PhD dissertation

Regulatory digestive proteases in the pathomechanism of chronic pancreatitis

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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
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<td>CF</td>
<td>cystic fibrosis</td>
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<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
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<td>CP</td>
<td>chronic pancreatitis</td>
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<td>CTRC</td>
<td>human chymotrypsinogen C</td>
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<td>CTRB1</td>
<td>human chymotrypsinogen B1</td>
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<tr>
<td>CTRB2</td>
<td>human chymotrypsinogen B2</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<td>ELA2A</td>
<td>human proelastase 2A</td>
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<td>ELA3A</td>
<td>human proelastase 3A</td>
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<td>ELA3B</td>
<td>human proelastase 3B</td>
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<td>ERCP</td>
<td>endoscopic retrograde cholangiopancreatography</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>HEK</td>
<td>human embrionic kidney</td>
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<td>HP</td>
<td>hereditary pancreatitis</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>MRCP</td>
<td>magnetic resonance cholangiopancreatography</td>
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<td>MWCO</td>
<td>molecular weight cut off</td>
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<td>OMIM</td>
<td>online Mendelian inheritance in man</td>
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<td>PAR</td>
<td>protease activated receptor</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PRSS1</td>
<td>human cationic trypsinogen</td>
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<td>PRSS2</td>
<td>human anionic trypsinogen</td>
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<td>PRSS3</td>
<td>human mesotrypsinogen</td>
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<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<td>SBTI</td>
<td>soybean trypsin inhibitor</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>SPINK1</td>
<td>serine protease inhibitor, Kazal type 1</td>
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<td>TAP</td>
<td>trypsinogen activation peptide</td>
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<td>TCA</td>
<td>trichloroacetic acid</td>
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<td>Tg</td>
<td>trypsinogen</td>
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<td>TLCK</td>
<td>tosyl-L-lysine chloromethyl ketone</td>
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<tr>
<td>TPCK</td>
<td>tosyl-L-phenylalanine chloromethyl ketone</td>
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INTRODUCTION

Chronic pancreatitis (CP) is a disease characterized by permanent destruction of the pancreatic parenchyma leading to maldigestion and diabetes mellitus. Unfortunately there is very little known about how to prevent or cure this debilitating disease to date. Recent progress in understanding the underlying causes came in 1996, when the genetic basis of the disorder was firmly established: Whitcomb and coworkers identified a germline mutation in the gene encoding trypsinogen to associate with the disease phenotype [Whitcomb, 1996b]. Hereditary pancreatitis is characterized by early onset episodes of acute pancreatitis with frequent progression to chronic pancreatitis and an increased risk for pancreatic cancer.

Animal models of experimental pancreatitis have long suggested that the initiating event in pancreatitis is premature activation of trypsinogen to active trypsin inside the acinar cells. However, direct evidence for this mechanism in human pancreatitis was lacking. Consequently, genetically determined pancreatitis, which includes classic hereditary pancreatitis and other forms of pancreatitis that are associated with trypsinogen or trypsin inhibitor gene mutations, emerged as the principal model of the human disease. Biochemical investigations suggested that mutations in the cationic trypsinogen gene upset the protease-antiprotease balance in the pancreas by promoting autoactivation of cationic trypsinogen to trypsin. Trypsin has the potential to activate the cascade of digestive enzymes prematurely within the pancreas, resulting in autodigestion of the organ. Consistent with the central pathophysiological role of trypsin, loss-of-function alterations in the trypsin inhibitor gene predispose to various forms of pancreatitis by lowering the protective levels of the inhibitor [Sahin-Toth, 2006].

Elucidation of the pathomechanism of genetically determined pancreatitis provided valuable insight into the pathophysiology of human chronic pancreatitis. The central role of trypsin has prompted researchers for decades to understand the way trypsin activity is regulated within the pancreas. Our goal was to identify and characterize enzymes capable of degrading trypsin, with the hope to get insight into the potential regulation of trypsin activity which may offer new therapeutic possibilities in pancreatitis.
REVIEW OF THE LITERATURE

CLINICAL ASPECTS OF CHRONIC PANCREATITIS

Chronic pancreatitis (CP) is an incurable condition characterized by progressive and ultimately irreversible damage to both exocrine and endocrine components of the pancreas, eventually manifesting clinically as significant exocrine insufficiency (maldigestion) and diabetes. CP also confers an increased risk for pancreatic cancer. The disease is characterized by permanent destruction of pancreatic acinar and islet cells, inflammatory cell infiltration, fibrosis and calcifications within the excretory ducts. The reported incidence and prevalence of CP in Western countries ranges from 10 to 100 per 100,000 population [Worning, 1990].

Symptoms

The three major clinical features of CP are pain, maldigestion and diabetes mellitus. The most common symptom is epigastric (upper abdominal) pain, which often radiates to the back and may be accompanied by nausea, vomiting and loss of appetite. Recurrent (type A) or continuous (type B) pain is considered to be the hallmark of CP, but some patients may have no pain at all, presenting instead with symptoms of pancreatic insufficiency [Witt, 2007]. While the course of pain in CP can be unpredictable, in general as the disease gets worse and more of the pancreas is destroyed, pain may actually become less severe.

A damaged pancreas cannot produce important digestive enzymes, therefore people with CP may develop problems with digesting and absorbing food and nutrients. This can lead to weight loss, vitamin deficiencies, diarrhea and steatorrhea. Steatorrhea is the symptom of advanced CP and does not occur until pancreatic lipase secretion is reduced to less than 10% of normal. Lipase secretion decreases more rapidly than protease or amylase secretion, therefore maldigestion of lipids occurs earlier than that of other nutrients.

In addition to problems with exocrine secretion, diabetes mellitus may develop in cases of long-standing disease. The diabetes is classified as type IIIc according to the American Diabetes Association [The Expert Committee, 2003] and is characterized by destruction of both insulin- and glucagon-producing cells.

The major complications of chronic pancreatitis are pseudocyst formation and mechanical obstruction of the common bile duct and duodenum. Less common complications
include pancreatic fistulas with pancreatic ascites, pleural or sometimes pericardial effusion; splenic vein thrombosis and development of gastric varices; and formation of a pseudoaneurysm, with hemorrhage or pain resulting from expansion and pressure on adjacent structures.

**Diagnosis**

The diagnosis of CP relies in relevant symptoms, imaging modalities to assess pancreatic structure, and assessment of pancreatic function. The diagnostic gold standard of early stage disease would be an adequate surgical biopsy, which is rarely available. The key histopathologic features of CP are pancreatic fibrosis, acinar atrophy, chronic inflammation, and distorted/blocked ducts. Distinctive histologic features are described in some forms of CP, such as extensive pancreatic calcification in tropical pancreatitis [Balakrishnan, 2006] and a prominent lymphocytic and plasma cell infiltrate in autoimmune pancreatitis [Ketikoglou, 2005; Okazaki, 2001].

The imaging modalities in the assessment of a patient with painful CP include upper gastrointestinal endoscopy, abdominal/endoscopic ultrasonography, and endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP) in order to detect potentially reversible cause of pain (e.g. peptic ulcer, pseudocyst, common bile duct stricture). ERCP is the best imaging procedure for assessing the severity and extent of ductal changes: dilatations, stenoses, and abnormalities of the side branches. ERCP eventually may be superseded by a noninvasive alternative, namely MRCP for the diagnosis of CP [Calvo, 2002].

The secretin-cerulein test is regarded as the method of choice for the detection of exocrine pancreatic insufficiency. The test is only available at specialized centers, therefore less invasive alternatives have been developed including fecal elastase, lipase or chymotrypsin; the pancreolauryl test; the bentiromide test; and a variety of breath tests using radiolabeled pancreatic substrates, usually triolein [Chowdhury, 2003].

**Treatment**

Conservative treatment goals of uncomplicated CP are pain control, relief of mechanical obstruction or complications, correction of malabsorption and diabetes. Pancreatic extracts
frequently are administered to improve absorption and reduce pain. Cessation of alcohol ingestion is essential because the 5-year mortality rate of chronic pancreatitis in patients who continue to abuse alcohol is 50 percent [Mergener, 1997]. If pain is increasing or intractable, imaging should be performed to assess for complications such as pseudocyst or mechanical obstruction. Surgery, either open or endoscopic, can be helpful in such cases. Celiac plexus nerve block is performed frequently for long-term pain control. Unfortunately, no causal treatment of CP is known today.

Prognosis

In patients monitored for more than 10 years, the mortality rate is 22% and pancreatitis-induced complications account for 13% of the deaths. The causes of death are alcoholic liver disease, postoperative complications, and cancer. Older age at diagnosis, cigarette smoking, and alcohol intake are major predictors of mortality among individuals with chronic pancreatitis [Steer, 1995].

The risk of developing pancreatic cancer is significantly higher in patients with CP than in the general population. Alcoholic CP and tropical pancreatitis are associated with a 15-fold and a 5-fold increased risk of pancreatic cancer, respectively [Chari, 1994; Lowenfels, 2005], whereas the cumulative lifetime risk of cancer in patients with hereditary pancreatitis is reported to be as high as 40% [Lowenfels, 2005]. Mortality in CP, particularly alcoholic, is approximately one-third higher compared to an age- and sex-matched general population [Levy, 1989].

Etiology

Alcohol abuse is the major risk factor of CP in industrialized countries accounting for almost 80% of cases of CP. On the other hand, only 5 to 10% of alcoholics develop chronic pancreatitis, suggesting that other factors must be important in the pathogenesis of the disease [Bisceglie, 1984]. Long-term obstruction of the pancreatic duct can also cause CP. The obstruction can be caused by a periampullary tumor, papillary stenosis, pseudocysts, stricture, or trauma. Pancreas divisum, an anatomic variant in which the head and body of the pancreas are separate glands, can cause CP as a result of obstruction at the lesser papilla. Interestingly CP differs from other inflammatory disorders in that infectious agents and autoimmune
processes are exceedingly rare causes of the disease. Factors such as genetic mutations (hereditary pancreatitis), drugs, hypertriglyceridemia, hypercalcemia (e.g. hyperparathyroidism), and cystic fibrosis also have been implicated [Ahmed, 2006; Etemad, 2001]. Tropical chronic pancreatitis is a juvenile form of chronic calcific nonalcoholic pancreatitis, a condition of unknown etiology that is seen commonly in South India and other parts of the tropics, where it is the most common cause of CP.

To date in the vast majority of cases of CP that are not related to alcohol abuse, no identifiable cause can be found, and the diagnosis of idiopathic pancreatitis is made. However, it is anticipated that with increasing identification of putative genetic/environmental factors, the number of true idiopathic cases of CP will diminish further.

GENETICS OF CHRONIC PANCREATITIS

More than 50 years ago, in 1952, it was reported that CP may cluster in selected families independent of additional environmental factors suggesting an inherited disease in these patients [Comfort, 1952]. The underlying genetic defect, however, remained obscure for more than 40 years. After genome-wide linkage analyses, three independent groups reported an association of the hereditary pancreatitis phenotype with the long arm of chromosome 7 in 1996 [Le Bodic, 1996; Pandya, 1996; Whitcomb, 1996a]. Within the same year the p.R122H mutation of the cationic trypsinogen gene was identified in all hereditary pancreatitis (HP) affected individuals and obligate carriers from five kindreds [Whitcomb, 1996b]. HP is an autosomal-dominant disorder with a clinical manifestation that is indistinguishable from other etiologic varieties of pancreatitis. The estimated penetrance of the disease is 70–80%. In affected patients, HP begins with recurrent attacks of acute pancreatitis that usually start in childhood or young adulthood. The attacks of pancreatitis frequently progress to chronic disease at an early age and are associated with a 40- to 50-fold increased lifetime risk for the development of pancreatic cancer [Lowenfels, 1997].

For a long time, genetic factors in the pathogenesis of pancreatitis were thought to be rare; however, the finding of PRSS1 mutations causing HP and mutations in new genes (SPINK1 and CFTR) in patients with so-called idiopathic CP indicate that cases of CP with genetic risk factors in the background are much more common than originally envisioned. Recent data suggests that besides the “classic” autosomal dominant way of inheritance (HP),
the autosomal recessive and multigenic inheritance pattern is also a common genetic background of the disease. Considerable research effort has been directed toward understanding the mechanism of genetic abnormalities that predispose to CP, mutations of several candidate genes related to trypsinogen activation/inactivation are increasingly recognized for their potential disease-modifier role in distinct forms of CP [Teich, 2006].

**Hereditary pancreatitis and mutations in the cationic trypsinogen gene (PRSS1)**

Trypsinogen is the most abundant digestive proteolytic pro-enzyme in the pancreatic juice. In humans it is secreted in three isoforms, cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen encoded by the *PRSS1, PRSS2* and *PRSS3* genes, respectively. Cationic trypsinogen (~50–70%) and anionic trypsinogen (~30–40%) make up the bulk of trypsinogens in the pancreatic juice, while mesotrypsinogen accounts for 2–10% [Rinderknecht, 1979; Rinderknecht, 1984; Rinderknecht, 1985]. The names have been designated according to the electrophoretic mobility of the proteins.

Genetic variants of the gene encoding cationic trypsinogen (protease, serine 1; *PRSS1*) have been identified in patients with different forms of clinically idiopathic CP: hereditary pancreatitis (HP), familial pancreatitis or sporadic pancreatitis. The EUROPAC (European Registry of Hereditary Pancreatitis and Pancreatic Cancer) study states that the diagnosis of HP should be made on the basis of two first-degree relatives or three or more second degree relatives, in two or more generations with recurrent acute pancreatitis, and/or chronic pancreatitis for which there were no precipitating factors [Howes, 2004]. Cases in which these strict criteria were not met, but more than one family member was carrying the disease, mostly within the same generation, are generally classified as familial chronic pancreatitis; the term sporadic refers to disease carriers with a negative family history.

The *PRSS1* gene is about 3.6 kilobases long with five exons; more than 25 variants of the gene have been identified in patients with various forms of CP. Mutations are located in three clusters within the cationic trypsinogen sequence: in the trypsinogen activation peptide (TAP), in the N-terminal part of trypsin or in the longest peptide segment not stabilized by disulfide bridges between Cys^64^ and Cys^139^. Three *PRSS1* mutations, namely p.R122H (~70%), p.N29I (~25%), and p.A16V (~4%), have been found with relatively high frequency in multiple families, whereas additional genetic variants have been identified only in very few
patients [Teich, 2006]. The two most frequently occurring, thereby clinically relevant, p.R122H and p.N29I variants, which display a penetrance of 70–80%, are found exclusively in patients with a clear family history of pancreatitis, whereas p.A16V is a sporadic mutation, a genetic risk factor in CP [Witt, 1999].

Although many pedigrees have been reported, not all families described with clinically defined HP carry mutations in the PRSS1 gene. Consequently, the involvement of other yet unidentified genes may be significant in the disease pathogenesis.

Anionic trypsinogen (PRSS2)

Due to the big number of mutations found in the gene encoding cationic trypsinogen, it was postulated that there are disease-causing mutations in the other major trypsinogen isoenzyme, anionic trypsinogen encoded by the PRSS2 gene. However, a recent study indicated that the PRSS2 gene does not contain any mutation that causes or enhances the risk of CP, but strikingly a protective variant has been identified. The variant of codon 191 (p.G191R) was found in 220 of 6459 (3.4%) controls but only in 32 of 2466 (1.3%) patients with idiopathic or alcoholic CP. Although the overall contribution of p.G191R to disease pathogenesis is low, the variant is the first example in pancreatitis for a disease-protective mutation [Witt, 2006].

Serine protease inhibitor, Kazal type 1 (SPINK1)

SPINK1, also known as pancreatic secretory trypsin inhibitor, was first isolated from the bovine pancreas in 1948 [Kazal, 1948]. It is regarded as a first-line defense system thought to be capable of inhibiting intrapancreatic trypsin activity that could result from accidental premature trypsinogen activation within acinar cells. The human SPINK1 gene is located on chromosome 5 and is comprised of approximately 7.1 kilobases, which contain four exons [Horii, 1987]. In 2000 Witt and coworkers screened the SPINK1 gene in 96 unrelated children and adolescents with idiopathic CP who did not have PRSS1 mutations and found mutation p.N34S in 23% of patients [Witt, 2000]. Data taken from eight larger studies in Europe and the United States indicate that 12.6% of patients with CP were heterozygous for p.N34S, and 3.6% were homozygous. Heterozygosity was detected at 1.9% in controls on average [Kiraly, 2007a]. Thus the majority of carriers never develop pancreatitis, but the p.N34S mutation represents an important risk factor in CP. In addition to these studies, p.N34S was also
recognized as a major genetic risk factor in tropical pancreatitis in the Indian subcontinent and as a minor susceptibility factor in alcoholic CP, in two diseases where PRSS1 mutations have no described role today. Taken together, the p.N34S alteration is considered to be a relatively frequent genetic susceptibility factor to CP, which exerts its effect in the context of other genetic and environmental factors, resulting in the highly variable penetrance and expression of the disease. Several other SPINK1 alterations have been described in recent years, mainly in single patients of families only (for detailed information of the different variants see www.uni-leipzig.de/pancreasmutation).

Cystic fibrosis transmembrane conductance regulator (CFTR)
CFTR is an apical membrane chloride channel critical for fluid and electrolyte secretion in the respiratory and digestive tracts. In the pancreas, CFTR is localized to centroacinar and proximal intralobular duct cells and also in the apical membranes of acinar cells [Zeng, 1997], and is responsible for the regulation of bicarbonate secretion. Abnormal function as a result of mutations in the CFTR gene is associated with cystic fibrosis (CF), the most frequent autosomal-recessive disease in the White Caucasian population, characterized by pulmonary dysfunction and pancreatic insufficiency. Only the minority of CF patients suffer from recurrent pancreatitis.

The CFTR gene is about 250 kilobases long with 27 exons on chromosome 7; more than 1500 variants of the gene have been found to date. In 1998, two groups reported independently an association between idiopathic CP and mutations in the CFTR gene [Cohn, 1998; Sharer, 1998]. One of the studies tested 134 patients with CP, including alcoholic and idiopathic etiologies, for the 22 most frequent mutations [Sharer, 1998]. 13.4% of the patients, most of them from the idiopathic CP group (20% mutation frequency), were heterozygous for a CFTR mutation, as compared with a frequency of 5.3% in healthy controls. The frequency of CFTR mutations in idiopathic CP was 4 times enriched relative to controls. Subsequent studies analyzing the complete CFTR coding sequence as well as the SPINK1 and PRSS1 status found that 25–30% of CP patients carried at least one mutation, but only a few patients were compound heterozygous. Several patients, however, were found to be transheterozygous for a CFTR alteration and a SPINK1 or PRSS1 variant, highlighting the significance of the combination of mutations in different genes in disease pathogenesis [Noone, 2001].
Alcoholic chronic pancreatitis

Alcohol remains one of the most important risk factors associated with CP. The correlation between alcohol abuse and CP is not linear because it seems that less than 10% of severe alcoholics develop pancreatitis as a consequence of their excessive ethanol consumption [Bisceglie, 1984]. It was though that the variability of individual susceptibility to alcohol may be due to genetic predisposition. Several studies investigating PRSS1, CFTR, pancreatitis associated protein and alcohol metabolizing or detoxifying enzymes have yielded negative or conflicting results [Witt, 2007]. In a large multicenter study, an association of the p.N34S mutation of the \textit{SPINK1} gene and alcoholic CP was described: the mutation was found in 16 of 274 (5.8%) patients, but only 4 of 540 (0.8%) healthy controls and in 1 of 98 (1.0%) alcoholic control individuals without CP [Witt, 2001]. Although the \textit{SPINK1} mutation may represent a minor genetic risk factor for the development of alcoholic pancreatitis, it does not seem to be the main triggering event.

PATHOPHYSIOLOGICAL SIGNIFICANCE OF INTRAPANCREATIC DIGESTIVE PROTEASE ACTIVATION IN CHRONIC PANCREATITIS

In 1896, Chiari speculated that pancreatitis is a result of autodigestion of the gland [Chiari, 1896]. A key role has been attributed to trypsin, the most abundant protein in the acinar cell of the pancreas, that is capable of activating all proteolytic proenzymes to their active form. Early conversion of pancreatic zymogens to active enzymes within the pancreatic parenchyma was proposed to initiate the inflammatory process.

Physiologic activation of trypsinogen to trypsin takes place in the duodenum by enteropeptidase (enterokinase), a highly specialized serine protease in the brush-border membrane of enterocytes, and therefore the cascade of enzymes is activated in the duodenum under normal conditions. Trypsin can also activate trypsinogen, a process termed “autoactivation”, which in the duodenum may have a physiological role in facilitating zymogen activation, whereas inappropriate autoactivation in the pancreas might cause pancreatitis. Theoretically, premature activation of large amounts of trypsinogen can overwhelm the protective mechanisms (described below), leading to damage of the zymogen-confining membranes and the release of activated proteases into the cytosol. The suggestion
that prematurely activated digestive enzymes play a central role in the pathogenesis of pancreatitis comes from experimental animal models and biochemical models of genetically determined pancreatitis.

**Experimental animal models of pancreatitis**

Various animal models indicate that a pathological increase in trypsin activity in the pancreatic acinar cells is one of the early events in the development of pancreatitis [Saluja, 2007]. The two most frequently used experimental animal models of acute pancreatitis administer the cholecystokinin analogue cerulein or L-arginine [Hegyi, 2004]. Trypsin activity measured with antibodies directed against the activation peptide of trypsin (TAP) was located to the secretory pathway during experimental pancreatitis, where trypsinogen and lysosomal enzymes co-localize [Hofbauer, 1998]. The lysosomal cysteine protease cathepsin B is thought to have a role in the intrapancræatic activation of digestive enzymes by mediating premature trypsinogen activation [Gorelick, 1995]. The largely circumstantial evidence for the cathepsin B hypothesis is based on the fact that cathepsin B has been shown to activate trypsinogen *in vitro* and the redistribution of cathepsin B into the zymogen granule-containing subcellular compartment and the secretory organelles was observed during the initial phase of acute pancreatitis in several animal models [Kukor, 2002b; Saluja, 1987]. A cathepsin B-deficient mouse has also been generated and interestingly trypsinogen activation was lowered significantly in experimental pancreatitis in the absence of cathepsin B [Halangk, 2000].

Much of our current knowledge regarding the onset of pancreatitis comes from animal and isolated cell models. The results, although they are highly reproducible and recapitulate many of the cellular events, can be hardly related to human disease mainly because none of the models are caused by factors that we currently believe cause human pancreatitis [Pandol, 2007]. As pancreatic tissue or juice from patients is not readily available in significant amounts, the analysis of genetic alterations found in association with CP provide a useful model of pancreatitis in humans. A growing body of indirect biochemical evidence also supports the hypothesis that elevated intraacinar trypsin activity is highly significant in the disease pathogenesis.
Biochemical models of genetically determined pancreatitis

Genetic and biochemical evidence defines a pathological pathway in which a sustained imbalance between intrapancreatic trypsinogen activation and trypsin inactivation results in the development of chronic pancreatitis. *Gain-of-function* variants in *PRSS1* have been linked to autosomal dominant hereditary pancreatitis and subsequently also to idiopathic chronic pancreatitis [Teich, 2006]. Recently, triplication of the *PRSS1* locus has been observed in a subset of families with hereditary pancreatitis [Le Marechal, 2006]. *In vitro* biochemical studies revealed that the majority of disease predisposing *PRSS1* variants increase autocatalytic conversion of trypsinogen to active trypsin and probably promote premature intrapancreatic trypsin activation *in vivo* [Sahin-Toth, 2006]. The importance of *PRSS1* mutations as pathogenic mediators in hereditary pancreatitis is also supported by a recent study using a transgenic mouse model expressing mutant p.R122H mouse trypsinogen. The pancreas of these mice displayed early onset acinar injury, inflammatory cell infiltration, and enhanced response to cerulein-induced pancreatitis; with progressing age, pancreatic fibrosis and acinar cell dedifferentiation developed [Archer, 2006]. Consistent with the central pathophysiological role of trypsin, p.N34S and other *loss-of-function* alterations in the trypsin inhibitor *SPINK1* predispose to idiopathic, tropical, and alcoholic chronic pancreatitis [Bhatia, 2002; Kiraly, 2007b; Witt, 2000].

Taken together, mutations in *PRSS1* or in *SPINK1* lead to an imbalance of proteases and their inhibitors within the pancreatic parenchyma, resulting in an inappropriate activation of pancreatic zymogens with subsequent autodigestion and inflammation. Hereditary pancreatitis associated mutations in the PRSS1 gene stimulate activation of trypsinogen to trypsin (Figure 1). Mutations in the SPINK1 gene reduce inhibitor levels and thus compromise trypsin inhibition. In contrast to pathogenic *PRSS1* and *SPINK1* variations, the p.G191R *PRSS2* variant affords protection against chronic pancreatitis due to rapid autodegradation [Witt, 2006]. Mutations of CFTR also may disturb the delicate balance between proteases and antiproteases, by intrapancreatic acidification or by defective trafficking of zymogen granules, thereby facilitating the intrapancreatic activation of digestive enzymes [Witt, 2007].
Figure 1. The trypsin-dependent pathological model of chronic pancreatitis.

Protective trypsinogen degrading enzymes in the human pancreas

Several mechanisms protect the functional unit of the exocrine pancreas, the acinar cell. Zymogens are packaged into granules with localized environments inside the cell reducing the risk of early activation and secreted as proenzymes (inactive zymogens), which are only activated in the duodenum under physiological conditions. The earlier described serine protease inhibitor (SPINK1) is also an important safeguard to protect from the delirious effects of active digestive enzymes within the cell (Figure 1).

Inactivation of intrapancreatic trypsin through trypsin-mediated trypsin degradation (autolysis) or by an unidentified serine protease (enzyme Y) were also proposed to be protective against pancreatitis [Halangk, 2002; Rinderknecht, 1984; Rinderknecht, 1988; Varallyay, 1998; Whitcomb, 1996b]. This notion received support from the discovery that the p.R122H mutation, which eliminates the Arg^{122} autolytic site in cationic trypsinogen, causes autosomal dominant hereditary pancreatitis in humans [Whitcomb, 1996b]. Autolytic cleavage of the Arg^{122}-Val^{123} peptide bond was suggested to trigger rapid trypsin degradation by increasing structural flexibility and exposing further tryptic sites. On the basis of in vitro experiments, a theory was put forth that digestive enzymes are generally resistant to each other and undergo degradation through autolysis only [Bodi, 2001; Varallyay, 1998]. However, human cationic trypsin was shown to be highly resistant to autolysis, and appreciable auto-degradation was observed only with extended incubation times in the complete absence of Ca^{2+} and salts [Kukor, 2003; Sahin-Toth, 2000b; Szilagyi, 2001].
Taken together, the in vitro studies indicate that autolysis alone cannot be responsible for the inactivation and degradation of human cationic trypsin and suggest that other pancreatic enzymes might be important in this process. The identity of these pancreatic proteases remains to be solved (Figure 1). In humans mesotrypsin has been labeled a candidate for this function [Rinderknecht, 1984]. Later, the presence of another unknown enzymatic activity effective in the degradation of pancreatic zymogens was also observed in human pancreatic juice. This uncharacterized activity was named enzyme Y and was proposed as one of the protective factors against CP [Rinderknecht, 1988].

**Mesotrypsin**

Mesotrypsin is a minor digestive protease secreted by the human pancreas. It was reported between 3 and 10% of total trypsinogen content in normal pancreatic juice [Rinderknecht, 1979; Rinderknecht, 1984; Rinderknecht, 1985]. Mesotrypsin was first discovered as a new inhibitor-resistant protease found in human pancreatic tissue and fluid [Rinderknecht, 1978], and a systematic characterization was published in 1984 [Rinderknecht, 1984]. A cDNA coding for mesotrypsinogen was cloned from human pancreas in 1997 [Nyaruhucha, 1997], and the crystal structure of mesotrypsin complexed with benzamidine was solved in 2002 [Katona, 2002]. An alternatively spliced form of mesotrypsinogen in which the signal-peptide is replaced with a novel sequence encoded by an alternative exon 1 is expressed in the human brain [Wiegand, 1993]. Although usually referred to as “brain trypsinogen”, there is no evidence for the activation of this novel chimeric molecule, which might have a function unrelated to proteolytic activity [Chen, 2003].

The most intriguing property of mesotrypsin is its resistance to polypeptide trypsin inhibitors, such as the Kunitz-type soybean trypsin inhibitor (SBTI) or the Kazal-type pancreatic secretory trypsin inhibitor (SPINK1, serine protease inhibitor, Kazal type 1) [Katona, 2002; Nyaruhucha, 1997; Rinderknecht, 1984]. Analysis of the recent crystal structure of mesotrypsin provided compelling evidence that the presence of an arginine residue in place of the highly conserved Gly$^{198}$ might be responsible for the peculiar inhibitor resistance of mesotrypsin [Katona, 2002]. Arg$^{198}$ occupies the S2’ subsite and its long side-chain sterically clashes with protein inhibitors and possibly substrates. Furthermore, the charge of the guanidino group contributes to the strong clustering of positive charges around
the primary specificity pocket of mesotrypsin. However, no direct experimental evidence has ever been presented for the proposed role of Arg^{198}.

Despite the high-resolution crystal structure, the biological function of mesotrypsin has remained mysterious. In two clearly conflicting theories, it was proposed that premature activation of mesotrypsin in the pancreas might cause or protect against pancreatitis, as the inhibitor-resistant trypsin activity can freely activate or degrade other pancreatic zymogens [Rinderknecht, 1984].

**ENZYME Y**

Enzyme Y was described by Heinrich Rinderknecht in 1988 [Rinderknecht, 1988]. He initially alleged that mesotrypsin can degrade trypsinogens, but later he withdrew this conclusion and attributed the trypsinogen degrading activity to an unidentified serine protease, which he named enzyme Y [Rinderknecht, 1984; Rinderknecht, 1988]. This enigmatic activity developed when human cationic trypsinogen, purified by native gel electrophoresis, was incubated at 37 °C. The activity degraded all human trypsinogen isoforms, and millimolar Ca^{2+} concentrations blocked degradation. Enzyme Y became very popular among pancreas researchers and has been highlighted in almost every significant article discussing defense mechanisms against intrapancreatic trypsin activity. Rinderknecht himself believed that enzyme Y was probably a degradation fragment of cationic trypsin [Rinderknecht, 1988], perhaps complexed with pancreatic secretory trypsin inhibitor [Whitcomb, 1999], although he acknowledged the possibility of contamination with an unknown protease [Rinderknecht, 1988].

Almost 20 years after its initial description this enzyme has remained elusive because no matching gene or protein has been identified. Interestingly, our laboratory recently discovered that a minor human chymotrypsin, chymotrypsin C facilitates autoactivation of human cationic trypsinogen by limited proteolysis of the trypsinogen activation peptide in the presence of millimolar Ca^{2+} concentrations [Nemoda, 2006]. These experiments focused our attention onto this less-known chymotrypsin, and we characterized its interaction with human cationic trypsinogen in more detail. Strikingly, our preliminary observations suggested that chymotrypsin C may be responsible for the protective trypsinogen degradation in the micromolar Ca^{2+} concentration range.
AIMS OF THE STUDY

Trypsin degradation has been discussed as a possible protective mechanism against pancreatitis for decades, numerous digestive proteases for this putative protective function have been proposed. The aim of this work was to study and characterize biochemically two candidates, namely mesotrypsin and chymotrypsin C, to elucidate their role in the human pancreas and investigate their possible impact on pancreatic disease.

The characterization of mesotrypsin [I]
Mesotrypsin is a minor trypsin isoform resistant to natural trypsin inhibitors in the human pancreatic juice. It was suggested that the inhibitor resistance of mesotrypsin was due to Arg198. Despite our detailed structural knowledge, the biological function of mesotrypsin has remained mysterious.

Our specific aims were:

1. To provide evidence that the inhibitor resistance of mesotrypsin is caused by Arg198.
2. To analyze the potential of mesotrypsin to activate or degrade pancreatic zymogens.
3. To find the biological and pathological function of mesotrypsin.
4. To provide a biochemical basis for intrapancreatic mesotrypsinogen activation.

The characterization of chymotrypsin C [II]
Our preliminary observations suggested that chymotrypsin C may be the long-elusive digestive enzyme (enzyme Y) responsible for trypsin degradation in the gut and may serve as a protective protease in the pancreas to curtail premature trypsin activation.

Our specific aims were:

1. To elucidate the role of chymotrypsin C in the degradation of human cationic trypsin.
2. To investigate the Ca^{2+} concentration dependence of the reaction.
3. To determine the sites of cleavage and the exact mechanism of trypsin degradation.
4. To prove that chymotrypsin C is identical to enzyme Y, the trypsinogen degrading activity from human pancreatic juice.
EXPERIMENTAL PROCEDURES

MATERIALS

Ultrapure bovine enterokinase was from Biozyme Laboratories, and reagent grade bovine serum albumin was from Biocell Laboratories. Bovine chymotrypsinogen A, TLCK-treated bovine chymotrypsin and TPCK-treated bovine trypsin was obtained from Worthington Biochemical Corporation. The concentration of bovine trypsin was determined by active-site titration with p-nitrophenyl-p’-guanidinobenzoate (Sigma) as described in [Chase, 1967]. Soybean (Glycine max) trypsin inhibitor (Kunitz type) was from Fluka, and was further purified on an affinity column containing immobilized S200A mutant human cationic trypsin. Human SPINK1 was expressed in Saccharomyces cerevisiae and purified on the S200A affinity column. Inhibitor concentrations were determined by titration with bovine trypsin. Human recombinant cathepsin B was a gift from Paul M. Steed (Novartis Pharmaceuticals).

NOMENCLATURE

The genetic abbreviations PRSS1 (protease, serine, 1), PRSS2 and PRSS3 are used to denote human cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen, respectively. The genetic abbreviations SPINK1 (serine protease inhibitor, Kazal type 1) and CTRC (chymotrypsinogen C) are also used in my thesis. The abbreviations are put in italics throughout the text when referring to the genes encoding these digestive proteases. Amino acid residues in the trypsinogen sequence are numbered according to their position in the native pre-proenzyme, starting with Met1. The first amino acid of the mature cationic trypsinogen is Ala16.

CONSTRUCTION OF EXPRESSION PLASMIDS

Human trypsinogens

The pTrapT7 expression plasmids harboring the human cationic trypsinogen and human anionic trypsinogen genes were made earlier in our laboratory [Kukor, 2003; Sahin-Toth, 2000a; Sahin-Toth, 2000b]. The R122A and S200A cationic trypsinogen mutants were also
constructed previously [Nemoda, 2005b; Sahin-Toth, 2000b; Szepessy, 2006b] and mutations L81A and E82A were created by oligonucleotide-directed overlap-extension PCR mutagenesis.

The gene encoding mesotrypsinogen was PCR-amplified from the IMAGE clone #2659811 (GenBank #AW182356, purchased from Incyte Genomics Reagents & Services) and ligated into the expression plasmid pTrap [Graf, 1987] behind the alkaline phosphatase promoter and signal-sequence. Mutation R198G was introduced via oligonucleotide-directed site-specific mutagenesis, using the overlap-extension PCR mutagenesis method. A typical polymerase chain reaction (PCR) mixture contained 200 µM dNTP, 2 µM of each primer, approximately 1 ng DNA template, 0.05 U/µl Deep Vent DNA polymerase (New England BioLabs) and 1x ThermoPol reaction buffer in a total volume of 50 µL. 35 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 55 °C and 1 min extension at 72 °C were performed. PCR products were analyzed by conventional submarine horizontal agarose gel electrophoresis.

**Human proelastases**

The pTrapT7 expression plasmid harboring the human elastase 2A gene was constructed previously [Szepessy, 2006a]. The cDNA for proelastase 3A (ELA3A) was PCR-amplified from IMAGE clone #3950453 (GenBank accession BC007028) using the ELA3A-EcoRI sense primer 5’-AAA TTT GAA TTC CCT ATC ATC ACA AAA CTC ATG ATG CTC-3’ and the ELA3 BamHI antisense primer 5’-TTT TTT GGA TCC GAG AGA TCT TTA TTC TTT ATT CAG GAT-3’. The cDNA for proelastase 3B (ELA3B) was PCR-amplified from IMAGE clone #3949903 (GenBank accession BC005216) using the ELA3B-EcoRI sense primer 5’-AAA TTT GAA TTC CCT ATC ATC GCA AAA CTC ATG ATG CTC-3’ and the ELA3 BamHI antisense primer. The ELA3A and ELA3B PCR products were digested with EcoRI and BamHI and cloned into the pcDNA3.1(−) plasmid.

**Human chymotrypsinogens**

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) with the CTRC-XhoI sense primer 5’-GGA ATT CTC GAG CAC CTA ACC CTG TCT TCT CTC ATC CTC ATG AGA TCT TTA TTC ATT CAG GAT-3’. 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) with the CTRC-XhoI sense primer 5’-GGA ATT CTC GAG CAC CTA ACC CTG TCT TCT CTC ATC CTC ATG AGA TCT TTA TTC ATT CAG GAT-3’.
Experimental procedures

TTG GGC ATC ACT GTC-3’ and CTRC-EcoRI antisense primer 5’-TTT TTT GAA TTC GAG GAG AAG GAA GTT TAT TGC TGT TGC-3’. The PCR product was digested with Xho I and EcoR I and subcloned into the pcDNA3.1(−) plasmid (Invitrogen).

The cDNA for chymotrypsinogen B1 (CTRB1) was PCR-amplified from IMAGE clone #3950220 (GenBank accession BC005385) with CTRB1-XhoI sense primer 5’-AAA TTT CTC GAG GGG ACC GGC AGA CAG GCG TCC TAC ACC CCT-3’ and CTRB1-BamHI antisense primer 5’-AAA TTT GGA TCC CAT GGG TTT ACT GAG GCT CTG TGG GGA GCA-3’. The cDNA for chymotrypsinogen B2 (CTRB2) was PCR-amplified from IMAGE clone #5225186 (GenBank accession BI838552) with CTRB2-XhoI sense primer 5’-AAA TTT CTC GAG GGC AGC GGC ATG GCT TCC TCT GTC CTC CTC-3’ and CTRB2-BamHI antisense primer 5’-AAA TTT GGA TCC CAT GGG TTT ACT GAG GCT CTG TGG GGA GCA-3’. The CTRB1 and CTRB2 PCR products were digested with Xho I and BamH I and cloned into the pcDNA3.1(−) plasmid.

**Expression of Digestive Proenzymes**

**Human trypsinogens**

Mesotrypsinogen was expressed in *E. coli* SM138 [Graf, 1987; Sahin-Toth, 1999], typically, 2.4 L cultures of SM138/pTrap in Luria-Bertani medium with 100 µg/mL ampicillin were grown to saturation overnight and periplasmic fractions were isolated by osmotic shock. The propeptide sequence of the mature secreted trypsinogen expressed from the pTrap plasmid was Ile-Gln-Ala-Phe-Pro-Val-(Asp)_4-Lys.

In an attempt to increase yield, the mesotrypsinogen gene was transferred to the pTrap-T7 expression plasmid, which has been constructed for the high-level expression of human cationic and anionic trypsinogens [Sahin-Toth, 2000a; Sahin-Toth, 2000b]. The pTrap-T7 plasmid harboring the different trypsinogen genes was transformed into the *E. coli* Rosetta(DE3) strain (Novagen), which is a BL21(DE3) derivative strain carrying a chromosomal copy of T7 RNA polymerase under the control of the *lacZ* promoter. 50 mL cultures were grown in Luria-Bertani medium with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol to an OD_{600 nm} of 0.5, induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside, and grown for an additional 5 h. Cells were harvested by centrifugation
and inclusion bodies were isolated by sonication and centrifugation. The propeptide sequence of recombinant mesotrypsinogen expressed from the pTrap-T7 plasmid was Met-Val-Pro-Phe-(Asp)₄-Lys.

In vitro refolding of human trypsinogens

The inclusion body pellet was washed twice with 1.5 mL of 0.1 M Tris-HCl (pH 8.0) and dissolved in 500 µl of 4 M guanidine-HCl / 0.1 M Tris-HCl, pH 8.0. Dithiothreitol was added to a final concentration of 30 mM, and trypsinogen (Tg) was completely reduced at 37 °C for 30 min. Denatured Tg was then rapidly diluted into 50 mL of refolding buffer (0.9 M guanidine-HCl, 0.1 M Tris-HCl (pH 8.0), 30 mM L-cysteine, 30 mM L-cystine), slowly stirred under argon for 5 min at room temperature, and kept at 4 °C overnight before purification.

Expression of human chymotrypsinogens and proelastases

Human embrionic kidney (HEK) 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS), and 4 mM L-glutamine in 75 cm² flasks to 95% confluence. Transfections were carried out in Opti-MEM 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 covered with 20 mL of the same medium. Alternatively, 20 mL of the protein-free Pro293a-CDM medium (BioWhittaker) was used to facilitate purification. Conditioned media were harvested after 48 h incubation.

Human proelastase 2A was successfully expressed in *E. coli* and *in vitro* refolding from inclusion bodies was accomplished as described for the expression of trypsinogens.

**Purification of the digestive proenzymes**

*Ecotin affinity chromatography*

Human trypsinogens and proelastase 2A were purified using ecotin affinity chromatography, utilizing the column-bound inhibitor ecotin. After refolding of the proteins Tris-HCl (pH 8.0) and NaCl were added to a final concentration of 20 mM and 0.2 M, respectively, and the
samples were applied directly to an ecotin affinity column. The column was washed with
20 mM Tris-HCl (pH 8.0) / 0.2 M NaCl, and the zymogen was eluted with 50 mM HCl.

Ecotin affinity chromatography was used to purify chymotrypsinogen C, proelastase 3A
and proelastase 3B as well. Conditioned medium (40–60 mL pooled from 2–3 parallel
transfections) was directly applied to a 2 mL ecotin column equilibrated with 20 mM
Tris-HCl (pH 8.0) / 0.2 M NaCl and the column was washed with the same buffer. Zymogens
were eluted with 50 mM HCl and Tris-HCl (pH 8.0) was added to 0.1 M final concentration.

**Ion-exchange and gel-filtration chromatography**

Chymotrypsinogen B1 and B2 did not bind to the ecotin column, therefore, these zymogens
were purified by a combination of ion-exchange and gel-filtration chromatography. First,
conditioned media (40–60 mL) were concentrated to about 4 mL using a 10,000 MWCO
Vivaspin 20 concentrator and washed twice in the concentrator with 15 mL 20 mM
Na-acetate (pH 5.0) and concentrated again to 4 mL. The 4 mL sample was then loaded onto a
Mono S HR 5/5 column (Pharmacia) and the column was developed with a 0–1 M NaCl
gradient. Fractions containing chymotrypsinogens B1 and B2 were pooled and concentrated
to about 50 µL using a 10,000 MWCO Vivaspin 500 concentrator. The 50 µL zymogen
sample was then loaded onto a Superose 6 HR 10/30 gel-filtration column (Pharmacia)
equilibrated with 20 mM Tris-HCl (pH 8.0) / 0.2 M NaCl. The column was eluted at a flow-
rate of 0.5 mL/min and pure chymotrypsinogens peaked at about 18 mL.

**Protein concentrations**

Concentrations of the purified zymogen solutions were calculated from their ultraviolet
absorbance at 280 nm, using the following theoretical extinction coefficients
(http://ca.expasy.org/tools/protparam.html). Cationic trypsinogen, 36,160 M⁻¹ cm⁻¹; anionic
trypsinogen, 37,440 M⁻¹ cm⁻¹; mesotrypsinogen, 40,570 M⁻¹ cm⁻¹; proelastase 2A,
73,505 M⁻¹ cm⁻¹; proelastase 3A and 3B, 76,025 M⁻¹ cm⁻¹; chymotrypsinogen B1 and B2,
47,605 M⁻¹ cm⁻¹ and chymotrypsinogen C, 64,565 M⁻¹ cm⁻¹.
PROTEASE ACTIVITY ASSAYS

Measurement of enzyme activity
Trypsin activity was measured with the synthetic chromogenic substrate, N-CBZ-Gly-Pro-Arg-p-nitroanilide (Sigma; 0.14 mM final concentration) in 200 µL final volume. One-minute time courses of the release of the yellow p-nitroaniline were followed at 405 nm in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂, at 22 ºC using a Spectramax Plus 384 microplate reader (Molecular Devices). To verify the activity of the other recombinant pancreatic enzymes, chymotrypsins were assayed with Suc-Ala-Ala-Pro-Phe-p-nitroanilide (Bachem, 0.15 mM concentration); elastase 2A activity was measured with Glt-Ala-Ala-Pro-Leu-p-nitroanilide (0.5 mM concentration). Elastase 3A and 3B were assayed with DQ-elastin fluorescent substrate (Molecular Probes, EnzCheck elastase assay kit) according to the manufacturer’s instructions using a SpectraMax Gemini XS fluorescent microplate reader (Molecular Devices).

Activation of proenzymes
Trypsinogens (2 µM concentration) were activated to trypsin with human enteropeptidase (28 ng/mL concentration) for 30 min at 37 ºC, in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂. Chymotrypsinogens and proelastases (1–5 µM concentration) were activated in 0.1 M Tris-HCl (pH 8.0) and 10 mM CaCl₂ for 20 min at 37 ºC, with 100 nM cationic trypsin (final concentration), with the exception of proelastase 2A, which was activated with 100 nM anionic trypsin. Before use, cathepsin B was activated with 1 mM dithiothreitol (final concentration) for 30 min on ice.

Inhibitor assays
Tight-binding inhibition of trypsin by SBTI or SPINK1 was measured by incubating 15-50 nM trypsin with given concentrations of the inhibitor in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and 1.5 mg/mL bovine serum albumin for 10 min at room temperature. Residual activity was then determined with N-CBZ-Gly-Pro-Arg-p-nitroanilide as described above. With the exception of mesotrypsin, no significant dissociation of the inhibitor-trypsin complex was detectable during the 1 min assay time.
**Experimental procedures**

**Inhibitor degradation**

SBTI or SPINK1 were incubated with mesotrypsin or the indicated protease in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and 1 mg/mL bovine serum albumin at 37 °C. Aliquots were withdrawn at indicated times, mixed with bovine trypsin at a concentration slightly above the initial inhibitor concentration and incubated at room temperature for 1 min before the residual trypsin activity was measured with N-CBZ-Gly-Pro-Arg-p-nitroanilide. Because association of cleaved ("modified") SBTI to bovine trypsin is considerably slower than association of the intact ("virgin") SBTI, the SBTI activity detected after 1 min incubation with bovine trypsin is a specific measure of the virgin SBTI concentration [Luthy, 1973].

**Visualization of proteins**

**Gel electrophoresis**

Samples were precipitated with trichloroacetic acid (10% final concentration), the precipitate was dissolved in Laemmli sample buffer containing 100 mM dithiothreitol (final concentration) and were heat-denatured at 95 °C for 5 min. Electrophoretic separation was performed on 13% SDS-PAGE mini gels in standard Tris-glycine buffer. SPINK1 samples were precipitated with 20% trichloroacetic acid (final concentration), the precipitate was dissolved in sample buffer containing 200 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.04% Coomassie Blue G-250 and 100 mM dithiothreitol (final concentrations). Samples were heat-denatured at 95 °C for 5 min and electrophoretic separation was performed on 16% SDS-PAGE mini gels in Tris-tricine buffer. Gels were stained with Coomassie Brilliant Blue R for 30 min, and destained with 30% methanol, 10% acetic acid overnight.

**N-terminal sequencing**

The procedure utilises the well-established Edman degradative chemistry, sequentially removing amino acid residues from the N-terminus of the protein and identifying the N-terminal amino acids by reverse-phase HPLC. Samples were run on 13% Tris-glycine gels under reducing conditions and transferred onto a sequencing-grade PVDF (polyvinylidene difluoride) membrane (Sequi-Blot PVDF Membrane, Bio-Rad Laboratories) at 300 mA for 1h. The membrane was stained with Coomassie Brilliant Blue R and was washed thoroughly with 50% methanol and dried at room temperature.
Results

THE ROLE OF MESOTRYPSIN [I]

Activation with enterokinase and catalytic properties of mesotrypsin

Mesotrypsinogen was completely activated by bovine enterokinase, albeit at a slower rate than anionic or cationic trypsinogen (Figure 2). Catalytic parameters of activated mesotrypsin were determined with the chromogenic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide, and mesotrypsin exhibited an approximately 3-fold higher turnover number ($k_{cat}$) with a comparable $K_M$ value relative to cationic or anionic trypsin (Table 1).

![Figure 2](image_url)

*Figure 2. Activation of human cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2), wild-type and R198G mutant mesotrypsinogen (PRSS3) with bovine enterokinase. Trypsinogen samples at 2 µM final concentration were incubated with 200 ng/mL enterokinase at 37 °C, in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and 2 mg/mL bovine serum albumin in a final volume of 100 µL. Trypsin activity was expressed as percentage of potential maximal activity.*

Arg¹⁹⁸ is responsible for the inhibitor resistance of mesotrypsin

On the basis of sequence alignments [Nyaruhucha, 1997] and a crystal structure [Katona, 2002], it has been suggested that mesotrypsin is resistant to proteinaceous trypsin inhibitors because of the presence of the Arg¹⁹⁸ side chain, which sterically impairs inhibitor binding to the enzyme. However, this has never been tested experimentally so far. We have expressed and purified the R198G mesotrypsin mutant, in which the characteristic Gly¹⁹⁸ residue – universally found in chymotrypsin-like serine proteases – has been restored. Surprisingly,
activation of mesotrypsinogen mutant R198G with enterokinase under physiological conditions (pH 8.0, 1 mM Ca\(^{2+}\), 37 °C) yielded only 20% of the expected activity (Figure 2). Activation in the presence of high Ca\(^{2+}\) concentrations (50 mM) at room temperature (22 °C) increased the mesotrypsin yield, and eventually pure R198G-mesotrypsin preparation could be obtained by separation on a benzamidine affinity column. Catalytic parameters of R198G-mesotrypsin were essentially identical to those of cationic or anionic trypsin (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>K(_{M}) (µM)</th>
<th>k(_{cat}) (s(^{-1}))</th>
<th>k(<em>{cat}/K</em>{M}) (M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRSS1</td>
<td>15 ± 1</td>
<td>50 ± 1</td>
<td>3.3 (\times) 10(^6)</td>
</tr>
<tr>
<td>PRSS2</td>
<td>11 ± 1</td>
<td>41 ± 1</td>
<td>3.7 (\times) 10(^6)</td>
</tr>
<tr>
<td>PRSS3</td>
<td>22 ± 2</td>
<td>148 ± 4</td>
<td>6.7 (\times) 10(^6)</td>
</tr>
<tr>
<td>R198G</td>
<td>10 ± 2</td>
<td>37 ± 1</td>
<td>3.7 (\times) 10(^6)</td>
</tr>
</tbody>
</table>

*Table 1. Kinetic parameters of human trypsins on the synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide at 22 °C. PRSS1, human cationic trypsin; PRSS2, anionic trypsin; PRSS3, mesotrypsin; R198G, mesotrypsin mutant Arg\(^{198}\) →Gly. Data for PRSS1 and PRSS2 were taken from reference [Sahin-Toth, 2000b].

Strikingly, R198G-mesotrypsin fully regained its sensitivity to protein trypsin inhibitors, and formed tight inhibitory complexes with SBTI or SPINK1 (Figure 3).

*Figure 3. Inhibition of human cationic trypsin (PRSS1), anionic trypsin (PRSS2), wild-type and R198G mesotrypsin (PRSS3) with soybean trypsin inhibitor (SBTI, panel A) and human pancreatic secretory trypsin inhibitor (SPINK1, panel B). For a detailed description of inhibitory assays see Experimental Procedures.*

As expected, wild-type mesotrypsin was resistant to these inhibitors. The experiments confirmed that the unique inhibitor resistance of mesotrypsin was the result of a single
evolutionary amino-acid change, which replaced the small conserved Gly\(^{198}\) residue with a bulky Arg. Notably, in addition to rendering mesotrypsin resistant to inhibitors, the evolutionary selection of the potentially trypsin-sensitive Arg\(^{198}\) side-chain also stabilized mesotrypsin(ogen) against autocatalytic degradation (the R198G mutant is degraded by autolysis). This apparent paradox is resolved if we assume that Arg\(^{198}\) blocks access to mesotrypsin’s active site not only for protein inhibitors, but also for protein substrates, and thus renders mesotrypsin relatively inactive towards its own trypsin-sensitive sites.

**Role of mesotrypsin in pancreatic zymogen activation**

Next, we tested the hypothesis that because of its inhibitor resistance mesotrypsin can activate pancreatic zymogens unopposed by SPINK1, and this mechanism might play a role in the development of human pancreatitis [Nyaruhucha, 1997; Rinderknecht, 1984]. This theory was contradicted by our experiments in which mesotrypsin was used to activate human cationic (Figure 4A) and anionic trypsinogen (not shown). Both zymogens autoactivated spontaneously to trypsin as a function of time, and addition of cationic trypsin, anionic trypsin or R198G-mesotrypsin markedly enhanced this process.

![Figure 4](image.png)

**Figure 4.** Activation of pancreatic zymogens with wild-type (PRSS3) and R198G mutant mesotrypsin. Human cationic trypsinogen (PRSS1-trypsinogen, panel A) and bovine chymotrypsinogen A (panel B) were incubated in the absence of added trypsin (control) or in the presence of cationic trypsin (PRSS1), anionic trypsin (PRSS2), wild-type mesotrypsin (PRSS3) or R198G mesotrypsin mutant.
In contrast, inclusion of mesotrypsin had no appreciable effect on trypsinogen activation, and mesotrypsinogen itself exhibited no autoactivation either. Wild-type mesotrypsin did not activate bovine chymotrypsinogen A (Figure 4B) or human proelastase 2A (not shown), whereas R198G-mesotrypsin was essentially as active as cationic or anionic trypsin. When rates of chymotrypsinogen activation were compared quantitatively, mesotrypsin proved to be 500–1000-fold less efficient than cationic trypsin.

These experiments convincingly rule out a possible role for mesotrypsin in promoting intrapancreatic zymogen activation. Furthermore, our notion that the presence of Arg^{198} renders mesotrypsin catalytically impaired toward protein substrates gained clear experimental support. In terms of cleaving the activation peptide bonds of pancreatic zymogens, mesotrypsin appears to be 2–3 orders of magnitude less active than the major trypsin isoforms, and this defect can be fully repaired by the restoration of Gly^{198}.

**Mesotrypsin cannot degrade pancreatic trypsinogens**

Previous work by Rinderknecht et al. indicated that mesotrypsin can degrade bovine trypsinogen and possibly other pancreatic zymogens [Rinderknecht, 1984]. This observation spawned the theory, that inappropriate trypsinogen activation in the pancreas is curbed not only by SPINK1, which mops up active trypsin, but also by the SPINK1-resistant mesotrypsin, which eliminates trypsinogen, and thus limits further escalation of the activation cascade and protects against CP [Nyaruhucha, 1997; Rinderknecht, 1984].

To test this model, we examined the activity of mesotrypsin on the degradation of human cationic and anionic trypsinogen (Figures 5 and 6). For these experiments we used activation-resistant mutant trypsinogens, in which Lys^{23} in the activation site was replaced with Gln (K23Q). K23Q-trypsinogen is an ideal model substrate to study trypsinogen degradation without interference from trypsinogen activation [Kukor, 2002a; Kukor, 2003]. In the absence of Ca^{2+} (in 1 mM EDTA, pH 8.0, 37 °C) mesotrypsin slowly cleaved the Arg^{122}-Val^{123} peptide bond in cationic K23Q-trypsinogen. We demonstrated previously that this peptide bond is the most sensitive trypsinolytic site in cationic trypsinogen [Kukor, 2002a]. Cleavage at this site does not result in any further degradation or inactivation, but yields a double-chain trypsin(ogen) species, which is functionally equivalent to its single-chain parent enzyme. Furthermore, cleavage never proceeds to completion, but due to trypsin-mediated re-synthesis
of the Arg
\textsuperscript{122}-Val\textsuperscript{123} peptide bond an equilibrium is established between the single-chain and
the double-chain species. Mesotrypsin-mediated digestion of the Arg\textsuperscript{122}-Val\textsuperscript{123} peptide bond
in 1 mM EDTA resulted in the expected hydrolysis equilibrium with approximately 18% single-chain and 82% double-chain trypsinogen present (Figure 5A and C). Addition of 1 mM Ca\textsuperscript{2+} significantly decreased the rate of cleavage, and at 240 min only 20% double-chain trypsinogen was observed (Figure 5B and C). The results clearly demonstrate that mesotrypsin is capable of cleaving the Arg\textsuperscript{122}-Val\textsuperscript{123} peptide bond. However, comparison of mesotrypsin and cationic trypsin in their ability to cleave cationic K23Q-trypsinogen revealed that mesotrypsin was at least 500-fold less active. Other than cleaving at Arg\textsuperscript{122}-Val\textsuperscript{123}, mesotrypsin did not degrade single-chain or double-chain cationic K23Q-trypsinogen to any extent.

**Figure 5.** Cleavage of the Arg\textsuperscript{122}-Val\textsuperscript{123} peptide bond in human cationic trypsinogen (PRSS1) by mesotrypsin (PRSS3). Cationic K23Q-trypsinogen (2 μM final concentration) was digested with mesotrypsin (200 nM final concentration) at 37 °C in 0.1 M Tris-HCl (pH 8.0) containing 1 mM EDTA (panel A) or 1 mM Ca\textsuperscript{2+} (panel B). Samples were analyzed by SDS-PAGE. Panel C. Densitometric quantitation of the double-chain cationic trypsinogen forms.

In contrast to cationic K23Q-trypsinogen, digestion of the proteolytically less stable anionic K23Q-trypsinogen by mesotrypsin resulted in complete zymogen degradation, with a \(t_{1/2}\) of approximately 50 min (Figure 6A and C). Under similar conditions (pH 8.0, 1 mM EDTA, 37 °C, 1:10 trypsin-to-zymogen ratio), cationic trypsin degraded anionic K23Q-trypsinogen 22-fold more rapidly, with a half-life of 2.25 min [Kukor, 2003]. Addition of 1 mM Ca\textsuperscript{2+} almost completely stabilized anionic K23Q-trypsinogen and only approximately
20% mesotrypsin-mediated degradation was detected over the 240 min time course studied (Figure 6B and C).

**Figure 6.** Degradation of human anionic trypsinogen (PRSS2) by mesotrypsin (PRSS3). Anionic K23Q-trypsinogen (2 µM final concentration) was digested with mesotrypsin (200 nM final concentration) at 37 °C in 0.1 M Tris-HCl (pH 8.0) containing 1 mM EDTA (panel A) or in 1 mM Ca²⁺ (panel B). Samples were analyzed by SDS-PAGE. Panel C. Densitometric quantitation of the intact anionic K23Q-trypsinogen band.

Taken together, the results demonstrate that the two major human trypsinogen isoforms are poor substrates for mesotrypsin, and dispute a protective role for mesotrypsin-mediated zymogen degradation in pancreatic physiology. However, the slow but measurable degradation of anionic trypsinogen by mesotrypsin also indicates that the loss of affinity towards protein substrates is not always several orders of magnitude, and a “specific” mesotrypsin substrate might exist, which can avoid the guarding side-chain of Arg198.

**Mesotrypsin rapidly cleaves the reactive-site peptide bond of SBTI**

Seminal work from the Laskowski laboratory demonstrated that in the complexes of proteases and canonical protease inhibitors, the reactive-site peptide bond of the inhibitor gets slowly cleaved, resulting in an equilibrium mixture of double-chain “modified” inhibitor and single-chain “virgin” inhibitor [Laskowski, 2000]. We hypothesized that due to their low but still significant affinity, mesotrypsin recognizes protein trypsin inhibitors as substrates and may rapidly hydrolyze their reactive-site peptide bonds. In the experiment shown in Figure 7A, we incubated 500 nM SBTI with 10 nM mesotrypsin (final concentrations) and at indicated times aliquots were taken and the virgin (intact) SBTI concentration was determined using bovine
Results

Remarkably, a rapid decrease in the concentration of virgin SBTI was observed, and the reaction reached a plateau in about 20 min, with approximately 40% of the original virgin inhibitor remaining. Analysis of the digestion reactions on reducing SDS-PAGE revealed that mesotrypsin cleaved SBTI at a single site (Figure 7B), resulting in the appearance of two new bands on the gels. N-terminal sequencing of the major fragment yielded a sequence of Ile-Arg-Phe-Ile-Ala, confirming that the cleaved peptide bond was the Arg^{88}-Ile^{89} reactive site of SBTI (numbering starts with Met^1). Densitometric quantitation of the virgin SBTI band indicated that approximately 40–44% virgin (intact) and 56–60% modified (cleaved) inhibitor was present in the equilibrium mixture, which was in good agreement with the results of the functional assay in Figure 7A. In control experiments 50 nM cationic or anionic trypsin was incubated with 500 nM SBTI (final concentrations), and after the initial 50 nM decrease due to complex formation between trypsin and SBTI, no further change in the concentration of free virgin SBTI was detectable for 70 hours (not shown).

Figure 7. Hydrolysis of the reactive site of SBTI by mesotrypsin. Panel A. SBTI (500 nM) was incubated with 10 nM mesotrypsin. Cleavage of the reactive site was followed by measuring the decrease in the association rate of modified SBTI with bovine trypsin, as described in Experimental Procedures. Panel B. Samples were subjected to reducing SDS-PAGE. The asterisk indicates the band subjected to N-terminal protein sequencing.
Mesotrypsin degrades human SPINK1

In contrast to SBTI, which forms stable complexes with trypsin, SPINK1 is a so-called “temporary inhibitor”, because trypsin-SPINK1 complexes irreversibly dissociate over time [Laskowski, 1953]. First, reversible digestion of the Lys\textsuperscript{41}-Ile\textsuperscript{42} reactive site peptide bond occurs, which is followed by the irreversibly inactivating cleavage of the Arg\textsuperscript{67}-Gln\textsuperscript{68} bond. In addition, peptide bonds Arg\textsuperscript{28}-Glu\textsuperscript{29}, Arg\textsuperscript{65}-Lys\textsuperscript{66} and Lys\textsuperscript{75}-Ser\textsuperscript{76} are also subject to trypsic attack [Kikuchi, 1989; Schneider, 1973; Schneider, 1974]. In our experiments, temporary inhibition followed a relatively rapid time-course (t\textsubscript{1/2} 2–4 hours) when human cationic or anionic trypsin was in some excess to SPINK1, however, at lower trypsin-to-SPINK1 ratios the reaction proceeded dramatically slower. Thus, when cationic or anionic trypsin (50 nM, final concentration) was reacted with SPINK1 (500 nM, final concentration) at a 1:10 ratio, no trypsin activity was detectable up to 90 hours, and free SPINK1 levels did not change measurably either (not shown). In sharp contrast, incubation of 50 nM mesotrypsin with 500 nM SPINK1 (final concentrations) resulted in a much more rapid decrease of active SPINK1 concentration (Figure 8A), which eventually resulted in complete elimination of SPINK1 activity.

**Figure 8.** Degradation of human SPINK1 by mesotrypsin. Panel A. SPINK1 (500 nM) was incubated with 50 nM mesotrypsin, 500 nM bovine chymotrypsin or 500 nM human elastase 2. Residual inhibitory activity was measured with bovine trypsin, as described in Experimental Procedures. Panel B. SPINK1 (15 µM) was incubated with 0.75 µM mesotrypsin. Samples were precipitated with 20% trichloroacetic acid at indicated times; separated on 16% tricine-SDS gels under reducing conditions. Panel C. Sites of mesotrypsin cleavage in SPINK1.
In control experiments, incubation of 500 nM bovine chymotrypsin or human elastase 2A with 500 nM SPINK1 (final concentrations) did not result in any appreciable SPINK1 degradation up to 20 h, indicating that mesotrypsin can be regarded as a specific degrading enzyme for SPINK1. To visualize the degradation of SPINK1, an experiment was performed using high concentrations of inhibitor (15 µM) and 20-fold less mesotrypsin (0.75 µM). Under these conditions, the reaction proceeded faster, and more than 50% of SPINK1 activity was abolished in 40 min (not shown). Samples were analyzed under reducing conditions on SDS-tricine polyacrylamide gels, where gradual disappearance of the SPINK1 band was apparent (Figure 8B). Only a faint and transiently visible cleavage product was detected, indicating that SPINK1 suffered more extensive proteolysis beyond the cleavage of the reactive-site peptide bond. To confirm this notion, a sample of the digestion mixture taken at 40 min was subjected to Edman-degradation. In addition to the native N terminus, two major new N termini were identified, which indicated cleavages at peptide bonds Lys$^{41}$-Ile$^{42}$ (the reactive site) and Lys$^{75}$-Ser$^{76}$. Two other N termini were found in smaller yield, which revealed cleavages at Arg$^{28}$-Glu$^{29}$ and Arg$^{67}$-Gln$^{68}$ (Figure 8C). Although we could not establish the exact order of cleavages from these data, the results clearly confirmed that one of the major targets of mesotrypsin was the reactive site of SPINK1, and the other tryptic cleavages corresponded to those previously described. Therefore, we conclude that mesotrypsin-mediated SPINK1 degradation followed the established mechanism of “temporary inhibition”, but at a markedly higher rate.

**Cathepsin B is a potential pathological activator of mesotrypsinogen**

The observations described above directly suggest the idea that premature activation of mesotrypsinogen in the pancreas can degrade protective SPINK1 and eventually cause pancreatitis. Since we found that mesotrypsin cannot autoactivate (Figure 2), we were left with the dilemma of identifying a possible activating enzyme for mesotrypsinogen in the pancreas. In this regard, the two major trypsin isoforms appeared to be good candidates. However, when mesotrypsinogen was activated with cationic trypsin, anionic trypsin or enterokinase under physiologically optimal conditions (37 °C, pH 8.0, 1 mM Ca$^{2+}$), human trypsins generated less than 20% of the potentially maximal mesotrypsin activity in 2 hours, and a considerable fraction of mesotrypsinogen was degraded, despite the presence of 1 mM
Results

Ca^{2+} (not shown). The results indicate that cationic and anionic trypsin are more likely to play a role in mesotrypsinogen degradation than activation.

The lysosomal cysteine protease cathepsin B was shown to play a critical role in various experimental pancreatitis models as the intracellular catalyst of pathological trypsinogen activation [Halangk, 2000]. Cathepsin B is also found in the secretory pathway of the human pancreas, where it is co-localized with trypsinogen [Kukor, 2002b]. *In vitro*, cathepsin B is a potent activator of cationic trypsinogen, with a pH optimum of 4.0 [Figarella, 1988; Kukor, 2002b; Luthy, 1973]. When activation of the three human trypsinogen isoforms was compared at pH 4.0, an interesting picture emerged (Figure 9A). Rapid activation of mesotrypsinogen was apparent, which was followed by slow degradation. Compared to cationic and anionic trypsinogen, the initial rate of mesotrypsinogen activation was approximately 2-fold and 6-fold higher, respectively (Figure 9B). Therefore, under certain conditions, cathepsin B can rather selectively activate mesotrypsinogen, and potentially initiate the mesotrypsin-mediated degradation of SPINK1.

![Figure 9. Activation of mesotrypsinogen by cathepsin B. Panel A. Human cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2) and mesotrypsinogen (PRSS3) were activated at 2 µM concentration with human cathepsin B (90 µg/mL) at 37 °C in 0.1 M Na-acetate buffer (pH 4.0) in the presence of 1 mM dithiothreitol, 2 mg/mL bovine serum albumin, 1 mM EDTA, and 300 µM benzamidine. Aliquots (2.5 µL) were withdrawn at indicated times and trypsin activity was measured. Panel B. The diagram depicts the calculated initial rates of trypsinogen activation.](image-url)
THE ROLE OF CHYMOTRYPSIN C [II]

Chymotrypsin C promotes degradation of human cationic trypsin

Incubation of human cationic trypsin with chymotrypsin C at pH 8.0; 37 °C, in the presence of 25 µM Ca\(^{2+}\), resulted in rapid loss of trypsin activity, with a half-life (t\(_{1/2}\)) of about 15 min (Figure 10A). Under these conditions, autocatalytic degradation (autolysis) of cationic trypsin was negligible. Degradation of cationic trypsin by chymotrypsin C proved to be highly specific, as chymotrypsin B1, chymotrypsin B2, elastase 2A, elastase 3A or elastase 3B had no significant degrading activity at pH 8.0 (Figure 10A) or at pH 6.0 (not shown).

SDS-PAGE analysis of the spontaneous autolysis reaction of cationic trypsin showed no appreciable degradation over the 1 h time course studied. As described previously [Kukor, 2002a], human cationic trypsin consists of an equilibrium mixture of single-chain and double-chain forms cleaved at the Arg\(^{122}\)-Val\(^{123}\) peptide bond, and both forms remained stable under the experimental conditions. Double-chain trypsin is cleaved at the Arg\(^{122}\)-Val\(^{123}\) peptide bond and runs as two bands on reducing gels; the upper band corresponds to the C-terminal chain (Val\(^{123}\)-Ser\(^{247}\)) and the lower band is the N-terminal chain (Ile\(^{24}\)-Arg\(^{122}\)) (Figure 10B). In stark contrast, when chymotrypsin C was included in the reaction, SDS-PAGE showed the time-dependent disappearance of both the single-chain and double-chain trypsin bands and the appearance of degradation fragments, which were eventually further degraded to peptides too small to resolve on the 15% gels used (Figure 10C). Surprisingly, when a catalytically inactive Ser\(^{200}\rightarrow\)Ala (S200A) cationic trypsin mutant was digested with chymotrypsin C, the overall rate of trypsin degradation was markedly slower, indicating that trypsin activity is required for efficient chymotrypsin C mediated cationic trypsin degradation (Figure 10D).

N-terminal sequencing of the visible bands in Figure 10C revealed that the primary chymotrypsin C cleavage site was the Leu\(^{81}\)-Glu\(^{82}\) peptide bond within the Ca\(^{2+}\) binding loop of cationic trypsin (Figure 10E, Figure 11). The C-terminal chymotryptic fragment (Glu\(^{82}\)-Ser\(^{247}\)) underwent rapid trypsic (autolytic) cleavage at the Arg\(^{122}\)-Val\(^{123}\) peptide bond, resulting in 3 peptides, corresponding to the Ile\(^{24}\)-Leu\(^{81}\); Glu\(^{82}\)-Arg\(^{122}\) and Val\(^{123}\)-Ser\(^{247}\) segments. The same 3 peptides were generated when chymotrypsin C cleaved the Leu\(^{81}\)-Glu\(^{82}\) peptide bond in the double-chain cationic trypsin, already cleaved after Arg\(^{122}\) (Figure 10E and Figure 11). Finally, a peptide generated in low yields by chymotryptic cleavage of the
Leu$^{41}$-Asn$^{42}$ peptide bond was also identified among the lower molecular weight bands (Figure 10C).

**Figure 10.** Degradation of human cationic trypsin by chymotrypsin C. Panel A. Cationic trypsin (2 µM) was incubated alone (control) or with 300 nM of the indicated proteases in 0.1 M Tris-HCl (pH 8.0) and 25 µM CaCl$_2$ (final concentrations), in 100 µL final volume. At the indicated times, aliquots (2 µL) were withdrawn and residual trypsin activity was measured and expressed as percentage of the initial activity. Panels B, C and D. SDS-PAGE analysis of autolysis and chymotrypsin C mediated degradation of cationic trypsin. Wild-type cationic trypsin (B and C) or S200A mutant cationic trypsin (D) were incubated at 2 µM concentration in the absence (B) or presence (C and D) of 300 nM chymotrypsin C in 0.1 M Tris-HCl (pH 8.0) and 25 µM CaCl$_2$ (final concentrations). Panel E. Major proteolytic cleavage sites in cationic trypsin determined from N-terminal sequencing of the visible bands in Panel C.

**Ca$^{2+}$ protects cationic trypsin against chymotrypsin C mediated degradation**

Because the primary chymotrypsin C cleavage site is located within the calcium binding loop of cationic trypsin, we speculated that Ca$^{2+}$ might be protective against chymotryptic cleavage and subsequent trypsin degradation (Figure 11).
Indeed, increasing the Ca\(^{2+}\) concentration from 25 µM to 1 mM progressively inhibited the degradation of cationic trypsin by chymotrypsin C, with essentially complete protection observed at 1 mM Ca\(^{2+}\) (Figure 12A). The half-maximal protective Ca\(^{2+}\) concentration was 40 µM, which probably corresponds to the K\(_D\) of Ca\(^{2+}\) binding to cationic trypsin.

**Results**

![Figure 11. Primary structure of human cationic trypsin. Individual amino-acids are represented by circles. The Leu\(^{81}\) is highlighted in red and Arg\(^{122}\) in blue. The catalytic triad consisting of His\(^{63}\); Asp\(^{107}\) and Ser\(^{200}\) is shown in green. Note that Asp\(^{107}\) is located on the yellow peptide segment which is released upon cleavage of the Leu\(^{81}\)-Glu\(^{82}\) and Arg\(^{122}\)-Val\(^{123}\) peptide bonds. The 5 disulfide bridges and the interactions between the calcium ion and amino acids within the calcium binding loop are indicated. The yellow section corresponds to the yellow peptide in Figure 10E.](https://example.com/figure11)

**Figure 11.** Primary structure of human cationic trypsin. Individual amino-acids are represented by circles. The Leu\(^{81}\) is highlighted in red and Arg\(^{122}\) in blue. The catalytic triad consisting of His\(^{63}\); Asp\(^{107}\) and Ser\(^{200}\) is shown in green. Note that Asp\(^{107}\) is located on the yellow peptide segment which is released upon cleavage of the Leu\(^{81}\)-Glu\(^{82}\) and Arg\(^{122}\)-Val\(^{123}\) peptide bonds. The 5 disulfide bridges and the interactions between the calcium ion and amino acids within the calcium binding loop are indicated. The yellow section corresponds to the yellow peptide in Figure 10E.

![Figure 12. Effect of calcium on the chymotrypsin C mediated degradation of human cationic trypsin. Wild-type cationic trypsin (Panel A) or the E82A mutant (Panel B) were incubated in 0.1 M Tris-HCl (pH 8.0) and the indicated CaCl\(_2\) concentrations.](https://example.com/figure12)

**Figure 12.** Effect of calcium on the chymotrypsin C mediated degradation of human cationic trypsin. Wild-type cationic trypsin (Panel A) or the E82A mutant (Panel B) were incubated in 0.1 M Tris-HCl (pH 8.0) and the indicated CaCl\(_2\) concentrations.
To demonstrate that the protective effect of Ca\(^{2+}\) is exerted through binding to the Ca\(^{2+}\) binding loop in trypsin, we mutated Glu\(^{82}\) to Ala (E82A). Glu\(^{82}\) is one of three carboxylate side chains that bind the Ca\(^{2+}\) ion (Figure 11). In 25 µM Ca\(^{2+}\), both the E82A mutant (Figure 12B) and the corresponding wild-type (Figure 12A) cationic trypsins were degraded by chymotrypsin C with similar kinetics, indicating that Glu\(^{82}\) is not essential for efficient chymotrypsin C cleavage. Remarkably, however, degradation of E82A-trypsin was insensitive to Ca\(^{2+}\) up to 1 mM concentrations, confirming that an intact Ca\(^{2+}\) binding site in trypsin is required for the protective effect of Ca\(^{2+}\) against chymotrypsin C. Inhibition of trypsin degradation by Ca\(^{2+}\) was also quantified at pH 6.0, and a half-maximal protective concentration of 90 µM was obtained (Figure 13).

**Figure 13.** Effect of calcium on the chymotrypsin C mediated degradation of human cationic trypsin at pH 6.0. Wild-type cationic trypsin was incubated at 2 µM concentration with 500 nM chymotrypsin C (final concentration) in 0.1 M Na-MES (2-morpholinoethanesulfonic acid) at pH 6.0 and the indicated CaCl\(_2\) concentrations.

The significance of both the chymotryptic and tryptic cleavage in the degradation of human cationic trypsin

To investigate the relative significance of the chymotryptic cleavage after Leu\(^{81}\) and the autolytic cleavage after Arg\(^{122}\), both sites were mutated to Ala, individually, and the L81A and R122A mutant cationic trypsins were digested with chymotrypsin C. Strikingly, both mutations afforded essentially complete protection against chymotrypsin C mediated degradation (Figure 14A). SDS-PAGE analysis confirmed that mutant L81A remained intact
in the presence of chymotrypsin C, with both single-chain and double-chain species unaffected (Figure 14B). On the other hand, the R122A mutant was slowly cleaved by chymotrypsin C, at a rate that was similar to the chymotryptic cleavage of the S200A-trypsin in Figure 10D (Figure 14C). However, in the absence of autolytic cleavage at the Arg$^{122}$-Val$^{123}$ peptide bond, this slow chymotryptic cleavage was insufficient to produce appreciable degradation. Although not shown, mutation p.R122H protected against chymotrypsin C mediated trypsin degradation as well as mutation R122A. Taken together, the results indicate that chymotryptic cleavage of the Leu$^{81}$-Glu$^{82}$ peptide bond and autolytic cleavage of the Arg$^{122}$-Val$^{123}$ peptide bond are both essential for rapid trypsin degradation.

![Graph A](image1)

**Figure 14.** Mutations L81A and R122A stabilize cationic trypsin against chymotrypsin C mediated degradation. Wild-type, L81A and R122A cationic trypsins were incubated at 2 µM concentration with 300 nM chymotrypsin C in 0.1 M Tris-HCl (pH 8.0) and 25 µM CaCl$_2$ (final concentrations). Panel A. At the indicated times, residual trypsin activity was measured. Panels B and C. Aliquots (100 µL) were analyzed by 15% SDS-PAGE under reducing conditions.
The identity of chymotrypsin C with Rinderknecht’s enzyme Y

On the basis of its ability to degrade human cationic trypsin with high specificity and in a Ca\(^{2+}\) dependent manner, we speculated that chymotrypsin C might be identical to enzyme Y, the trypsinogen-degrading enzymatic activity isolated from human pancreatic juice by Heinrich Rinderknecht in 1988 [Rinderknecht, 1988]. Consistent with this assumption, chymotrypsin C was capable of degrading not only cationic trypsin, but also anionic trypsin and mesotrypsin (Figure 15A), and the trypsin precursors cationic trypsinogen, anionic trypsinogen and mesotrypsinogen as well (Figure 15B). Interestingly, trypsinogen degradation proceeded significantly faster (t\(_{1/2}\) ~2–5 min) than trypsin degradation. Millimolar Ca\(^{2+}\) concentrations inhibited trypsinogen degradation as well as trypsin degradation (not shown).

**Figure 15.** Degradation of the three human trypsin and trypsinogen isoforms by chymotrypsin C. Panel A. Human cationic trypsin, anionic trypsin and mesotrypsin were incubated at 2 µM concentration with 300 nM chymotrypsin C in 0.1 M Tris-HCl (pH 8.0) and 25 µM CaCl\(_2\) (final concentrations). Panel B. Human cationic trypsinogen, anionic trypsinogen and mesotrypsinogen (2 µM) were incubated with 300 nM chymotrypsin C in 0.1 M Tris-HCl (pH 8.0) and 25 µM CaCl\(_2\) (final concentrations). At the indicated times, 10 µL aliquots were withdrawn, CaCl\(_2\) was added to 10 mM final concentration and activable trypsinogen content was determined by incubating with 120 ng/mL human enteropeptidase for 15 min at room temperature and measuring trypsin activity. Activable trypsinogen was expressed as percentage of the initial activable trypsinogen content.

Figure 16 demonstrates the degradation banding pattern of human trypsinogens on gels and the chymotrypsin C cleavage sites deduced from N-terminal sequencing of the visible bands.
Although the Leu$^{81}$-Glu$^{82}$ peptide bond was readily cleaved in all three trypsinogens, additional isoform-specific chymotryptic cleavage sites were also observed.

In cationic trypsinogen, after cleavage of the Leu$^{81}$-Glu$^{82}$ peptide bond, the N-terminal cleavage fragment was further digested at the Leu$^{41}$-Asn$^{42}$ peptide bond. In anionic trypsinogen, besides the Leu$^{81}$-Glu$^{82}$ peptide bond, the Leu$^{148}$-Ser$^{149}$ peptide bond in the autolysis loop was also rapidly cleaved. The N-terminal cleavage fragment was further digested at the neighboring Tyr$^{37}$-Gln$^{38}$ and Leu$^{41}$-Asn$^{42}$ peptide bonds, whereas the C-terminal fragment was proteolyzed at the Leu$^{189}$-Glu$^{190}$ peptide bond. Note that wild-type

**Figure 16.** Degradation of human trypsinogens by chymotrypsin C. The degradation pattern of human cationic trypsinogen (panel A), anionic trypsinogen (panel B) and mesotrypsinogen (panel C) is shown.
Results

anionic trypsinogen suffered more extensive degradation than the other two isoforms. Therefore, to identify all the chymotrypsin C cleavage sites anionic trypsinogen mutants L81A and L148A were also digested with chymotrypsin C and the fragments analyzed by N-terminal sequencing (not shown). In mesotrypsinogen, the autolysis loop was first cleaved at two adjacent sites, in the Leu$^{148}$-Ser$^{149}$ and Phe$^{150}$-Gly$^{151}$ peptide bonds; followed by the digestion of the Leu$^{81}$-Glu$^{82}$ peptide bond.
DISCUSSION AND CONCLUSIONS

THE ROLE OF MESOTRYPSIN

Significance of Arg^{198}

First, on the basis of sequence alignment with other trypsins, it has been suggested that Arg^{198} is the major determinant of the inhibitor resistance exhibited by mesotrypsin against proteinaceous trypsin inhibitors [Nyaruhucha, 1997]. Subsequently, this notion gained support from the analysis of the mesotrypsin crystal structure [Katona, 2002]. Finally, direct experimental evidence for the role of Arg^{198} came from site-directed mutagenesis of Arg^{198} to Gly^{198} in mesotrypsin. The resulting R198G-mesotrypsin mutant became fully sensitive to soybean trypsin inhibitor (Kunitz) and human pancreatic secretory trypsin inhibitor (Kazal) (Figure 3). Furthermore, R198G-mesotrypsin activated chymotrypsinogen almost as well as cationic or anionic trypsin, indicating a complete restoration of the digestive capacity of mesotrypsin (Figure 4A). In conclusion, the evolutionary selection of Arg^{198} is solely responsible for the unique inhibitor resistance and restricted substrate specificity of mesotrypsin.

Physiological role of mesotrypsin

The results strongly argue that human mesotrypsin plays a unique and highly specialized role in the degradation of trypsin inhibitors. Obviously, such a function would be advantageous in the digestion of foods rich in naturally occurring trypsin inhibitors, and would provide a rationale for the evolution of this trypsin isoform. Such a specialized function can also explain the relatively low levels of mesotrypsin secretion, as opposed to the largely non-specific digestive enzymes. In most cases, cleavage of the reactive-site peptide bond already results in an apparent loss of inhibition, as the modified inhibitor associates with trypsin more slowly. Some inhibitors can be then further digested by mesotrypsin, while others might be degraded in concert with other digestive proteases. A known example is carboxypeptidase B, which can cleave off the newly generated C-terminal Lys or Arg residues after hydrolysis of the reactive-site peptide bonds and thus completely inactivate trypsin inhibitors. We hypothesized that mesotrypsin might be also responsible for the elimination of endogenous SPINK1.
secreted to the duodenum, however, model experiments indicated that at physiological concentrations SPINK1 was bound by cationic or anionic trypsin and the presence of mesotrypsin did not facilitate the irreversible dissociation of SPINK1 (data not shown). Thus, elimination of unwanted SPINK1 seems to occur through the “temporary inhibition” mechanism by major trypsin isoforms. Similarly, elimination of endogenous pancreatic secretory trypsin inhibitor secreted to the duodenum probably occurs through the “temporary inhibition” mechanism by major trypsin isoforms and not by mesotrypsin-mediated degradation.

Finally, because of its digestive defect, mesotrypsin cannot play a significant role in the degradation of dietary proteins, given the higher catalytic efficiencies and larger abundance of cationic and anionic trypsins. Interestingly, however, the extent of the “digestive defect” varies greatly between protein substrates tested. Pancreatic zymogens (chymotrypsinogen, proelastase, trypsinogen) are activated by mesotrypsin to their respective active enzymes at rates that are 500–1000-fold lower than activation by cationic or anionic trypsin. Mesotrypsinogen does not exhibit any appreciable autoactivation either. Mesotrypsin-mediated cleavage of the Arg^{122}-Val^{123} peptide bond in human cationic trypsinogen is also 500-fold slower when compared to cationic trypsin.

On the other hand, mesotrypsin degrades human anionic trypsinogen only 20-fold slower than human cationic trypsin. Mesotrypsin was also shown to cleave selectively the Arg^{79}-Thr^{80} and Arg^{97}-Thr^{98} peptide bonds in the lipid bound form of human myelin basic proteins [Medveczky, 2006]. Recent work in our laboratory provided further evidence that mesotrypsin is not a defective protease on polypeptide substrates in general, but exhibits a relatively high specificity for Lys/Arg – Ser/Thr (P1’ position) peptide bonds. Canonical activation sites of pancreatic protease zymogens generally contain Ile or Val at the P1’ position resulting in a poor mesotrypsin action on these substrates [Szepessy, 2006b].

Is mesotrypsin restricted to the human pancreas? Is mesotrypsin restricted to the human pancreas?

It is unclear whether a secretory form of mesotrypsin is expressed in extrapancreatic tissues; and properties of the presumably intracellular “trypsinogen IV” are even more obscure. A recent study suggested that extrapancreatic mesotrypsin might be a specific activator of protease activated receptors (PAR) 2 and 4 on epithelial cells [Cottrell, 2004]. The authors
based their conclusion on the identification of trypsinogen IV in epithelial cell lines and the
demonstration that mesotrypsin activates protease-activated receptors. Later, these findings
were disputed, but PAR-1 in the brain was shown to be activated by mesotrypsin [Grishina,
2005]. Thus, this question remains controversial, awaiting further studies. Paneth cells, the
secretory epithelial cells of the small intestine, were also shown to express trypsinogen IV and
anionic trypsinogen mRNA. The Paneth cell trypsins were proposed to be the processing
enzymes for the antimicrobial peptide defensin-5 [Ghosh, 2002].

Mesotrypsin-like enzymes seem to be widespread in the animal kingdom, suggesting that
protease-inhibitor degradation is a mechanism of general physiological significance. At the
time of its discovery, mesotrypsin seemed to be specific for humans, because
Rinderknecht et al. reported that no inhibitor resistant trypsin activity was found in pancreatic
extracts from dog, cow, pig, rat, mouse or hamster or in pancreatic juice from dog or hamster
[Rinderknecht, 1984]. With the ongoing sequencing of several vertebrate genomes, orthologs
of mesotrypsin have been identified and more can be expected in the near future. Mesotrypsin-like enzymes in animals can be divided into structural orthologs, which carry the
mesotrypsin signature mutation of Gly198, and functional orthologs, which exhibit inhibitor
resistance.

As to structural orthologs, the recently sequenced chimpanzee genome contains an
authentic mesotrypsinogen gene, which is 93% identical to human PRSS3 at the amino-acid
level. Both forms of the rat tryspinogen V mRNA code for a bulky tyrosine residue at position
198 [Kang, 1992]. While there is no evidence that functional tryspinogen V is expressed at the
protein level, this trypsin would be expected to exhibit mesotrypsin-like inhibitor resistance
and inhibitor-degrading properties. The corresponding mouse gene (trypsinogen 3) is a
pseudogene, which contains a frame-shift due to a single nucleotide deletion.

Among the functional orthologs of mesotrypsin, tryspin-1 from Dermasterias imbricata
[Estell, 1980a; Estell, 1980b] is a well-documented example, however, the amino-acid or
DNA sequence has not been determined for this protein yet. Rat tryspinogen IV was described
as partially inhibitor resistant, presumably due to the presence of the negatively charged
aspartic acid 198 (corresponding to Gln197 in mesotrypsinogen), which replaced the conserved neutral glutamine residue found at this position in other mammalian tryspinogens [Fukuoka,
2002]. This tryspinogen is hardly detectable in the normal pancreas, but cerulein stimulation
results in significant upregulation [Schick, 1984]. Two potential orthologs (94% identity at the amino-acid level) can be found in the mouse genome (trypsinogen 4 and 5), with no data on their expression or function. Finally, the expression of inhibitor resistant trypsin isoforms has been implicated in the adaptation of lepidopteran insects to plant proteinase inhibitors, but the amino-acid determinants of the inhibitor resistance have not been identified yet [Mazumdar-Leighton, 2001].

**Pathophysiological role of mesotrypsin**

The idea of mesotrypsin roaming the pancreas unopposed by trypsin inhibitors has fascinated scientist since its discovery. In two conflicting theories, it was proposed that premature activation of mesotrypsin in the pancreas might cause or protect against pancreatitis, as the inhibitor resistant trypsin activity can freely activate or degrade other pancreatic zymogens [Nyaruhucha, 1997; Rinderknecht, 1984]. Experimental data clearly indicate that mesotrypsin cannot activate pancreatic zymogens (chymotrypsinogen, proelastase, trypsinogen) and degradation of human trypsinogens by mesotrypsin is also defective. Thus, mesotrypsin is unlikely to play a role in activation or degradation of pancreatic zymogens.

The observation that mesotrypsin digests trypsin inhibitors raises the possibility that mesotrypsin could contribute to the pathogenesis of human pancreatitis by reducing the protective levels of pancreatic secretory trypsin inhibitor. Natural mutations of this inhibitor are associated with chronic pancreatitis, indicating that protease inhibition is an important safeguard against pancreatitis [Pfutzer, 2000; Witt, 2000]. Should mesotrypsinogen get converted to mesotrypsin prematurely in the pancreas, the ensuing degradation of SPINK1 might represent an immediate risk factor for the development of pancreatitis. Such a model of disease onset would require a relatively specific activator for mesotrypsinogen in the pancreatic acinar cells. *In vitro* cathepsin B robustly activated mesotrypsinogen, with a measurable preference over cationic or anionic trypsinogen. Thus, the biochemical basis clearly exists for a putative mesotrypsin-induced pancreatitis model. To confirm or rule out the medical significance of such a mechanism, identification of pancreatitis-associated mutations that stabilize mesotrypsin or *loss-of-function* mutations that protect against pancreatitis could provide particularly strong evidence.


**Genetic variants of mesotrypsin**

A variant of mesotrypsinogen, in which Glu$^{32}$ is deleted (p.E32del), was first described as the “b-form” of an alternatively spliced mesotrypsinogen cDNA cloned from the human brain [Wiegand, 1993]. The p.E32del variant has been ruled out as a potential disease causing mutation in patients with both hereditary [Chen, 1999] and alcoholic CP [Nemoda, 2005a]. The latter study also showed that the biochemical properties of the p.E32del mutant were essentially identical to the wild-type protein, which is in agreement with the genetic results identifying it as a frequent polymorphic variant. Although strong biochemical evidence qualified the *PRSS3* gene as a potential candidate gene for CP, a recent study has identified no variants enriched either in CP patients or in the control group after sequencing the complete gene of 320 unrelated patients and 330 controls [Rosendahl et al., unpublished results].

**THE ROLE OF CHYMOTRYPSIN C**

**Physiological role of chymotrypsin C**

The present study identifies chymotrypsin C as the pancreatic digestive enzyme that can promote degradation and inactivation of human cationic trypsin. These results resolve the contradiction between the *in vivo* documented intestinal trypsin degradation [Bohe, 1986; Borgstrom, 1957; Layer, 1986] and the *in vitro* observed resistance of human cationic trypsin against autolysis [Kukor, 2003; Sahin-Toth, 2000b; Szilagyi, 2001] and strongly suggest that chymotrypsin C is responsible for the elimination of trypsin activity in the lower small intestines. Furthermore, the observations clearly negate the notion that degradation of digestive serine proteases is always autocatalytic in nature [Bodi, 2001] and provide a clear example for heterolytic degradation. The specificity of chymotrypsin C in mediating trypsin degradation is striking. All other chymotrypsins and elastases tested were completely ineffective in this respect. Trypsin degradation is initiated by selective cleavage of the Leu$^{81}$-Glu$^{82}$ peptide bond within the Ca$^{2+}$ binding loop, followed by trypsin-mediated autolytic cleavage of the Arg$^{122}$-Val$^{123}$ peptide bond. Millimolar Ca$^{2+}$ concentrations inhibit chymotrypsin C mediated cleavage after Leu$^{81}$ by stabilizing the Ca$^{2+}$ binding loop and thus protect against degradation.
Autolytic degradation of cationic trypsin was postulated to play a protective role in the pancreas against inappropriate trypsin activation [Varallyay, 1998]. This assumption was based on the observation that human hereditary pancreatitis is associated with the p.R122H cationic trypsin mutation, which destroys the Arg\textsuperscript{122} autolytic site [Whitcomb, 1996b]. However, the unusually high resistance of human cationic trypsin to autolysis in vitro has called into question the presumed protective role of trypsin autolysis and the proposed pathomechanism of the p.R122H mutation. The results presented here support a putative defense mechanism, in which chymotrypsin C mediated trypsin degradation mitigates unwanted intrapancreatic trypsin activity. The conditions at the site of intraacinar trypsinogen activation (pH \textasciitilde 6.0, Ca\textsuperscript{2+} concentration \textasciitilde 40 \mu M) are conducive for such a mechanism to be operational [Sherwood, 2007]. Impairment of this mechanism by the p.R122H mutation may contribute to the pathogenesis of hereditary pancreatitis.

Chymotrypsin C was first isolated from pig pancreas and was shown to exhibit a preference for leucyl peptide bonds, which distinguished it from chymotrypsin A or chymotrypsin B [Folk, 1965a; Folk, 1965b]. Chymotrypsin C seems to be the same protein as caldecrin, a serum-calcium decreasing protein isolated from porcine and rat pancreas and later cloned from rat and human pancreas [Tomomura, 1995; Tomomura, 2001]. Recently, it has been reported by our laboratory that chymotrypsin C regulates autoactivation of cationic trypsinogen through limited proteolysis of its activation peptide at the Phe\textsuperscript{18}-Asp\textsuperscript{19} peptide bond [Nemoda, 2006]. The N-terminally truncated cationic trypsinogen autoactivates 3-fold faster than its intact form. Taken together with these previous observations, the present results identify chymotrypsin C as a key regulator of trypsinogen activation and trypsin degradation in humans. Thus, in the activation pathway, chymotrypsin C cleaves the Phe\textsuperscript{18}-Asp\textsuperscript{19} bond in the trypsinogen activation peptide, which in turn facilitates tryptic cleavage of the Lys\textsuperscript{23}-Ile\textsuperscript{24} activating peptide bond, resulting in increased autoactivation. In the degradation pathway, chymotrypsin C cleaves the Leu\textsuperscript{81}-Glu\textsuperscript{82} peptide bond, and subsequent tryptic cleavage of the Arg\textsuperscript{122}-Val\textsuperscript{123} peptide bond destines trypsin for degradation and inactivation. Clearly, chymotrypsin C accelerates already existing auto-regulatory functions of cationic trypsin (i.e. autoactivation and autolysis) (Figure 17).
Discussion and Conclusions

Figure 17. Chymotrypsin C stimulates trypsin-mediated trypsinogen activation (autoactivation, in red) and trypsin-mediated trypsin degradation (autolysis, in blue). In the presence of millimolar Ca\(^{2+}\) concentrations, the trypsin degradation pathway is blocked and only the trypsinogen activation pathway is operational. Both pathways are affected by certain hereditary pancreatitis-associated mutations. Mutation p.A16V stimulates the activation pathway [Nemoda, 2006], whereas mutation p.R122H inhibits the degradation pathway.

The balance between activation and degradation is regulated by the prevailing Ca\(^{2+}\) concentrations. In the presence of high Ca\(^{2+}\) concentrations (>1 mM) characteristic of the upper small intestines, the degradation pathway is blocked and trypsinogen activation is predominant. As the Ca\(^{2+}\) concentration falls below millimolar in the lower intestines, trypsin degradation prevails. Although intestinal Ca\(^{2+}\) absorption has been studied extensively, reliable data on the ionized Ca\(^{2+}\) concentrations along the small intestines is lacking [Bronner, 2003]. It is noteworthy that ionized Ca\(^{2+}\) concentrations in the gut are largely determined by luminal pH and insoluble complex formation, which becomes more significant at the more alkaline pH of the lower intestines, where trypsin degradation was shown to occur [Duflos, 1995].

The identity of the mysterious enzyme Y

An unexpected bonus from these studies was the identification of chymotrypsin C as enzyme Y, a so far obscure trypsinogen-degrading activity from human pancreatic juice. The observations presented in this study seem fully consistent with the conclusion that chymotrypsin C is in fact enzyme Y, which contaminated the cationic trypsinogen preparations in Rinderknecht’s experiments. Thus, chymotrypsin C is a serine protease, which can rapidly degrade all three human trypsinogens in a Ca\(^{2+}\)-dependent manner. No other
pancreatic protease tested in this study had trypsin or trypsinogen degrading activity. It is unlikely that chymotrypsin C mediated trypsinogen degradation has any physiological role in digestion, as high Ca\(^{2+}\) concentrations in the duodenum would inhibit trypsinogen degradation and favor trypsinogen activation.

On the other hand, Rinderknecht’s theory that enzyme Y protects the pancreas by decreasing trypsinogen concentrations during inappropriate zymogen activation might be valid (Figure 18), and the presence of loss-of-function mutations in the CTRC gene, that are associated with CP, would provide evidence for the proposed protective role of chymotrypsin C.

**Figure 18.** Pancreatic defense mechanisms against unwanted trypsin activity include trypsin inhibition by SPINK1 and trypsin degradation by CTRC.

**Genetic variants of chymotrypsin C are associated with chronic pancreatitis**

We analyzed the gene encoding the trypsin-degrading enzyme chymotrypsin C in subjects with chronic pancreatitis. Genetic analysis included 901 individuals with the diagnosis of idiopathic or hereditary chronic pancreatitis and 2804 control subjects. Preliminary results show that there are several alterations in the CTRC gene of pancreatitis patients with an overall frequency of 4.8%. The two most frequent alterations, a missense mutation and an in-frame deletion of eight amino acids, were significantly overrepresented in the pancreatitis group (3.5%) relative to healthy controls (0.7%). Heterologous expression of CTRC variants in HEK 293T and AR42J cells showed reduced activity and/or secretion, indicating that the mutations result in a loss of CTRC function.
The genetic and functional data presented identify *CTRC* as a novel pancreatitis associated gene. Our early observations provide further support for the trypsin-dependent pathogenic model of chronic pancreatitis in humans by demonstrating that trypsin/trypsinogen degradation by CTRC is an important mechanism in the maintenance of the physiological protease-antiprotease balance in the pancreas.
SUMMARY

Chronic pancreatitis is an incurable, persistent inflammatory disease, characterized by progressive and ultimately irreversible damage to both exocrine and endocrine functions of the pancreas. Genetic and biochemical evidence defines a pathological pathway in which a sustained imbalance between intrapancreatic trypsinogen activation and trypsin inactivation results in the development of the disease. Inactivation of potentially harmful intrapancreatic trypsin by proteolytic degradation has been discussed as a possible protective mechanism against chronic pancreatitis for decades. In humans the inhibitor resistant mesotrypsin has been labeled a candidate for this function. Later, the presence of another unknown enzymatic activity referred to as enzyme Y was found effective in the degradation of pancreatic zymogens in human pancreatic juice.

The aim of this work was to study and characterize the protective trypsin-degrading activity of mesotrypsin and chymotrypsin C, and to investigate their possible impact on pancreatic disease. Human digestive proteases were purified with ion-exchange and inhibitor affinity chromatography. Enzymatic interactions were followed by activity assays, SDS-PAGE, and N-terminal sequencing.

Our results demonstrate that human trypsinogens are not degraded by mesotrypsin, and dispute a protective role for mesotrypsin-mediated trypsin degradation in pancreatic physiology. However, we identified a unique role for mesotrypsin in the degradation of trypsin inhibitors. Furthermore, we found that this distinctive enzymatic activity was endowed by the mesotrypsin-specific amino-acid Arg\textsuperscript{198}. The observations not only indicate a physiological role for mesotrypsin in the degradation of dietary trypsin inhibitors, but also suggest that premature activation of mesotrypsinogen could contribute to the pathogenesis of human pancreatitis by reducing the protective levels of pancreatic secretory trypsin inhibitor.

Furthermore, we identified chymotrypsin C as enzyme Y, the so far obscure trypsinogen-degrading activity from human pancreatic juice. Chymotrypsin C can specifically promote inactivation of all human trypsin and trypsinogen isoforms by selective cleavage of the Leu\textsuperscript{81}-Glu\textsuperscript{82} peptide bond within the Ca\textsuperscript{2+} binding loop. Our results support a defense mechanism against pancreatitis, in which chymotrypsin C mediated trypsin degradation mitigates unwanted intrapancreatic trypsin activity.
ÖSSZEFoglalás

A krónikus pankreatitisz a hasnyálmirigy állományának fokozatos pusztulásával járó gyulladásos megbetegedés, amely elhúzódó lefolyása során a táplálék emésztésének és felszívódásának zavarához, valamint a cukorházartartás felborulásához vezet. A genetikai és biokémiai eredmények az intrapankreatikus tripszinogén aktiválódás vagy a tripszin inaktiválódás közötti elhúzódó aránytalanság fontosságát támasztják alá a betegség patomechanizmusában. A szakirodalom régóta tárgyalja a kórosan aktiválódott tripszin enzimatisk lebontását mint lehetséges védőmechanizmust a krónikus pankreatitisz kialakulása ellen. Az emberi szervezetben az inhibitor rezisztens mezotripszin volt az első jelölt erre a funkcióra. Később, egy a humán hasnyálban kimutatott enzimatikus aktivitás bizonyult hatékonynak a zimogének lebontásában, az ismeretlen aktivitást enzim Y-nak nevezték el.

A munkánk célja két hasnyálmirigy proteáz, a mezotripszin és a kimotripszin C, biokémiai jellemzése volt, továbbá lehetséges védő szerepük felderítése a hasnyálmirigy működésében. A rekombináns humán emésztőenzimeket ekotin affinitás kromatográfia segítségével tisztítottuk. Az enzimatikus folyamatokat aktivitás méréssel, gélelektroforézissel és N-terminális szekvenálással vizsgáltuk.

Eredményeink azt mutatják, hogy a mezotripszin defektív a humán tripszinogének lebontásában, emiatt kizárható a mezotripszin általi tripszin lebontás védő szerepe a hasnyálmirigy működésében. Ugyanakkor a mezotripszin a tripszin inhibitorok gyors lebontására képes, ezt a különleges enzimatikus aktivitást a mezotripszin-specifikus Arg^{198} okozza. Megfigyeléseink alapján a mezotripszin fiziológiás szerepe a táplálékban található tripszin inhibitorok emésztése. Patológiai körülmények között, a mezotripszinogén korai aktiválódása esetén, az enzim hozzájárulhat a krónikus pankreatitisz kialakulásához a védő funkciójú pankreatikus szekretoros tripszin inhibitor lebontása által.

Továbbá elmondhatjuk, hogy a kimotripszin C az eddig tisztázatlan identitású enzim Y, amely a tripszinogének lebontására képes a humán hasnyálban. A kimotripszin C specifikusan inaktiválja az összes humán tripszin és tripszinogén izoformát, a Ca^{2+}-kötő hurokban elhelyezkedő Leu^{81} -Glu^{82} peptidkötés szelektív hasítása által. A kimotripszin C tehát egy eddig ismeretlen védő hatású tényező a krónikus pankreatitisz ellen, minthogy a hasnyálmirigyen belül aktiválódott tripszin lebontására képes.
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ABSTRACTS


REFERENCES


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Human Mesotrypsin Is a Unique Digestive Protease Specialized for the Degradation of Trypsin Inhibitors*

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Mesotrypsin is an enigmatic minor human trypsin isoform, which has been recognized for its peculiar resistance to natural trypsin inhibitors such as soybean trypsin inhibitor (SBTI) or human pancreatic secretory trypsin inhibitor (SPINK1). In search of a biological function, two conflicting theories proposed that due to its inhibitor-resistant activity mesotrypsin could prematurely activate or degrade pancreatic zymogens and thus play a pathogenic or protective role in human pancreatitis. In the present study we ruled out both theories by demonstrating that mesotrypsin was grossly defective not only in inhibitor binding, but also in the activation or degradation of pancreatic zymogens. We found that the restricted ability of mesotrypsin to bind inhibitors or to hydrolyze protein substrates was solely due to a single evolutionary mutation, which changed the serine protease signature glycine 198 residue to arginine. Remarkably, the same mutation endowed mesotrypsin with a novel and unique function: mesotrypsin rapidly hydrolyzed the reactive-site peptide bond of the Kunitz-type trypsin inhibitor SBTI, and irreversibly degraded the Kazal-type temporary inhibitor SPINK1. The observations suggest that the biological function of human mesotrypsin is digestive degradation of trypsin inhibitors. This mechanism can facilitate the digestion of foods rich in natural trypsin inhibitors. Furthermore, the findings raise the possibility that inappropriate activation of mesotrypsinogen in the pancreas might lower protective SPINK1 levels and contribute to the development of human pancreatitis. In this regard, it is noteworthy that the well known pathological trypsinogen activator cathepsin B exhibited a preference for the activation of mesotrypsinogen of all three human trypsinogen isoforms, suggesting a biochemical mechanism for mesotrypsinogen activation in pancreatic acinar cells.

The human pancreas secretes three isoforms of trypsinogen, which are encoded by the PRSS (protease, serine) genes PRSS1 (OMIM 276000), PRSS2 (OMIM 601564), and PRSS3. On the basis of their electrophoretic mobility, they are commonly referred to as cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen. The two major isoforms, cationic and anionic trypsinogen, constitute the bulk of secreted trypsinogen, whereas levels of mesotrypsinogen were reported between 3 and 10% of total trypsinogen content in normal pancreatic juice (see Table I in Ref. 1; Fig. 8 in Ref. 2; and Table I in Ref. 3). Rinderknecht et al. (4) first discovered mesotrypsin in 1978 as a new inhibitor-resistant protease found in human pancreatic tissue and fluid, and a systematic characterization was published in 1984 (2). A cDNA coding for mesotrypsinogen was cloned from human pancreas in 1997 (5), and the crystal structure of mesotrypsin complexed with benzamidine was solved in 2002 (6). An alternatively spliced form of mesotrypsinogen in which the signal peptide is replaced with a novel sequence encoded by an alternative exon 1 is expressed in the human brain (7). Although usually referred to as “brain trypsinogen,” there is no evidence for the activation of this novel chimeric molecule, which might have a function unrelated to proteolytic activity (8).

The most intriguing property of mesotrypsin is its resistance to polypeptide trypsin inhibitors (see Table V in Ref. 2), such as the Kunitz-type soybean trypsin inhibitor (SBTI) or the Kazal-type pancreatic secretory trypsin inhibitor (SPINK1, serine protease inhibitor, Kazal type 1, OMIM 167790) (2, 5, 6). Analysis of the recent crystal structure of mesotrypsin provided compelling evidence that the presence of an arginine residue in place of the highly conserved Gly198 (Gly193 in the chymotrypsin numbering system) is responsible for the peculiar inhibitor resistance of mesotrypsin (6). Arg198 occupies the S2′ subsite and its long side-chain sterically clashes with protein inhibitors and possibly substrates. Furthermore, the charge of the guanidino group contributes to the strong clustering of positive charges around the primary specificity pocket of mesotrypsin. However, no direct experimental evidence has ever been presented for the proposed role of Arg198.

Despite the high resolution crystal structure, the biological function of mesotrypsin has remained mysterious. In two clearly conflicting theories, it was proposed that premature activation of mesotrypsin in the pancreas might cause or protect against pancreatitis, as the inhibitor-resistant trypsin activity can freely activate or degrade other pancreatic zymogens (2, 5). It was also suggested that mesotrypsin might have been “abandoned by the process of evolution,” and has no important role in digestion or pancreatic physiology (2, 8). In the present study we identified a unique and specific role for mesotrypsin in the degradation of trypsin inhibitors. Furthermore, we found that this distinctive enzymatic activity was endowed by the evolutionary selection of Arg198. Finally, we showed that the lysosomal cysteine protease cathepsin B activated mesotrypsinogen at a higher rate, relative to the activation of human cationic and anionic trypsinogens. The observations not only indicate a physiological role for mesotrypsin, but also suggest

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1 The abbreviations used are: SBTI, soybean trypsin inhibitor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

This paper is available on line at http://www.jbc.org
Human Mesotrypsinogen

EXPERIMENTAL PROCEDURES

Materials—N-CBZ-Gly-Pro-Arg-p-nitroanilide was purchased from Sigma. Suc-Ala-Ala-Pro-Phe-p-nitroanilide was from Bachem (King of Prussia, PA), ultrapure bovine enterokinase was from Biozyme Laboratories (San Diego, CA), and reagent grade bovine serum albumin was from Biocell Laboratories (Rancho Dominguez, CA). Bovine chymotrypsinogen A, TLCK-treated bovine chymotrypsin and TPCK-treated bovine trypsin was obtained from Worthington Biochemical Corp. (Lakewood, NJ). The concentration of bovine trypsin was determined by incubating 15 μl of bovine trypsin (final concentration) for 30 min on ice.

Expression and Purification of Mesotrypsinogen—The gene encoding mesotrypsinogen was PCR-amplified from the IMAGE clone 2659811 (GenBank accession number AW192265), purchased from Incyte Genomics (Reagents & Services, St. Louis, MO) and ligated into the expression plasmid pTrp14 (14) behind the alkaline phosphatase promoter and signal-sequence. Mutation R198G was introduced via oligonucleotide-directed site-specific mutagenesis, using the overlap extension PCR mutagenesis method. Mesotrypsinogen was expressed in E. coli SM138 [F−, araD139 (lacIqZΔM15), rhaD, rpaS, phi80] as described previously (14, 15). Typically, 2.4-liter cultures of SM138/pTrp14 in Luria-Bertani medium with 100 μg/ml ampicillin were grown to saturation overnight and the --260 ml periplasm was applied directly to an affinity column. Human SPINK1 was expressed in Pichia pastoris (Krynica, Prussia, PA), ultrapure bovine enterokinase was from Biozyme Laboratories (Rancho Dominguez, CA), and reagent grade bovine serum albumin was from Biocell Laboratories (Rancho Dominguez, CA). SPINK1 was measured by incubating 15 μl of SPINK1 samples were precipitated with 20% trichloroacetic acid (final concentration), the precipitate was dissolved in sample buffer containing 100 mM dithiothreitol (final concentration), and samples were heat-denatured at 95 °C for 5 min. Electrophotores separation was performed on 13% SDS-PAGE mini gels in standard Tris-glycine buffer. SPINK1 samples were precipitated with 20% trichloroacetic acid (final concentration), the precipitate was dissolved in sample buffer containing 200 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.04% Coomassie Blue G-250, and 100 mM dithiothreitol (final concentrations). Samples were heat-denatured at 95 °C for 5 min and electrophoretic separation was performed on 16% SDS-PAGE mini gels in Tris-Tricine buffer. Gels were stained with Coomassie Brilliant Blue R for 30 min, and destained with 30% methanol, 10% acetic acid overnight. Densitometric quantitation of bands was carried out as described in Ref. 18.

Expression of Mesotrypsinogen, Activation with Enterokinase, and Catalytic Properties of Mesotrypsin—Recombinant human mesotrypsinogen was expressed in two routinely used E. coli expression systems. In the SM138/pTrp14 expression host/plasmid system trypsinogen is constitutively secreted to the periplasmic space where it acquires its native fold. In the Rosetta(DE3)/pTrpT7 expression system trypsinogen is produced in a denatured form as inclusion bodies, which are solubilized and renatured in vitro. Details of expression and purification are given under “Experimental Procedures.” Importantly, recombinant mesotrypsinogen preparations purified from the two bacterial expression systems were indistinguishable in their functional properties studied here. Mesotrypsinogen was completely activated by bovine enterokinase, albeit at a slower rate than anionic or cationic trypsinogen (Fig. 1, A and B). Catalytic parameters of activated mesotrypsinogen were determined with the chromogenic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide, and mesotrypsin exhibited an --3-fold higher turnover number (kcat) with a comparable Km value relative to cationic or trypsinogen (Table I).

Inhibitor Resistance of Mesotrypsin Is Caused by Arg198—On the basis of sequence alignments (5) and a crystal structure (6), it has been suggested that mesotrypsin is resistant to proteinaceous trypsin inhibitors because of the presence of the Arg198 side chain, which sterically impairs inhibitor binding to the enzyme. However, this notion has never been tested experimentally so far. We have expressed and purified the R198G mesotrypsin mutant, in which the characteristic Gly198 residue, universally found in chymotrypsin-like serine proteases, has been restored. Surprisingly, activation of mesotrypsinogen mutant R198G with enterokinase under physiological conditions (pH 8.0, 1 μM Ca++, 37 °C) yielded only 20% of the expected activity (Fig. 1A). As shown on the gel in Fig. 1C, the lack of activity was due to massive degradation of the R198G-zymogen during the activation process. When the reaction was performed in the presence of SPINK1, which inhibited trypsin but not enterokinase, R198G-mesotrypsinogen was quantita-
tively converted to trypsin, indicating that the degradation was mediated by trypsin and not enterokinase (not shown). Activation in the presence of high Ca\textsuperscript{2+}/H\textsubscript{11001} concentrations (50 mM) at room temperature (22 °C) increased the trypsin yield, and eventually pure and active R198G-mesotrypsin preparation could be obtained by separating the degradation products on a benzamidine affinity column. Catalytic parameters of R198G-mesotrypsin on N-CBZ-Gly-Pro-Arg-p-nitroanilide were essentially identical to those of cationic or anionic trypsin (Table I). Strikingly, R198G-mesotrypsin fully regained its sensitivity to protein trypsin inhibitors, and formed tight inhibitory complexes with SBTI or SPINK1 (Fig. 2). As expected, wild-type mesotrypsin was resistant to these inhibitors. The experiments confirmed that the unique inhibitor resistance of mesotrypsin was the result of a single evolutionary amino acid change, which replaced the small conserved Gly198 residue with a bulky Arg. Notably, in addition to rendering mesotrypsin resistant to inhibitors, the evolutionary selection of the potentially trypsin-sensitive Arg198 side chain also stabilized mesotrypsin(ogen) against autocatalytic degradation. This apparent paradox is resolved if we assume that Arg 198 blocks access to the mesotrypsin active site not only for protein inhibitors, but also for protein substrates, and thus renders mesotrypsin relatively inactive toward its own trypsin-sensitive sites. This notion will be further explored by the subsequent experiments in this report.

Mesotrypsin Cannot Activate Pancreatic Zymogens—Next,
we tested the hypothesis that because of its inhibitor resistance mesotrypsin can activate pancreatic zymogens unopposed by SPINK1, and this mechanism might play a role in the development of human pancreatitis (2, 5). This theory was already contradicted by sporadic observations indicating that mesotrypsin was defective in activating bovine chymotrypsinogen (2) or human cationic and anionic trypsinogen (16). As shown in Fig. 3, these previous findings were fully confirmed by our experiments in which mesotrypsin was used to activate human anionic and cationic trypsinogen. Both zymogens autoactivated spontaneously to trypsin as a function of time, and addition of cationic trypsin, anionic trypsin or R198G-mesotrypsin markedly enhanced this process. In contrast, inclusion of mesotrypsin had no appreciable effect on trypsinogen activation, and mesotrypsinogen itself exhibited no autoactivation either.

Wild-type mesotrypsin did not activate bovine chymotrypsinogen A, whereas R198G-mesotrypsin was essentially as active as cationic or anionic trypsin. When rates of chymotrypsinogen activation were compared quantitatively, mesotrypsin proved to be 500–1000-fold less efficient than cationic trypsin (not shown). Finally, the activation experiments were extended to human pro-elastase 2 (ELA2A), which was efficiently activated by human cationic trypsin, and even better by R198G mesotrypsin mutant. Bovine chymotrypsinogen A and human pro-elastase 2 were activated with 32 and 75 nM trypsin, respectively. Although not shown, in other experiments mesotrypsin at 125 nM concentration activated ~5% of bovine chymotrypsinogen in 90 min, but had no measurable activating effect on human pro-elastase 2. Protease activities were determined as described under “Experimental Procedures,” and expressed as percent of maximal activity.
impaired toward protein substrates gained clear experimental support. In terms of cleaving the activation peptide bonds of pancreaticzymogens, mesotrypsin appears to be 2–3 orders of magnitude less active than the major trypsin isoforms, and this defect can be fully repaired by the restoration of Gly198.

Role of Mesotrypsin in Trypsinogen Degradation—Previous work by Rinderknecht et al. (2) indicated that mesotrypsin can degrade bovine trypsinogen and possibly other pancreaticzymogens. This observation spawned the theory, that inappropriately trypsinogen activation in the pancreas is curbed not only by SPINK1, which mops up active trypsin, but also by the SPINK1-resistant mesotrypsin, which eliminates trypsinogen, and thus limits further escalation of the activation cascade (2, 5). To test this model, we examined the activity of mesotrypsin on the degradation of human cationic and anionic trypsinogens. For these experiments we used activation-resistant mutant trypsinogens, in which Lys-23 in the activation site was replaced with Gln (K23Q). K23Q-trypsinogen is an ideal model substrate to study trypsinogen degradation without interference from trypsinogen activation (13, 18). Fig. 4 shows that in the absence of Ca$^{2+}$ (in 1 mM EDTA, pH 8.0, 37 °C) mesotrypsin slowly cleaved the Arg$^{122}$–Val$^{123}$ peptide bond in cationic K23Q-trypsinogen. We demonstrated previously that this peptide bond is the most sensitive tryspinolytic site in cationic trypsinogen (18). In contrast to a widely held belief, cleavage at this site does not result in any further degradation or inactivation, but yields a double-chain tryspinogen species, which is functionally equivalent to its single-chain parent enzyme. Furthermore, cleavage never proceeds to completion, but due to trypsin-mediated re-synthesis of the Arg$^{122}$–Val$^{123}$ peptide bond an equilibrium is established between the single-chain and the double-chain species. Mesotrypsin-mediated digestion of the Arg$^{122}$–Val$^{123}$ peptide bond in 1 mM EDTA resulted in the expected hydrolysis equilibrium with ~18% single chain and 82% double chain trypsinogen present (Fig. 4, A and C). Addition of 1 mM Ca$^{2+}$ significantly decreased the rate of cleavage, and at 240 min only 20% double chain trypsinogen was observed (Fig. 4, B and C). This was not a true equilibrium yet, because previous studies indicated that in the presence of Ca$^{2+}$ the equilibrium mixture should contain 40% double chain and 60% single chain trypsinogen (18). The results clearly demonstrate that mesotrypsin is capable of cleaving the Arg$^{122}$–Val$^{123}$ peptide bond. However, comparison of mesotrypsin (Fig. 4) and cationic trypsin (cf. Fig. 4 in Ref. 18) in their ability to cleave cationic K23Q-trypsinogen revealed that mesotrypsin was at least 500-fold less active. Other than cleaving at Arg$^{122}$–Val$^{123}$ mesotrypsin did not degrade single chain or double chain cationic K23Q-trypsinogen to any extent.

In contrast to cationic K23Q-trypsinogen, digestion of the proteolytically less stable anionic K23Q-trypsinogen by mesotrypsin resulted in complete zymogen degradation, with a $t_{1/2}$ of ~50 min (Fig. 5, A and C). Under similar conditions (pH 8.0, 1 mM EDTA, 37 °C, 1:10 trypsin-to-zymogen ratio), cationic trypsin degraded anionic K23Q-trypsinogen 22-fold more rapidly, with a half-life of 2.25 min (cf. Fig. 3 in Ref. 13). Addition of 1 mM Ca$^{2+}$ almost completely stabilized anionic K23Q-trypsinogen and only ~25% mesotrypsin-mediated degradation was detected over the 240-min time course studied (Fig. 5, B and C). Taken together, these data demonstrate that the two major human trypsinogen isoforms are poor substrates for mesotrypsin, and dispute a protective role for mesotrypsin-mediated zymogen degradation in pancreatic physiology. Furthermore, the slow but measurable degradation of anionic trypsinogen by mesotrypsin also indicates that the loss of affinity toward protein substrates is not always several orders of magnitude, and a “specific” mesotrypsin substrate might exist, which can avoid the guarding side chain of Arg$^{198}$.

**Mesotrypsin Is Weakly Inhibited by SPINK1 or SBTI**—We hypothesized that although mesotrypsin degrades human anionic trypsinogen very slowly, this process might still be significant under conditions when all other trypsins are inhibited by SPINK1 and only mesotrypsin is active. To demonstrate the feasibility of such a scenario, we digested anionic K23Q-trypsinogen with mesotrypsin in the presence of SPINK1. To our surprise, SPINK1 inhibited mesotrypsin activity even in submicromolar concentrations, and no degradation of anionic trypsinogen was detectable (not shown). These data seemed to contradict the observation in Fig. 2 that SPINK1 does not bind to mesotrypsin. However, the experimental setup used in Fig. 2 only tests for tight binding inhibition, which withstands dissociation during the assay. Relatively weak inhibitory complexes would quickly dissociate when the sample was diluted into the substrate-containing assay buffer, and no inhibition would be apparent. Thus, the assay used in Fig. 2 is not appropriate for testing weak inhibition. To characterize the relatively weaker inhibition of mesotrypsin by SPINK1, catalytic parameters ($K_m$, $k_{cat}$) of mesotrypsin were measured on N-CBZ-Gly-Pro-Arg-p-nitroanilide in the presence of increasing concentrations of inhibitor. From [inhibitor] versus $K_m$ plots a $K_i$ value of 1.5 mM was estimated (not shown). Similar experiments with SBTI

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**Fig. 4. Cleavage of the Arg$^{122}$–Val$^{123}$ peptide bond in human cationic trypsinogen (PRSS1) by mesotrypsin (PRSS3).** Cationic K23Q-trypsinogen (2 μM final concentration) was digested with mesotrypsin (200 nM final concentration) at 37 °C in 0.1 M Tris-HCl (pH 8.0) containing 1 mM EDTA (panel A) or 1 mM Ca$^{2+}$ (panel B). Samples were precipitated with trichloroacetic acid (10% final concentration) at the indicated times and analyzed by SDS-PAGE and Coomassie Blue staining. Panel C, densitometric quantitation of the double chain cationic trypsinogen forms.

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**Human Mesotrypsinogen**
yielded a $K_i$ value of 0.42 $\mu M$ (not shown), which was identical to the $K_i$ value determined by Katona et al. (6) using progress curve analysis. Interestingly, while SBTI acted in a purely competitive fashion, SPINK1 in the higher concentration range also exhibited non-competitive inhibition of mesotrypsin, for reasons that are not readily apparent. In any event, the results clearly indicate that mesotrypsin retained low but significant affinity toward trypsin inhibitors, and challenge the notion that mesotrypsin can act uncontrolled in the presence of SPINK1.

Mesotrypsin Rapidly Cleaves the Reactive Site Peptide Bond of SBTI—Seminal work from the Laskowski laboratory demonstrated that in the complexes of proteases and canonical protease inhibitors, the reactive site peptide bond of the inhibitor gets slowly cleaved, resulting in an equilibrium mixture of double chain “modified” inhibitor and single-chain “virgin” inhibitor (Ref. 19 and references therein). We hypothesized that due to the low but still significant affinity, mesotrypsin recognizes protein trypsin inhibitors as substrates and may rapidly hydrolyze their reactive site peptide bonds. A precedent for this notion was found by Estell and Laskowski (20, 21), who demonstrated that trypsin-1 from the starfish Dermasterias imbricata (leather star) cleaved the reactive sites of SBTI and bovine trypsin, as described under “Experimental Procedures.” Panel B, alternatively, samples were precipitated with 10% trichloroacetic acid (10% final concentration) and analyzed by reducing SDS-PAGE and Coomassie Blue staining. Panel C, densitometric quantitation of the intact anionic K23Q-trypsinogen band.

Fig. 5. Degradation of human anionic trypsinogen (PRSS2) by mesotrypsin (PRSS3). Anionic K23Q-trypsinogen (2 $\mu M$ final concentration) was digested with mesotrypsin (200 $n M$ final concentration) at 37 °C in 0.1 M Tris-HCl (pH 8.0) containing 1 mM EDTA (panel A) or in 1 mM Ca$^{2+}$ (panel B). Samples were precipitated with trichloroacetic acid (10% final concentration) at the indicated times and analyzed by SDS-PAGE and Coomassie Blue staining. The asterisk indicates the band subjected to N-terminal protein sequencing.

Fig. 6. Hydrolysis of the reactive site of SBTI by mesotrypsin. Panel A, SBTI (500 nM) was incubated with 10 nM mesotrypsin (final concentration) at 37 °C in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl$_2$, and 1 mg/ml bovine serum albumin. Cleavage of the reactive site was followed by measuring the decrease in the association rate of modified SBTI with bovine trypsin, as described under “Experimental Procedures.” Panel B, alternatively, samples were precipitated with 10% trichloroacetic acid (final concentration) and analyzed by reducing SDS-PAGE and Coomassie Blue staining. The asterisk indicates the band subjected to N-terminal protein sequencing.

was determined using bovine trypsin (see “Experimental Procedures” for a description of the assay). Remarkably, a rapid decrease in the concentration of virgin SBTI was observed, and the reaction reached a plateau in about 20 min, with ~40% of the original virgin inhibitor remaining. Analysis of the digestion reactions on reducing SDS-PAGE revealed that mesotrypsin cleaved SBTI at a single site (Fig. 6A), resulting in the appearance of two new bands on the gels. N-terminal sequencing of the major fragment yielded a sequence of Ile-Arg-Phe-Ala, confirming that the cleaved peptide bond was the Arg$^{48}$-Ile$^{68}$ reactive site of SBTI (numbering starts with Met$^1$). Densitometric quantitation of the virgin SBTI band indicated that ~40–44% virgin (intact) and 56–60% modified (cleaved) inhibitor was present in the equilibrium mixture, which was in good agreement with the results of the functional assay in Fig. 6A. At the same pH but under somewhat different assay conditions (0.5 M KCl, 21 °C) the Laskowski laboratory reported a hydrolysis equilibrium containing 78% modified SBTI after digestion with Dermasterias imbricata trypsin-1 or bovine trypsin (20). In control experiments 50 nM cationic or anionic trypsin was incubated with 500 nM SBTI (final concentrations), and after the initial 50 nM decrease due to complex formation between trypsin and SBTI, no further change in the concentration of free virgin SBTI was detectable for 70 h.

Mesotrypsin Degrades Human SPINK1—In contrast to SBTI, which forms stable complexes with trypsin, SPINK1 is a so-called “temporary inhibitor,” because trypsin-SPINK1 complexes irreversibly dissociate over time (22). First, reversible digestion of the Lys$^{41}$-Ile$^{42}$ reactive site peptide bond occurs, which is followed by the irreversibly inactivating cleavage of
the Arg$^{67}$–Gln$^{68}$ bond (23–25). In addition, peptide bonds Arg$^{28}$–Glu$^{29}$, Arg$^{65}$–Lys$^{66}$, and Lys$^{75}$–Ser$^{76}$ are also subject to tryptic attack (23–25). In our experiments, temporary inhibition followed a relatively rapid time course ($t_{1/2}$, 2–4 h) when human cationic or anionic trypsin was in some excess to SPINK1, however, at lower trypsin-to-SPINK1 ratios the reaction proceeded dramatically slower. Thus, when cationic or anionic trypsin (50 nM, final concentration) was reacted with SPINK1 (500 nM, final concentration) at a 1:10 ratio, no trypsin activity was detectable up to 90 h, and free SPINK1 levels did not change measurably either (not shown). In sharp contrast, incubation of 50 nM mesotrypsin with 500 nM SPINK1 (final concentrations) resulted in a much more rapid decrease of active SPINK1 concentration (Fig. 7A), which eventually resulted in complete elimination of SPINK1 activity. In control experiments, incubation of 500 nM bovine chymotrypsin or human elastase 2 with 500 nM SPINK1 (final concentrations) did not result in any appreciable SPINK1 degradation up to 20 h, indicating that mesotrypsin can be regarded as a specific degrading enzyme for SPINK1.

To visualize the degradation of SPINK1, an experiment was performed using high concentrations of inhibitor (15 μM) and 20-fold less mesotrypsin (0.75 μM). Under these conditions, the reaction proceeded faster, and more than 50% of SPINK1 activity was abolished in 40 min (not shown). Samples were analyzed under reducing conditions on SDS-Tricine polyacrylamide gels, where gradual disappearance of the SPINK1 band was apparent (Fig. 7B). Only a faint and transiently visible cleavage product was detected, indicating that SPINK1 suffered more extensive proteolysis beyond the cleavage of the reactive-site peptide bond. To confirm this notion, a sample of the digestion mixture taken at 40 min was applied to a ProSorb PVDF cartridge (Applied Biosystems) and subjected to Edman degradation. In addition to the native N terminus, two major
new N termini were identified, which indicated cleavages at peptide bonds Lys$^{41}$–Ile$^{42}$ (the reactive site) and Lys$^{75}$–Ser$^{76}$. Two additional N termini were found in smaller yield, which revealed cleavages at Arg$^{28}$–Glu$^{29}$ and Arg$^{67}$–Gln$^{68}$. Although we could not establish the exact order of cleavages from these data, the results clearly confirmed that one of the major targets of mesotrypsin was the reactive site of SPINK1, and the other tryptic cleavages corresponded to those previously described. Therefore, we conclude that mesotrypsin-mediated SPINK1 degradation followed the established mechanism of “temporary inhibition,” but at a markedly higher rate.

**Cathepsin B Is a Potential Pathological Activator of Mesotrypsinogen**—The observations described above directly suggest the idea that premature activation of mesotrypsinogen in the pancreas can degrade protective SPINK1 and eventually cause pancreatitis. Since we found that mesotrypsin cannot autoactivate, we were left with the dilemma of identifying a possible activating enzyme for mesotrypsinogen in the pancreas. In this regard, the two major trypsin isoforms appeared to be good candidates. However, when mesotrypsinogen was activated with cationic trypsin, anionic trypsin, or enterokinase under physiologically optimal conditions (37 °C, pH 8.0, 1 mM Ca$^{2+}$), human trypsins generated less than 20% of the potentially maximal mesotrypsin activity in 2 h (Fig. 8A). Gel analysis indicated that the mesotrypsinogen band was slowly converted to trypsin, and after 2 h approximately half of the mesotrypsinogen remained unactivated. In addition, a considerable fraction of mesotrypsinogen was degraded, despite the presence of 1 mM Ca$^{2+}$ (not shown). In the absence of Ca$^{2+}$, mesotrypsinogen was highly susceptible to degradation by cationic or anionic trypsin, and half-lives of 2.3 min and 0.5 min were measured, respectively. The $t_{1/2}$ value of degradation by cationic trypsin was almost identical to the previously determined half-life of anionic K23Q-trypsinogen in the presence of cationic trypsin (2.25 min, see Fig. 3 in Ref. 13). Thus, mesotrypsinogen seems to be similar in its proteolytic stability to anionic trypsinogen, while cationic trypsinogen is at least 20-fold more stable (cf. Fig. 3 in Ref. 13). In conclusion, the results indicate that cationic and anionic trypsin are more likely to play a role in mesotrypsinogen degradation than activation.
The lysosomal cysteine protease cathepsin B was shown to play a critical role in various experimental pancreatitis models as the intracellular catalyst of pathological trypsinogen activation (Refs. 20, 27, and references therein). Cathepsin B is also found in the secretory pathway of the human pancreas, where it is co-localized with trypsinogen (27). In vitro, cathepsin B is a potent activator of cationic trypsinogens, with a pH optimum of 4.0 (16, 27, 28). When activation of the three human trypsinogen isoforms was compared at pH 4.0, an interesting picture emerged (Fig. 8B). Rapid activation of mesotrypsinogen was apparent, which was followed by slow degradation. Compared with cationic and anionic trypsinogen, the initial rate of mesotrypsinogen activation was ~2-fold and 6-fold higher, respectively. Therefore, under certain conditions, cathepsin B can rather selectively activate mesotrypsinogen, and potentially initiate the mesotrypsin-mediated degradation of SPINK1.

DISCUSSION

The experiments presented in this study provide three important observations: (1) Mesotrypsin can rapidly hydrolyze the reactive-site peptide bond of Kunitz-type trypsin inhibitors and degrade Kazal-type temporary inhibitors (2). This unique activity is caused by the evolutionary selection of Arg198, which also rendered mesotrypsin resistant to protein inhibitors and limited its capacity to cleave protein substrates (3). Cathepsin B can preferentially activate mesotrypsinogen of the three human trypsinogen isoforms, under certain conditions. These observations clearly define a physiological purpose for mesotrypsin and also suggest a potential pathological role, in which prematurely activated mesotrypsin can degrade protective SPINK1 and cause pancreatitis.

The results strongly argue that human mesotrypsin plays a unique and highly specialized role in the degradation of trypsin inhibitors. Obviously, such a function would be advantageous in the digestion of foods rich in naturally occurring trypsin inhibitors, and would provide a rationale for the evolution of this trypsin isoform. Such a specialized function can also explain the relatively low levels of mesotrypsin secretion, as opposed to the largely nonspecific digestive enzymes. Conceivably, mesotrypsin exerts its effect in concert with other digestive proteases, which might attack the partially digested protease inhibitors. A known example is carboxypeptidase B, which can cleave off the newly generated C-terminal Lys or Arg residues after hydrolysis of the reactive-site peptide bonds and thus completely inactivate trypsin inhibitors (see also discussion in Ref. 19). We hypothesized that mesotrypsin might be also responsible for the elimination of endogenous SPINK1 secreted to the duodenum, however, model experiments indicated that at physiological concentrations SPINK1 was bound by cationic or anionic trypsin and the presence of mesotrypsin did not facilitate the irreversible dissociation of SPINK1 (data not shown). Thus, elimination of unwanted SPINK1 seems to occur through the "temporary inhibition" mechanism by major trypsin isoforms. From the experimental data it appears that mesotrypsin-mediated degradation of inhibitors becomes significant at high inhibitor concentrations, while in the absence of inhibitors mesotrypsin is probably degraded by the true digestive trypsin.

Undoubtedly, the most exciting implication of the observations presented here is that inappropriate activation of human mesotrypsinogen in the pancreas might degrade protective SPINK1 levels and cause pancreatitis. The role of SPINK1 in protecting against pancreatitis is clearly supported by the association of SPINK1 mutations with certain forms of chronic pancreatitis (e.g. Refs. 29–31). Should mesotrypsinogen get converted to mesotrypsin prematurely in the pancreas, the ensuing degradation of SPINK1 might represent an immediate risk factor for the development of pancreatitis. Such a model of disease onset would require a relatively specific activator for mesotrypsinogen in the pancreatic acinar cells. Remarkably, in our in vitro experiments cathepsin B robustly activated mesotrypsinogen, with a measurable preference over cationic or anionic trypsinogen. Thus, the biochemical basis clearly exists for a putative mesotrypsin-induced pancreatitis model, and future research will confirm or rule out the medical significance of such a mechanism. In this regard, identification of pancreatitis-associated mutations that stabilize mesotrypsin or loss-of-function mutations that protect against pancreatitis could provide particularly strong evidence.

Is mesotrypsin unique to humans? Rinderknecht et al. (2) reported that no inhibitor-resistant trypsin activity was found in pancreatic extracts from dog, cow, pig, rat, mouse, or hamster or in pancreatic juice from dog or hamster. Nonetheless, examination of the numerous rat trypsinogen genes found in databanks revealed that trypsinogen V contains a bulky tyrosine residue at position 198. While there is no evidence that functional protein is expressed from the rat trypsinogen V mRNA, this protein would be expected to exhibit mesotrypsin-like inhibitor-resistance and inhibitor-degrading properties. Interestingly, the corresponding mouse gene is probably a pseudogene with a 9 amino acid deletion in the N-terminal half of trypsinogen. No other sequenced trypsinogen gene carries the mesotrypsin signature mutation. Trypsin-1 from Dermasterias imbricata (20, 21) is clearly a mesotrypsin-like enzyme, however, the protein or DNA sequence has not been determined for this trypsin yet. With the ongoing sequencing of several vertebrate genomes, the discovery of new mesotrypsin orthologs can be expected in the near future. Finally, evolutionary mutations in trypsin at positions other than 198 can also result in mesotrypsin-like properties. In this regard, rat trypsinogen IV was described as partially inhibitor resistant, presumably due to the presence of a negatively charged aspartic acid, which replaced the conserved neutral glutamine residue found at position 197 (mesotrypsin numbering) in other mammalian trypsinogens (32).

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REFERENCES

Chymotrypsin C (caldecrin) promotes degradation of human cationic trypsin: Identity with Rinderknecht’s enzyme Y

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Digestive trypsins undergo proteolytic breakdown during their transit in the human alimentary tract, which has been assumed to occur through trypsin-mediated cleavages, termed autolysis. Autolysis was also postulated to play a protective role against pancreatitis by eliminating prematurely activated intrapancreatic trypsin. However, autolysis of human cationic trypsin is very slow in vitro, which is inconsistent with the documented intestinal trypsin degradation or a putative protective role. Here we report that degradation of human cationic trypsin is triggered by trypsinogen C, which selectively cleaves the Leu81-Glu82 peptide bond within the Ca2⁺ binding loop. Further degradation and inactivation of cationic trypsin is then achieved through trypsic cleavage of the Arg122-Val123 peptide bond. Consequently, mutation of either Leu81 or Arg122 blocks chymotrypsin C-mediated trypsin degradation. Calcium affords protection against chymotrypsin C-mediated cleavage, with complete stabilization observed at 1 mM concentration. Chymotrypsin C is highly specific in promoting trypsin degradation, because chymotrypsin B1, chymotrypsin B2, elastase 2A, elastase 3A, or elastase 3B are ineffective. Chymotrypsin C also rapidly degrades all three human trypsinogen isoforms and appears identical to enzyme Y, the enigmatic trypsinogen-degrading activity described by Heinrich Rinderknecht in 1988. Taken together with previous observations, the results identify chymotrypsin C as a key regulator of activation and degradation of cationic trypsin. Thus, in the high Ca²⁺ environment of the duodenum, chymotrypsin C facilitates trypsinogen activation, whereas in the lower intestines, chymotrypsin C promotes trypsin degradation as a function of decreasing luminal Ca²⁺ concentrations.

Chromotrypsin C (caldecrin) promotes degradation of human cationic trypsin. Incubation of human cationic trypsin with chymotrypsin C at pH 8.0 and at 37°C in the presence of 25 μM Ca²⁺ resulted in rapid loss of trypsin activity, with a t₁/₂ of ~15 min (Fig. L4). Under these conditions, autocatalytic degradation (autolysis) of cationic trypsin was negligible. Degradation of cationic trypsin by further trypsic sites (10). Autolysis was also proposed to play an essential role in physiological trypsin degradation in the lower intestines. A number of studies in humans have demonstrated that trypsin becomes inactivated during its intestinal transit, and in the terminal ileum only ~20% of the duodenal trypsin activity is detectable (12–14). On the basis of in vitro experiments, a theory was put forth that digestive enzymes are generally resistant to each other and undergo degradation through autolysis only (10, 15). However, human cationic trypsin was shown to be highly resistant to autolysis, and appreciable autodegradation was observed only with extended incubation times in the complete absence of Ca²⁺ and salts (16–18). Furthermore, we demonstrated that trypsic cleavage of the Arg₁²²-Val₁²³ peptide bond in human cationic trypsinogen (ogen) does not result in rapid degradation. Instead, due to trypsin-mediated resynthesis of the peptide bond, a dynamic equilibrium is reached between the single-chain (intact) and double-chain (cleaved) forms, which are functionally equivalent (19). Taken together, the in vitro studies indicate that autolysis alone cannot be responsible for the inactivation and degradation of human cationic trypsin and suggest that other pancreatic enzymes might be important in this process.

Recently, we serendipitously discovered that a minor human chromotrypsin, chromotrypsin C, regulates autoactivation of human cationic trypsinogen by limited proteolysis of the trypsinogen activation peptide (20). These experiments focused our attention onto this less-known chromotrypsin, and we characterized its interaction with human cationic trypsin in more detail. Surprisingly, we found that at low Ca²⁺ concentrations, chromotrypsin C cleaves a peptide bond with high selectivity within the Ca²⁺ binding loop of cationic trypsin, which results in rapid degradation and loss of trypsin activity. The observations suggest that chromotrypsin C may be the long-elusive digestive enzyme responsible for trypsin degradation in the gut and may serve as a protective protease in the pancreas to curtail premature trypsin activation. In this study, compelling evidence is presented in support of this contention.

Results

Chromotrypsin C Promotes Degradation of Human Cationic Trypsin. Incubation of human cationic trypsin with chromotrypsin C at pH 8.0 and at 37°C in the presence of 25 μM Ca²⁺ resulted in rapid loss of trypsin activity, with a t₁/₂ of ~15 min (Fig. L4). Under these conditions, autocatalytic degradation (autolysis) of cationic trypsin was negligible. Degradation of cationic trypsin by
chymotrypsin C proved to be highly specific, because chymotrypsin B1, chymotrypsin B2, elastase 2A, elastase 3A, or elastase 3B had no significant degrading activity at pH 8.0 (Fig. 1A) or at pH 6.0 (data not shown).

SDS/PAGE analysis of the spontaneous autolysis reaction of cationic trypsin showed no appreciable degradation over the 1 h time course studied (Fig. 1B). As described in ref. 19, human cationic trypsin consists of an equilibrium mixture of single-chain and double-chain forms cleaved at the Arg122-Val123 peptide bond, and both forms remained stable under the experimental conditions. In stark contrast, when chymotrypsin C was included in the reaction, SDS/PAGE showed the time-dependent disappearance of both the single-chain and double-chain trypsin bands and the appearance of degradation fragments, which were eventually further degraded to peptides too small to resolve on the 15% gels used (Fig. 1C). Surprisingly, when a catalytically inactive Ser200→Ala (S200A) cationic trypsin mutant was digested with chymotrypsin C, the overall rate of trypsin degradation was markedly slower, indicating that trypsin activity is required for efficient chymotrypsin C-mediated cationic trypsin degradation (Fig. 1D).

N-terminal sequencing of the visible bands in Fig. 1C revealed that the primary chymotrypsin C cleavage site was the Leu81-Glu82 peptide bond within the Ca2+ binding loop of cationic trypsin (Figs. 1E and 2). The C-terminal chymotryptic fragment (Glu82-Ser247) underwent rapid trypsinolytic cleavage at the Arg122-Val123 peptide bond, resulting in three peptides, corresponding to the Ile124-Leu81; Glu82-Arg122 and Val123-Ser247 segments (see Figs. 1E and 2). The same three peptides were generated when chymotrypsin C cleaved the Leu81-Glu82 peptide bond in the double-chain cationic trypsin, already cleaved after Arg122. Finally, a peptide generated in low yields by chymotryptic cleavage of the Leu81-Asn82 peptide bond was also identified among the lower molecular weight bands (Fig. 1C).

Ca2+ Protects Cationic Trypsin Against Chymotrypsin C-Mediated Degradation. Because the primary chymotrypsin C cleavage site is located within the calcium binding loop of cationic trypsin (see Fig. 2), we speculated that Ca2+ might be protective against chymotryptic cleavage and subsequent trypsin degradation. Indeed, increasing the Ca2+ concentration from 25 μM to 1 mM progressively inhibited the degradation of cationic trypsin by chymotrypsin C, with essentially complete protection observed at 1 mM Ca2+ (Fig. 3A). The half-maximal protective Ca2+ concentration was 40 μM, which probably corresponds to the Kd of Ca2+ binding to cationic trypsin. As shown in supporting information (SI) Fig. 7, inhibition of trypsin degradation by Ca2+ was also quantified at pH 6.0, and a half-maximal protective concentration of 90 μM was obtained. To demonstrate that the protective effect of Ca2+ is exerted through binding to the Ca2+ binding loop in trypsin, we mutated Glu82 to Ala (E82A). Glu82 is one of three carboxylate side chains that bind the Ca2+ ion
In 25 μM Ca\(^{2+}\), both the E82A mutant (Fig. 3B) and the corresponding wild-type (Fig. 3A) cationic trypsins were degraded by chymotrypsin C with similar kinetics, indicating that Glu\(^{82}\) is not essential for efficient chymotrypsin C cleavage. Remarkably, however, degradation of E82A-trypsin was insensitive to Ca\(^{2+}\) up to 1 mM concentrations, confirming that an intact Ca\(^{2+}\) binding site in trypsin is required for the protective effect of Ca\(^{2+}\) against chymotrypsin C.

Both Chymotryptic Cleavage After Leu\(^{81}\) and Autolytic Cleavage After Arg\(^{122}\) Are Required for Degradation of Cationic Trypsin. To investigate the relative significance of the chymotryptic cleavage after Leu\(^{81}\) and the autolytic cleavage after Arg\(^{122}\), both sites were mutated to Ala individually, and the L81A and R122A mutant cationic trypsins were digested with chymotrypsin C. Strikingly, both mutations afforded essentially complete protection against chymotrypsin C-mediated degradation (Fig. 4). SDS/PAGE analysis confirmed that mutant L81A remained intact in the presence of chymotrypsin C, with both single-chain and double-chain trypsin species unaffected (Fig. 4B). The R122A mutant was slowly cleaved by chymotrypsin C at a rate that was similar to the chymotryptic cleavage of the S200A-trypsin in Fig. 1D (Fig. 4C). However, in the absence of autolytic cleavage at the Arg\(^{122}\)-Val\(^{123}\) peptide bond, this slow chymotryptic cleavage was insufficient to produce appreciable degradation. Although not shown, mutation R122H protected against chymotrypsin C-mediated trypsin degradation as well as mutation R122A. Taken together, the results indicate that chymotryptic cleavage of the Leu\(^{81}\)-Glu\(^{82}\) and Arg\(^{122}\)-Val\(^{123}\) peptide bonds are both essential for rapid trypsin degradation.

Chymotrypsin C Is Identical to Enzyme Y, the Trypsinogen Degrading Activity from Human Pancreatic Juice. On the basis of its ability to degrade human cationic trypsin with high specificity and in a Ca\(^{2+}\)-dependent manner, we speculated that chymotrypsin C might be identical to enzyme Y, the trypsinogen-degrading enzymatic activity isolated from human pancreatic juice by Heinrich Rinderknecht in 1988 (see Discussion for more on enzyme Y) (8). Consistent with this assumption, chymotrypsin C was capable of degrading not only cationic trypsin but also anionic trypsin and mesotrypsin (Fig. 5A), as well as the trypsin precursors cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen (Fig. 5B). Interestingly, trypsinogen degradation proceeded significantly faster (t\(_{1/2}\) ~ 2–5 min) than trypsin degradation. Millimolar Ca\(^{2+}\) concentrations inhibited trypsinogen degradation as well as trypsin degradation (data not shown).

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**Fig. 2.** Structural determinants of chymotrypsin C-mediated trypsin degradation. (A) Ribbon diagram of human cationic trypsin [Protein Data Bank ID code 1TRN; chain B of the crystallographic dimer shown here (21)] with the Leu\(^{81}\) (red) and Arg\(^{122}\) (blue) side chains indicated. The catalytic triad consisting of His\(^{63}\) (His\(^{57}\) in the conventional chymotrypsin numbering), Asp\(^{107}\) (chymotrypsin no. Asp\(^{102}\)) and Ser\(^{200}\) (chymotrypsin no. Ser\(^{195}\)) are shown in green. Note that Asp\(^{107}\) is located on the yellow peptide segment, which is released upon cleavage of the Leu\(^{81}\)-Glu\(^{82}\) and Arg\(^{122}\)-Val\(^{123}\) peptide bonds. See text for details. The image was rendered using DeepView/Swiss-PdbViewer version 3.7. (B) Primary structure of human cationic trypsin. Individual amino acids are represented by circles. The catalytic triad is highlighted in green, Leu\(^{81}\) is in red, and Arg\(^{122}\) is in blue. The five disulfide bridges and the interactions between the calcium ion and amino acids within the calcium binding loop are indicated. Note that both the Leu\(^{81}\)-Glu\(^{82}\) and the Arg\(^{122}\)-Val\(^{123}\) peptide bonds are located in a long peptide segment not stabilized by disulfide bonds. The yellow section corresponds to the yellow peptide in A.
SI Fig. 8 demonstrates the degradation banding pattern of human trypsinogens on gels and the chymotrypsin C cleavage sites deduced from N-terminal sequencing of the visible bands. Although the Leu81-Glu82 peptide bond was readily cleaved in all three trypsinogens, additional isoform-specific chymotryptic cleavage sites were also observed.

Discussion
The present study identifies chymotrypsin C as the pancreatic digestive enzyme that can promote degradation and inactivation of human cationic trypsin. These results resolve the contradiction between the in vivo documented intestinal trypsin degradation (12–14) and the in vitro observed resistance of human cationic trypsin against autolysis (16–18) and strongly suggest that chymotrypsin C is responsible for the elimination of trypsin activity in the lower small intestines. Furthermore, the observations clearly negate the notion that degradation of digestive serine proteases is always autocatalytic in nature (15) and provide a clear example for heterolytic degradation. The specificity of chymotrypsin C in mediating trypsin degradation is striking. All other chymotrypsins and elastases tested were completely ineffective in this respect. Trypsin degradation is initiated by selective cleavage of the Leu81-Glu82 peptide bond within the Ca\(^{2+}\) binding loop, followed by trypsin-mediated autolytic cleavage of the Arg122-Val123 peptide bond. Millimolar Ca\(^{2+}\) concentrations inhibit chymotrypsin C-mediated cleavage after Leu81 by stabilizing the Ca\(^{2+}\) binding loop and thus protect against degradation.

Autolytic degradation of cationic trypsin was postulated to play a protective role in the pancreas against inappropriate trypsin activation (9–10). This assumption was based on the observation that human hereditary pancreatitis is associated with the R122H cationic trypsin mutation, which destroys the Arg122 autolytic site (9). However, the unusually high resistance of human cationic trypsin to autolysis in vitro has called into question the presumed protective role of trypsin autolysis and the proposed pathomechanism of the R122H mutation. Hereditary pancreatitis follows an autosomal dominant inheritance pattern with incomplete penetrance and variable disease expression, and it is typically characterized by early onset episodes of acute pancreatitis with frequent progression to chronic pancreatitis and an increased risk for pancreatic cancer (9, 22). Besides mutation R122H, to date >20 other cationic trypsinoen variants have been identified in pancreatitis patients, but mutation R122H is responsible for the large majority of cases (~70%) (22). The results presented here support a putative defense mechanism in which chymotrypsin C-mediated trypsin degradation mitigates unwanted intrapancreatic trypsin activity. The conditions at the site of intraacinar trypsinogen activation (pH...
Incubated with 300 nM chymotrypsin C in 0.1 M Tris HCl (pH 8.0) and 25 μM CaCl$_2$ (final concentrations). At the indicated times, residual trypsin activity was measured as described in Fig. 1A. (B) Human cationic trypsinogen, anionic trypsinogen, and mesotrypsinogen (2 μM) were incubated with 300 nM chymotrypsin C in 0.1 M Tris HCl (pH 8.0) and 25 μM CaCl$_2$ (final concentrations). At the indicated times, 10-μl aliquots were withdrawn, CaCl$_2$ was added to 10 mM final concentration and activable trypsinogen content was determined by incubating with 120 ng/ml human enteropeptidase for 15 min at room temperature and measuring trypsin activity. Activable trypsinogen was expressed as a percentage of the initial activable trypsinogen content.

$\approx$6.0, Ca$^{2+}$ concentration $\approx$40 μM are conducive for such a mechanism to be operational (23). Impairment of this mechanism by the R122H mutation may contribute to the pathogenesis of hereditary pancreatitis.

Chymotrypsin C was first isolated from pig pancreas and was shown to exhibit a preference for leucyl peptide bonds, which distinguished it from chymotrypsin A or chymotrypsin B (24, 25). Chymotrypsin C seems to be the same protein as caldecrin, a calcium-decreasing protein isolated from porcine and rat pancreas and later cloned from rat and human pancreas (26, 27). Recently, we reported that chymotrypsin C regulates autodegradation of cationic trypsinogen through limited proteolysis of its activation peptide at the Phe$^{18}$-Asp$^{19}$ peptide bond (20). The N-terminally truncated cationic trypsinogen autoactivates 3-fold faster than its intact form. Taken together with these previous observations, the present results identify chymotrypsin C as a key regulator of trypsinogen activation and trypsin degradation in humans (Fig. 6). Thus, in the activation pathway, chymotrypsin C cleaves the Phe$^{18}$-Asp$^{19}$ bond in the trypsinogen activation peptide, which in turn facilitates trypsic cleavage of the Lys$^{23}$-Ile$^{24}$-activating peptide bond, resulting in increased autoactivation. In the degradation pathway, chymotrypsin C cleaves the Leu$^{81}$-Glu$^{82}$ peptide bond, and subsequent tryptic cleavage of the Arg$^{122}$-Val$^{123}$ peptide bond destines trypsin for degradation and inactivation. Clearly, chymotrypsin C accelerates already existing autoregulatory functions of cationic trypsin (i.e., autoactivation and autolysis). The balance between activation and degradation is regulated by the prevailing Ca$^{2+}$ concentrations. In the presence of high Ca$^{2+}$ concentrations (>1 mM) characteristic of the upper small intestines, the degradation pathway is blocked and trypsinogen activation is predominant. As the Ca$^{2+}$ concentration falls below millimolar levels in the lower intestines, trypsin degradation prevails. Although intestinal Ca$^{2+}$ absorption has been studied extensively, reliable data on the ionized Ca$^{2+}$ concentrations along the small intestines is lacking (28). It is noteworthy that ionized Ca$^{2+}$ concentrations in the gut are largely determined by luminal pH and insoluble complex formation, which becomes more significant at the more alkaline pH of the lower intestines, where trypsin degradation was shown to occur (29).

An unexpected bonus from these studies was the identification of chymotrypsin C as enzyme Y, a so-far obscure trypsinogen-degrading activity from human pancreatic juice. Enzyme Y was described by the late Heinrich Rinderknecht, the renowned pancreatologist, in 1988 (8). Rinderknecht was also the first to identify the inhibitor-resistant human mesotrypsin in 1984 (4). He initially alleged that mesotrypsin can degrade trypsinogens, but later he withdrew this conclusion and attributed the trypsinogen-degrading activity to an unidentified serine protease, which he named enzyme Y (7, 8). This enigmatic activity developed when human cationic trypsinogen, purified by native gel electrophoresis, was incubated at 37°C. This activity degraded all human trypsinogen isoforms, and millimolar Ca$^{2+}$ concentrations blocked degradation. Enzyme Y became very popular among pancreas researchers and has been highlighted in almost every significant article discussing defense mechanisms against intrapancreatic trypsin activity. Rinderknecht himself believed that enzyme Y was probably a degradation fragment of cationic trypsin (8), perhaps complexed with pancreatic secretory trypsin inhibitor (30), although he acknowledged the possibility of contamination with an unknown protease (8). The observations presented in this study seem fully consistent with the conclusion that chymotrypsin C is in fact enzyme Y, which contaminated the
cationic trypsinogen preparations in Rinderknecht’s experiments. Thus, chymotrypsin C is a serine protease, which can rapidly degrade all three human trypsinogens in a Ca²⁺-dependent manner. No other pancreatic protease tested in this study had trypsin- or trypsinogen-degrading activity. It is unlikely that chymotrypsin C-mediated trypsinogen degradation has any physiological role in digestion, as high Ca²⁺ concentrations in the duodenum would inhibit trypsinogen degradation and favor trypsinogen activation (see Fig. 6). However, Rinderknecht’s theory that enzyme Y protects the pancreas by decreasing trypsinogen concentrations during inappropriate zymogen activation might be valid, and our present study should stimulate further research in this direction.

Methods

Nomenclature. Amino acid residues in the trypsinogen sequence are numbered according to their position in the native preproenzyme, starting with Met¹. The first amino acid of the mature cationic trypsinogen is Ala¹⁶.

Construction of Expression Plasmids and Expression and Purification of Digestive Proenzymes. See SI Text for more information about expression plasmids and purification of digestive proenzymes.


Activation of Digestive Proenzymes. Chymotrypsinogens and pro-elastases (1–5 μM concentration) were activated in 0.1 M Tris-HCl (pH 8.0) and 10 mM CaCl₂ for 20 min at 37°C, with 100 nM cationic trypsin (final concentration), with the exception of pro-elastase 2A, which was activated with 100 nM anionic trypsin. Trypsinogens (2 μM concentration) were activated to trypsin with human enteropeptidase (28 ng/ml concentration) for 30 min at 37°C, in 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl₂.

Enzymatic Assays. Trypsin activity was measured with the synthetic chromogenic substrate, N-CBZ-Gly-Pro-Arg-p-nitroanilide (0.14 mM final concentration) in 200 μl final volume. One-minute time courses of p-nitroaniline release were followed at 405 nm in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂, at room temperature. To verify the activity of the other recombinant pancreatic enzymes, chymotrypsins were assayed with Suc-Ala-Ala-Ala-Pro-Phe-p-nitroanilide (0.15 mM concentration), elastase 2A activity was measured with Glu-Ala-Ala-Pro-Leu-p-nitroanilide (1 mM concentration), and elastase 3A and 3B were assayed with DQ elastin (Invirotegen, Carlsbad, CA).

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