Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene

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Hereditary pancreatitis (HP) is a rare, early-onset genetic disorder characterized by epigastric pain and often more serious complications. We now report that an Arg–His substitution at residue 117 of the cationic trypsinogen gene is associated with the HP phenotype. This mutation was observed in all HP affected individuals and obligate carriers from five kindreds, but not in individuals who married into the families nor in 140 unrelated individuals. X-ray crystal structure analysis, molecular modelling, and protein digest data indicate that the Arg 117 residue is a trypsin-sensitive site. Cleavage at this site is probably part of a fail-safe mechanism by which trypsin, which is activated within the pancreas, may be inactivated; loss of this cleavage site would permit autodigestion resulting in pancreatitis.

Hereditary pancreatitis (HP) is an autosomal dominant disorder with 80% penetrance and variable expressivity[1–4]. Nearly 100 kindreds have been reported worldwide since the genetic nature of this disorder was recognized by Comfort and Steinberg in 1952 (refs 2,5). The majority of the families are of white European ancestry, but affected kindreds have been reported in Japan, India, and among other ethnic groups[1]. HP is characterized by recurrent bouts of severe epigastric pain with onset usually before ten years of age. The clinical, laboratory and pathologic features of HP are indistinguishable from attacks of pancreatitis from other causes. In addition to recurrent acute attacks, many HP patients progress to complicated chronic pancreatitis characterized by pancreatic calcifications, pseudocysts, chronic abdominal pain, pancreatic exocrine failure, diabetes mellitus and/or pancreatic cancer[1,2]. Despite years of research, no unique morphologic or biochemical markers have been identified for HP, and the pathophysiologic mechanisms that lead to intermittent attacks of acute pancreatitis remain obscure. Therefore, no rational or effective preventative strategies have been developed, and treatment consists solely of supportive care.

Because of the absence of biochemical markers specific for HP, attention has focused on identifying the HP disease gene. The availability of a high-density map of the human genome, based on polymorphic simple tandem repeat (STR) markers, and familial linkage analysis made it possible to identify an HP gene locus on chromosome 7q35 (refs 3,4). We therefore sought to identify and sequence the HP gene to determine the site of the disease-causing mutation(s) in an effort to understand the molecular mechanism leading to HP. Several previously mapped

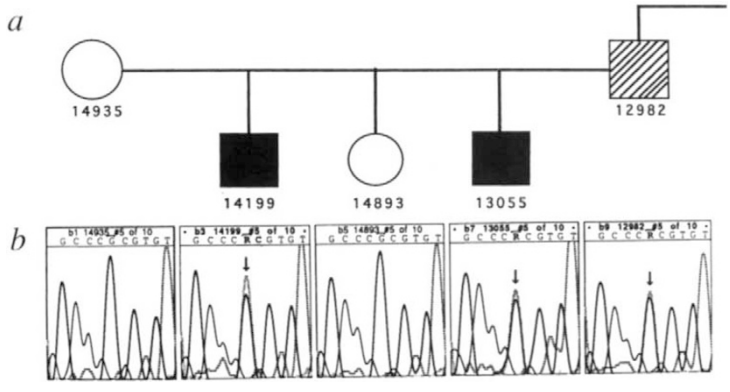

Fig. 1 a, Partial pedigree of one of the HP kindreds. The integers below the boxes are laboratory accession numbers which correspond with the electropherograms in panel *b*. *b*, DNA sequencing electropherograms, aligned with the pedigree in the top panel, showing the DNA sequence of the human cationic trypsinogen gene in the region of the HP mutation. Specimens from HP affected family members (14199 & 13055) and an obligate carrier family member (12982) demonstrate heterozygosity at the fifth nucleotide in the frame (see arrows in the 2nd, 4th, and 5th panels). The sequencing signal is almost exactly 50% G and 50% A for each of these three specimens. In contrast, the signal observed for the two unaffected family members (14935 & 14893) is 100% G which is in keeping with the published sequence for cationic trypsinogen. All DNA amplifications and sequencing were performed as described[11,12].

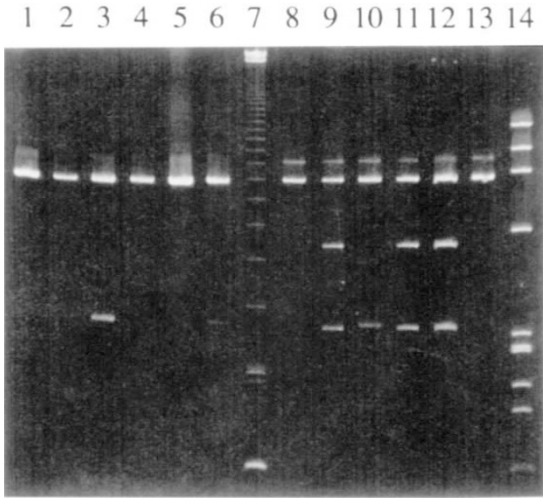

Fig. 2 Ethidium bromide-stained agarose gel of PCR-amplified exon 3 products from the human cationic trypsinogen gene using the two external primers[12]. Lanes 1–5, Undigested exon 3 amplified DNAs (911 bp) from the same HP family members depicted in Fig. 1 (all specimens are in the same order). Lane 6, Amplified exon 3 DNA from an unrelated control specimen. Lanes 7,14, contains DNA molecular weight markers. Lanes 8–13, The same amplified DNAs as in lanes 1–6 following digestion with *Afl*III which recognizes a novel site created by the HP G:A mutation. Note the appearance of the two lower molecular weight bands (565 and 346 bp, respectively) in lanes 9, 11, and 12 which correspond to the *Afl* III digestion products from the two HP affected individuals (14199 & 13055) and the obligate HP carrier individual (12982). Together these two bands account for the HP mutant allele, and the remaining high molecular weight band (911 bp) corresponds to the normal allele. There is a faint artifactual band that appears in some of the undigested and some of the digested samples that is generated during the PCR amplification process which can be seen migrating just above the smaller of the two *Afl*III digest products. This is not a cleavage product and demonstrates the necessity of using a nested approach for sequencing. All restriction enzyme digests were performed overnight according to the manufacturer's (New England Biolabs) instructions following concentration of the amplified DNA using Microcon-50 microconcentrators (Amicon). The agarose gel composition was 1% SeaKem agarose and 1% Nuseive agarose (FMC).

genes on chromosome 7q were considered candidates for the HP disease gene because they are known to be expressed in the exocrine pancreas and encode enzymes that could potentially activate digestive enzymes within the pancreas. The hypothesis that pancreatitis results from inappropriate activation of pancreatic proenzymes was first promulgated 100 years ago and subsequently demonstrated to be an experimental model for pancreatitis[6,7]. Although carboxypeptidase A1 (*CPA1*) was considered the primary candidate[4], this gene mapped centromeric to the HP locus defined by obligate recombinations in our HP linkage study[3,8–11] and was, therefore, excluded by our laboratory from further consideration. However, at least eight trypsinogen genes are located on chromosome 7q35 between markers *D7S495* and *D7S498* and within the V and D-C segments of the complex T-cell receptor β chain gene (*TCRβ*)[12]. Trypsinogen is an inactive proenzyme for trypsin, which becomes active when an eight amino acid amino-terminal peptide is removed. Although small amounts of trypsin are normally generated within the pancreas, this active trypsin is usually rapidly inactivated before pancreatic autodigestion occurs. Thus, the trypsinogen genes were considered primary candidates for the HP disease gene.

Trypsinogen genes exist with the TCRβ locus

The entire 685-kilobase (kb) TCRβ-trypsinogen locus has recently been sequenced as part of the largest human genome sequencing project completed so far[12]. Eight trypsinogen-like genes were sequenced and identified that map within the TCRβ locus[12]. Three were located at the 5' end of the locus and were determined by sequence analysis to be pseudogenes. Another group of five trypsinogen genes, including the cationic and anionic pancreatic trypsinogen genes[13–15], were found to be in a cluster located between the $V_8$4S1 and the $D_8$1 elements near the 3' end of the TCRβ locus. Based on comparisons with the pancreatic cDNAs in Genbank, one of the newly identified trypsinogen-like genes (T6 or TRYC, nomenclature according to ref. 12) may also be functional, but no corresponding cDNAs have been identified so far.

These five trypsinogen genes are highly homologous, each residing within a tandemly duplicated 10 kb seg-

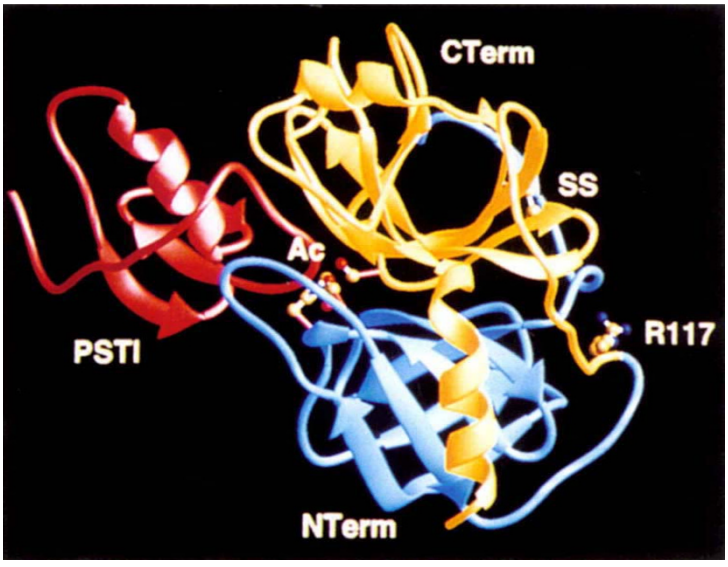

Fig. 3 Ribbon diagram for the trypsinogen-trypsinogen inhibitor complex. The N-terminal domain (N Term, blue) and C-terminal domain (C Term, yellow) of trypsinogen are shown relative to Arg 117 (R117, shown with the amino acid side chain). The side chains of the amino acids of the catalytic site (His 57, Asp 102, Ser 195) are seen on the opposite face of the trypsinogen molecule from Arg 117 and below trypsin inhibitor (PSTI, red). An Arg117His mutation would prevent trypsin-like hydrolysis of the chain connecting the two domains that are required for trypsin activity.

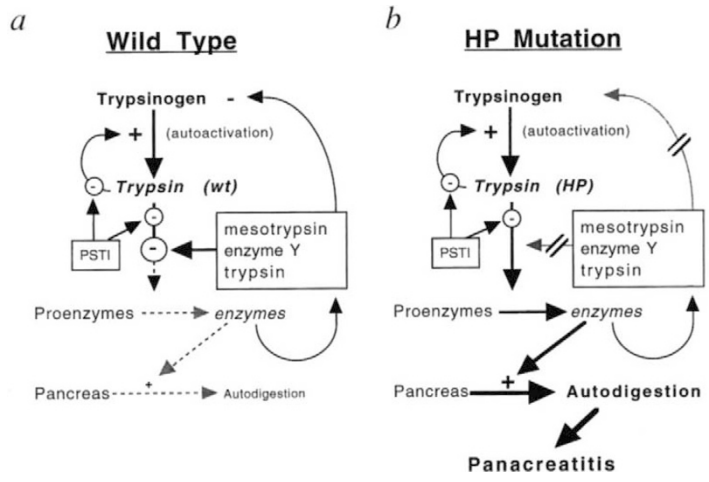

Fig. 4 Model of trypsin self-destruct mechanism to prevent pancreatic autodigestion. *a*, Autoactivation and enzymatic activation of trypsinogen generate trace amounts of active trypsin within pancreatic acinar cells. Active trypsin is inhibited by a limited supply of trypsin inhibitor (PSTI). If trypsin activity exceeds the inhibitory capacity of PSTI, then proenzymes, including mesotrypsin and enzyme Y are activated. The activation of these enzymes is postulated to be part of a feed-back mechanism for inactivating wild-type (wt) trypsinogen, trypsin and other zymogens. *b*, Model of mutant (HP) trypsin activation, in amounts that exceed the inhibitory capacity of PSTI, that results in unchecked activation of proenzymes. Since the Arg 117 cleavage site for mesotrypsin, enzyme Y and trypsin is replaced by His in the HP mutant trypsin, trypsin continues to activate trypsinogen and other zymogens unabated, leading to autodigestion of the pancreas and pancreatitis.

ment and each being composed of five exons. The extremely high degree of DNA sequence homology (>91%) present among this cluster of five trypsinogen genes demanded that highly specific sequence analysis strategies be developed for mutational screening. This was necessary to ensure that each sequencing run contained only the two alleles corresponding to a single gene, thereby permitting detection of heterozygotes, and not a dozen or more alleles from multiple related trypsinogen-like genes which would make detection of heterozygotes nearly impossible. The initial DNA sequencing effort used members of the S-family and focused on the trypsinogen genes that were known to be expressed, specifically cationic trypsinogen and anionic trypsinogen[13,14]. This strategy was accomplished by sequencing each of the five exons from the specifically cationic trypsinogen and anionic trypsinogen genes individually using a gene-specific, nested PCR strategy.

Mutational analysis of the trypsinogen genes

Mutational screening analyses for each of the exons from the cationic and anionic trypsinogen genes were performed for multiple affected and unaffected HP family members. A single G to A transition mutation was identified in the third exon of cationic trypsinogen (Fig. 1) from all of the HP affected individuals and the obligate carriers examined from the S-family. This mutation is predicted to result in an Arg(CGC) to His(CAC) substitution at amino acid residue 105 of trypsin (#117 in the more common chymotrypsin numbering system). Subsequently, a total of 42 family members including: 20 HP affected; 6 obligate carriers; and 16 unaffected family members from five different HP kindreds (four from the USA and one from Italy) were tested for the presence of this mutation using PCR amplification and cycle sequencing of the third exon of cationic trypsinogen. All affected and obligate carriers examined from each of these five kindreds displayed the same G:A transition in codon 117, but none of the obligate unaffected members (individuals who married into the family) carried the mutation. Thus, the Arg117His mutation represents a consistent mutation in all cases of HP examined so far. The finding of identical mutations in five separate kindreds raised the possibility that these families might be distantly related and that the mutation is centuries old. Subsequent haplotyping studies revealed that all four of the American families displayed the same high risk haplotype over a 4-centiMorgan region encompassing seven STR markers, confirming the likelihood that these kindreds share a common ancestor, although no link could be found through eight generations. The fifth family, from Naples, Italy, displayed a unique haplotype indicating that the same mutation had occurred on at least two occasions[17].

To rule out the minor possibility that the observed G:A mutation seen in the HP kindreds might be a present in the population-at-large as a natural polymorphism, we performed a population-based study. The G:A mutation, at Arg 117, creates a novel restriction enzyme recognition site for *Afl*III (New England Biolabs) which provided a facile means to screen for the presumptive HP mutation (Fig. 2). Using the cationic trypsinogen exon 3-specific nested primer pairs, 140 unrelated individuals chosen from our clinical database (no repeated surnames) were evaluated for the presence of the presumptive HP mutation. As with the obligate unaffected members of the HP kindreds, none of the 140 controls possessed the G:A mutation as assayed by the lack of *Afl*III digestion of the amplified exonic DNA. These data strongly suggest that the observed mutation is HP-specific.

Modelling the effects of the HP mutation

Our examination of the X-ray crystal structure of cationic trypsinogen-trypsin inhibitor complex (Brookhaven Protein Data Bank, and refs 18,19) revealed that the three-dimensional position of Arg117 was on the face of the trypsin molecule opposite the catalytic site and trypsin inhibitor binding site (Fig. 3). This suggested that an Arg117His mutation would have little effect on the tertiary structure of trypsin, alter the catalytic activity, or interfere with trypsin inhibitor binding (S. Swaminathan, unpublished results) However, it is also evident that Arg 117 is in a critical position in the peptide chain connecting the two globular domains of the trypsin molecule. As trypsin-like serine proteases hydrolyse peptide chains at arginine and lysine residues, the Arg 117 of the wild-type protein could be susceptible to hydrolysis by these enzymes. Indeed molecular modelling predicts that trypsinogen can adopt a tertiary structure formation

that would create a trypsin-like cleavage site at this residue[20], and *in vitro* experiments have demonstrated that Arg 117 is the primary site for proteolysis of trypsin[21,22]. Although cleavage between Arg 117 and Val 118 *in vitro* does not immediately inactivate trypsin, possibly because the two globular domains remain linked by a disulphide bond[21,22]. Disruption of the disulphide bond after the Arg 117 cleavage does, however, permanently inactivate trypsin[23]. Furthermore, in human pancreatic juice trypsin is rapidly hydrolysed suggesting that the Arg 117 cleavage may expose additional proteolytic sites, within what is termed the autolysis loop, to other active proteolytic enzymes[19,24–26]. The Arg117His mutation would render this site resistant to trypsin-like proteases preventing trypsin from being inactivated by this mechanism.

Although trace amounts of trypsin can be identified within the normal pancreas, a more general activation of digestive enzymes within the pancreas leads to autodigestion and acute pancreatitis[6,7]. The pancreas employs an array of mechanisms to prevent this problem[27]. All proteolytic enzymes, phospholipase A2 and colipase are synthesized as inactive proenzymes (zymogens). These proenzymes are sequestered from other subcellular components within membrane-bound zymogen granules. Activation of these digestive enzymes normally occurs outside the pancreas when intestinal enterokinase hydrolyses trypsinogen to form active trypsin. Trypsin subsequently catalyses its own conversion from trypsinogen to trypsin, and converts all other proenzymes to their active form within the intestine. As trace amounts of trypsin normally become activated within pancreatic acinar cells, two protective mechanisms are available to prevent the digestive enzyme activation cascade and pancreatic autodigestion (Fig. 4*a*). The first line of defense is pancreatic secretory trypsin inhibitor (PSTI), a 56-amino acid peptide that reversibly inhibits up to 20% of potentially available trypsin activity[27–30]. If trypsin activity overwhelms the inhibitory potential of PSTI then trypsin-like enzyme(s)[27] are activated that hydrolyse trypsin and other zymogens, thereby serving as a second or 'fail-safe' line of defense against initiating autodigestion and pancreatitis. Thus, excessive activation of trypsin would result in self-destruction of the zymogen granules' content.

Based on this model, individuals with the HP allele would be protected from pancreatic autodigestion and pancreatitis as long as the level of trypsin activity was less than the inhibitory capacity of PSTI. Once trypsin activity exceeds the inhibitory potential of PSTI, then unopposed trypsin activity would ensue because the final self-destruct mechanism for inappropriately activated trypsin would be blocked by the absence of the Arg 117 cleavage site (Fig. 4*b*).

Discussion

The process of inappropriate trypsin activation may be especially important in humans. Cationic trypsinogen, which represents two-thirds of trypsin activity in normal human pancreatic juice, differs from the cationic trypsinogen of other species by its propensity to autoactivate, especially below pH 6 (refs 31–33). As the rate of trypsinogen activation is greatly increased by trypsin, a trypsin-sensitive inactivation mechanism would appear to be critical. At least two trypsin-like proteases have been described, mesotrypsin and enzyme Y[23–27], that are found in the exocrine pancreas, are activated by trypsin, and in turn rapidly degrade trypsin and other zymogens to inert products in *in vitro* systems. Mesotrypsin represents a minor trypsin species (5%) that is completely resistant to PSTI neutralization. When activated by trypsin, mesotrypsin hydrolyses zymogens to inert products, even in the presence of PSTI. Enzyme Y appears to be a serine protease that differs from all other known pancreatic enzymes. When added to pancreatic juice with low PSTI content enzyme Y causes rapid inactivation of zymogens without significant activation of proenzymes or reduction of PSTI. In addition, trypsin itself will catalyse trypsin degradation[23–25]. Thus, at least three pancreatic enzymes have been identified that are activated by trypsin and that may serve as feedback inhibitors by digesting trypsin at arginine or lysine residues. Mutation of trypsin at Arg 117 would render trypsin resistant to hydrolysis at this site by any of these three enzymes.

Our findings and subsequent model offer a rational explanation for many of the clinical characteristics of HP. As the HP mutation is predicted to permit uncontrolled trypsin activity by nature of its inability to be cleaved, once the enzyme is activated, the phenotype should be present in heterozygotes. This is in agreement with the autosomal dominant pattern of inheritance observed for HP. Furthermore, this model would predict that attacks of acute pancreatitis would occur only occasionally in patients carrying the HP allele, such as when rates of intrapancreatic activation of trypsinogen overwhelm the protective effects of PSTI. Indeed, in HP patients, attacks of acute pancreatitis occur intermittently, often at times of unusual pancreatic stress such as after large meals or with excessive alcohol use[1–3]. The reason for incomplete penetrance of symptoms is under investigation.

Our data suggest that HP results from a genetic defect that disrupts a critical component of a self-protective mechanism that permits non-affected individuals to digest their food without the risk of pancreatitis from autodigestion. HP represents an important and serious disease in which determination of the pathophysiologic mechanism will be central to the development of strategies for prevention and treatment. The rapid progress made in this field since the mapping of HP, just weeks ago, was made possible by the availability of large-scale genomic sequence data. These findings demonstrate the utility of long range DNA sequencing projects[12] for aiding in the positional cloning of inherited disease gene alleles. Now that the disease gene is known, individuals who carry the gene can be identified before pancreatitis begins. Furthermore, the predicted mechanism for HP will aid in the design of strategies for preventing or controlling the development of the clinical manifestations of the disease. Finally, HP represents an excellent, relevant human model of acute and chronic pancreatitis that may provide insights into the pathophysiology of alcoholic, idiopathic and other types of pancreatitis in man.

Methods

Family ascertainment. Diagnostic criteria for the HP patients and unaffected relatives from the 'S-family'[16] and the HP kindreds in New York; North Carolina; Florida; and Naples, Italy were as described[3,16]. Patient recruitment and interview were

conducted by the Midwest Multicenter Pancreatic Study Group and members of the International Hereditary Pancreatitis Study Group (Lowenfels unpublished data).

Positional cloning. A nested PCR strategy was employed for each trypsinogen gene wherein exon-specific amplification was achieved. Therefore, each of the five exons, with intronic flanking sequences, for the cationic and anionic trypsinogen genes were amplified individually. Both the inner and outer sets of primers were designed such that the 3' base for each of the four primers used to amplify a given exon was positioned on a gene-specific intronic polymorphism in an attempt to minimize amplification of related trypsinogen genes from within the cluster. This provided for the direct amplification and sequencing of each exon from both the cationic and anionic trypsinogen genes, in their entirety, from genomic DNA. Comparison of the DNA sequences generated from the PCR amplifications of control specimens with the published sequence from these regions revealed 100% concordance, thus confirming the utility of the nested primer approach for gene-specific mutational screening.

Reaction conditions for all amplifications consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 µM of each of the 4 dNTPS (dATP, dCTP, dGTP, and dTTP). Cycling conditions for all PCR reactions consisted of an initial 3 min denaturation step at 95 °C, followed by 40 cycles at 94 °C for 10 s; 64 °C for 10 s; 72 °C for 2 min, with a final 10 min elongation step at 72 °C.

The primers used to support the nested PCR for exon 3 of the human cationic trypsinogen gene were: U306, 5'–GGTC-CTGGGTCTCATACCTT–3' (5' outer primer); L1197, 5'–GG-GTAGGAGGCTTCACACTT–3' (3' outer primer); U329, 5'–TGACCCACATCCCTCTGCTG–3' (5' inner/sequencing primer); L924, 5'–TCTCCATTTGTCCTGTCTCT–3' (3' inner/sequencing primer). DNA sequencing reactions were performed using cycle sequencing kits and dye-terminator chemistry according (Perkin Elmer) to the manufacturer's recommendations with modifications[33]. All DNA sequencing was performed on an 373A automated DNA sequencer equipped with a five colour wheel (Applied Biosystems). Sequence alignments were performed using DNA Sequencher™ (GeneCodes).

Molecular modelling. X-ray crystallographic analysis of the human trypsinogen protein structure was performed on a silicon graphics IRIS indigo R 4000 using 1 TGS for the trypsinogen pancreatic secretion trypsin inhibitor complex. Modelling of the trypsin-like cleavage at the Arg 117 site of cationic trypsinogen was performed using the Kinemage File# MMDB 3116 from the NCBI using MAGE software.

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