THE ROLE OF CATIONIC TRYPSINOGEN AND ITS MUTATIONS IN PANCREATITIS

Ph.D. Thesis

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

I. **Schnúr A**, Beer S, Witt H., Hegyi P., Sahin-Tóth M. Functional effects of 13 rare *PRSS1* variants presumed to cause chronic pancreatitis. *Gut* 2013 Mar 1 (Epub ahead of print) **IF_{2013}: 10.111**

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III. Farkas K, Yeruva S, Rakonczay Z Jr, Ludolph L, Molnár T, Nagy F, Szepes Z, **Schnúr A**, Wittmann T, Hubricht J, Riederer B, Venglovecz V, Lázár G, Király M, Zsembery Á, Varga G, Seidler U, Hegyi P. New therapeutic targets in ulcerative colitis: the importance of ion transporters in the human colon. *Inflamm Bowel Dis* 2011; 17(4):884-898. **IF_{2011}: 4.855**

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1 INTRODUCTION

Chronic pancreatitis (CP) is a long-term progressive inflammatory disease of the pancreas characterized by repeated attacks of abdominal pain and irreversible morphological changes which leads to the impairment of the exocrine as well as the endocrine component of the gland and eventually result in maldigestion and diabetes. Currently, there is no definitive medical treatment for CP. Treatment options are limited to enzyme replacement therapy, pain relief and surgical interventions.

Trypsin was assigned to play the key role in initiating the disease by triggering an activation cascade of the digestive zymogens in the pancreas leading to the self-digestion of the gland. This pathogenic concept was supported by *gain-of function* mutations found in the cationic trypsinogen. Consistently, *loss-of-function* variants in serine protease inhibitor, Kazal type 1 (SPINK1) gene encoding a pancreatic trypsin inhibitor, and the trypsin-degrading enzyme chymotrypsin C (CTRC) are strongly associated with idiopathic CP. Protective mechanisms evolved to curtail premature activation of zymogens. One of the defense mechanisms might be the hypersecretion of the bicarbonate-rich, alkaline pancreatic juice. Besides neutralizing the acidic chyme entering the duodenum from the stomach, pancreatic fluid is responsible for flushing out digestive enzymes down the ductal tree and thereby preventing their early activation. The pancreatic juice has an alkaline pH, achieved by high concentration of bicarbonate secreted by the duct cells, that is considered important for the prevention of trypsin activation in the ductal space. It has been suggested that the fall of intraductal pH may increase the risk of developing pancreatitis.

Hereditary pancreatitis (HP) is defined by the presence of a proteinase serine 1 gene *PRSS1* mutation, encodes the cationic trypsinogen. In the large majority of HP families, the causative *PRSS1* mutation is either p.R122H (~70%) or p.N29I (~20%). Other less frequent *PRSS1* mutations documented to cause pancreatitis with a family history include p.A16V, p.N29T, p.V39A, p.R116C and p.R122C. Interestingly, mutation p.A16V exhibits variable penetrance and was often found in sporadic cases as well. Over the past decade, worldwide screening of *PRSS1* broadened the mutational spectrum. More than 50 additional *PRSS1* variants have been reported, the majority of which were found in patients with sporadic CP

(for a complete list with references see www.pancreasgenetics.org) and often assigned by disease causing mutation despite the lack of phenotypic characterization.

The mechanism of action of HP-associated mutations involves increased autoactivation of mutant trypsinogens resulting in elevated intrapancreatic trypsin activity level. Recent studies uncovered that these *PRSS1* mutations alter the regulation of activation and degradation of cationic trypsinogen by CTRC. Pancreatitis-associated mutations render trypsinogen resistant to CTRC-dependent degradation and/or increase N-terminal processing by CTRC and thereby elevate trypsin levels generated through autoactivation.

The unifying pathological mechanism described above does not seem to apply to some mutations that alter the number of cysteine residues in cationic trypsinogen. HP-associated mutation p.R116C was shown to induce protein misfolding with intracellular retention and degradation, which may represent an alternative disease-causing mechanism unrelated to trypsinogen activation and trypsin activity. Mutation-dependent misfolding can elicit Endoplasmic reticulum (ER) stress which might be responsible for increased pancreatitis risk, although the mechanism remains unclear.

2 AIMS

I. The secretion of the alkaline pancreatic juice is considered to prevent intraductal trypsin activation. Inhibition of fluid secretion, accompanied by a decrease in intraductal pH, was proposed to increase the risk of developing CP. Therefore, we investigated the effect of pH on the autoactivation of human cationic trypsinogen.

Our specific aim was:

- *To investigate the autoactivation of human trypsinogen at different pH*

II. Numerous rare *PRSS1* mutations with unknown clinical significance were identified in subjects with idiopathic CP. Despite lack of evidence, some of these variants have been described as pancreatitis-associated. To clarify the possible pathogenic role of these mutations, our aim was to functionally characterize 13 published trypsinogen variants:

1. Our primary objective was to test whether these variants also exhibit increased activation in the presence of CTRC, as previously seen with disease-causing mutants in HP.

Our specific aims were:

- *To examine the kinetic parameters of variants*
- *To investigate their autoactivation*
- *To determine the effect of the CTRC on their autoactivation*
- *To examine their degradation by CTRC*

2. Mutation-induced changes in folding and secretion may be another disease relevant mechanism, therefore, our second objective was to assess whether mutation-induced misfolding is a more common phenotype of *PRSS1* variants than previously appreciated.

Our specific aim was:

- *To assess cellular secretion of the mutants*

3 MATERIALS AND METHODS

3.1 Nomenclature

Nucleotide numbering reflects coding DNA numbering with +1 corresponding to the A of the ATG translation initiation codon in *PRSS1*. Amino acid residues are numbered starting with the initiator methionine of the primary translation product for human cationic trypsinogen. The abbreviations are in *italics* throughout the text when referring to genes encoding digestive zymogens

3.2 Mutagenesis, expression and purification of human cationic trypsinogens

The expression plasmids harboring the human cationic trypsinogen gene, was constructed previously in our laboratory. Mutations were introduced by oligonucleotide-directed overlap-extension PCR mutagenesis. PCR products were analyzed by agarose gel electrophoresis and DNA sequencing. Trypsinogen variants were cloned in fusion with a 154 amino acid long mini-intein. This construct was expressed in an aminopeptidase P deficient *Escherichia coli* strain (LG-3) as inclusion bodies.

Inclusion bodies, containing the trypsinogens were isolated by sonication and centrifugation and *in vitro* refolding of trypsinogens was performed. Trypsinogens were purified using ecotin-affinity chromatography, utilizing the column-bound ecotin. Protein concentration of eluted trypsinogen was calculated from the UV absorbance at 280 nm using the extinction coefficient 37,525 $M^{-1}cm^{-1}$.

3.3 Measurements of enzyme activities and kinetic parameters of trypsin

Trypsin activity was 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. To obtain kinetic parameters, activity measurements were performed at varying substrate concentrations (0-180μM) and initial rates were plotted as a function of substrate concentration. Kinetic parameters (V_{max}, K_M) were determined from hyperbolic fits to the Michaelis-Menten equation.

3.4 Trypsinogen autoactivation in the presence and absence of chymotrypsin C

Trypsinogen at 1 μM concentration was incubated with or without 5 nM human CTRC and with 10 nM cationic trypsin. At given times, aliquots were withdrawn and activites were measured as described above. For autoactivation experiments at varying pH ranging from 6 to 8.5, a polybuffer system (100 mM MES, 100 mM HEPES and 100 mM Tris) was used. The pH of the polybuffer was adjusted to given values with HCl. (pH 6.0 and 6.5) or NaOH (pH 7.0, 7.5, 8.0 and 8.5).

3.5 Secretion measurements of human cationic trypsinogens

Human Embryonic Kidney cells (HEK 293T)cells were transiently transfected with plasmid harboring the wild-type or mutant trypsinogen gene and conditioned media were harvested after 24 hours. Expressed protein levels were qualitatively analyzed on Coomassie Blue stained SDS-polyacrylamide gels, and quantitatively determined by Western blots and densitometry.

For western blot analysis 5 μL Samples were subjected to SDS-PAGE and blotted onto a Immobilon-P membrane. Media were assayed using an antibody raised against the native trypsinogen. After the antibody against the native protein raised in sheep, Horseradhish-peroxidase (HRP)-conjugated donkey polyclonal anti-sheep IgG was used. HRP was detected using the SuperSignal West Pico Chemiluminescent Substrate. Quantitation of bands was carried out with Image Lab 3.0 software.

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

4 RESULTS

4.1 Autoactivation of human cationic trypsinogen is pH dependent

We examined the autoactivation of human cationic trypsinogen *in vitro* at pH values ranging from 6.0 to 8.5. The rate at which cationic trypsinogen autoactivates was increased when the pH was reduced from 8.5 to 7.0 in the presence of 1 mM $CaCl_2$ and in the absence of NaCl. However, decreasing the pH from 7.0 to 6.0 had a slight effect only. To exclude that the differences observed in autoactivation were due to the different ionic strength of the buffers used, we performed the experiments in the presence of higher concentration of sodium (100 mM NaCl) or lower concentration of calcium (0.1 mM $CaCl_2$). Although the overall autoactivation rates were much slower in the presence of 100 mM NaCl, the pH profile of autoactivation in the presence of 100 mM NaCl was essentially identical to that observed in the absence of added salt. The pH dependent changes in the autoactivation of trypsinogen were still detectable when the experiments were performed using a low calcium buffer.

4.2 *PRSS1* missense variants are exceedingly rare in the general population

We surveyed the functional properties of 13 rare *PRSS1* missense mutations (p.P36R, p.G83E, p.I88N, p.K92N, p.Q98K, p.D100H, p.V123M, p.S124F, p.T137M, p.C139F, p.K170E, p.S181G and p.G208A) found in patients with sporadic CP. The 13 *PRSS1* variants were found in patients with idiopathic CP without family history. The variants were found only in one to three cases each, therefore, the genetic information was insufficient to determine whether these variants are pathogenic or neutral.

To get an estimate of the prevalence of missense *PRSS1* variants in healthy individuals, we sequenced exon-3 of the *PRSS1* gene in 1000 German subjects without any pancreatic disease. Exon-3 was selected because most of the studied variants (9 of 13) were found in this region. With the exception of a novel c.367G>T (p.V123L) variant found in one subject, no other alterations were identified. We consulted the NHLBI Exome Sequencing ProjectExome Variant Server (http://evs.gs.washington.edu) which lists aggregate exome-

sequencing data for approximately 4300 European-American and 2200 African-American individuals. We found *PRSS1* variants p.V123M and p.T137M in one European-American subject each and variant p.S181G in one African-American subject. However, interpretation of these findings is difficult, because the database also lists the disease-causing mutation p.R122C found in two European-American individuals, suggesting that the studied cohort may have contained pancreatitis patients. We also surveyed published sequencing data, which showing the absence of these 13 *PRSS1* variants in 200 French, 82 German, 420 Chinese and 28 Korean control subject.

4.3 Enzyme kinetic parameters of trypsin and autoactivation of trypsinogens

Trypsinogen variants were characterized for catalytic activity and their ability to autoactivate in the absence of CTRC. Only one mutant, p.D100H, exhibited increased autoactivation, which was about 2.5-fold faster relative to wild type. Five mutants (p.Q98K, p.T137M, p.K170E, p.S181G, and p.G208A) autoactivated comparable to wild-type trypsinogen, while two (p.K92N and p.S124F) exhibited a decreased autoactivation rate, although they reached the same activity level. In contrast four of the investigated mutants (p.P36R, p.G83E, p.I88N, and p.V123M) exhibited decreased rates of autoactivation with reduced final level, suggesting that these variants may become partially degraded during autoactivation.

Enzyme kinetic parameters of trypsin variants were comparable to those of wild-type cationic trypsin. This finding is in accordance with previous observations that natural *PRSS1* variants almost never affect catalytic function of trypsin.

4.4 Autoactivation of wild-type and mutant trypsinogens in the absence and presence of chymotrypsin C

Cationic trypsinogen and trypsin are under the regulation of CTRC. CTRC promotes proteolytic degradation of trypsinogen and trypsin and also stimulates trypsinogen autoactivation. To determine the effect of CTRC on trypsinogen variants, we measured autoactivation in the absence and presence of CTRC.

Only one of the 12 variants tested, p.D100H exhibited increased activation in the presence of CTRC. A similar phenotype was observed with this mutant when autoactivation was performed in the absence of CTRC, indicating that the mutation increases autoactivation independent of CTRC. Six mutants (p.K92N, p.Q98K, p.T137M, p.K170E, p.S181G, and p.G208A) autoactivated in the presence of CTRC in a manner that was comparable to wild type. Surprisingly, however, five mutants (p.P36R, p.G83E, p.I88N, p.V123M and p.S124F) reached markedly reduced trypsin activity levels during autoactivation, suggesting increased susceptibility to CTRC-dependent degradation.

4.5 Degradation of wild type and mutant trypsinogens/trypsins by chymotrypsin C

Five mutants (p.P36R, p.G83E, p.I88N, p.V123M and p.S124F) reached markedly reduced trypsin activity levels during autoactivation in the presence of CTRC, suggesting increased susceptibility to CTRC-dependent degradation. We performed direct degradation experiments to confirm this notion. We tested degradation of trypsin and trypsinogen by CTRC. Indeed these five mutants exhibited an increased trypsin as well as trypsinogen degradation by CTRC.

4.6 Secretion of *PRSS1* variants from HEK 293T cells

To identify trypsinogen variants defective in folding, we evaluated their secretion from transiently transfected HEK 293T cells.

Six of 13 mutants tested (p.P36R, p.G83E, p.Q98K, p.V123M, p.T137M, and p.S181G) showed trypsinogen secretion close to wild-type levels (~70-120%). Severe secretion defects (~20% of wild type) were observed with mutants p.D100H and p.C139F. Moderate reduction in secretion (~40-50% of wild type) was noted in case of three mutants (p.K92N, p.S124F and p.G208A). Two mutants (p.I88N and p.K170E) showed increased secretion levels, 170% and 140% of wild type, respectively. Although increased secretion of variant p.I88N may represent a gain of function, the rapid degradation of this variant by CTRC (see above) would cancel out this effect and result in a *loss-of-function* phenotype. For the majority of the variants, the trypsin activity levels in the conditioned medium correlated

well with the protein levels secreted. Four variants (p.P36R, p.G83E, p.K92N, and p.C139F) had considerably lower enzyme activity relative to their protein levels. Variant p.C139F may be misfolded and catalytically defective, which explains its lower activity. The other three variants exhibited normal catalytic activity in enzymatic tests (see above); suggesting that degradation or incomplete activation may underlie their lower activity.

5 DISCUSSION

A growing body of evidence supports the hypothesis that elevated intrapancreatic trypsin activity is highly significant in the pathogenesis of pancreatitis. There are protective mechanisms, such as the continuous ductal fluid secretion, responsible for the arrest of early trypsin activation. Impairment of bicarbonate secretion leads to the increase in the transit time of zymogens down the ductal tree and also decreases luminal pH ($pH_{L)}$. In light of these view, we tested whether trypsinogen autoactivation was affected by pH over the range 6.0 to 8.5.

Autoactivation of trypsinogen was relatively slow at pH 8.5, but decreasing the pH from 8.5 to 7 stimulated autoactivation. These results suggest that under physiological conditions bicarbonate secretion by pancreatic ductal epithelial cells is not only important for elevating the pH in the duodenum, but also for keeping digestive enzymes in an inactive state. On the other hand, our results indicate that not only the increased time but the decreased pH_L will contribute to the early trypsin activation

We investigated the functional properties of 13 rare *PRSS1* variants detected in patients with CP. The clinical significance of such variants has been a contentious issue because their very low frequency did not allow statistical determination of genetic association with the disease phenotype. Segregation with pancreatitis within families could not be observed either, because these variants were found in cases with no family history. In this work, we used functional analysis as a tool to identify phenotypic similarities between rare variants and well-characterized known disease-causing *PRSS1* mutations. The common biochemical phenotype of *PRSS1* mutations associated with HP is the generation of greatly increased trypsin levels during autoactivation in the presence of CTRC. Simply put, if a rare variant exhibits similar properties as HP associated mutations, then this variant is very likely pathogenic. Conversely, phenotypically neutral variants are likely to be clinically irrelevant. Unexpectedly, we observed that only one (p.D100H) of 13 mutants had a gain-of-function phenotype, which might contribute to the development of pancreatitis. However, this variant also exhibits a severe secretion defect and is therefore less likely to reach significant intrapancreatic trypsin activity. Contrariwise, half of the trypsinogen variants showed an

increased propensity to CTRC degradation, resulting in lower trypsin levels during autoactivation relative to wild-type trypsinogen. This biochemical phenotype is inconsistent with the trypsin-dependent model of HP and suggests that these variants are not pathogenic (p.P36R, p.G83E, p. I88N, p.V123M).

ER-stress is proposed to represent a different pathomechanism leading to pancreatitis. Mutation-induced protein misfolding can cause ER-stress, especially if the protein is made in huge amounts, as it is the case of digestive enzymes in the pancreatic acinar cells. Furthermore, it can be hypothesized that the threshold of pathological ER-stress correlates with the abundance of each protein made. As cationic trypsinogen is the most abundant protease secreted from the pancreatic acinar cell, misfolding might have a significant impact. Although, most mutants investigated in this study did not cause a pancreatitis-associated gain-of-function phenotype, secretion defect, likely caused by mutation-induced misfolding, was observed in the present study for variants p.D100H and p.C139F and, to a smaller extent, variants p.K92N, p.S124F and p.G208A, suggesting that these variants are pathogenic. Increased secretion of mutant trypsinogens may result in higher trypsinogen levels in the pancreatic juice with consequently increased risk for autoactivation. Copy number mutations in trypsinogen genes may exert their pathogenic effect via this mechanism. In this study, we observed slightly increased secretion with two mutants, p.I88N and p.K170E. While the rapid degradation of mutant p.I88N by CTRC would counteract the effect of higher trypsinogen concentrations, the phenotype of mutant p.K170E may indicate a true gain of function with potentially elevated risk of pancreatitis. Finally, three trypsinogen variants (p.Q98K, p.T137M and p.S181G) proved functionally neutral in this study, indicating that *PRSS1* variants of no clinical significance may be incidental findings when subjects with CP are screened for underlying genetic defects.

Taken together, our experimental results indicate that most of the investigated rare *PRSS1* variants do not phenocopy the disease causing HP-associated *PRSS1* mutations (p.R122H, p.R122C, p.N29I.). Instead, loss-of-function characteristics related to increased degradation or reduced secretion were observed mostly. Variants with decreased secretion may increase pancreatitis risk through an alternative pathological pathway related to mutation-induced misfolding and consequent ER stress.

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