Hospital and the Danish Data Protection Agency. The diagnosis of chronic pancreatitis (CP) was based on the criteria used by The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) which require two first degree relatives or three or more second degree relatives in two or more generations with recurrent acute pancreatitis, and/or CP for which there are no known precipitating factors.

3.3 Genetic analyses

Blood samples were drawn from the index patient, his brother and his father into tubes with ethylene diamine tetraacetic acid (EDTA) and stored at -20°C . Genomic DNA was extracted from full blood using the Maxwell® DNA purification robot. The samples were tested for small deletions, insertions and point mutations in all exons and the exon-intron boundaries of the *PRSSI* (GenBank NM_002769.3) and *SPINK1* (GenBank NM_003122.3) genes using DHPLC.

3.4 Plasmid construction and mutagenesis

The pTrapT7 intein and pcDNA3.1(-) expression plasmids harboring the coding DNA for human pancreatic digestive proteases were constructed previously in our laboratory. To increase expression levels in cell transfection experiments, the TAP mutations were transferred into the pcDNA3.1(-) p.K237D/p.N241D mutant *PRSSI* background. Mutations in *PRSSI* were generated by overlap extension PCR mutagenesis with designed mutagenic primers.

3.5 Expression and purification of protease zymogens

Wild-type and mutant trypsinogens were expressed in the aminopeptidase P deficient LG-3 *Escherichia coli* strain as fusions with a self-splicing mini-intein. This expression system produces recombinant trypsinogen with uniform, authentic N termini. Refolding and purification of trypsinogen by ecotin affinity chromatography was then carried out. Concentrations of trypsinogen preparations were determined from the UV absorbance at 280 nm using the extinction coefficient $37,525 \text{ M}^{-1} \text{ cm}^{-1}$.

Histidine-tagged forms of human chymotrypsinogens CTB1, CTB2, CTB3 and CTRL1 and proelastases ELA3A and ELA3B were expressed in Human Embryonic Kidney 293T (HEK 293T) cells and purified from the conditioned medium using nickel-affinity chromatography. ELA2A was activated using 10 nM human anionic trypsin and other proteases were activated with immobilized bovine trypsin in and the trypsin beads were removed by centrifugation. Active protease concentrations were determined by active site titration with ecotin.

3.6 Trypsinogen autoactivation

Trypsinogen at 1 μM concentration was incubated with or without 25 nM CTB3 and with 10 nM cationic trypsin. At given times, aliquots were withdrawn and activities were measured with synthetic chromogenic substrate. The release of the yellow p-nitroaniline upon proteolytic cleavage was followed at 405 nm for 1 min using a SpectraMax plus384 microplate reader. Reaction rates were calculated from fits to the initial linear portions of the curves.

3.7 Gel electrophoresis and densitometry

Trypsinogen samples were subjected to 15% SDS-PAGE under reducing conditions. The samples of trypsinogen N-terminal processing experiments were analyzed by 15% non-reducing SDS-PAGE. Quantitation of bands was carried out with the Quantity One 4.6.9 software. Rectangles were drawn around each band of interest, and an identical rectangle was used in each lane for background subtraction.

3.8 Secretion measurements of human cationic trypsinogens

HEK 293T cells were transiently transfected with plasmid harboring the wild-type or mutant trypsinogen gene and conditioned media were harvested after 4, 8, 12, 24, 32 and 48 hours.

For western blot analysis, aliquots of conditioned media (20 μ L per lane) were directly mixed with sample buffer, electrophoresed on SDS-PAGE and blotted onto a Immobilon-P membrane. Trypsinogen was detected with a sheep polyclonal antibody followed by horse-radish peroxidase (HRP)-conjugated donkey polyclonal anti-sheep IgG.

For trypsin activity measurements aliquots of conditioned media were collected, trypsinogens were activated by enteropeptidase and trypsin activity was determined as described above.

4 RESULTS

4.1 A novel intragenic duplication in the *PRSSI* gene

There were three affected members within the study family which satisfy the formal criteria of autosomal dominant hereditary pancreatitis. The DNA sequence analysis of the *PRSSI* gene in the index patient showed a so far unreported duplication in exon 2. The identified c.63_71dup mutation creates an insertion of the Ile-Asp-Lys (IDK) sequence within the activation peptide between amino acids Lys23 and Ile24 (K23_I24insIDK). No other mutations were identified in the *PRSSI* gene and all affected family members were negative for *SPINK1* mutations and a select panel of *CFTR* mutations.

4.2 Activation characteristics of K23_I24insIDK mutant cationic trypsinogen

To study the effect of the K23_I24insIDK mutation on the activation of cationic trypsinogen, we have generated recombinant versions of wild-type and mutant trypsinogens and purified them to homogeneity. When autoactivation of wild-type and mutant trypsinogens were compared in a quantitative manner (pH 8.0, 37 °C), the mutant enzyme autoactivated at rates that were >10-fold higher relative to wild-type trypsinogen.

When we monitored the enteropeptidase-mediated activation of the insertional mutant, we found that activation of the K23_I24insIDK was comparable to that of wild-type (pH 8.0, 37 °C). This result is in accord with our previous studies showing that the TAP plays no significant role in the recognition of human cationic trypsinogen by human enteropeptidase.

We tested the effect of the K23_I24insIDK mutation on cathepsin B-mediated trypsinogen activation at pH 4.0, where autoactivation is minimal. Remarkably, the mutant was activated by cathepsin B at a markedly elevated rate, which seemed approximately 5-10-fold higher than that of wild-type.

We found no change in the degradation of the K23_I24insIDK mutant by cathepsin L (pH 4.0, 37 °C) as compared to wild-type cationic trypsinogen.

4.3 Autoactivation of hereditary pancreatitis associated activation peptide mutants in the absence and presence of chymotrypsin C

A number of recent investigations revealed that cationic trypsinogen and trypsin are under the regulation of CTRC which draws the conclusion that pancreatitis associated mutants may exert their effect in a CTRC dependent manner. To find out whether increased autoactivation is caused directly by mutational impact or influenced by the regulatory effect of CTRC we investigated the autoactivation properties of TAP mutations. Autoactivation of human cationic trypsinogen in the presence of 25 nM CTRC results in a slight increase in the rate accompanied by a marked reduction in final trypsin levels attained. The increased rate is due to N-terminal processing of the activation peptide by CTRC, whereas the reduced trypsin levels are a consequence of CTRC-dependent trypsinogen degradation. When autoactivation of mutants D19A, D22G, K23R and K23_I24insIDK were tested in the absence and presence of CTRC we found that all four mutations increased the rate of trypsinogen autoactivation robustly (3.9-fold, 9.2-fold, 5.7-fold and 17.5-fold, respectively) even in the absence of CTRC, in agreement with previous observations. Similarly, in the presence of CTRC, all four mutants autoactivated markedly faster than wild-type trypsinogen and reached much higher trypsin levels, which then slowly decreased due to CTRC-mediated trypsin degradation. Peak trypsin levels correlated with the rate of autoactivation and were higher in mutants D22G and K23_I24insIDK versus D19A and K23R.

4.4 Chymotrypsin C mediated degradation: Cleavage of the Leu81-Glu82 peptide bond in the activation peptide mutants

Mutation caused defect in the CTRC-dependent degradation during autoactivation can be crucial in the trypsin activity regulation. Because the activation peptide is not in the proximity of the calcium binding loop, mutations D19A, D22G, K23R and K23_I24insIDK are unlikely to affect CTRC-mediated trypsinogen degradation. We tested this assumption experimentally and found that cleavage of the Leu81-Glu82 peptide bond by CTRC was unchanged in the activation peptide mutants compared to wild-type cationic trypsinogen.

4.5 Elevated autoactivation by chymotrypsin C: N-terminal processing of activation peptide mutants

CTRC cleaves the Phe18-Asp19 peptide bond in the TAP and removes three amino acids from the N terminus. This, in turn, results in increased autoactivation of cationic trypsinogen. To assess whether the activation peptide mutations altered CTRC-mediated N-terminal processing, we incubated wild-type and mutant trypsinogens with 50 nM CTRC at pH 8.0, in 1 mM CaCl₂, to minimize cleavage after Leu81. Mutant D19A exhibited 4-fold increased N-terminal processing, whereas mutants D22G, K23R and K23_I24insIDK were processed at rates comparable with wild type. Surprisingly, the enhanced processing of mutant D19A did not translate to a more robust autoactivation increase than seen in mutants D22G or K23R. Similarly, even though mutant K23_I24insIDK was processed normally, this modification had no impact on autoactivation.

CTRC-mediated cleavage of the Phe18-Asp19 peptide bond in the TAP is highly specific and other human chymotrypsins (CTRB1, CTRB2, CTRL1) and elastases (ELA2A, ELA3A, ELA3B) do not catalyze this reaction. We considered the possibility that activation peptide mutations might allow for cleavages by proteases other than CTRC, however, this was not the case. None of the mutant activation peptides was cleaved by any of the chymotrypsins or elastases tested.

4.6 Effect of the activation peptide mutations on calcium binding

The tetra-Asp motif in the TAP binds Ca²⁺, which stimulates autoactivation in a concentration-dependent manner. To determine the effect of the activation peptide mutations on the calcium binding affinity of the activation peptide, we measured the rate of N-terminal processing by CTRC as a function of increasing calcium concentrations. We found that calcium dependence of N-terminal processing by CTRC was comparable for the activation peptide mutants as the calculated K_D values for mutants D19A, D22G, K23R and K23_I24insIDK were 1.1 mM, 1.3 mM, 2.6 mM and 1.3 mM, respectively.

4.7 Effect of calcium on autoactivation of the activation peptide mutants

Calcium binding to the tetra-Asp motif in the TAP stimulates autoactivation. Even though the activation peptide mutations appear to bind calcium normally, the effect of calcium on autoactivation may be altered. When autoactivation of wild-type and mutant trypsinogens were measured in the presence of 0, 1, 5 and 10 mM calcium, wild-type

trypsinogen and mutants D19A and K23R were stimulated in a concentration dependent manner, whereas autoactivation of mutants D22G and K23R_I24insIDK was insensitive to calcium.

4.8 Secretion of trypsinogen activation peptide mutants from transiently transfected HEK 293T cells

We previously found that secretion of activation peptide mutants from transfected cells was reduced, although the four mutants have never been studied in a comparative manner within the same experiment. To establish whether this secretion defect might be a mechanism that partly offsets the drastically increased autoactivation, we quantified secretion of wild-type and mutant trypsinogens from transiently transfected HEK 293T cells. We measured trypsin activity in the conditioned media after enteropeptidase-mediated activation and trypsinogen levels by western blotting. We found that the activation peptide mutants were secreted at significantly lower levels than wild-type trypsinogen, in agreement with previous observations. Importantly, secretion rates for the mutants were inversely proportional with their ability to autoactivate (D19A>K23R>D22G ≈K23_I24insIDK).

5 DISCUSSION

We identified first an intragenic duplication within the *PRSSI* gene in association with HCP. In contrast to the previously reported large-scale gene duplications, in our study family the duplication was confined only to a nine-nucleotide segment within exon 2 without any evidence of more extensive genetic changes. Interestingly, the duplication was identified only in the father and his two sons, while it was absent in the grandparents or in the father's siblings, indicating that it was *de novo* generated in the father. Ours is the second report on capturing a mutational event leading to hereditary pancreatitis.

Intragenic duplications are likely to result in a frame shift and truncated, non-functional protein. In this case, however, the reading frame was kept and at the amino-acid level the duplication generated an insertion within the TAP. As expected from this alteration, the activation properties of cationic trypsinogen have been affected in profound ways. Trypsin-mediated trypsinogen activation (autoactivation) and cathepsin B-mediated trypsinogen activation were both increased by an order-of-magnitude; an effect size never before seen with the known *PRSSI* mutants. With respect to autoactivation, the K23_I24insIDK mutant's phenotype is consistent with the reported properties of other *PRSSI* mutations affecting the activation peptide (D19A, D22G, and K23R), which all result in markedly increased autoactivation. Mechanistically, the increased autoactivation of K23_I24insIDK is explained by the disruption of inhibitory interactions between the negatively charged tetra-Asp motif in the activation peptide and trypsin. In the K23_I24insIDK mutant Asp21 is replaced with a hydrophobic Ile residue and Asp20 by a positively charged Lys.

Cathepsin B has long been known as a pathological activator of trypsinogen in experimental models of acute pancreatitis, cerulein-induced pancreatitis in particular. The effect of hereditary pancreatitis-associated mutations on cathepsin B-mediated trypsinogen activation has been studied in detail previously. Mutant K23_I24insIDK is the first cationic trypsinogen variant that exhibits increased sensitivity to cathepsin B-mediated activation and thus stands in contrast with all other *PRSSI* mutants studied to date. We believe this property is related to the longer activation peptide which allows extended contacts with the activating enzyme.

Another important objective of the present work was to investigate the effect of CTRC on the autoactivation of trypsinogen activation peptide mutants D19A, D22G,

K23R and K23_I24insIDK found in hereditary pancreatitis. Previously, we demonstrated that wild-type cationic trypsinogen is largely degraded by CTRC during autoactivation, whereas hereditary pancreatitis associated mutants N29I, N29T, V39A, R122C and R122H exhibited resistance to CTRC-mediated degradation and autoactivated to higher trypsin levels. In addition, we found that mutations A16V and N29I increased CTRC-mediated cleavage of the activation peptide, and thereby accelerated autoactivation. We speculated that activation peptide mutants might also affect processing of the activation peptide by CTRC and/or alter the effect of this cleavage, perhaps even resulting in decreased autoactivation. This would then offer an explanation for the puzzling observation that the robust autoactivation of activation peptide mutants is not accompanied by a more severe clinical picture.

Our results confirmed that even in the absence of CTRC activation peptide mutants autoactivated at markedly increased rates (~4-18-fold). In the presence of CTRC, activation rates were further increased slightly (D19A, D22G, K23R) or remained unchanged (K23_I24insIDK), indicating that the robust autoactivation of the activation peptide mutants is mostly independent of CTRC. Peak trypsin levels attained during autoactivation positively correlated with the rate of autoactivation, indicating that faster conversion of trypsinogen to trypsin results in higher trypsin levels, as trypsin is less sensitive to CTRC-mediated degradation than trypsinogen. As expected, the mutations had no effect on CTRC cleavage of the Leu81-Glu82 peptide bond in the calcium binding loop and consequent trypsinogen degradation. Importantly, when compared to the most common pancreatitis-associated mutant R122H, in the presence of CTRC the activation peptide mutants still exhibited much higher rates of autoactivation (~3-10-fold).

N-terminal processing of the activation peptide at Phe18 by CTRC was unchanged in mutants D22G, K23R and K23_I24insIDK, whereas mutant D19A was processed at 4-fold increased rate. Surprisingly, however, the increased processing of mutant D19A was paralleled only with a slight increase in the rate of autoactivation, suggesting that Asp19 is important for mediating the functional effect of N-terminal processing. Thus, shortening the activation peptide by CTRC may increase autoactivation by partly relieving the inhibitory interaction between Asp218 and Asp19. This effect is probably due to neutralization of the negative charge on Asp19 by the newly created proximity of the positively charged amino terminus. Similarly, mutation D19A would increase autoactivation by neutralizing Asp19 but at the same time it would diminish the effect of N-terminal processing by CTRC. This notion is supported by the observations that the

extent of the autoactivation increase either by CTRC processing or by mutation D19A is comparable, approximately 3-4-fold. The stimulatory effect of CTRC cleavage on autoactivation was also abolished in mutant K23_I24insIDK, which may be readily explained by sterical uncoupling of the original tetra-Asp motif from the activation site by the three-amino-acid insertion. Furthermore, previous experiments indicated that trypsin-mediated activation (i.e. autoactivation) of the K23_I24insIDK mutant most likely proceeds by sequential cleavage of the two lysyl peptide bonds found in the mutated activation peptide. In this case, cleavage of the N-terminal Lys-Ile peptide bond would eliminate the N-terminal eight amino acids together with any effect of CTRC-dependent processing.

Calcium in millimolar concentrations stimulates autoactivation presumably by binding to the activation peptide and shielding the inhibitory negative charges of the tetra-Asp motif. Using the CTRC-dependent processing of the activation peptide as readout, we determined the calcium binding affinity of wild-type and mutant activation peptides. Unexpectedly, mutant and wild-type trypsinogens bound calcium at the activation peptide with comparable affinities, 1.6 mM on average. This observation suggests that calcium probably engages only two or three Asp side chains and elimination of one of the four Asp residues either by mutation D19A or D22G is tolerated. When the functional effect of calcium binding was investigated, however, we found that autoactivation of mutants D22G and K23_I24insIDK were insensitive to calcium. The results suggest that the stimulatory effect calcium on autoactivation is mediated through binding to Asp22 and neutralizing its negative charge. During autoactivation, Asp22 in the activation peptide binds to the S2 subsite on trypsin, a conserved hydrophobic pocket formed by His63, Leu104 and Trp216, and this unfavorable interaction may be alleviated by calcium. In the K23_I24insIDK mutant, calcium likely binds only to the original tetra-Asp motif, which is now further removed from the activation peptide bond. The Asp-Ile-Lys-Asp sequence preceding the activation site does not seem competent to bind calcium. Furthermore, as pointed out above, during autoactivation the N-terminal eight amino acids are removed before cleavage at the activation peptide takes place.

The biochemical properties of the TAP mutants do not explain why the markedly increased autoactivation is not associated with a more severe clinical presentation of hereditary pancreatitis. Previously, we observed that secretion of activation peptide mutants was compromised from transfected cells due to intracellular autoactivation and ensuing degradation. We extended these studies here and quantitatively compared, for the

first time, cellular secretion of all four activation peptide mutants to determine whether reduced secretion could offset the effect of increased autoactivation. Indeed, we confirmed not only that all four activation peptide mutants were secreted at lower levels than wild-type trypsinogen, but also that the secretion defect inversely correlated with the rates of autoactivation. Our observations indicate that nature carefully titrated trypsinogen secretion against autoactivation propensity, to curb the risk for excessive pathological intra-pancreatic trypsinogen activation in carriers of the activation peptide mutations.

Our results define the TAP mutations D19A, D22G, K23R and K23_I24insIDK as a special subset of hereditary-pancreatitis associated mutations which stimulate autoactivation largely independently of CTRC and this robust effect is compensated by their reduced secretion. Taken together with previous studies, the observations indicate that human cationic trypsinogen mutations may increase autoactivation by several independent but not mutually exclusive mechanisms in HCP: (i) increased secretion, as seen with copy number mutations; (ii) resistance to CTRC-mediated degradation; (iii) increased processing of the activation peptide by CTRC and (iv) direct stimulation of autoactivation, as demonstrated here for the activation peptide mutations.

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