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Hereditary pancreatitis caused by mutation induced misfolding of human cationic trypsinogen - a novel disease mechanism

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Abstract

We investigated the biochemical properties and cellular expression of the c.346C>T (p.R116C) human cationic trypsinogen (PRSS1) mutant, which we identified in a German family with autosomal dominant hereditary pancreatitis. This mutation leads to an unpaired Cys residue with the potential to interfere with protein folding via incorrect disulfide bond formation. Recombinantly expressed p.R116C trypsinogen exhibited a tendency for misfolding *in vitro*. Biochemical analysis of the correctly folded, purified p.R116C mutant revealed unchanged activation and degradation characteristics compared to wild type trypsinogen. Secretion of mutant p.R116C from transfected 293T cells was reduced to ~20% of wild type. A similar secretion defect was observed with another rare PRSS1 variant, p.C139S, whereas mutants p.A16V, p.N29I, p.N29T, p.E79K, p.R122C, and p.R122H were secreted normally. All mutants were detected in cell extracts at comparable levels but a large portion of mutant p.R116C was present in an insoluble, protease-sensitive form. Consistent with intracellular retention of misfolded trypsinogen, the endoplasmic reticulum (ER) stress markers BiP and *XBP1s* were elevated in cells expressing mutant p.R116C. The results indicate that mutation induced misfolding and intracellular retention of human cationic trypsinogen causes hereditary pancreatitis in carriers of the p.R116C mutation. ER stress triggered by trypsinogen misfolding represents a new potential disease mechanism for chronic pancreatitis.

Keywords

chronic pancreatitis; trypsinogen mutation; PRSS1; protein misfolding; endoplasmic reticulum stress; ER; unfolded protein response

INTRODUCTION

Chronic pancreatitis is a persistent inflammatory disease of the pancreas typically caused by alcohol abuse, metabolic disorders or unknown etiology. Inherited susceptibility to chronic pancreatitis is a complex genetic condition which can involve alterations in several genes conferring various degrees of risk (Witt et al., 2007). Most patients exhibit no family history of the disease. Autosomal dominant hereditary pancreatitis (MIM# 167800) is an interesting exception, in which a single genetic risk factor is causative and patients usually present with

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a strong family history following a dominant Mendelian inheritance pattern with characteristically incomplete penetrance and variable expressivity. Hereditary pancreatitis shows a strong association with the *PRSSI* gene (protease, serine 1; MIM# 276000), mutations of which are found in about 30–50% of affected families (Whitcomb et al., 1996; Applebaum-Shapiro et al., 2001; Simon et al., 2002b; Keim et al., 2003; Howes et al., 2004; Teich et al., 2006; Rebours et al., 2008). The *PRSSI* gene encodes cationic trypsinogen, the most abundant digestive proenzyme in humans. Mutations p.R122H and p.N29I are found in about 70% and 25% of hereditary pancreatitis families, respectively, and these two variants cause clinically indistinguishable disease (Whitcomb et al., 1996; Applebaum-Shapiro et al., 2001; Howes et al., 2004). Worldwide screening of the *PRSSI* gene to date resulted in the identification of 24 additional rare variants, either in families with hereditary pancreatitis or in idiopathic cases with no apparent family history (Teich et al., 2006, and references therein).

The functional effects of the pancreatitis-associated mutations have been studied on recombinant human cationic trypsinogen preparations *in vitro* (for recent reviews see Sahin-Tóth, 2006; Teich et al., 2006). The most consistently observed biochemical defect was an increased propensity for trypsin-mediated trypsinogen activation, commonly referred to as autoactivation. In addition, mutation p.R122H was shown to render cationic trypsin resistant to degradation by chymotrypsin C (Szmola and Sahin-Tóth, 2007). On the basis of these findings it was proposed that most *PRSSI* variants are gain-of-function mutations which cause hereditary pancreatitis by promoting premature trypsinogen activation in the pancreas.

In the present study we examined the effect of the p.R116C mutation on the function and secretion of cationic trypsinogen. This relatively rare mutation has been previously identified among patients of widely varying ethnicities such as Thais, Turks and Europeans (Le Maréchal et al., 2001; Tautermann et al., 2001; Teich et al., 2002; Pho-Iam et al., 2005) and here we confirm its association with autosomal dominant hereditary pancreatitis in a German kindred. Moreover, we demonstrate that mutation induced misfolding of cationic trypsinogen results in intracellular retention and decreased secretion. Finally, we find that intracellular misfolding of mutant p.R116C causes endoplasmic reticulum (ER) stress and elicits the unfolded protein response. The results point to an entirely novel mechanism whereby *PRSSI* mutations cause hereditary pancreatitis, which is unrelated to trypsinogen activation but involves mutation induced proenzyme misfolding and consequent ER stress.

METHODS

Mutation screening

A nuclear family of an 11 year old girl with recurrent pancreatitis (presently aged 17), an unaffected brother, an unaffected mother and a father with chronic pancreatitis presented at our clinic in Germany. The father had overcome an alcohol problem and the remaining family members were teetotalers. Other risk factors such as gallstone disease, pulmonary symptoms indicative of cystic fibrosis, hyperparathyroidism and hyperlipidemia were excluded. Hereditary pancreatitis was suspected and the family members, including the mother's father who also suffers from chronic relapsing pancreatitis, gave their informed consent for genetic screening. The entire coding region of *PRSSI* (GenBank NM_002769.3) was sequenced and only a heterozygous c.346C>T (p.R116C) variation was identified in the indicated family members (Fig 1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. Other potential genetic risk factors such as the c.101A>G (p.N34S) mutation in the *SPINK1* gene (MIM# 167790; GenBank NM_003122.2) (Witt et al., 2000) and the c.760C>T (p.R254W) and c.738_761del24 (p.K247_R254del) mutations in the *CTRC* gene (MIM# 601405; GenBank NM_007272.2) (Rosendahl et al., 2008) were ruled out. The mother's

paternal grandfather, who was unavailable for genetic testing, also had a history of chronic pancreatitis.

Plasmid construction and mutagenesis

The pTrapT7_PRSS1 and pcDNA3.1(-)_PRSS1 expression plasmids were constructed previously (Sahin-Tóth, 2000; Sahin-Tóth and Tóth, 2000; Nemoda and Sahin-Tóth, 2006). Missense mutations p.A16V, p.N29I, p.N29T, p.E79K, p.R116A, p.R116C, p.R122C, p.R122H, and p.C139S as well as Glu-Glu-tagged constructs were generated by overlap extension PCR mutagenesis.

Expression and purification of cationic trypsinogen

Wild type, p.R116C and p.R116A cationic trypsinogens were expressed in *Escherichia coli* Rosetta (DE3) as cytoplasmic inclusion bodies. Refolding and purification of trypsinogen on immobilized ecotin was carried out as reported previously (Lengyel et al., 1998; Sahin-Tóth, 2000; Sahin-Tóth and Tóth, 2000). Concentrations of trypsinogen solutions were determined from the UV absorbance at 280 nm using the extinction coefficient $36,160 \text{ M}^{-1} \text{ cm}^{-1}$ (<http://ca.expasy.org/tools/protparam.html>).

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells and Paca44 pancreatic adenocarcinoma cells were cultured in 6-well tissue culture plates (10^6 cells per well) in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO₂. Transfections were performed using 4 µg pcDNA3.1(-)_PRSS1 plasmid and 10 µL Lipofectamine 2000 (Invitrogen) in 2 mL DMEM. After overnight incubation at 37 °C, cells were washed and the transfection medium was replaced with 2 mL OptiMEM. Time courses of expression were measured starting from this medium change and were followed for 24 h. We found that autoactivation of secreted cationic trypsinogen and consequent trypsinization of cells was not significant within this time and inclusion of a trypsin inhibitor was not necessary.

Preparation of cell lysates

Transfected cells were washed twice with phosphate buffered saline. Two-hundred µL reporter lysis buffer (Promega) and 4 µL protease inhibitor cocktail (Sigma, #P8340) was added to each well and the cells were scraped and briefly vortexed. After 15 min incubation at room temperature the lysates were cleared by centrifugation. The protein concentration of the supernatant was measured with Micro BCA™ Protein Assay Kit (Thermo Scientific).

RT-PCR analysis

RNA was isolated from HEK 293T cells transfected with given plasmids using the RNAqueous kit (Ambion) and 1 µg RNA was reverse-transcribed with the SMART PCR cDNA Synthesis Kit (Clontech). Semi-quantitative measurements of *XBP1* and *XBP1s* (spliced form) cDNAs were performed by PCR using the following primers that amplify both forms (Hirota et al., 2006). *XBP1* sense primer, 5'-CCT TGT AGT TGA GAA CCA GG -3', *XBP1* antisense primer, 5'-GGG CTT GGT ATA TAT GTG G -3'. The *XBP1* primers generate 441 nt and 415 nt amplicons from the unspliced and spliced cDNAs, respectively. As a reference control, a 261 nt fragment of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was also amplified using pseudogene-free amplification conditions (Harper et al., 2003). *GAPDH* sense primer 5'-GTC CAC TGG CGT CTT CAC CA -3', *GAPDH* antisense primer 5'-GTG GCA GTG ATG GCA TGG AC -3'.

Western blot analysis

To facilitate specific immunodetection of cationic trypsinogen, a Glu-Glu epitope tag was incorporated at the C terminus of the PRSS1 mutants. This tag contains the EYMPME peptide sequence derived from the polyoma virus medium T antigen (Grussenmeyer et al., 1985). Aliquots of conditioned media (10 µL per lane) or cell lysates (20 µg protein per lane) were electrophoresed on Tris-glycine minigels and transferred onto Immobilon-P membranes (Millipore). The membranes were blocked with 5% milk powder solution overnight and incubated with given primary and secondary antibodies for 1 h at room temperature. To detect the Glu-Glu-tag, a horseradish peroxidase (HRP)-conjugated goat polyclonal antibody (Abcam, #ab1267) was used at a dilution of 1:10,000. BiP was detected with a rabbit polyclonal antibody against a synthetic peptide of mouse BiP; which reacts with mouse, rat and human isoforms (Abcam, #ab21685). The anti-BiP antibody was used at a dilution of 1:5,000, followed by HRP-conjugated goat polyclonal anti-rabbit IgG (Pierce, #31460) at a dilution of 1:20,000. Actin was detected with a mouse monoclonal antibody (Sigma, #A4700, clone AC-40) used at a dilution of 1:1,000 followed by HRP-conjugated goat polyclonal anti-mouse IgG (Abcam, #ab6789) at a dilution of 1:2,500. HRP was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

The c.346 C>T (p.R116C) cationic trypsinogen mutation is associated with hereditary pancreatitis

Mutation c.346 C>T (p.R116C) in the *PRSS1* gene was originally described by three independent groups in 2001–2002. Tautermann et al. (2001) identified this variant in four members of a Turkish family with hereditary pancreatitis. The p.R116C mutation was present in three generations (grandfather – father – son and daughter), however, only the father and his daughter were affected by chronic pancreatitis, indicating incomplete penetrance. Le Maréchal et al. (2001) found the p.R116C mutation in two unrelated French patients affected by idiopathic chronic pancreatitis. Teich et al. (2002) identified the p.R116C mutation in a pediatric patient with recurrent acute pancreatitis and in her unaffected mother. More recently, Pho-Iam et al. (2005) described a Thai family with hereditary pancreatitis in which the p.R116C mutation was present in three affected (mother, son, mother's sister) and one unaffected (mother's brother) members.

Here we present an extended pedigree and detailed genetic analysis of a German family with hereditary pancreatitis carrying the p.R116C mutation (Fig 1), first reported by Teich et al. (2002). The mutation was found in the 11 year old proband, her unaffected mother and affected grandfather. All three carriers were negative for the p.N34S *SPINK1* variant (Witt et al., 2000) and the p.R254W and p.K247_R254del *CTRC* mutations (Rosendahl et al., 2008). The proband's great-grandfather also suffered from chronic pancreatitis, but he was not available for genetic testing. Interestingly, the father of the proband did not carry the mutation but was still affected by chronic pancreatitis, which was probably of alcoholic origin. Taken together, the previous reports and the present data confirm that mutation p.R116C associates with classic hereditary pancreatitis, exhibiting an autosomal dominant inheritance pattern with the characteristic incomplete penetrance.

Modeling the effects of the p.R116C mutation on the structure and function of cationic trypsinogen

To predict why mutation of Arg116 to Cys causes hereditary pancreatitis, we examined the three-dimensional model of human cationic trypsin (Supplementary Fig S1). Arg116 is located on a surface loop extending between Ser115 and Gly136, which connects the two globular halves of trypsin. The same loop harbors the Arg122-Val123 peptide bond, which

is highly sensitive to tryptic (autolytic) cleavage. Mutation of Arg122 was shown to stabilize human cationic trypsin against degradation either by autolysis or by chymotrypsin C (Sahin-Tóth and Tóth, 2000; Szmola and Sahin-Tóth, 2007). Similarly, mutation of Arg116 also might stabilize trypsinogen against proteolytic degradation. Alternatively, the newly generated unpaired Cys side chain in the p.R116C mutant may lead to misfolding through incorrect disulfide bond formation or dimerization. In the experiments presented below these predictions will be explored.

Expression and purification of p.R116C recombinant human cationic trypsinogen

To study the effect of the p.R116C mutation on cationic trypsinogen, we expressed wild type and p.R116C mutant trypsinogens in *E. coli* as inclusion bodies. To determine whether functional effects seen with the p.R116C mutant are due to the loss of the Arg residue or the gain of a reactive Cys amino-acid, we also constructed and expressed mutant p.R116A, in which Arg116 is replaced with the small, apolar Ala residue. Inclusion bodies were solubilized in guanidine-HCl and subjected to *in vitro* refolding followed by affinity purification on immobilized ecotin (Lengyel et al., 1998; Sahin-Tóth, 2000; Sahin-Tóth and Tóth, 2000; Nemoda and Sahin-Tóth, 2006). Surprisingly, relative to wild type or p.R116A mutant trypsinogen, yields of the p.R116C mutant eluted from the ecotin column were reduced by about 65%, even though expression levels in the inclusion bodies were comparable for all three trypsinogens (Supplementary Fig S2). Consistent with the lower yield in the eluate, the flow-through of the p.R116C mutant contained higher levels of unbound trypsinogen, indicating that a larger portion of the p.R116C mutant was misfolded during the *in vitro* refolding procedure and the misfolded protein did not bind to the affinity column. A similar phenomenon was observed previously with the p.R122C mutant, which also contained an unpaired Cys residue (Simon et al., 2002a). We conclude that the presence of an unpaired Cys residue might interfere with the correct disulfide-bond formation in cationic trypsinogen, at least under the experimental conditions. Despite the lower yields, the correctly folded fraction of mutant p.R116C was purified in amounts sufficient for biochemical analysis.

Activation of p.R116C cationic trypsinogen

The physiological activator enterokinase (enteropeptidase) activated wild type, p.R116C and p.R116A trypsinogens at essentially identical rates at pH 8.0 (not shown). Similarly, activation kinetics with the pathological activator cathepsin B at pH 4.0 were comparable (not shown). Previously, we demonstrated that the majority of pancreatitis-associated cationic trypsinogen mutations stimulated trypsin-mediated trypsinogen activation (autoactivation) (Sahin-Tóth, 2006; Teich et al., 2006). In contrast to these earlier findings, mutations p.R116C or p.R116A had no effect on the autoactivation of human cationic trypsinogen at pH 8.0 (Supplementary Fig S3A) or at pH 5.0 (not shown).

Catalytic properties and inhibition of p.R116C trypsin

When tested on the synthetic peptide substrate *N*-benzyloxycarbonyl-Gly-Pro-Arg-*p*-nitroanilide, enzyme kinetic parameters of wild type cationic trypsin and mutants p.R116C and p.R116A were essentially identical (Supplementary Table S1). The physiological trypsin inhibitor SPINK1 inactivated wild type, p.R116C and p.R116A trypsins stoichiometrically with comparable inhibition constants (not shown).

Degradation of p.R116C cationic trypsin

We speculated that elimination of a potentially trypsin-sensitive peptide bond by the p.R116C mutation might stabilize cationic trypsin against degradation. First, we tested trypsin-mediated trypsin degradation, i.e. autolysis. Human cationic trypsin undergoes

autolysis at a very slow rate, and measurable degradation at pH 8.0 is observed only in the presence of the Ca^{2+} chelator EDTA. Under these conditions, no difference was detected in the autolysis of wild type, p.R116C and p.R116A trypsins (Supplementary Fig S3B). We recently showed that chymotrypsin C promotes degradation of human cationic trypsin with a mechanism that involves chymotryptic cleavage of the Leu81-Glu82 peptide bond and tryptic cleavage of the Arg122-Val123 peptide bond (Szmola and Sahin-Tóth, 2007). This degradation pathway was also unaffected by the p.R116C or p.R116A mutations at pH 8.0, in 25 μM Ca^{2+} (Supplementary Fig S3C).

Secretion of p.R116C cationic trypsinogen from transiently transfected HEK 293T cells

In the previous experiments the only phenotypic alteration detected was the tendency of the p.R116C mutant to exhibit misfolding in the test tube. To investigate whether or not the p.R116C mutation induces misfolding of cationic trypsinogen inside living cells, we expressed wild type cationic trypsinogen and mutant p.R116C in HEK 293T cells via transient transfection. For comparison, expression of a panel of pancreatitis-associated mutants were also tested, which included p.A16V, p.N29I, p.N29T, p.E79K and p.R122H (see Teich et al., 2006, and references therein). Secretion of cationic trypsinogen into the incubation medium of transfected cells was first measured by activity assays. Remarkably, the rate of secretion of mutant p.R116C was reduced to about 20% of the wild type, whereas all other mutants tested were secreted normally (Fig 2A). The reduced secretion of mutant p.R116C was clearly related to the presence of the unpaired Cys residue, because mutant p.R116A was secreted at wild type rate (Fig 2B). To test whether or not unpaired Cys residues always lead to reduced secretion we also transfected 293T cells with mutant R122C and the rare variant p.C139S (Keiles and Kammesheidt, 2006). Secretion of cationic trypsinogen variant p.C139S was markedly reduced, whereas secretion of p.R122C was only moderately decreased (Fig 2B), indicating that unpaired Cys residues interfere with folding in a position-specific manner. SDS-PAGE of conditioned media followed by Coomassie Blue staining confirmed the defective secretion of mutants p.R116C and p.C139S and the moderate decrease in the levels of mutant p.R122C, whereas all other mutants were present at wild type levels (Fig 2C). Trypsinogen mutants were also electrophoresed under non-reducing conditions to determine whether the Cys mutants formed disulfide-linked dimers or higher-order aggregates, but this was not observed (Supplementary Fig S4).

Pancreatic secretory trypsin inhibitor (SPINK1) is co-secreted with trypsinogens in the pancreas and is responsible for curtailing premature trypsin activation. Co-transfection of SPINK1 with the PRSS1 mutants had no appreciable effect on their secretion, indicating that intracellular trypsinogen activation is not responsible for the reduced secretion of the p.R116C mutant (not shown).

Secretion of p.R116C cationic trypsinogen from transiently transfected Paca44 cells

To confirm that the secretion defect observed with mutants p.R116C and p.C139S is not cell line specific, we have analyzed secretion of the various trypsinogen mutants from transiently transfected PaCa44 pancreatic adenocarcinoma cells (Hucl et al., 2007). In this cell line, cationic trypsinogen is secreted to lower levels than in HEK 293T cells, therefore, epitope-tagged trypsinogen constructs were used to allow detection of trypsinogen in the conditioned medium by western blotting. As shown in Fig 3, mutants p.R116C and p.C139S were secreted at significantly reduced levels, whereas secretion of mutants p.R116A, p.N29I and p.R122H was comparable to wild type.

Intracellular retention of misfolded p.R116C trypsinogen

To investigate the intracellular fate of p.R116C trypsinogen, HEK 293T cells were transfected with the epitope-tagged trypsinogen constructs and western blot analysis was

used to compare the trypsinogen content in the medium and in cell lysates. Fig 4 demonstrates secretion of the various trypsinogen mutants into the growth medium of transfected HEK 293T cells. Again, mutants p.R116C and p.C139S were detected only at low levels, whereas mutants p.N29I, p.R116A, p.R122C and p.R122H were secreted to wild type levels or higher. Despite the diminished secretion of p.R116C and p.C139S trypsinogens, both mutants were detectable in cell lysates at wild type levels (Fig 4). These observations indicate that mutants p.R116C and p.C139S are synthesized normally inside the cell; however, they become retained, most likely due to misfolding suffered in the ER.

To test whether aggregation plays a role in the intracellular retention of misfolded trypsinogen, we subjected cell lysates containing wild type or p.R116C trypsinogen to ultracentrifugation and compared the trypsinogen content of the pellet and the supernatant (Fig 5A). A significant portion of both trypsinogens was recovered in the pellet, but this insoluble fraction of the p.R116C mutant was noticeably larger, indicating that intracellularly the p.R116C mutant is more prone to aggregation than wild type trypsinogen is. In a control experiment, conditioned medium containing wild type or p.R116C trypsinogen was subjected to ultracentrifugation and essentially all trypsinogen was recovered from the supernatant, confirming that secreted trypsinogen is fully soluble (Fig 5B).

To provide independent evidence for the misfolded state of the p.R116C mutant, we subjected cell lysates to trypsin digestion and compared the protease resistance of wild type and p.R116C trypsinogens. As shown in Fig 5C, a large portion of the p.R116C mutant present in cell lysates was rapidly digested by trypsin, whereas wild type trypsinogen was almost completely resistant to the protease.

The unfolded protein response in HEK 293T cells expressing p.R116C trypsinogen

Retention and accumulation of misfolded proteins in the ER can lead to ER stress that triggers the unfolded protein response (Marciniak and Ron, 2006; Ron and Walter, 2007). To investigate the possibility that the p.R116C mutant causes ER stress, we measured the levels of immunoglobulin-binding protein (BiP) in cell lysates by western blot. BiP is one of the major ER chaperones which is upregulated in ER stress. As shown in Fig 6A, BiP levels in extracts from cells expressing mutants p.R116C and p.C139S were elevated 1.8- and 1.5-fold, respectively, compared to cells transfected with wild type trypsinogen, whereas BiP levels in cells expressing mutants p.R116A, p.N29I or p.R122H were comparable to wild type.

ER stress is sensed by the luminal domain of the intramembrane transducer inositol-requiring enzyme-1 (IRE1), which upon activation catalyzes the cytoplasmic splicing of the X-box binding protein-1 (*XBPI*) mRNA and generates a 26 nt shorter variant (*XBPIs*), which is then translated into an active transcription factor that induces the transcription of ER stress-related genes (Marciniak and Ron, 2006; Ron and Walter, 2007). We used RT-PCR to measure the extent of *XBPI* splicing. Fig 6B demonstrates that expression of mutants p.R116C and p.C139S caused an increase in *XBPIs* compared to the lower levels observed in cells expressing wild type trypsinogen or mutants p.R116A, p.N29I and p.R122H.

Although data are not shown, we did not find a significant change in the mRNA levels of the C/EBP homologous protein (CHOP) in cells expressing p.R116C trypsinogen, as measured by RT-PCR. Similarly, no increase in the activity of caspase-3 was measurable in lysates of cells transfected with the p.R116C mutant, indicating that apoptosis was not triggered by the ER stress.

DISCUSSION

Genetic alterations that increase the risk to chronic pancreatitis or protect against the disease have been found mainly in genes that encode digestive proteases or their inhibitor and are specifically expressed by the acinar cells. First, *PRSS1* mutations were identified in families affected by hereditary pancreatitis and later also in patients with familial or sporadic cases of idiopathic chronic pancreatitis (Whitcomb et al., 1996; Applebaum-Shapiro et al., 2001; Keim et al., 2003; Howes et al., 2004; Teich et al., 2006). Triplication and duplication of the *PRSS1* locus was also observed in idiopathic and hereditary chronic pancreatitis (Le Maréchal et al., 2006; Masson et al., 2008b). *In vitro* biochemical investigations suggested that *PRSS1* mutations upset the protease-antiprotease balance in the pancreas by promoting autoactivation of cationic trypsinogen (Sahin-Tóth, 2006; Teich et al., 2006). This concept received further support when association of the p.N34S variant in the *SPINK1* gene with chronic pancreatitis was described (Pfützer et al., 2000; Witt et al., 2000). The *SPINK1* gene encodes the pancreatic secretory trypsin inhibitor protein, which is responsible for the inactivation of prematurely activated trypsin in the pancreas. The clinically common p.N34S variant is most likely a loss-of-function mutation, however, the exact functional defect has not been demonstrated experimentally so far (Kuwata et al., 2002; Király et al., 2007b). On the other hand, several rare missense *SPINK1* mutations have been identified in patients with hereditary or idiopathic chronic pancreatitis and were shown to diminish inhibitor secretion due to intracellular retention and degradation (Király et al., 2007a; 2007b; Boulling et al., 2007).

In contrast to the pathogenic *PRSS1* and *SPINK1* mutations, the p.G191R variant in the *PRSS2* gene (MIM# 601564) was found to afford protection against chronic pancreatitis (Witt et al., 2006). *In vitro* biochemical studies revealed that the p.G191R variant caused rapid autodegradation of anionic trypsinogen. Conceptually, these observations were noteworthy because they highlighted the protective role of trypsinogen degradation against chronic pancreatitis and eventually led to the identification of chymotrypsin C (CTRC) as the digestive enzyme responsible for regulating trypsinogen degradation (Szmola and Sahin-Tóth, 2007). Subsequent genetic association studies demonstrated that loss-of-function variants in the *CTRC* gene that reduced catalytic activity or secretion of chymotrypsin C were significantly enriched in patients with chronic pancreatitis relative to unaffected controls, suggesting that impairment of the trypsin-degrading activity of chymotrypsin C increases the risk to chronic pancreatitis (Rosendahl et al., 2008; Masson et al., 2008a).

Taken together, the genetic and biochemical evidence gathered to date defined a pathological pathway in which a sustained imbalance between intrapancreatic trypsinogen activation and trypsin elimination through inhibition or degradation results in the development of chronic pancreatitis. This model assumes that gain or loss of catalytic or inhibitory activity of the participant proteins is critical in disease pathogenesis. However, it is also conceivable that mutation induced folding problems result in intracellular perturbations of the cell homeostasis regardless of the actual function of the enzyme or inhibitor. One such example in which the protein degradation defense against accumulating protein products is perturbed and results in an inflammatory pancreatic disorder is the Johanson-Blizzard syndrome associated with mutations in the *UBR1* gene (Zenker et al., 2005). Accumulation of unfolded/misfolded proteins in the ER lumen represents a cellular stress in which intracellular signaling pathways are activated aimed at relieving the ER burden and curtailing proteotoxicity (Marciniak and Ron, 2006; Ron and Walter, 2007). Collectively, these pathways are called the unfolded protein response. ER stress is sensed by transmembrane transducers containing luminal sensor domains and cytoplasmic effector domains, which signal the cell to lower protein synthesis and translocation into the ER and to upregulate expression of various gene products that facilitate protein folding. In the

present study a convincing example of this mechanism is documented in hereditary pancreatitis associated with the p.R116C cationic trypsinogen mutation. The p.R116C trypsinogen mutant exhibits no phenotypic alteration at the biochemical level. This stands in contrast to other pancreatitis-associated mutations, which typically stimulate autoactivation of cationic trypsinogen. On the other hand, mutation p.R116C induces misfolding of cationic trypsinogen both in the test tube and in living cells. As a result of misfolding, secretion of mutant p.R116C is diminished and the proenzyme is retained intracellularly, most likely in the ER in an insoluble, protease-sensitive form. Misfolding of the p.R116C mutant is dependent on the presence of the unpaired Cys residue, since the p.R116A mutant exhibits no misfolding *in vitro* and is secreted from transfected cells in the same manner as wild type cationic trypsinogen. As a consequence of ER retention, the unfolded protein response is activated and commonly measured ER stress markers (BiP, *XBPIs*) become elevated.

Two studies using experimental animal models of pancreatitis have recently established a link between the activation of ER stress regulators and the onset of acute pancreatitis induced by either arginine injection or supramaximal secretagogue stimulation (Kubisch et al., 2006; Kubisch and Logsdon, 2007). With respect to the human disease, hereditary chronic pancreatitis in association with the p.R116C cationic trypsinogen mutation is the first example in which intracellular accumulation of a folding-deficient protease leads to the loss of catalytically competent proenzyme secretion and causes ER stress. Retention of mutant trypsinogen and/or the resultant ER stress could render the pancreas susceptible to other pathological stimuli such as supramaximal stimulation by secretory hormones or other insults leading to a secretory block. Alternatively, the ER stress *per se* may be responsible for direct and immediate pancreatic injury.

In summary, these results indicate that mutation induced misfolding and intracellular retention of human cationic trypsinogen causes hereditary pancreatitis in carriers of the p.R116C mutation. The resulting ER stress represents a new disease mechanism for pancreatitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Association of the c.346C>T (p.R116C) cationic trypsinogen mutation with hereditary pancreatitis in a German family. Subjects affected with idiopathic chronic pancreatitis are indicated by solid black symbols. The crossed symbol indicates the proband's father suffering from alcohol related pancreatitis. The asterisk marks the index patient; arrows point to heterozygous carriers of the c.346C>T (p.R116C) mutation. A limited pedigree of the same family was reported by Teich et al. (2002).

Figure 2.

Secretion of pancreatitis-associated cationic trypsinogen mutants from transiently transfected HEK 293T cells. **A** and **B**. At 8, 12 and 24 hours after transfection aliquots of conditioned media were collected and trypsin activity was measured after enterokinase activation. Trypsin activities were expressed as percentage of the 24 h wild type trypsin activity. The average of 3 independent transfection experiments is shown. For clarity, the error bars have been omitted; the standard error of the mean was always within 15%. **C**. Twenty-four hours after transfection 400 μ L aliquots of conditioned media were precipitated with 10% trichloroacetic acid (final concentration), heat denatured at 95 $^{\circ}$ C for 5 min in reducing Laemmli sample buffer and electrophoresed on 15% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue. Representative gels of three independent experiments are shown.

Figure 3. Western blot analysis of conditioned media of PaCa44 cells expressing pancreatitis-associated cationic trypsinogen mutants carrying the Glu-Glu epitope tag. Media were harvested and processed 24 h after transfection. Aliquots of conditioned media (100 μ L) were loaded on a 15% SDS-polyacrylamide gel, transferred to Immobilon-P membrane and trypsinogen was detected with an anti-Glu-Glu-tag antibody. A representative immunoblot of two independent experiments is shown.

Figure 4.

Western blot analysis of conditioned media and cell lysates of HEK 293T cells expressing pancreatitis-associated cationic trypsinogen mutants carrying the Glu-Glu epitope tag. Cells and media were harvested and processed 24 h after transfection. Aliquots of conditioned media (10 μ L) and cell lysates (20 μ g total protein) were loaded on a 15% SDS-polyacrylamide gel, transferred to Immobilon-P membrane and trypsinogen was detected with an anti-Glu-Glu-tag antibody. A representative immunoblot of three independent experiments is shown.

Figure 5.

Intracellular aggregation and protease-sensitivity of wild type and p.R116C mutant cationic trypsinogen. Glu-Glu tagged forms of wild type trypsinogen and mutant p.R116C were expressed in HEK 293T cells. Twenty-four hours after transfection, cells were harvested and lysates were prepared as described in *Methods*. Representative immunoblots of two or three independent experiments are shown. **A.** Cell lysates (10 μ g) were centrifuged at 50,000 g for 15 min at 4 °C. The distribution of trypsinogen between the supernatant and pellet was then analyzed by western blotting using an antibody against the Glu-Glu-tag. **B.** As a control experiment, conditioned media containing Glu-Glu-tagged forms of wild type (20 μ L medium) or p.R116C trypsinogen (100 μ L medium) were centrifuged and the supernatant and pellet were subjected to western blot analysis with the anti-Glu-Glu tag antibody. **C.** Cell lysates (10 μ g total protein) were supplemented with 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl_2 (final concentrations) and incubated with 100 nM human cationic trypsin (final concentration) in 20 μ L volume, for the indicated times. Trypsinogen degradation was followed by western blot analysis using the anti-Glu-Glu tag antibody.

Figure 6.

ER stress markers in HEK 293T cells expressing wild type or mutant trypsinogens. **A.** BiP protein levels in cell lysates (20 μ g total protein) were analyzed by western blotting as described in *Methods*. Actin was measured as loading control. Representative immunoblots of three independent experiments are shown. The band intensities were quantitated by densitometry and BiP/actin ratios are shown as bar graphs. Statistical analysis was performed with the Tukey-Kramer Multiple Comparisons Test. Two asterisks, $p < 0.01$; three asterisks, $p < 0.001$. **B.** The extent of *XBP1* splicing was determined by measuring the unspliced (*XBP1*) and spliced (*XBP1s*) forms by RT-PCR. Expression of *GAPDH* was measured as a reference control, as described in *Methods*. Representative ethidium-stained agarose gels of two independent experiments are shown.