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Comparative *in Vitro* Studies on Native and Recombinant Human Cationic Trypsins

CATHEPSIN B IS A POSSIBLE PATHOLOGICAL ACTIVATOR OF TRYPSINOGEN IN PANCREATITIS*

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Hereditary pancreatitis, an autosomal dominant disease is believed to be caused by mutation in the human trypsinogen gene. The role of mutations has been investigated by *in vitro* studies using recombinant rat and human trypsinogen (TG). In this study we compare the enzymatic properties and inhibition by human pancreatic secretory trypsin inhibitor (hPSTI) of the native, postsynthetically modified and recombinant cationic trypsin, and found these values practically identical. We also determined the autolytic stability of recombinant wild type (Hu1Asn21) and pancreatitis-associated (Hu1Ile21) trypsin. Both forms were equally stable. Similarly, we found no difference in the rate of activation of the two zymogens by human cationic and anionic trypsin. Mesotrypsin did not activate either form. The rate of autocatalytic activation of Hu1Asn21 TG and Hu1Ile21 TG was also identical at pH 8 both in the presence and absence of Ca^{2+}. At pH 5 Hu1Ile21 TG autoactivated about twice as fast as Hu1Asn21 TG. The presence of physiological amount of hPSTI completely prevented autoactivation of both zymogens at pH 8 and at pH 5 as well. Cathepsin B readily activated both zymogens although Hu1Ile21 TG was activated about 2.5–3 times as fast as Hu1Asn21 TG. The presence of hPSTI did not prevent the activation of zymogens by cathepsin B. Our results underlie the central role of cathepsin B in the development of different forms of pancreatitis.

It has long been known that pancreatitis is initiated by uncontrolled, premature activation of trypsinogen in the pancreas, followed by the activation of other proteolytic and lipolytic enzymes of the pancreatic juice. Recent genetic studies on families with hereditary pancreatitis have revealed three specific mutations, R117H, N21I, and K15R, respectively, in the gene encoding cationic TG[1] (1–3). As to the R117H mutation it has been proposed that pancreatitis may be caused by the elimination of a natural defense mechanism, the autolytic cleavage of trypsin at this site, that prevents the accumulation of prematurely activated trypsin (1). The K15R mutation significantly facilitates the tryptic cleavage of the propeptide region, as shown by the tryptic digestion of synthetic dodecapeptide bearing the above mentioned amino acid substitution (3). Mutation N21I, however, does not involve trypsin-sensitive sites. Modeling the effect of the mutation in the x-ray structure of human cationic trypsin (4), it was proposed that the asparagine to isoleucine substitution would give rise to conformational changes in the loop formed by residues Glu^{23}-Ser^{26}. This change, according to the hypothesis, might allow the formation of a Glu^{23}-Arg^{117} salt bridge rendering Arg^{117} resistant to tryptic cleavage (4, 5). Accordingly, both R117H and N21I mutations would induce pancreatitis by similar mechanisms.

An obvious way to approach this question is to generate recombinant forms of these mutant enzymes and to study their behavior under *in vitro* conditions. Using a rat anionic trypsinogen II model, it has been shown that indeed the change of Arg^{117} to Asn or His protects trypsin (6) and TG (7) against autodigestion.

A proposal for the mechanism by which the N21I mutation might cause pancreatitis is based on experiments with an anionic rat trypsinogen/trypsin model system as well. To model the mutation of human cationic TG at position 21, Thr^{21} of rat anionic TG was replaced with Asn or Ile. It was found that Ile at position 21 significantly decreased the rate of autoactivation and zymogen degradation without affecting trypsin activity and stability (8). More recently, however, it has been shown that the Asn^{21} to Ile mutation in the human cationic TG, contrary to the effects observed in rat anionic TG, did not change the stability of the zymogen (9).

There are two caveats, however, to this simplistic approach. First, it has been shown that human cationic trypsin is postsynthetically modified, presumably phosphorylated (10), but there are no data showing how this modification influences its properties. Second, in the previous *in vitro* models aimed at the molecular pathomechanism of pancreatitis, an important factor, the presence of human pancreatic secretory inhibitor (hPSTI), has been neglected. Therefore we isolated native human cationic trypsin and hPSTI from human pancreatic juice, compared the enzymic properties of native and recombinant

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[1] The abbreviations used are: TG, trypsinogen; Hu1Asn21 TG, recombinant wild type human cationic trypsinogen; Hu1Ile21 TG, pancreatis-associated N21I mutant of human cationic trypsinogen; hPSTI, human pancreatic secretory trypsin inhibitor; Z-GPR-pNA, N-carbobenzyloxy-glcyl-prolyl-arginyl *p*-nitroanilide; Z-K-SBzl, N^{α}-carbobenzyloxy-lysine thiobenzylester; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

cationic trypsin, and investigated the stability of zymogens in the presence of hPSTI as well. To achieve this goal, we cloned human cationic, anionic, and mesotrypsinogen from a commercial human pancreatic adenocarcinoma-derived cDNA library and worked out their large scale expression by using a T7 promoter driven system in *Escherichia coli*. Our study was extended to Hu1Ile21 (pancreatitis associated) TG and trypsin, also.

EXPRIMENTAL PROCEDURES

Cloning and Expression of Human Cationic Trypsinogen—Full-length human anionic, cationic TG, and mesotrypsinogen genes (without the signal peptide) were amplified by polymerase chain reaction from a pancreatic cDNA library (Stratagen number 937208) with oligonucleotide pairs matching the amino-terminal sequence of the propeptide region and the 3′-flanking region of the coding sequence, respectively. Human cationic trypsinogen clones were selected by restriction mapping, and identified by automated DNA sequencing. The oligonucleotides used for amplification introduced a 5′ *Hin*dIII and a 3′ *Sal*I site, thereby allowing facile subcloning into the bacterial expression vector, pTRAP (11). This manipulation, however, have changed the sequence of the propeptide region. The native Ala-Pro-Phe-$(Asp)_4$-Lys^{15} was transformed into Ala-Phe-Pro-Val-$(Asp)_4$-Lys^{15} which was identical to the rat trypsinogen II propeptide sequence.

The periplasmic expression vector pTRAP (11) transformed into SM 138 cells was satisfactory for the expression of human anionic TG and mesotrypsinogen, but the yield of human cationic TG was extremely low. To obtain higher levels of expression the coding region of human TG was introduced into a modified pET17b vector (Novagen) resulting in expression vector pET17_Hu1. Mutagenesis was performed by the megaprimer method (12).

To express TG, 250-ml cultures of BL21(DE3)pLysS transformed with pET17_Hu1 were grown 37 °C in Luria-Bertrami medium with 100 μg of ampicillin to an $A_{600\ nm}$ of 0.5 then induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and grown for an additional 4 h. Cells were harvested with centrifugation, and lysed by sonication. The inclusion body was washed three times with 50 ml of 0.1 M Tris-HCl, 2 mM EDTA, pH 8.0. The pellet was dissolved in 4 ml of 6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.5, 2 mM EDTA containing 30 mM dithiothreitol and was reduced for 30 min at 37 °C. Denatured and reduced TG was rapidly diluted into 125 ml of ice-cold, deaerated re-folding buffer (0.9 M guanidine hydrochloride, 0.1 M Tris-HCl, pH 8.5, 2 mM EDTA, 1 mM L-cysteine, and 1 mM L-cystine). The solution was slowly stirred under N_2 at 4 °C for 16 h.

Purification of Trypsinogen and Trypsin—TG solution was exhaustively dialyzed against 2.5 mM HCl, then the precipitate was removed by centrifugation. The clear supernatant was loaded onto a column of SP-Superose FF equilibrated with 25 mM LiOAc, pH 4.0. TG was eluted in the same buffer with a linear gradient of 0–1 M NaCl. TG was further purified on a MonoQ column (5/5) in 20 mM Tris-HCl, pH 8.8, 1 mM EDTA, then by passing it through a soybean trypsin inhibitor-Sepharose column to remove trypsin contamination. The solution was immediately acidified by adding HCl to 30 mM final concentration, and kept in aliquots at −20 °C. Trypsin was obtained by adding bovine enterokinase (1/50 w/w) in 50 mM ϵ-aminocaproic acid, pH 7.0, 10 mM $CaCl_2$, followed by affinity purification on an immobilized soybean trypsin inhibitor-Sepharose column.

Isolation of Trypsin from Pancreatic Juice—Human pancreatic juice was collected by catheterization of the Wirsung duct after surgery for pancreatic diseases. To 20 ml of pancreatic juice equal volume of 50 mM LiOAc, pH 4.5, 10 mM $CaCl_2$ was added, and the pH was set to 4.5 by acetic acid. The precipitate was removed by centrifugation (25,000 rpm, 30 min) and the clear supernatant was loaded onto a MonoS column (5/5) equilibrated with 25 mM LiOAc, pH 4.5, 5 mM $CaCl_2$. TG was eluted with a linear gradient of NaCl (0–0.5 M). Trypsin was isolated as described above.

Isolation of Human Pancreatic Trypsin Inhibitor from Pancreatic Juice—20 ml of pancreatic juice was exhaustively dialyzed against 1 mM HCl. The precipitation was removed by ultracentrifugation and the clear supernatant was lyophilized. The dry protein mixture was dissolved in 2 ml of 0.5 M NaCl and 10 mM HCl, and was loaded in 0.5-ml aliquots onto a Superose 12 column equilibrated with the same buffer. The flow rate was 0.75 ml/min. hPSTI was eluted at 26 min as a sharp symmetric peak. Its purity was validated by reverse phase-high performance liquid chromatography.

High Performance Liquid Chromatography and Mass Spectrometry—Analysis of protein mixtures was performed with a Smart System equipped with a UV-M II monitor connected to an electronspray-single quadrupole mass spectrometic detector (Hewlett Packard Series 1100 MSD). Separation of protein was made using a 2.1 × 150-mm Zorbax 300SB-C18 reversed-phase chromatography column termostatted at 10 °C. The mobile phase consisted of water containing 0.05% (v/v) trifluoroacetic acid (A) and acetonitrile (B). Proteins were separated using the following gradient system at a flow rate of 150 μl/min: isocratic at 20% B, 0–10 min; linear from 20 to 80% B, 10–25 min. Electronspray mass spectra in the positive ion mode were acquired over a mass/charge range of 500–3000 every 1.5 s during the chromatographic separation. The following instrumental parameters were used for mass spectrum detection: gain, 5; fragmentor voltage, 40 V; step size, 0.15; threshold, 300; drying gas, 10 liters/min; nebulizer pressure, 25 psi; drying gas temperature, 300 °C; capillary voltage, 3500 V.

Determination of Kinetic Constants—Active site concentration of trypsins was determined by titration with *methyl*-umbelliferyl guanidinobenzoate (13). Enzymatic activity was measured in activity buffer (0.1 M NaCl, 50 mM Tricine, pH 8.0, 10 mM $CaCl_2$, and 0.05% Triton X-100) with the synthetic substrates *N*-carbobenzyloxy-glycyl-prolyl-arginyl *p*-nitroanilide (Z-GPR-pNA, Bachem) or N^{α}-carbobenzyloxy-lysine thiobenzylester (Z-K-SBzl, Sigma) at 37 °C. Color development for Z-K-SBzl substrate was achieved with 200 μM 2,2′-dithiopyridine (Sigma). Kinetic constants were determined by analyzing the progress curves for chromophore release by KINSIM and FITSIM (14) according to the Michaelis-Menten equation.

$$E + \mathrm{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\mathrm{S} \xrightarrow{k_{+2}} E + \mathrm{P} \qquad \text{(Eq. 1)}$$

Inhibition by hPSTI—Equilibrium inhibitor constants (K_i) were determined according to a published method (15). A constant amount of trypsin (0.5 nM) was incubated with incremental amounts of hPSTI (0.1–10 nM) at 25 °C. The incubation mixture consisted of 50 mM Tricine, pH 8, 10 mM $CaCl_2$, 0.1 M NaCl, 0.005% Triton X-100, 0.05% bovine serum albumin. After the equilibrium was reached (>6 h), the residual trypsin activity was determined by using Z-K-SBzl as described above. Rate constants for the association and dissociation of the enzyme-inhibitor complex were also determined by progress curve analysis (16). In this case, Equation 1 was supplemented in the mechanism file of KINSIM with a term describing the reversible formation of the complex.

$$E + I \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} EI \qquad \text{(Eq. 2)}$$

Autolysis and Autoactivation—Time course experiments were performed in a 200-μl final volume at 37 °C, other conditions are described in the legends to the figures. Aliquots of 5 μl were withdrawn from the reaction mixture at the indicated times and assayed for trypsin activity at 25 °C in 1 ml of activity buffer containing 100 μM Z-K-SBzl and 200 μM 2,2′-dithiopyridine. Enzymatic measurements were performed in triplicates using at least two different zymogen preparations. Trypsin activity was expressed as percentage of the total activity determined by rapid activation of an aliquot of trypsinogen with high concentration (1/10, w/w) of enterokinase. Standard error was less than 10%, except when it is directly indicated.

RESULTS

Isolation Proteins from Human Pancreatic Juice

There are several published methods for isolation of human cationic trypsin (17–19). On the basis of earlier studies we developed a rapid method for isolation of cationic trypsin from pancreatic juice. The first step was the isolation of trypsinogen by fast protein liquid chromatography on a MonoS column at pH 4.5. Trypsinogen was activated by enterokinase, and trypsin was isolated by affinity chromatography on STI-Sepharose column. The preparation was homogeneous in SDS-PAGE and reverse phase chromatography (Fig. 1). Electron spray mass spectrometric analysis of the column effluent gave a mass averaged molecular mass of 24,184 Da for the separated protein (Fig. 2). This value is 80 Da higher than the one calculated on the basis of the cDNA sequence of the mature human cationic trypsin. Our result is in agreement with the earlier obser-

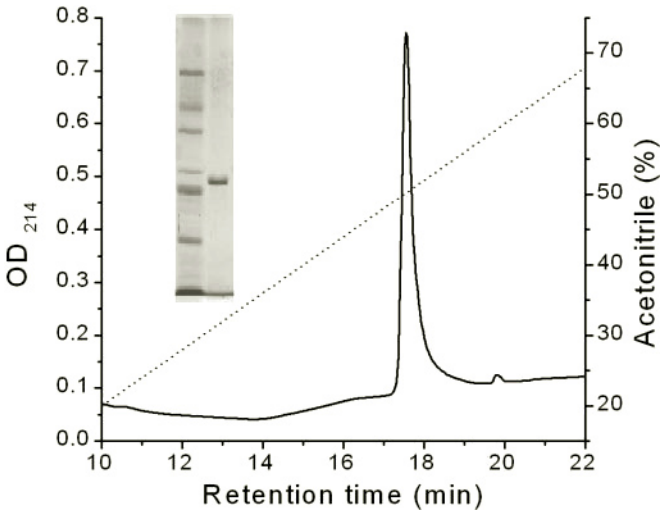

FIG. 1. **Reverse phase chromatography of modified native human cationic trypsin.** Approximately 10 pmol of native human cationic trypsin was injected into a Zorbax 300SB-C18 (2.1 × 150 mm) column attached to a Smart System thermostated at 10 °C. *Inset* shows the SDS-PAGE pattern of the sample before chromatography. Molecular weight markers from top to bottom: bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, bovine trypsinogen, and trypsin inhibitor.

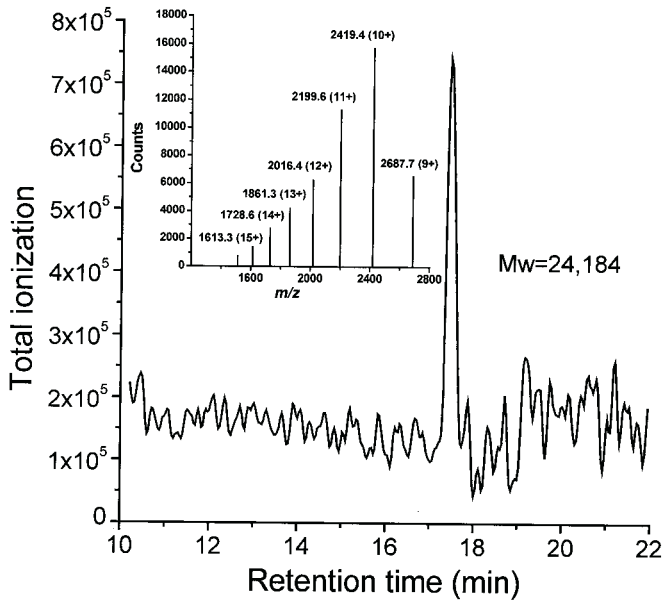

FIG. 2. **Total ion chromatogram of native human cationic trypsin.** *Inset* shows the average mass spectrum calculated for the major peak. *m/z* values and net charges of the individual ions are also indicated.

vation (10), and supports the notion about the post-synthetic modification of human cationic trypsin.[2]

Interestingly, from a different patient's pancreatic juice we were able to obtain the non-modified (M_r = 24,104 Da) trypsin. In this case the isolation procedure contained prolonged exposure to acidic environment before the cation exchange chromatography. (Trypsinogen was actually a by-product of the chromatography aimed to isolate hPSTI.) It was not clear whether the lower molecular weight was the result of hydrolytic loss of the modifying group, or trypsinogen was in fact unmodified in this sample.

Cloning and Expression of Human Trypsinogens

We cloned the coding sequences for human cationic, human anionic TG, and mesotrypsinogen from a commercial human pancreatic library. According to the nomenclature of the human trypsinogen cDNA sequences, recombinant human cationic TG was labeled as Hu1 TG, while the enzyme isolated from human pancreatic juice was referred to as cationic trypsin.

The coding sequences were inserted into a T7 promoter driven expression vector. In this case the yield of the expression was 30–60 mg/liter of culture. The recombinant protein accumulated in the form of inclusion bodies from which oxidative refolding (20) in the presence of a cysteine-cystine redox system we were able to renature it with high yield. To evaluate the efficiency of our refolding method the enzymic properties of the recombinant enzyme were compared with those of cationic trypsinogen isolated from human pancreatic juice.

Characterization of Native and Recombinant Human Cationic Trypsin

Enzymic Properties of Native and Recombinant Human Cationic Trypsin—Table I shows the catalytic constants of native trypsins (both modified and unmodified) and of the two recombinant forms (Hu1Asn21 and Hu1Ile21) on two different synthetic substrates. These data show that our refolding process results in enzymes identical with the natural one, and that the N21I mutation has no effect on the catalytic properties of cationic trypsin.

Inhibition Studies with hPSTI—We measured the inhibition constants of hPSTI against native trypsins (both modified and unmodified) and of the two recombinant forms (Hu1Asn21 and Hu1Ile21). Two methods were used for the determination of the inhibition constants. The equilibrium method is based on the measurement of the residual enzymic activity of trypsin after equilibrating it with different amounts of hPSTI for 12–16 h. The kinetic method utilizes the analysis of progress curves recorded in the absence and presence of different concentrations of hPSTI. Progress curves were analyzed with the KINSIM/FITSIM software. To reflect the difference of the methods, constants obtained by the equilibrium method were referred to as K_i, while the constants derived from the rate constants k_{on} and k_{off} were labeled as K_d. The equilibrium and kinetic methods gave identical inhibition constants. The pH dependence of the K_d was also investigated. We found a small but significant difference between the constants of the modified and the non-modified native trypsin. The constants for the non-modified trypsin are about 2-fold higher, indicating a weaker interaction with hPSTI. The constants for non-modified human cationic trypsin and for recombinant Hu1Asn21 and Hu1Ile21 trypsin were identical at all pH studied.

Comparison of Wild Type (Hu1Asn21) and Pancreatitis-associated (Hu1Ile21) Trypsin and Trypsinogen

Autolytic Stability of Trypsins—To test the assumption according to which a salt bridge formed between Glu^{23}-Arg^{117} in mutant N21I would make Arg^{117} resistant to tryptic cleavage (5, 7), we compared the autolysis of Hu1Asn21, Hu1Ile21, human anionic trypsin, and rat trypsin II in the presence (Fig. 3*A*) and absence (Fig. 3*B*) of Ca^{2+} ions. Remarkably, both Hu1Asn21 and Hu1Ile21 trypsins proved to be extremely stable in the presence of Ca^{2+} and there was no difference in the rates of activity loss in the absence of Ca^{2+} either. The data in Fig. 3 are in agreement with earlier observations (17) regarding the high stability of cationic trypsin in the presence of Ca^{2+}, and the considerable loss of this stability in the absence of Ca^{2+}.

[2] According to Ref. 10, human cationic trypsin is phosphorylated at tyrosine 151. Our preliminary studies suggest that the modifying group is not phosphate but sulfate. Because of this ambiguity we do not specify the modifying group.

TABLE I
Kinetic parameters for native and recombinant human cationic trypsins

	Z-K-SBzl			Z-GPR-*p*NA		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$s^{-1}\ M^{-1}$	s^{-1}	μM	$s^{-1}\ M^{-1}$
Native modified cationic trypsin	97.5 ± 3.2	9.95 ± 1.5	(9.80 ± 1.5) + 10^6	82.8 ± 4.6	10.8 ± 1.3	(7.62 ± 1.0) + 10^6
Native non-modified cationic trypsin 1	94.3 ± 2.7	10.1 ± 1.9	(9.34 ± 1.8) + 10^6	87.4 ± 3.5	11.3 ± 1.1	(7.73 ± 0.8) + 10^6
Hu1Asn21	102.1 ± 4.5	12.3 ± 2.2	(8.36 ± 1.5) + 10^6	76.6 ± 3.2	9.92 ± 1.8	(7.72 ± 1.4) + 10^6
Hu1Ile21	106.6 ± 2.6	11.2 ± 2.0	(9.6 ± 1.7) + 10^6	75.4 ± 2.8	8.81 ± 0.9	(8.57 ± 0.9) + 10^6

Autoactivation of Trypsinogens—To test another hypothesis, according to which the N21I mutation might increase the stability of the zymogen, we compared rates of autoactivation for Hu1Asn21 and Hu1Ile21 zymogens at pH 8.0 in the presence and absence of Ca^{2+} (Fig. 4). The error of these experiments was large; the error bar shown in Fig. 4 was calculated from the data of 10 independent experiments, five Hu1Asn21 TG and five Hu1Ile21 TG activation. Neither the rate of autoactivation nor the SDS-PAGE pattern at various stages of autoactivation (not shown) revealed any difference between the wild type and N21I mutant zymogens. The lag period of autoactivation was rather long; it took about 3 h in the presence and about 7 h in the absence of Ca^{2+} until the activity reached 10% of the theoretical maximum.

It is known that the pancreatic juice contains trypsin inhibitor. The molar ratio of TG to hPSTI is about 5:1 (21). When the autoactivation experiments were performed in the presence of 15 M percent of hPSTI, we found no increase in trypsin activity up to 24 h either with Hu1Asn21 or with Hu1Ile21 TG.

Activation of Trypsinogens—We compared the rates of activation of the two zymogens with bovine enterokinase, and found that Hu1Asn21 was activated about twice as fast as Hu1Ile21 (data not shown). This is in agreement with earlier observations on rat TG (8), but may have little relevance to hereditary pancreatitis. Premature and preferential catalytic activation of the trypsinogen mutant by other available proteases like the different forms of human trypsin may be another possibility to consider. We determined the rate of activation of Hu1Asn21 (Fig. 5*A*) and Hu1Ile21 (Fig. 5*B*) TG with human cationic trypsin, human anionic trypsin, and mesotrypsin. Activation was initiated by adding 1.5% of the total activity to be released from the zymogen in the case of cationic and anionic trypsin and 5% in the case of mesotrypsin. Both cationic and anionic trypsin readily activated the two zymogens and there was no difference in the rate of activation. On the other hand a recombinant mesotrypsin preparation possessing full tryptic activity on synthetic substrates did not activate either Hu1Asn21 or Hu1Ile21 TG at all.

The Role of Acidic pH and Cathepsin B—In animal models of pancreatitis (22–24) abnormal co-localization of digestive enzymes and lysosomal hydrolases were observed. It was shown that cathepsin B, a lysosomal enzyme, readily activated human cationic and anionic TG *in vitro* (25). To simulate the milieu in the lysosomes, experiments were performed at pH 5.0 both in the absence and presence of cathepsin B.

In agreement with earlier observations we found that cationic TG readily autoactivates at pH 5.0 (9, 25, 26, 28). The lag time of the autoactivation was considerably shorter than at pH 8.0, both in the presence and absence of Ca^{2+} (Fig. 6). Pancreatitis-associated Hu1Ile21 TG autoactivated about twice as fast as the wild type form. Fig. 6 also shows, that when autoactivation was followed in the presence of 15% (mole/mole) hPSTI, simulating the condition in the pancreatic juice, no activation was observed.

Fig. 7 shows the activation of Hu1Asn21 and Hu1Ile21 TG with cathepsin B at pH 5.0. There was no lag phase, and the activation was very fast. The presence of hPSTI was not able to prevent the activation. Obviously hPSTI does not inhibit cathepsin B. Comparing the slopes of the linear phases of activation (between 2 and 8 min) in the presence of hPSTI, Hu1Ile21 TG was activated about 2.5–3 times faster than Hu1Asn21 TG.

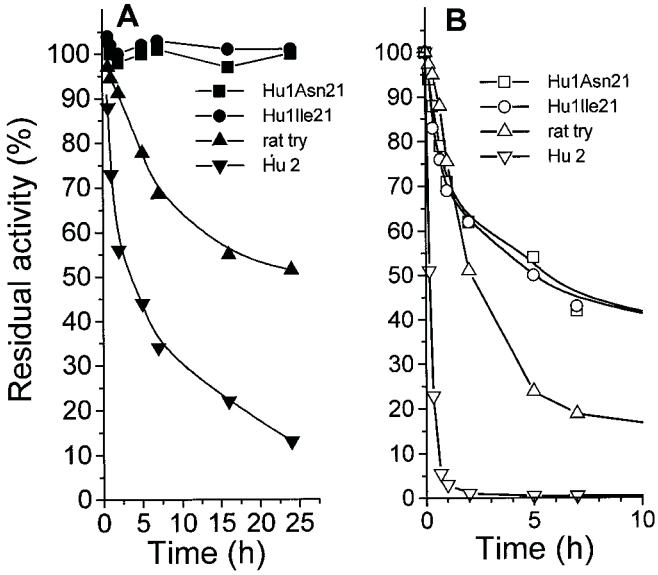

FIG. 3. **Autolysis of trypsins.** 1 μM Hu1Asn21 trypsin (*square*), Hu1Ile21 trypsin (*circle*), rat anionic trypsin (*up triangle*), or human anionic trypsin (*down triangle*) was incubated at 37 °C in 0.1 M NaCl, 50 mM Tris-HCl in the presence of: *A*, 20 mM $CaCl_2$; or *B*, 1 mM EDTA. At the indicated time points 5-μl aliquots were assayed for trypsin activity as described under "Experimental Procedures."

DISCUSSION

Our comparative studies on native human cationic trypsin and the unmodified native and recombinant forms show that the modification does alter neither the amidolytic nor the esterolytic activity of the enzyme (Table I). At the same time hPSTI inhibited modified trypsin slightly better than non-modified trypsin (Table II). This is in agreement with the results of modeling the interaction between human cationic trypsin and hPSTI (10) but it also indicates, that the contribution of the modifying group to the stability of the complex is not very significant.

The fact that non-modified native trypsin and the two recombinant forms Hu1Asn21 and Hu1Ile21 have identical enzymic activities and inhibition constants indicates that our refolding method results in properly folded enzymes. It also justifies the use of recombinant enzymes in studies to clarify the eventual role of the N21I and other mutations in the pathomechanism of hereditary pancreatitis.

A hypothesis explaining the harmful effects of the N21I mutation was based on the x-ray structure of human cationic trypsin (10). Modeling indicated a possibility to form a salt bridge between Glu^{24} and Arg^{117} as a consequence of the Asn to Ile mutation at position 21. This salt bridge, according to the hypothesis, would render Arg^{117} resistant to autolytic degra-

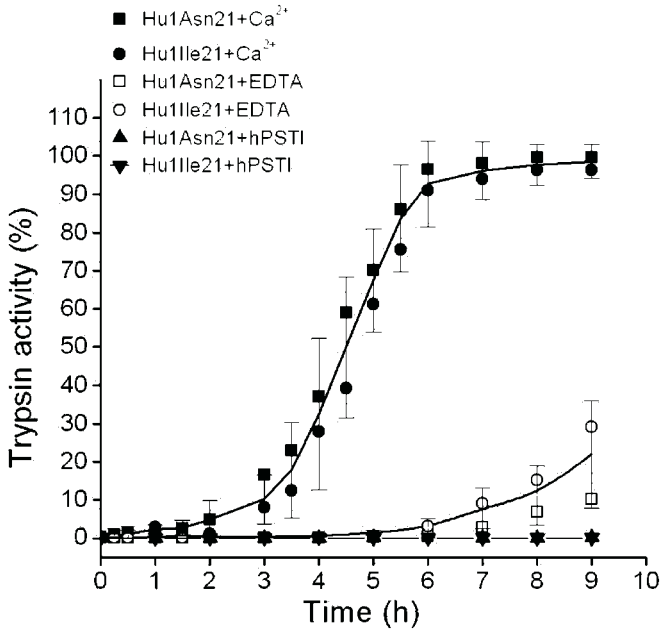

FIG. 4. **Autoactivation of trypsinogens at pH 8.0.** 5 μM Hu1Asn21 trypsinogen (square) or Hu1Ile21 trypsinogen (*circle*) was incubated at 37 °C in 0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, in the presence of 20 mM $CaCl_2$ (*closed symbols*) or 1 mM EDTA (*open symbols*). Incubations were also performed in the presence of 0.8 μM hPSTI: *up triangle*, Hu1Asn21+hPSTI; *down triangle*, Hu1Ile21+hPSTI. *Continuous lines* and *error bars* represent the average and S.E., respectively, of 10 independent experiments; *symbols* show one series of experiment. At the indicated time points 5-μl aliquots were assayed for trypsin activity as described under "Experimental Procedures." Activity was expressed as percentage of potential total activity determined by rapid enterokinase activation.

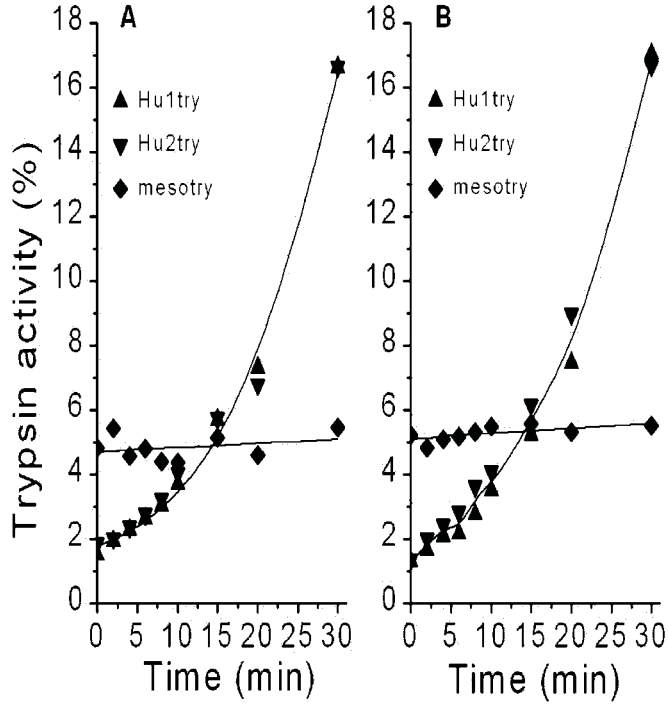

FIG. 5. **Activation of zymogens by trypsins.** 5 μM Hu1Asn21 trypsinogen (*A*) or Hu1Ile21 trypsinogen (*B*) was activated at 37 °C with 75 nM human cationic trypsin (*up triangle*), 75 nM human anionic trypsin (*down triangle*), and 250 nM mesotrypsin (*diamond*) in the presence of 0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, 20 mM $CaCl_2$. At the indicated time points 5-μl aliquots were assayed for trypsin activity as described under "Experimental Procedures." Activity was expressed as percentage of potential total activity determined by rapid enterokinase activation.

dation (4, 5). Our experiments as well as a recent observation (9) show that there is no difference between the autolytic stability of wild type and mutant trypsin. Both forms are surprisingly stable, and we observed no cleavage at Arg^{117} in the presence of Ca^{2+}. Thus, our results do not support the hypothesis that the N21I mutation renders cationic human trypsin resistant to autolysis.

Besides the loss of the natural protection presented by self-degradation, another possibility to consider is the premature activation of TG either autocatalytically or by other proteinases. At pH 8 we find no difference between the autocatalytic activations of wild type and mutant zymogens (Fig. 4). At pH 5, where the autocatalytic activation of cationic TG is known to be faster than at pH 8 (9, 25–27) there is a difference between the two forms (Fig. 6). Pancreatitis-associated Hu1Ile21 TG exhibited autoactivation about twice as fast as wild type TG. In a recent paper (9) it was suggested that this enhanced autoactivation of Hu1Ile21 TG in acidic environment might play a role in the pathomechanism of hereditary pancreatitis. On the other hand, when autoactivation experiments were performed in the presence of a physiological amount of hPSTI (Figs. 4 and 6), we observed no activation of trypsinogens at either pH. At pH 8 the inhibition constants of both enzymes are in the nanomolar range and they increase by about 50–70-fold at pH 5 (Table II). Our experiments show that even this weaker inhibition provides sufficient protection against autocatalytic activation. It is noteworthy that recent animal experiments demonstrate parallel regulation of trypsinogen and pancreatic secretory trypsin inhibitor expression (28). We have no data regarding the expression of hPSTI in hereditary pancreatitis patients bearing the N21I mutation, but it is highly unlikely that this mutation in the cationic trypsinogen gene would alone disrupt this concerted regulation. On the other hand, our data

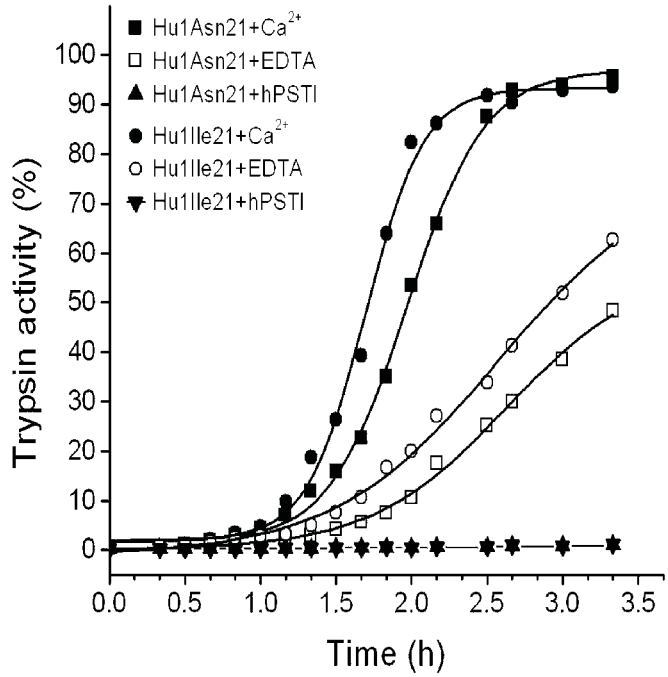

FIG. 6. **Autoactivation of trypsinogens at pH 5.0.** 5 μM Hu1Asn21 trypsinogen (*square*) or Hu1Ile21 trypsinogen (*circle*) was incubated at 37 °C in 0.1 M KOAc, pH 5.0, in the presence of 20 mM $CaCl_2$ (*closed symbols*) or 1 mM EDTA (*open symbols*). Incubations were also performed in the presence of 0.8 μM hPSTI: *up triangle,* Hu1Asn21+hPSTI; *down triangle,* Hu1Ile21+hPSTI. At the indicated time points, 5-μl aliquots were assayed for trypsin activity as described under "Experimental Procedures." Activity was expressed as percentage of potential total activity determined by rapid enterokinase activation.

presented here suggest that zymogen autoactivation experiments performed in the absence of the naturally occurring inhibitor (8, 9, 29) might be relevant only if we assumed that autoactivation of trypsinogen would occur in a compartment of the acinar cell that does not also contain inhibitor. Such a scenario, however, could not be a physiological one, because it is unlikely that trypsinogen and trypsin inhibitor would follow different secretory pathways. One might consider this as a consequence of the mutation. Nevertheless, earlier experiments demonstrated that the first 12 amino acids of rat trypsinogen were not essential for sorting to the regulated secretory pathway in transformed mouse anterior pituitary tumor cell line AtT-20 (30). Unfortunately, trypsin used in those experiments was truncated after position 19, so we cannot exclude the possibility that a mutation at position 21 could still induce missorting of Hu1Ile21 TG. Such a possibility seems to be unlikely.

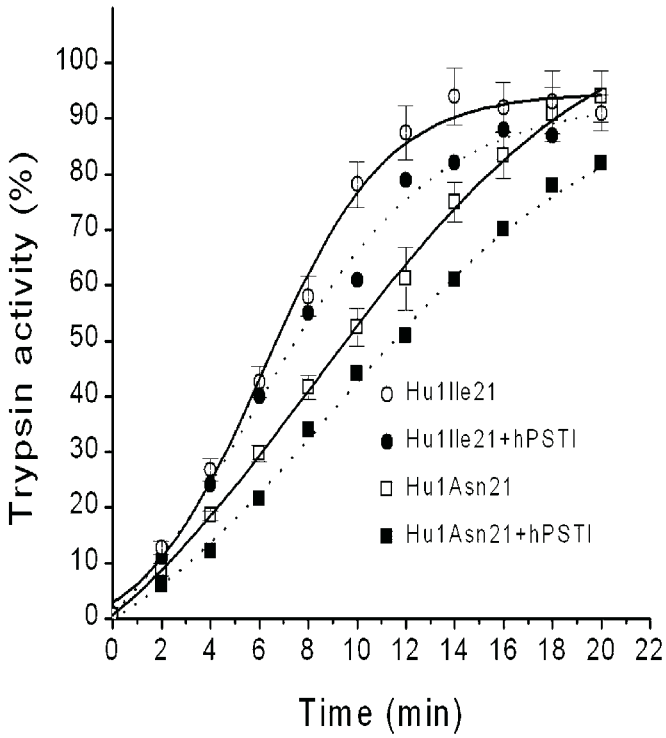

FIG. 7. **Activation of trypsinogens by cathepsin B.** 5 μM Hu1Asn21 trypsinogen (*square*) or Hu1Ile21 trypsinogen (*circle*) was activated with human cathepsin B in 0.1 M KOAc pH 5.0 at 37 °C. Human cathepsin B (from Sigma) was dissolved in 0.1 M KOAc, pH 5.0, 5 mM L-cysteine, and 2 mM EDTA to make a 25 units/ml stock solution. To initiate digestion aliquots containing 0.1 unit of cathepsin B was added to 200 μl of trypsinogen solution. At the indicated time points 5-μl aliquots were assayed for trypsin activity as described under "Experimental Procedures." Activity was expressed as percentage of potential total activity determined by rapid enterokinase activation.

Fig. 5 models the situation when free trypsin together with the zymogen is present in the pancreas. Human pancreatic juice contains three trypsin isoenzymes, cationic trypsin, anionic trypsin, and mesotrypsin (31, 32). Cationic and anionic trypsin activate both the wild type and the mutant trypsinogen equally well, while mesotrypsin does not activate trypsinogen at all. This later observation is especially important, because it is well documented, that mesotrypsin cannot be inhibited with hPSTI (32, 33) so theoretically this enzyme might break through the protective barrier of hPSTI.

Experiments shown in Fig. 7 represent another possibility of trypsinogen activation in the presence of hPSTI. It has long been known that cathepsin B activates trypsinogen (25). Here we show that a significant difference, an about 3-fold increase in the rate of activation in favor of Hu1Ile21 TG compared with Hu1Asn21 TG can be observed when cathepsin B is the activator at pH 5. The process is extremely fast, and hPSTI cannot prevent zymogen activation. For such an accidental activation to occur we do not have to suppose a derailment of the sorting mechanism, because mature cathepsin B was shown to be present not only in the lysosomes of adult rat acinar cells but also in the condensing vacules of the trans-Golgi system, the zymogen granules, and the pancreatic juice of the intralobal ducts (34).

Previous works (8, 9, 29) suggested that the enhanced autoactivation at pH 5, as a result of a gain-of-function mutation, was a common mechanism in the pathological accumulation of active trypsin in hereditary pancreatitis. Here we demonstrate, however, that the presence of human secretory trypsin inhibitor completely prevents the autoactivation of both the native and the mutant trypsinogen. At the same time we show that cathepsin B, a lysosomal enzyme, preferentially activates the pancreatitis-associated Hu1Ile21 zymogen even in the presence of human secretory trypsin inhibitor. Thus, our *in vitro* study on a hereditary pancreatitis-associated trypsinogen provides evidence for the notion that intracellular and/or extracellular activation of the digestive enzymes by cathepsin B (or by an enzyme which cannot be inhibited by hPSTI) may play a crucial role in the pathomechanism of different forms of human pancreatitis. We strongly suggest, however, subsequent studies on more complex biological systems to further clarify the role of trypsinogen mutations in the pathomechanism of hereditary pancreatitis. Recent findings on the mechanism of regulated apical secretion of zymogens in rat pancreas open new possibilities in this field (35).

TABLE II
Inhibition constants of native and recombinant human cationic trypsins for hPSTI

		K_i[a]	k_{on}	k_{off}	K_d[b]
		nM^{-1}	$M^{-1}\,s^{-1}$	s^{-1}	nM^{-1}
Native modified cationic trypsin	(pH8)	0.89 ± 0.05	(2.16 ± 0.1) + 10^6	(1.8 ± 0.1) + 10^{-3}	0.77 ± 0.06
Native non-modified cationic trypsin	(pH8)	1.45 ± 0.09	(7.90 ± 0.2) + 10^5	(1.3 ± 0.18) + 10^{-3}	1.62 ± 0.23
Hu1 Asn21	(pH8)	1.85 ± 0.07	(5.82 ± 0.31) + 10^5	(1.07 ± 0.28) + 10^{-3}	1.84 ± 0.49
Hu1 Asn21	(pH7)	ND[c]	(7.11 ± 0.37) + 10^5	(1.87 ± 0.24) + 10^{-3}	2.63 ± 0.35
Hu1 Asn21	(pH6)	ND	(6.88 ± 0.21) + 10^5	(5.53 ± 0.33) + 10^{-3}	8.05 ± 0.54
Hu1 Asn21	(pH5)	ND	(1.26 ± 0.08) + 10^6	0.25 ± 0.03	126 ± 17
Hu1Ile21	(pH8)	1.99 ± 0.1	(6.22 ± 0.39) + 10^5	(1.17 ± 0.19) + 10^{-3}	1.89 ± 0.33
Hu1Ile21	(pH7)	ND	(4.11 ± 0.25) + 10^5	(9.37 ± 0.11) + 10^{-4}	2.28 ± 0.14
Hu1Ile21	(pH6)	ND	(8.85 ± 0.18) + 10^5	(6.28 ± 0.54) + 10^{-3}	7.1 ± 0.63
Hu1Ile21	(pH5)	ND	(1.41 ± 0.17) + 10^6	0.14 ± 0.04	101 ± 31

[a] Values measured by the equilibrium method (15).
[b] $K_d = k_{off}/k_{on}$, rate constants were obtained by progress curve analysis. For further details see "Experimental Procedures."
[c] ND, not determined.

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REFERENCES

1. Whitcomb, D. C., Gorry, M. C., Preston, R. A., Furey, W., Sossenheimer, M. J., Ulrich, C. D., Martin, S. P., Gates, L. K., Jr., Amann, S. T., Toskes, P. P., Liddle, R., McGrath, K., Uomo, G., Post, J. C., and Ehrlich, G. D. (1996) *Nat. Genet.* **14,** 141–145
2. Gorry, M. C., Gabbaizedeh, D., Furey, W., Gates, L. K., Jr., Preston, R. A., Aston, C. E., Zhang, Y., Ulrich, C., Ehrlich, G. D., and Whitcomb, D. C. (1997) *Gastroenterology* **113,** 1063–1068
3. Teich, N., Ockenga, J., Hoffmeister, A., Manns, M., Mossner, J., and Keim V. (2000) *Gastroenterology* **119,** 461–5
4. Nishimory, I., Kamakura, M., Fujikawa-Adachi, K., Morita, M., Onishi, S., Yokohama, K., Makino, I., Ishida, H., Yamamoto, M., Watanabe, S., and Ogawa, M. (1999) *Gut* **44,** 259–263
5. Whitcomb, D. C. (1999) *Gut* **45,** 317–322
6. Várallyay, É., Pál, G., Patthy, A., Szilágyi, L., and Gráf, L. (1998) *Biochem. Biophys. Res. Commun.* **243,** 56–60
7. Sahin-Tóth, M., Gráf, L., and Tóth, M. (1999) *Biochem. Biophys. Res. Commun.* **264,** 505–508
8. Sahin-Tóth, M. (1999) *J. Biol. Chem.* **274,** 29669–29704
9. Sahin-Tóth, M. (2000) *J. Biol. Chem.* **275,** 22750–22755
10. Gabriaud, C., Serre, L., Guy-Crotte O., Forest, E., and Fontecilla-Camps, J-C. (1996) *J. Mol. Biol.* **259,** 995–1010
11. Graf, L., Craik, C. S., Patthy, A., Roczniak, S., Fletterick, R. J., and Rutter, W. J. (1987) *Biochemistry* **26,** 2616–2623
12. Sarkar, G., and Sommer, S. S. (1990) *BioTechniques* **8,** 404–7
13. Coleman, P. L., Latham, H. G., Jr., and Shaw, E. N. (1976) *Methods Enzymol.* **45,** 12–26
14. Dang, Q. D., and Frieden, C. (1997) *Trends Biochem. Sci.* **22,** 317
15. Empie, M. W., and Laskowski, M. (1982) *Biochemistry* **21,** 2274–2284
16. Frieden, C., Kurz, L. C., and Gilbert, H. R. (1980) *Biochemistry* **19,** 5303–5309
17. Feinstein G., Hofstein, R., Koifman, J., and Sokolovsky, M. (1974) *Eur. J. Biochem.* **43,** 569–581
18. Colomb, E., Guy, O., Deprez, P., Michel, R., and Figarella, C. (1978) *Biochim. Biophys. Acta* **525,** 186–193
19. Guy-Crotte, O., Amouric, M., and Figarella, C. (1984) *Biochem. Biophys. Res. Commun.* **125,** 516–23
20. Orsini G., and Goldberg, M. E. (1978) *J. Biol. Chem.* **253,** 3453–3458
21. Rinderknecht, H. (1993) in *The Pancreas: Biology, Pathobiology, and Disease* (Go, V. L. W., DiMagno, E. P., and Gardner, J. D., eds) 2nd Ed., pp. 219–251, Raven, New York
22. Koike, H., Steer, M. L., and Meldolesi, J. (1982) *Am. J. Physiol.* **242,** 297–307
23. Steer, M. L., Meldolesi, J., and Figarella, C. (1984) *Dig. Dis. Sci.* **29,** 934–938
24. Watanabe, O., Bacciono, F. M., Steer, M. L., and Meldolesi, J. (1984) *Am. J. Physiol.* **246,** 457–467
25. Figarella, C., Miszczuk-Jamska, B., and Barret, A. J. (1988) *Biol. Chem. Hoppe-Seyler* **369,** 293–298
26. Brodrick, J. W., Largman, C., Johnson, J. H., and Geokas, M. C. (1978) *J. Biol. Chem.* 2732–2736
27. Ohta, T., Tajima, H., Fushida, S., Kitagawa, H., Kayahara, M., Nagakawa, T., Miwa, K., Yamamoto, M., Numata, M., Nakamura, Y., Kitamura, Y., and Terada, T. (1998) *J. Mol. Med.* **1,** 689–692
28. Graf, R., Valeri, F., Gassmann, R., Hailemariam, S., Frick, T. W., and Bimmler D. (2000) *Pancreas* **21,** 181–190
29. Sahin-Tóth, M., and Tóth, M. (2000) *Biochem. Biophys. Res. Commun.* **278,** 286–289
30. Burgress, T. L., Craik C. C., Matsuuchi, L., and Kelly, R. B. (1987) *J. Cell Biol.* **105,** 659–668
31. Rinderknecht, H., Renner, I. G., and Carmack, C. (1979) *Gut* **20,** 886–891
32. Rinderknecht, H., Renner, I. G., Abramson, S. V., and Carmack, C. (1984) *Gastroenterology* **86,** 681–692
33. Nyarahucha, C. N. M., Kito, M., and Fukuoka, S.-I. (1997) *J. Biol. Chem.* **272,** 10573–10578
34. Tooze, J., Hollinhead, M., Hensel, G., Kern, H. F., and Hoflack, B. (1991) *Eur. J. Cell Biol.* **56,** 187–200
35. Schmidt, K., Schrader, M., Kern, H. F., and Kleene R. (January 10, 2001) J. *Biol. Chem.* 10.1074/jbc. M006221200