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Trypsinogen Stabilization by Mutation Arg117→His: A Unifying Pathomechanism for Hereditary Pancreatitis?

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Mutations Arg117→His and Asn21→Ile of the human cationic trypsinogen have been recently identified in patients affected by hereditary pancreatitis (HP). The Arg117→His substitution is believed to cause pancreatitis by eliminating an essential autolytic cleavage site in trypsin, thereby rendering the protease resistant to inactivation through autolysis. Here we demonstrate that the Arg117→His mutation also significantly inhibits autocatalytic trypsinogen breakdown under Ca^{2+}-free conditions and stabilizes the zymogen form of rat trypsin. Taken together with recent findings demonstrating that the Asn21→Ile mutation stabilizes rat trypsinogen against autoactivation and consequent autocatalytic degradation, the observations suggest a unifying molecular pathomechanism for HP in which zymogen stabilization plays a central role.

Hereditary pancreatitis (HP) is a relatively rare genetic disorder, characterized by early onset episodes of acute pancreatitis with frequent progression to chronic pancreatitis (1–3). Recently, two mutations in the cationic trypsinogen (TG) gene, Arg117→His and Asn21→Ile, have been identified in patients affected by HP (2–7). It has been proposed that mutation Arg117→His leads to pancreatitis by rendering prematurely activated trypsin resistant to inactivation through autolysis (2–4). Degradation of trypsin by autolysis or by trypsin-like enzymes in the pancreas is believed to serve as a failsafe mechanism against excessive trypsin generation (2–7). Indeed, Arg117 has been shown a critical autolysis site in bovine (8) as well as rat trypsin (9, 10), and mutations of this residue stabilize trypsin against autocatalytic degradation (10). The mechanism of action of the Asn21→Ile mutation appears to be much more elusive. In an attempt to find a common mechanism for both mutations, it was proposed that the Asn21→Ile mutation may change the proteolytic accessibility of Arg117 by bringing Glu24 close enough to form a salt-bridge (2, 3). Such a conformational change would result in increased resistance against autolytic cleavage of the Arg117 site, thus it would mimic the effects of the Arg117→His mutation. An alternative theory suggested that the mutation might change the structure of the activation peptide region and lead to increased autoactivation and excessive trypsin formation (5, 7). Surprisingly, when these two theories were tested on the homologous recombinant rat TG, neither increased autolytic stability nor increased autoactivation was observed (11). In sharp contrast, the Asn21→Ile mutation significantly decreased autoactivation and zymogen degradation without affecting trypsin stability or activity. These findings suggested that unwanted zymogen stabilization by the Asn21→Ile mutation might play an important role in HP. We hypothesized that proteolytic inactivation of TG by trypsin generated during autoactivation might be another important safeguard mechanism of controlling excessive trypsin liberation in the pancreas (11). Mutations, which stabilize TG against autoactivation and/or autocatalytic degradation, would impair the efficacy of this protective mechanism and render the zymogen storage pool more susceptible to widespread activation. Since Arg117 is the primary cleavage site for autocatalytic breakdown of both trypsin (8–10) and TG (11), it is tempting to speculate that the Arg117→His mutation may exert its effect through proteolytic stabilization of both species. In the present study, we demonstrate that under Ca^{2+}-free conditions the Arg117→His substitution also affords significant zymogen stabilization, and this observation offers a unifying pathogenic scheme for the initiating steps of HP.

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EXPERIMENTAL PROCEDURES

Materials. Ecotin was over-expressed in *E. coli* BL21 as described by Pál *et al.* (12, 13), and purified to homogeneity using a trypsin affinity column. Purified ecotin was immobilized to ACTI-GEL ALD resin (Sterogene Bioseparations, Carlsbad, CA) as previously described (14). Ultrapure enterokinase was purchased from Biozyme Laboratories (San Diego, CA), and N-CBZ-Gly-Pro-Arg-*p*-nitroanilide from Sigma (St. Louis, MO).

Construction of TG mutants. Mutations were introduced into the rat anionic TG gene in the expression vector pTrap by oligonucleotide-directed site-specific mutagenesis, using the "overlap extension" PCR method (15), as previously described (11).

Expression and purification of recombinant TGs. Wild-type and mutant TGs were expressed to the periplasm in *E. coli* SM138 as previously described (11, 16, 17). Periplasmic fractions were isolated by osmotic shock, and zymogens were purified twice on an ecotin affinity column (11, 14). The second eluate was essentially homogenous TG, no other contaminating bands were detectable on Coomassie-blue stained gels. Concentrations of zymogen solutions were estimated from their ultraviolet absorbance using an extinction coefficient of 38,000 M^{-1} cm^{-1} at 280 nm (17).

Assay of trypsin activity. Trypsin activity was determined using the synthetic chromogenic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. Kinetics of the chromophor release were followed at 410 nm in 0.1 M Tris–HCl, pH 8.0, 1 mM $CaCl_2$, at 37°C.

Autoactivation of trypsinogen. TG (150 pmol) was incubated at 37°C in 0.1 M Tris–HCl (pH 8.0) in the presence of 5 mM $CaCl_2$ or 1 mM K–EDTA in a volume of 110 μL. At the indicated times samples of 5–10 μL were removed for trypsin activity assays. Alternatively, reactions were terminated by trichloroacetic-acid precipitation, proteins were separated on a 12% reducing SDS–PAGE and bands were visualized by Coomassie blue staining.

RESULTS AND DISCUSSION

Mutation Arg117→His was constructed in the rat anionic TG mutant Thr21→Asn (Asn21/His117), as previously described (11). We chose this background, because amino acid 21 in TG appears to be a critical determinant of autoactivation (11), and human cationic TG carries an Asn residue at this position. Catalytic properties or proteolytic stability of trypsin are not affected by the Thr21→Asn substitution to any extent whatsoever (11). Similarly, mutations of Arg117 have insignificant effects on the catalytic parameters of rat trypsin (10, 18). In contrast, as discussed above, resistance of rat trypsin against autolysis is significantly increased by mutations of Arg117 (10, 11, 18).

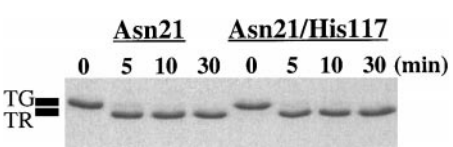

FIG. 1. Activation of mutant zymogens with enterokinase. Approximately 150 pmol trypsinogen (Asn21, mutant Thr21→Asn; Asn21/His117, mutant Thr21→Asn/Arg117→His) was incubated with 60 ng enterokinase at 22°C, in the presence of 0.1 M Tris–HCl, pH 8.0, and 5 mM $CaCl_2$ in a final volume of 110 μL. Reactions were terminated at indicated times (5, 10, and 30 min) by precipitation with trichloroacetic acid. Samples were then analyzed by SDS–PAGE and Coomassie blue staining. Although not shown, no other bands were observed on gels under these conditions. TG, trypsinogen; TR, trypsin.

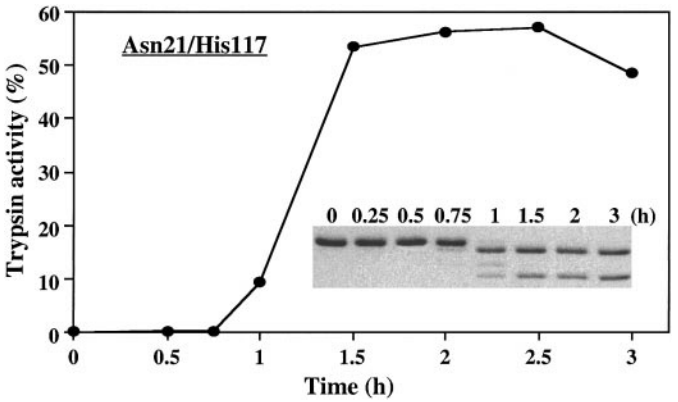

FIG. 2. Autoactivation and zymogenolysis of Asn21/His177 mutant trypsinogen in the presence of Ca^{2+}. Approximately 150 pmol trypsinogen (Asn21/His117, mutant Thr21→Asn/Arg117→His) was incubated at 37°C, in the presence of 0.1 M Tris–HCl, pH 8.0, and 5 mM $CaCl_2$ in a final volume of 110 μL. Aliquots of 5–10 μL were withdrawn from reaction mixtures at indicated times and trypsin activity was determined with the synthetic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide. Activity was expressed as percentage of the potential total activity, as determined on similar zymogen samples activated with enterokinase. (Inset) Reactions were terminated at indicated times (15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, and 3 h) by precipitation with trichloroacetic acid, and samples were analyzed by SDS–PAGE and Coomassie blue staining.

In the present study, we characterized the zymogen form of the Asn21/His117 mutant. First, time-courses of enterokinase-catalyzed conversion of TG to trypsin were followed by SDS–PAGE and Coomassie blue staining. As shown in Fig. 1, in the presence of 5 mM Ca^{2+} at 22°C, enterokinase activated zymogens with Asn21 or Asn21/His117 at identical rates, and the activation process was nearly complete within 5 min. Although not shown, no other degradation products were observed on the stained gels. The results indicate that the Arg117→His mutation does not change the proteolytic accessibility of the activation peptide to any appreciable extent. To confirm and extend these findings, autoactivation of the Asn21/His117 zymogen was characterized by activity assays and SDS–PAGE. When incubated at 37°C, pH 8.0, in the presence of 5 mM Ca^{2+}, Asn21/His117 TG underwent relatively rapid autoactivation and limited proteolytic degradation (Fig. 2). The kinetics and extent of trypsin generation were remarkably similar to those previously observed for the Asn21 mutant (see Fig. 3 in Ref. 11), confirming that the Arg117→His mutation does not influence the zymogen activation process. As shown by the inset in Fig. 2, two stable proteolytic products were detected on stained gels, which have been previously identified as the N-terminal fragments of TG and trypsin, respectively, after cleavage at the Lys188 site (11). No other degradation products were visible on the rou-

tinely used 12% gels. Although we have predicted that due to the lack of the Arg117 autolysis site significantly more trypsin will be generated, the enhanced cleavage at Lys188 seemed to provide a fairly efficient inactivating breakdown pathway, and only a slight increase in steady state trypsin levels were detectable relative to those previously observed with the Asn21 mutant (see 11). Taken together, the observations indicate that the Arg117→His mutation does not affect proteolytic cleavage of the Lys15 activation site and it does not increase zymogen stability in the presence of 5 mM Ca^{2+}. This stands in clear contrast to the effect of the Asn21→Ile mutation, which was shown to stabilize TG by inhibiting proteolysis at Lys15 in a Ca^{2+}-independent fashion (11). Remarkably, however, when the previous autoactivation experiment was performed under essentially Ca^{2+}-free conditions, significant zymogen stabilization was evident. In the experiment shown in Fig. 3, autoactivation profiles of TG mutants Asn21/His117 (Thr21→Asn/Arg117→His), Asn21 (Thr21→Asn), Ile21 (Thr21→Ile) and wild-type rat TG (Thr21) were followed up to 2 h at 37°C, pH 8.0, in the presence of 1 mM K-EDTA. Samples were visualized by SDS–PAGE and Coomassie blue staining, and trypsin activity was measured at the end of the 2 h incubation. Clearly, the Asn21/His117 and Ile21 mutants exhibited significant stability up to 2 h, while both Asn21 and Thr21 zymogens were almost completely degraded by this time. It is also obvious, that zymogen stabilization is due to the lack of cleavage at Arg117, since the major proteolytic fragment of this cleavage was completely lacking from gels with the Asn21/His117 mutant, while it was readily observed in the Asn21 samples. This band has been previously identified as the N-terminal fragment of TG cleaved at Arg117 (11). Finally, after the 2 h incubation, only wild-type rat TG (Thr21) exhibited significant trypsin activity (18%), while trypsin generation in the Asn21, Asn21/His117 and Ile21 mutants was below 10% of the total potential activity, as determined on enterokinase activated zymogen samples. This observation is in agreement with previous results indicating that under Ca^{2+}-free conditions autoactivation-coupled zymogen breakdown generally proceeds with minimal trypsin generation (11).

One of the intriguing aspects of the present findings is that while both HP-associated mutations cause zymogen stabilization, the effect of Arg117→His requires a Ca^{2+}-free milieu, while stabilization by Asn21→Ile is Ca^{2+}-independent. This difference highlights the dual regulatory effect of Ca^{2+} on autoactivation and autocatalytic degradation. Thus, proteolytic cleavage at the Lys15 (activation) and Arg117 (autolysis) sites are inversely controlled by millimolar concentrations of Ca^{2+}, which enhance proteolysis at Lys15 and inhibit at Arg117 (10, 11). The sum of these two opposing actions of Ca^{2+} appears to determine the overall reaction kinetics of zymogen degradation. In the presence of Ca^{2+}, autoactivation is favored, and significant amounts of trypsin are generated, which in turn cleaves TG at Arg117, Lys188 and possibly other cleavage sites, too. In the absence of Ca^{2+}, cleavage at Lys15 is inhibited, and only trace amounts of trypsin are produced from Asn21-TG. However, Ca^{2+}-free conditions increase proteolytic sensitivity at Arg117 significantly, and the minimal trypsin activity is adequate for efficient cleavage at this site. The result is relatively rapid zymogen breakdown with insignificant trypsin liberation. An interesting pattern is observed with wild-type rat TG (Thr21-TG), where in the absence of Ca^{2+}, inhibi-

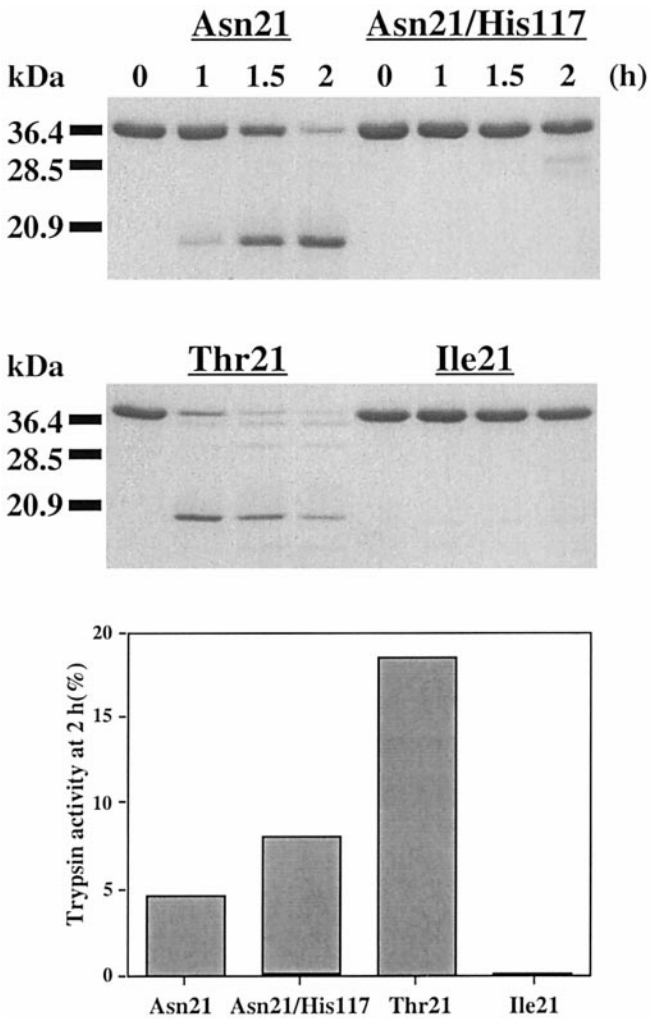

FIG. 3. Autoactivation and zymogenolysis of mutant trypsinogens in the absence of Ca^{2+}. Approximately 150 pmol trypsinogen (Asn21, mutant Thr21→Asn; Asn21/His117, mutant Thr21→Asn/Arg117→His; Thr21, wild-type rat anionic trypsinogen; Ile21, mutant Thr21→Ile) was incubated at 37°C, in the presence of 0.1 M Tris–HCl pH 8.0 and 1 mM K-EDTA in a final volume of 110 μL. Reactions were terminated at indicated times (1, 1.5, and 2 h) by precipitation with trichloroacetic acid, and samples were analyzed by SDS–PAGE and Coomassie blue staining. At 2 h, an aliquot of 5 μL was withdrawn from each reaction mixture and trypsin activity was determined with the synthetic substrate N-CBZ-Gly-Pro-Arg-*p*-nitroanilide. Activity was expressed as percentage of the potential total activity, as determined on similar zymogen samples activated with enterokinase.

tion of cleavage at Lys15 is less pronounced than in Asn21-TG. Consequently, somewhat more trypsin is liberated during autoactivation and proteolysis at Arg117 is extensive, resulting in significantly more rapid zymogen breakdown (11).

The physiological rationale behind the dual regulation by Ca^{2+} appears to be clear. In the low-Ca^{2+} intracellular environment non-desirable zymogen activation is inhibited, while sensitization of Arg117 provides a protective zymogen/trypsin degradation pathway to prevent widespread autoactivation. In contrast, the higher Ca^{2+} concentration of the pancreatic and intestinal juices allows for efficient activation of the zymogens at Lys15 and slows down TG/trypsin breakdown at Arg117. Importantly, in pancreatitis pathologic trypsin generation is initiated inside the acinar cells, in a low Ca^{2+} environment (19). Therefore, properties of mutant zymogens under Ca^{2+}-free conditions are presumably more relevant with respect to the pathomechanism of HP. Our observations clearly demonstrate that both Asn21→Ile and Arg117→His afford significant zymogen stabilization in the absence of Ca^{2+}, and this effect may represent a common initiating step in the pathogenesis of HP.

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