Hereditary Pancreatitis Caused by a Novel PRSS1 Mutation (Arg-122 → Cys) That Alters Autoactivation and Autodegradation of Cationic Trypsinogen*

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Hereditary pancreatitis has been found to be associated with germline mutations in the cationic trypsinogen (PRSS1) gene. Here we report a family with hereditary pancreatitis that carries a novel PRSS1 mutation (R122C). This mutation cannot be diagnosed with the conventional screening method using AflIII restriction enzyme digest. We therefore propose a new assay based on restriction enzyme digest with BstUI, a technique that permits detection of the novel R122C mutation in addition to the most common R122H mutation, and even in the presence of a recently reported neutral polymorphism that prevents its detection by the AflIII method. Recombinantly expressed R122C mutant human trypsinogen was found to undergo greatly reduced autoactivation and cathepsin B-induced activation, which is most likely caused by misfolding or disulfide mismatches of the mutant zymogen. The $K_m$ of R122C trypsin was found to be unchanged, but its $k_{cat}$ was reduced to 37% of the wild type. After correction for enterokinase activatable activity, and specifically in the absence of calcium, the R122C mutant was more resistant to autolysis than the wild type and autoactivated more rapidly at pH 8. Molecular modeling of the R122C mutant trypsin predicted an unimpaired active site but an altered stability of the calcium binding loop. This previously unknown trypsinogen mutation is associated with hereditary pancreatitis, requires a novel diagnostic screening method, and, for the first time, raises the question whether a gain or a loss of trypsin function participates in the onset of pancreatitis.

MATERIALS AND METHODS

Nomenclature—All data refer to the human cationic trypsinogen gene or protein (PRSS1 gene, GenBank™ accession no. U66081). To denote PRSS1 mutations, the chymotrypsinogen amino acid numbering system has been used in the past. Because genetic alterations in the PRSS1 sequence have been observed for which no corresponding amino acid exists in the chymotrypsinogen sequence, this practice has been abandoned and the actual human PRSS1 sequence is now used by convention (9). The previously named Asn-21 → Ile mutation is now referred to as Asn-29 → Ile, and the previous Arg-117 → His has become Arg-122 → His. In this report we only use the new nomenclature with single-letter abbreviations (e.g. N29I, R122H).

Kindred with Hereditary Pancreatitis—A 54-year-old patient was admitted for recurrent episodes of pancreatitis and changes consistent with chronic pancreatitis on endoscopic retrograde cholangio-pancre-
atography and computed tomography. His family originates from a region of Germany where hereditary pancreatitis is fairly common and over 20 extended kindreds have so far been identified. When his 24-year-old son was found to also suffer from recurrent episodes of pancreatitis and no other risk factors could be identified in both patients’ histories, his medical evaluation, including genetic testing, was subsequently performed at 60 °C. BstUI recognizes a single restriction site in the wild-type sequence of exon 3 that is destroyed by the R122H mutation.

Combinant Expression of Cys-122 Trypsin—Eracin was overexpressed in Escherichia coli BL21 (DE3) as described by Pål et al. (12, 13) and purified to homogeneity using a trypsin affinity column. Purified eracin was immobilized to Actigel ALD resin (Sterogene Bioseparations, Carlsbad, CA) as described previously (14). Plasmid pTrap was a genetic tool, which allows high level expression in E. coli strains expressing the terminal sequence of the cationic trypsinogen used in this study is Met-Ala-Pro-Phe-Asp-

Molecular Modeling—For modeling studies we used the program DeepView version 3.5.1 (17) was used, and the human trypsin crystal structure at 2.3 Å resolution was used as a template (Protein Data Bank entry 1trn) as published by Gabariaud and co-workers (18). The R122C mutation was introduced with the “mutation tool,” and the energetically most favorable rotamers were selected by direct visual comparison on a WinNT work station. The wild type and the mutant structures were energy-minimized to a total energy of −11,400 kJ/mol and −9200 kJ/mol, respectively, to which Arg-122 contributed −860 kJ/mol and Cys-122 12 kJ/mol.

RESULTS

The fact that our patient had developed chronic pancreatitis as an adult would not have suggested hereditary pancreatitis as a differential diagnosis, despite the fact that we could not identify any apparent risk factors for pancreatitis such as alcohol abuse or metabolic disorders. After it became apparent that his son also suffered from recurrent episodes of pancreatitis, we investigated the possibility of genetic alterations in the PRSS1 gene. When the most common hereditary pancreatitis-associated PRSS1 mutation (R122H) was not detected by standard A/JIII restriction enzyme digest, the entire coding sequence of the PRSS1 gene was sequenced. Both patients were found to be carriers of a previously unreported C to T transition mutation at position 133,282 of the published genomic sequence (GenBank accession no. U66061; Fig. 1). This mutation results in an arginine to cysteine amino acid substitution in position 122 (R122C) and therefore affects the same codon as the common R122H mutation. Among the first degree relatives of the patients, two more were found to be carriers of the same mutation (aged 25 and 45 years), but neither was aware of an episode of pancreatitis in the past (Fig. 1). In this particular kindred, this would indicate a disease penetrance of 50%, provided that neither of the two unaffected carriers develops pancreatitis later in life. The latter remains a distinct possibility because of the late symptom onset in both affected patients (at age 52 and 24 years, respectively).

The C to T transition eliminates a single BstUI site in the PCR fragment of exon 3, which permits rapid screening for the new R122C mutation by a simple BstUI restriction digest without the need of DNA sequencing.

To compare A/JIII and BstUI in their ability to recognize clinically relevant trypsinogen mutations, we used PCR to amplify exon 3 from DNA of healthy controls, from known heterozygous carriers of R122H mutation, and from our patients who carried the R122C mutation, and performed restriction digests with both enzymes (Fig. 2). As predicted by the sequence, the A/JIII digest detected a restriction site in the R122H carrier, but failed to detect the R122C mutation. On the other hand, a complete BstUI digest was found only in the healthy control cDNA, whereas, in carriers of the R122H as well as the R122C mutation, the restriction site on one allele was destroyed, reflected in the presence of an undigested PCR product in addition to the two restriction fragments of the wild-type allele. As indicated in Fig. 2, the complete BstUI digest would also detect the mutation in the presence of a recently reported neutral polymorphism in exon 3 that is not disease-relevant but makes the common R122H mutation inaccessible for screening with A/JIII (19, 20).

Characteristics of Recombinant Cys-122 Trypsin—Recombinant wild-type and Cys-122 mutant human cationic trypsinogen
gen (PRSS1) were generated and purified on ecotin affinity columns. The full-length sequence as well as the amino acid exchange at position 122 are indicated in Fig. 3. Under reducing conditions both proteins appear to have the appropriate and identical molecular mass (Fig. 3). Under nonreducing conditions, the Cys-122 mutant runs somewhat more slowly, which could indicate that a significant proportion of the mutant protein is present in a different conformation or that disulfide mismatches have formed. When equal protein amounts of wild-type and Cys-122 trypsinogen were activated with bovine enterokinase at pH 8.0 and in the presence of 5 mM Ca^{2+}, only ~40% of activity was generated from Cys-122 trypsinogen as compared with the wild type (end point measurements after 30-min incubation; data not shown). When we calculated the enzyme kinetics for wild-type and Cys-122 mutant trypsin, the $K_m$ values were not different between the two enzymes, but the $k_{cat}$ of Cys-122 trypsin amounted to only 37% of the wild-type (Table I). This suggests that only 37% of the mutantzymogens forms correctly folded active trypsin upon enterokinase activation and that the remaining portion is present in a conformation that renders the protein non-activatable. When equal protein amounts of wild-type and Cys-122 trypsinogen were studied for autoactivation in the presence of 5 mM Ca^{2+} at pH 8.0 or at pH 5.0, only 20% or less of the activity of wild-type trypsinogen was found for the Cys-122 mutant (Fig. 4, A and B). When, on the other hand, autoactivation of the two trypsinogens was compared in fractions that were corrected for potential activity (normalized for enterokinase activatable trypsin activity), the results were rather different. In the presence of 5 mM Ca^{2+}, autoactivation of Cys-122 trypsinogen proceeded only slightly faster than wild-type trypsinogen at either pH 5.0 or at pH 8.0 (Fig. 4, C and D). It must remain open whether this difference is biochemically meaningful in view of the wholly different protein concentrations that were used in the assay to correct for enterokinase activatable enzyme activity. In the presence of 1 mM EDTA, however, the Cys-122 trypsinogen autoactivated much more rapidly at pH 8.0, whereas autoactivation was found to proceed more or less at the same rate compared with wild-type trypsinogen at pH 5.0 (Fig. 4, E and F).

Experiments that were performed to study the activation of trypsinogen by human cathepsin B resulted in similar enzyme kinetics as seen for enterokinase activation of trypsinogen, i.e. when equal protein amounts of wild-type and Cys-122 trypsinogen were activated with cathepsin B, the activation of the Cys-122 mutant was found to be dramatically reduced and only when the amounts of protein were corrected for enterokinase activatable (potential) activity did the mutant activate marginally faster (Fig. 5A). These results were independent of whether or not calcium was present or chelated with EDTA (data not shown). Another property of trypsin that could po-
tentially confer a gain of function is the kinetic at which the active trypsin is inactivated or undergoes autolysis (15). In the presence of 5 mM Ca\(^{2+}\) or at pH 5.0, wild-type and Cys-122 trypsin were both found to be completely stable and did not undergo autolysis (data not shown; for wild type, see Refs. 7 and 8). However, in the presence of EDTA and at pH 8.0, the Cys-122 mutant trypsin was found to be considerably more resistant to autolysis than the wild-type enzyme (Fig. 5B).

Molecular Modeling—The structure of the wild-type PRSS1 in the front view (standard view, Fig. 6A) and side view (90° rotation along the y axis, Fig. 6B) shows that Arg-122 is located at a considerable distance from the active site or the activation domain of trypsin and would therefore be unlikely to directly affect its catalytic activity. In both orientations it can be seen in close proximity to the calcium binding loop (70-loop) of trypsin. In view of this location at the exposed back of the molecule, a replacement of Arg-122 by cysteine could not only affect a potential hydrolysis site of trypsin (9, 18) but would also permit the formation of disulfide bonds between two trypsinogen molecules or between trypsinogen and other proteins. The two molecules in the asymmetric unit of the reported PRSS1 structure (18) were superimposed and are shown together with the neighboring residues of Arg-122 within a distance of 8.0 Å (Fig. 6C). Both structures are remarkably similar with exception of the arginine side chains at position 122 (orange and purple in Fig. 6C), which show a high temperature factor (>70 Å\(^{2}\)). The fact that Arg-122 can exist in two different orientations in the crystal structure (18) is surprising and reflects structural flexibility at this position. A rotamer search shows that alternative orientations of the side chain of Arg-122 could form favorable interactions with neighboring residues (i.e. hydrogen bonds to Gly-74 or Asn-84 and a charged hydrogen bond to Glu-82), which, in turn, would affect the conformation of the calcium binding loop. When Arg-122 is replaced by cysteine (Fig. 6D), the formation of hydrogen bonds with the calcium binding loop is reduced. In the wild-type structure, Ca\(^{2+}\) could stabilize the conformation of the 70-loop. In the absence of Ca\(^{2+}\), however, the interaction of Arg-122 with its neighboring residues could result in a different conformation of the 70-loop and therefore

Table I

|          | kcat \(\text{s}^{-1}\) | K\(_{\text{m}}\) \(\mu\text{M}\) | kcat/K\(_{\text{m}}\) \(\text{s}^{-1}\mu\text{M}^{-1}\) |
|----------|----------------|----------------|----------------|
| Wild-type| 90.4 ± 1.1     | 2.1 ± 0.1 \times 10^{-5} | 4.3 \times 10^{6} |
| Cys-122  | 33.2 ± 0.5     | 2.0 ± 0.1 \times 10^{-5} | 1.6 \times 10^{6} |

FIG. 3. Protein sequence of recombinant human PRSS1 into which an arginine to cysteine mutation was introduced by site-directed mutagenesis at codon 122. The corresponding, Coomassie-stained SDS gels under reducing and nonreducing conditions are shown on the right. Note that the Cys-122 trypsin runs somewhat slower through the gel under nonreducing conditions.

FIG. 4. Biochemistry of recombinant Cys-122 trypsinogen. For autoactivation studies of wild-type (dashed line) and Cys-122 trypsinogen (solid line), equal amounts of purifiedzymogen (2.5 µM final concentration) were incubated at 37 °C in 0.1 M Tris-HCl at pH 8.0 (panel A) or at pH 5.0 (panel B) and in the presence of 5 mM CaCl\(_{2}\) (Ca\(^{2+}\)) in a final volume of 100 µl. Aliquots of 2.5 µl were withdrawn from reaction mixtures at the indicated time intervals, and trypsin activity was determined with the synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. Activity was expressed as a percentage of total wild-type trypsin activity. In panels C and D, the same experiments (at pH 8.0 in C, and at pH 5.0 in D) were performed in the presence of 5 mM CaCl\(_{2}\) and in panels E (pH 8.0) and F (pH 5.0) in the presence of 1 mM EDTA, but the amount of zymogen was corrected for enterokinase activatable trypsin activity; accordingly, the results were expressed as percentage of the potential total activity.
lead to an altered catalytic activity. Cysteine at position 122 could not participate in these predicted interactions, and the conformation of the 70-loop would thus remain unaltered in either the presence or absence of Ca$^{2+}$.

**DISCUSSION**

Why structural changes in the cationic trypsinogen gene lead to the onset of hereditary pancreatitis has been a matter of debate. Because pancreatitis has long been regarded as a disease that is caused by proteolytic autodigestion of the pancreas (21) and because trypsin is known to be a potent activator of other pancreaticzymogens in the gut (22), it has been suggested that the trypsinogen mutations that were found in association with hereditary pancreatitis confer a gain of enzymatic function (2, 9).

In vitro studies have analyzed the biochemistry of recombinant human trypsinosins, into which pancreatitis-associated mutations were introduced and found that, under defined experimental conditions, either a facilitated trypsinogen autoactivation or an extended trypsin activity can result (6–8, 10). Whether these experimental conditions reflect the highly compartmentalized situation under which protease activation begins intracellularly in vivo (23, 24) is presently unknown, but the above studies would suggest that either a more effective autoactivation of trypsinogen or an impaired inactivation of trypsin (by degradation or autolysis) would be involved in the onset of hereditary pancreatitis.

A number of arguments, however, have been raised against the gain of trypsin function hypothesis of hereditary pancreatitis. The increased autoactivation of recombinant trypsinogen in vivo studies can be completely abolished by adding physiological concentrations of pancreatic secretory trypsin inhibitor (0.15 μM pancreatic secretory trypsin inhibitor, 1 μM trypsinogen; Ref. 10) and may therefore have no biological relevance. Statistically, most hereditary disorders are associated with loss of function mutations that render a specific protein either defective or impair its intracellular processing and targeting (25). Moreover, at least five mutations: A16V (26), D22G (6), K23R (27), N29I (28), and R122H (2), have been found in association with hereditary pancreatitis, are located in different regions of the PRSS1 gene, and would thus be expected to have different structural effects on the trypsinogen molecule. It would therefore be easier to explain their common pathophysiology in terms of a loss of enzymatic function rather than through a gain of enzymatic function. In particular, one of these mutations (A16V) also affects the signal peptide cleavage site that is assumed to be involved in the correct processing of trypsinogen (26). Experiments in isolated pancreatic acini and lobules that studied the in vivo mechanisms of intracellularzymogen activation have shown that trypsin activity is neither required nor involved in the activation of other digestive proteases and that its most prominent role is in autodegradation (29). This, in turn, would suggest that intracellular trypsin activity has a role in the defense against other, potentially more harmful, digestive proteases and that structural alterations that impair the function of trypsin would eliminate a
protective mechanism rather than generate a triggering event for pancreatitis (30). Whether these experimental observations obtained on rodent pancreatic acini and lobules have any relevance to human hereditary pancreatitis is presently unknown and cannot be readily assumed without further evidence because human cationic trypsinogen has distinct characteristics in terms of its ability to autoactivate and to autodegrade (31, 32).

The kindred reported here is interesting in several respects. The single nucleotide exchange is only one position upstream of the one found in the most common variety of hereditary pancreatitis and leads to an amino acid exchange at the same codon (R122C versus R122H). In terms of the diagnostic detection of the R122C mutation in patients suspected of suffering from hereditary pancreatitis, this mutation escapes the conventional screening method with the A/III restriction enzyme digest (2). We therefore propose an alternative screening method using the restriction enzyme BstUI, which has the added advantage of simultaneously detecting the R122H and the R122C mutations, even in the presence of a recently reported neutral polymorphism that makes the R122H mutation inaccessible for screening with the A/III technique (19, 20).

In terms of the clinical manifestation of pancreatitis, the R122C mutation appears to have a milder phenotype than the most common N29I and R122H mutations, as indicated by the relatively late age of initial symptom onset in the two affected patients (with 24 and 52 years, respectively) and the fact that two family members are as yet unaffected carriers. Although the kindred is much too small to make a definitive assessment, the currently known carriers would suggest a disease penetrance of only 50%.

In terms of the question whether hereditary pancreatitis is caused by a gain of trypsin function or a loss of trypsin function, the biochemical characteristics of recombinant Cys-122 trypsinogen are compatible with both possibilities. Only when the amount of trypsinogen in the autoactivation and autolysis assays is corrected for enterokinase activable trypsin activity do the characteristics of Cys-122 trypsinogen resemble those of the previously investigated His-122 mutant (7). In contrast to His-122 trypsinogen, however, the autoactivation of Cys-122 trypsinogen in the absence of Ca2+ remains pH-dependent and, at pH 5.0, parallels the autoactivation of wild-type trypsinogen.

Although the differences between Cys-122 and wild-type trypsinogen are unremarkable in the presence of Ca2+, they are significant in the absence of Ca2+ and at pH 8.0, where a much more rapid autoactivation and a retarded autolysis can be found. Both the increased autoactivation and the decreased autolysis of Cys-122 trypsin could confer a gain of enzymatic function. Molecular modeling further predicted that the active site of trypsin would remain unaffected by a mutation in codon 122, and, consistent with this prediction, the $k_{cat}$ of Cys-122 trypsin remained unchanged from the wild-type enzyme. The retarded degradation of the active enzyme could be explained by interference with a potential autolysis site, as has also been suggested for His-122 trypsin (9).

When, on the other hand, equal amounts of protein were used for the biochemical studies, the enterokinase-induced activation and the autoactivation of Cys-122 trypsinogen were found to be significantly reduced by 60–70% compared with the wild-type enzyme. The $k_{cat}$ of Cys-122 trypsin, which amounts to only 37% of that of the wild type, suggests that the mutant trypsinogen is largely expressed in a conformation that compromises its activation. Whether this is caused by a destabilization of the interactions between codon 122 and the calcium-binding loop of trypsinogen, as suggested by the modeling studies, or by the formation of mismatched disulfide bonds between the cysteine residues, which would be compatible with the different band appearance in nonreducing SDS gels, remains presently unknown. Interestingly, the activation of Cys-122 trypsinogen by cathepsin B, a mechanism of activation that is not inhibitable by physiological pancreatic secretory trypsin inhibitor concentrations (10), was also dramatically reduced when equal amounts of trypsinogen protein were studied. This observation is of pathophysiological importance because the lysosomal protease cathepsin B is physiologically present in the secretory compartment not only of rodent (33) but also of human pancreas (34) and has been shown to play a crucial role in trypsinogen activation in vivo (33, 35).

The interpretation of these changes in terms of their in vivo consequences must, by definition, remain speculative. The classical view would be that the R122C mutation largely resembles the R122H mutation and leads to an increased intracellular autoactivation as well as a greatly impaired autolysis and therefore confers a gain of trypsin function. This, in turn, would permit recurrent episodes of proteolytic autodigestion and pancreatitis. In this model the suggested misfolding of Cys-122 trypsinogen would have to be disregarded as a phenomenon that only affects recombinant enzyme in vitro and does probably not occur under the compartmentalized intracellular conditions in vivo. At most, the misfolding would be considered as a factor that somewhat reduces the gain of enzymatic function and therefore accounts for the milder phenotype in comparison to the R122H mutation, as reflected by the later age of onset and the seemingly lower disease penetrance.

The alternative model would predict that Cys-122 trypsinogen also misfolds or forms mismatched disulfide bridges under intracellular in vivo conditions and therefore confers a dramatic loss of trypsin function that cannot be compensated for by the facilitated autoactivation or the impaired autolysis at a basic pH and in the absence of Ca2+. If this scenario should reflect the in vivo conditions within the pancreas, it would represent the first direct evidence from a human study for a protective role of trypsin activity in pancreatitis. Short of direct access to living human acini from carriers of PRSS1 mutations or a transgenic animal model into which the human PRSS1 polypeptide is stably expressed in vivo, it is presently not possible to predict the pathophysiological consequences of the R122C mutation in the human pancreas.

We conclude that our report of a novel, hereditary pancreatitis-associated mutation in the cationic trypsinogen gene confirms the genetic heterogeneity of the disease (26, 36) and documents the requirement for a novel diagnostic screening approach, which we propose to be the widely applicable restriction enzyme digest with BstUI that makes the inferior A/III method redundant. It further implies that the biochemical properties of Cys-122 trypsinogen are compatible with the interpretation that either a gain of enzymatic function or a loss of enzymatic function could represent the triggering mechanism for hereditary pancreatitis.

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Addendum—The timely Internet access to Journal of Biological Chemistry manuscripts in press permits other authors to comment on upcoming articles before they appear in print. In this way we were informed not only of an initial report of the R122H polymorphism (19) but also of manuscripts in which two other groups independently report hereditary pancreatitis patients with the R122C mutation (37, 38). This already makes the R122C mutation the fourth most common PRSS1 mutation in patients with hereditary pancreatitis.

Novel PRSS1 Mutation (R122C) Causes Hereditary Pancreatitis 5409
