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Presence of Cathepsin B in the Human Pancreatic Secretory Pathway and Its Role in Trypsinogen Activation during Hereditary Pancreatitis*

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The lysosomal cysteine protease cathepsin B is thought to play a central role in intrapancreatic trypsinogen activation and the onset of experimental pancreatitis. Recent *in vitro* studies have suggested that this mechanism might be of pathophysiological relevance in hereditary pancreatitis, a human inborn disorder associated with mutations in the cationic trypsinogen gene. In the present study evidence is presented that cathepsin B is abundantly present in the secretory compartment of the human exocrine pancreas, as judged by immunogold electron microscopy. Moreover, pro-cathepsin B and mature cathepsin B are both secreted together with trypsinogen and active trypsin into the pancreatic juice of patients with sporadic pancreatitis or hereditary pancreatitis. Finally, cathepsin B-catalyzed activation of recombinant human cationic trypsinogen with hereditary pancreatitis-associated mutations N29I, N29T, or R122H were characterized. In contrast to a previous report, cathepsin B-mediated activation of wild type and all three mutant trypsinogen forms was essentially identical under a wide range of experimental conditions. These observations confirm the presence of active cathepsin B in the human pancreatic secretory pathway and are consistent with the notion that cathepsin B-mediated trypsinogen activation might play a pathogenic role in human pancreatitis. On the other hand, the results clearly demonstrate that hereditary pancreatitis-associated mutations do not lead to increased or decreased trypsinogen activation by cathepsin B. Therefore, mutation-dependent alterations in cathepsin B-induced trypsinogen activation are not the cause of hereditary pancreatitis.

Hereditary pancreatitis is an inborn variety of acute and chronic pancreatitis that is most commonly associated with germ line mutation in the *PRSS1* gene encoding cationic trypsinogen (1). Several trypsinogen mutations (but as of today, no mutations in any of the other digestive enzymes) have been found to be associated with the disease phenotype. The mutations affect different regions of the gene and confer single amino acid substitutions such as A16V (2), D22G (3), K23R (4), N29I (4–6), N29T (7), R122H (1), or R122C (7–9). The cellular mechanisms through which these mutations trigger the onset of pancreatitis are still a matter of debate (for review, see Refs. 10 and 11). Intuitively, the mutations might lead to a gain of trypsin function either by increased intrapancreatic trypsinogen activation or by an extended activity of trypsin due to impaired inactivation. Trypsin would then, in analogy to the conditions in the small intestine, activate other digestive proteases in a cascade-like fashion and, thus, mediate acinar cell injury. Indeed, investigations into the biochemical properties of hereditary pancreatitis-associated trypsinogen mutants unraveled both possible mechanisms, increased autocatalytic activation (autoactivation) of trypsinogen and decreased autocatalytic degradation (autolysis) of trypsin (3, 8, 12–15). Enhanced autoactivation has been shown for several mutations (D22G, K23R, N29I, N29T, R122H), whereas increased trypsin stability was found for the N29T, R122H, and R122C mutations. In contrast, the clinically relevant and rather common N29I mutation has no effect on trypsin autolysis (12–15), and mutations located within the activation peptide region (A16V, K23R, D22G) would also not affect the stability of active trypsin. It is therefore likely that increased trypsin stability is either an accessory pathway that is present in only certain varieties of hereditary pancreatitis or simply a biochemical epiphenomenon. More recent experiments demonstrated that the surface loop containing Arg-122 in cationic trypsinogen is a low affinity inhibitor of trypsin and might play an important role in stabilizing intrapancreatic trypsinogen against autoactivation, particularly at high zymogen concentrations (16). Loss of this inhibitory activity due to the R122H mutation might also increase autoactivation of trypsinogen and contribute to the development of hereditary pancreatitis associated with this mutation.

A number of reports have challenged the gain-of-function hypothesis and suggested that a loss of trypsin function might impair the inactivation of other digestive enzymes (8, 17). Support for this idea comes from the observation that recombinant trypsinogen carrying the R122C mutation exhibits reduced activability (8) and that experiments in isolated pancreatic acini and lobules have shown that intracellular trypsin activity

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is neither required nor involved in trypsinogen activation, and its most prominent role is in autodegradation (17). 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 (10). All investigators, however, agree that a premature and intrapancreatic activation of trypsinogen is an early event in the course
of pancreatitis and is critically involved in the disease onset (1, 10, 11, 14, 18, 19).

The lysosomal cysteine protease cathepsin B was also shown capable of activating bovine (20) or human (21) trypsinogen *in vitro*. Furthermore, several studies employing animal models of the disease (22) or cathepsin B-deficient strains of mice (23) demonstrate that cathepsin B plays a critical role in intrapancreatic trypsinogen activation and the onset of experimental pancreatitis. Whether these observations have any relevance for the human pancreas or for the onset of pancreatitis associated with trypsinogen mutations is unknown. In this respect, recent experiments with recombinant human cationic trypsinogen demonstrate that cathepsin B activates the N29I mutant almost 3-fold faster than wild-type trypsinogen, indicating that a pathogenic role for cathepsin B in hereditary pancreatitis is a distinct possibility (15). On the other hand, several conditions need to be met before such a mechanism could be regarded of pathophysiological relevance. (i) Cathepsin B has to be intracellularly sorted into the secretory pathway, where it can interact with digestive pro-enzymes. This is clearly the case in the rat (24) and rabbit (25) pancreas, but it is unknown whether it occurs in the human pancreas. (ii) To activate protease zymogens, cathepsin B has to be present in the secretory compartment as mature, active protease. (iii) To play a relevant role in hereditary pancreatitis, cathepsin B has to activate recombinant trypsinogen with hereditary pancreatitis-associated mutations differently from wild-type trypsinogen. Increased susceptibility of trypsinogen mutants to cathepsin B activation would be consistent with the gain-of-function model, whereas decreased activation by cathepsin B would support the loss-of-function hypothesis. In the present study we have addressed all three issues and found that although cathepsin B is clearly sorted into the secretory compartment of the exocrine pancreas and secreted as an active enzyme, cathepsin B-induced trypsinogen activation is not affected by any of the three hereditary pancreatitis-associated trypsinogen mutations (N29I, N29T, R122H) we have investigated.

EXPERIMENTAL PROCEDURES

Materials—Cathepsin B was purchased from Calbiochem. This commercial preparation was purified from human liver by high performance liquid chromatography (catalog #219362, lot #B28549; 1.136 mg/ml protein concentration, 22 units/mg protein specific activity, in 20 mM sodium acetate (pH 5.0) and 1 mM EDTA; purity >95%). Where indicated, experiments were also carried out with recombinant human cathepsin B, expressed in sf9 insect cells, and purified as described previously (26). This enzyme preparation exhibited ~30% lower specific activity for human trypsinogen substrate than commercial cathepsin B purified from human liver. Before use, cathepsin B was activated with 1 mM dithiothreitol (DTT,[1] final concentration) for 30 min on ice. Benzamidine-HCl and *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide were from Sigma, bovine enterokinase was from Biozyme Laboratories (San Diego, CA), $[CBZ\text{-}Ile\text{-}Pro\text{-}Arg]_2$-rhodamine 110 and CBZ-Arg-Arg-aminomethylcoumarin was from Molecular Probes (Eugene, OR), reagent grade bovine serum albumin (BSA) was from Biocell Laboratories (Rancho Dominguez, CA), E64d (l-*trans*-epoxysuccinyl-Leu-3-methylbutylamide-ethyl-ester, lot 0542851) was from Bachem (Heidelberg, Germany), and AEBSF (pefabloc, lot B36440) was from Calbiochem. For the detection of cathepsin B three different antibodies were used: AB1, polyclonal cathepsin B antibody, which was a kind gift of Drs. H. Kirschke and E. Weber (Halle/Saale, Germany); AB2, polyclonal (catalog #PC41); and AB3, monoclonal (catalog #IM27L) cathepsin B antibodies obtained from Oncogene Research Products (San Diego, CA). A monoclonal antibody directed against anionic trypsinogen was generated as previously described (27). Enzymatic deglycosylation kit #170-6500 was purchased from Bio-Rad.

Nomenclature—Amino acid residues in the human cationic trypsinogen sequence were denoted according to their actual position in the native, wild-type preproenzyme. A comparison of this numbering system to the classic chymotrypsin numbering can be found in (28).

Ultrastructural Localization of Cathepsin B and Trypsinogen—To study the subcellular distribution of cathepsin B in relation to digestive enzymes in the exocrine pancreas, human pancreatic tissue and juice were used with informed patient consent and permission of the Ethics Committees of the Universities of Rostock or Münster. Small blocks of pancreas tissue from organ donors after explantation were fixed in iced 1.5% glutaraldehyde solution and embedded in LR-White resin. Thin sections were subsequently double-labeled as previously reported (29, 30) with polyclonal antibody directed against cathepsin B (AB1, final concentration 200 μg/ml) and monoclonal antibody directed against anionic trypsinogen (final concentration, 100 μg/ml). Antigen detection was achieved with specific secondary antibody coupled to immunogold particles of 15 nm for cathepsin B and of 5 nm for trypsinogen. Control sections were incubated with antibody that had been preincubated with an excess of human cathepsin B or trypsin, respectively, for 2 h at room temperature. Sections were stained with uranyl acetate for better contrast and viewed on a Phillips EM 10 or EM 109 electron microscope. To determine the relative content of cathepsin B in secretory granules and lysosomes, the surface labeling over cross-sections of 100 randomly selected organelles was evaluated at high magnification, and the absolute number of 15-nm gold particles was quantitated. Both types of organelles were identified by their typical morphological appearance, and the presence of trypsin in secretory granules and its complete absence in primary lysosomes served as an additional characteristic. Only secretory vesicles with a diameter of >1 μm were used for quantitation to ensure sectioning through the approximate center of the organelle and to avoid underestimation of the cathepsin B content.

Assay of Cathepsin B and Trypsin Activity in Pancreatic Juice—To establish whether cathepsin B and trypsinogen are secreted by the human exocrine pancreas and appear in the secretory compartment of patients with sporadic pancreatitis or hereditary chronic pancreatitis, informed consent was obtained to collect pancreatic juice during endoscopic retrograde cholangio-pancreatography (ERCP), a clinical imaging procedure during which the main pancreatic duct is selectively cannulated and collection of pure pancreatic juice is feasible. Two of the patients with chronic pancreatitis carried none of the known gene mutations associated with hereditary pancreatitis, whereas two others were both carriers of the R122H mutation. In the aliquots of pure pancreatic juice, spontaneous trypsin activity as well as enterokinase-activated (0.001 units/ml, 60 min, 37 °C) trypsin activity was determined using the specific fluorogenic substrate $[CBZ\text{-}Ile\text{-}Pro\text{-}Arg]_2$-rhodamine 110 in 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$, 10 μM substrate (final concentrations), and 0.1 μg of juice protein in a 150-μl final volume at an excitation wavelength of 485 nm and an emission wavelength of 530 nm at 37 °C. Initial rates of substrate hydrolysis were measured in arbitrary fluorescence units/min, as previously described (31, 32). Enzyme activity was then compared with the activity of bovine trypsinogen (Calbiochem #6502, lot B27456, 3194 units/mg) activated with enterokinase (0.001 units/ml, 60 min at 37 °C) as standard, and activity was expressed as units/mg juice protein. Cathepsin B activity in pancreatic juice samples was determined in 0.25 M sodium acetate buffer (pH 5.0) containing 2 mM EDTA, 1 mM DTT, 10 μM specific substrate CBZ-Arg-Arg-aminomethylcoumarin (final concentrations), and 1 μg of juice protein in a final volume of 150 μl at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Where indicated, the serine protease inhibitor pefabloc (0.1 mM final concentration) or the cathepsin B inhibitor E64d (10 mM final concentration) were included in the assays. Cleavage of the cathepsin B substrate was measured for 60 min in a microplate fluorescence reader (SPECTRAmax GEMINI, Molecular Devices, Sunnyvale, CA), compared with the activity of human cathepsin B (Calbiochem, see above) as standard, and expressed in units/mg of juice protein. To demonstrate the presence of pro-cathepsin B in pancreatic juice, the pro-enzyme was activated to mature cathepsin B by incubating with pepsin (1 mg/ml

[1] The abbreviations used are: DTT, dithiothreitol; BSA, bovine serum albumin.

porcine stomach mucosa pepsin, Calbiochem) in 0.1 M sodium formate buffer at pH 3.0 for 1 h, as described previously (Ref. 33 and references therein).

Western Blot Analyses—Pancreatic juice samples were diluted with ice-cold buffer (50 mM HEPES (pH 7.4), 5 mM EDTA, 150 mM NaCl, protease inhibitor mixture (1 ml/mg protein, containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.01 M sodium pyrophosphate, 0.1 M NaF, 1 mM L-phenylmethylsulfonyl fluoride, and 0.02% soybean trypsin inhibitor)) and adjusted to the same volume. Pancreatic tissue and liver tissue was homogenized by 10 strokes in lysis buffer containing 0.25 M sodium acetate, 2 mM EDTA, and 1 mM DTT using a glass Dounce S homogenizer. Samples were subsequently sonicated twice for 10 s at 30% power setting and centrifuged at 14,000 rpm for 15 min at 4 °C. Protein concentration was determined according to Bradford (Bio-Rad), and equal amounts of protein were used in subsequent experiments. Samples were boiled for 5 min in 4× SDS sample buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol, 0.2% bromphenol blue, 12% SDS, 5% mercaptoethanol) and electrophoresed on 12 or 4–15% SDS-polyacrylamide gels (15 μg of protein/lane, Bio-Rad, Criterion precast gel), and proteins were blotted onto nitrocellulose membranes (Hybond C, Amersham Biosciences). After overnight blocking in 0.2% NET-gelatin (1.5 M NaCl, 0.05 M EDTA, 0.5 M Tris-HCl (pH 7.5), 0.5% Triton X-100, 0.2% gelatin) immunoblot analysis was performed with a polyclonal anti-cathepsin B antibody (AB1, 1:10,000 dilution) followed by horseradish peroxidase-coupled goat anti-rabbit IgG (1:15,000 dilution) and enhanced chemiluminescence detection (Amersham Biosciences). For immunoprecipitation, a 1:1 mixture of protein A- and G-Sepharose (Amersham Biosciences) was preincubated with cathepsin B antibody (AB3) in 20 mM HEPES at pH 7.5. Tissue homogenates were added to the pre-coupled antibody and incubated for 1 h at 4 °C on a rotor wheel. Precipitates were washed with HNTG buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and boiled for 5 min in 2× SDS sample buffer, and SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described above.

Plasmids and Mutagenesis—Construction of expression plasmids with wild-type and mutant trypsinogen genes (N29I, N29T, R122H) was previously described (12–14).

Expression and Purification of Trypsinogen—Small scale expression and *in vitro* refolding of trypsinogen was carried out essentially as previously reported (12). Concentrations of zymogen solutions were measured from their ultraviolet absorbance using a calculated extinction coefficient of 36,160 M^{-1} cm^{-1} at 280 nm. Unless otherwise indicated, the activation peptide sequence of recombinant zymogen preparations used in this study was Met-Ala-Pro-Phe-$(Asp)_4$-Lys.

Cathepsin B Activation of Recombinant Trypsinogen—The standard activation mixture (50 μl) contained 2 μM cationic trypsinogen, 2 mg/ml BSA, 1 mM K-EDTA, 300 μM benzamidine, and 0.1 M sodium acetate buffer at the indicated pH (final concentrations). This mixture was preincubated at 37 °C for 1 min, and the reaction was initiated by adding 2 μl of activated cathepsin B solution. Incubations were carried out at 37 °C, and at the indicated times, 2.5-μl aliquots were removed for trypsin activity assays. Trypsin activity was determined using the synthetic chromogenic substrate *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide (200 μM final concentration) at 405 nm in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, at 22 °C.

RESULTS

Presence of Cathepsin B in the Secretory Compartment of the Human Pancreas—First, the specificity of the cathepsin B and trypsin antibodies used for the immunological detection of these enzymes was characterized. We compared two polyclonal (AB1 and AB2 in Fig. 1*A*) and one monoclonal (AB3 in Fig. 1*A*) anti-human cathepsin B antibody preparations in Western blot and immunoprecipitation experiments with homogenized human pancreatic or liver tissue (Fig. 1*A*). All three antibodies detected two specific bands in pancreatic tissue, and the same two bands were immunoprecipitated from pancreatic or liver homogenates. On the basis of their mobility, the 47- and 32-kDa bands were identified as pro-cathepsin B and the single-chain form of mature cathepsin B, respectively. Subjecting the immunoprecipitated material to enzymatic deglycosylation (Bio-Rad) did not alter the mobility of the two bands, indicating that they do not represent differently glycosylated forms of mature cathepsin B (not shown). Furthermore, treatment of pancreatic homogenates with pepsin, which converts pro-cathepsin B to mature cathepsin B (see Ref. 33 and references therein), resulted in a 1.5–2-fold increase in cathepsin B activity, confirming the presence of inactive pro-cathepsin B (not shown). It is also noteworthy that mobility of the two immunoreactive bands from pancreas or liver homogenates was essentially identical, suggesting that these two tissues contain the same cathepsin B isoforms. The monoclonal antibody against anionic trypsinogen detected a strong band representing anionic trypsinogen and a second weaker band corresponding to anionic trypsin (Fig. 1*B*) after isoelectric focusing and immunoblotting of purified anionic trypsinogen or pancreatic juice. This monoclonal antibody does not cross-react with any other pancreatic secretory enzyme or cathepsin B and has been previously been characterized in detail (27).

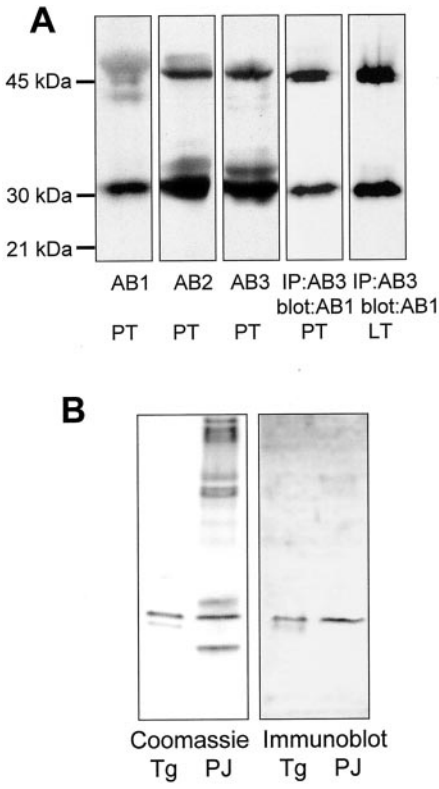

FIG. 1. **Antibody specificity for cathepsin B and anionic trypsinogen.** *Panel A*, three different antibodies (AB1 polyclonal, AB2 polyclonal, AB3 monoclonal) were used to detect cathepsin B in Western blot (*blot*) and immunoprecipitation (*IP*) studies using homogenized human pancreatic (*PT*) or liver (*LT*) tissue, as described under "Experimental Procedures." All 3 antibodies detected pro-cathepsin B (47-kDa band) and the mature single-chain form of cathepsin B (32 kDa). *Panel B*, a monoclonal antibody directed against anionic trypsinogen was used to detect anionic trypsinogen after isoelectric focusing and immunoblotting of a purified anionic trypsinogen preparation (*Tg*) or human pancreatic juice (*PJ*). Previously we found no cross-reaction with any other secretory pancreatic enzyme or cathepsin B (27). The *left lanes* in *panel B* indicate a Coomassie Blue-stained gel, and the *right lanes* show the corresponding immunoblot.

As predicted from previous animal studies, cathepsin B was detected by immunogold electron microscopy in acinar cells of the healthy human pancreas (Fig. 2). Control incubations with the AB1 anti-cathepsin B or anti-trypsinogen antibodies that had been preincubated with their respective antigens resulted in hardly any background gold decoration (Fig. 2*A*). Cathepsin B appeared in the endoplasmic reticulum (Fig. 2*B*) and in primary and secondary lysosomes (inset in Fig. 2*B*) but was detected neither in the nucleus nor in the cytosol of acinar cells. A prominent and consistent co-localization with trypsinogen was found in secretory vesicles of acinar cells (Fig. 2, *B–D*), and abundant cathepsin B was also detected in the acinar cell

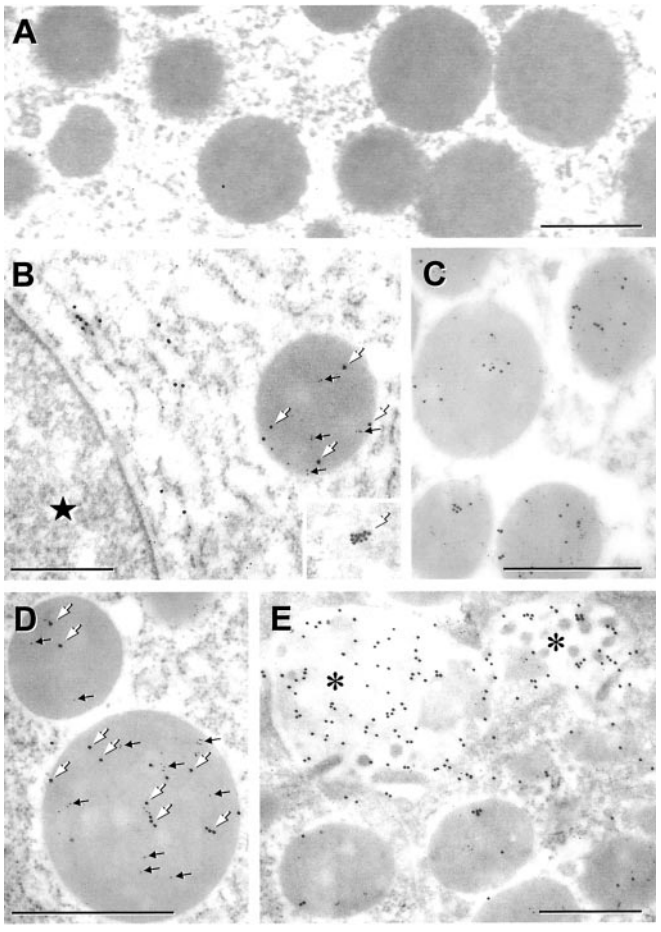

FIG. 2. **Ultrastructural localization of cathepsin B and trypsinogen.** Tissue blocks from two pancreas donors with no prior history of pancreatic disease were fixed, resin-embedded, and immunogold-labeled with antibodies directed against anionic trypsinogen (5-nm gold) and cathepsin B (15-nm gold) as described under "Experimental Procedures." Representative electron micrographs from the first donor pancreas are shown in *panels A*, *B*, and *C* and of the second in *panels D* and *E*. *Bars* indicate 1 μm. On control sections that were labeled with antibodies that had been preincubated with an excess of their respective antigens for 2 h practically no background gold decoration was detected (*panel A*). In labeled sections cathepsin B was found in the endoplasmic reticulum of acinar cells (*panel B*), in lysosomes (inset in *panel B*), and in zymogen granules where it co-localized with trypsinogen, but it was absent from the nucleus (*asterisk* in *panel B*). Co-localization of cathepsin B with trypsinogen could be consistently detected in the secretory compartment of both donor pancreata (*panels C* and *D*) and ultimately appeared in the acinar lumen (*asterisks* in *panel E*), which indicates its discharge from acinar cells. In *panels B* and *D* *white arrows* indicate cathepsin B-specific 15-nm gold labeling, and *black arrows* point at trypsin-specific 5-nm gold label.

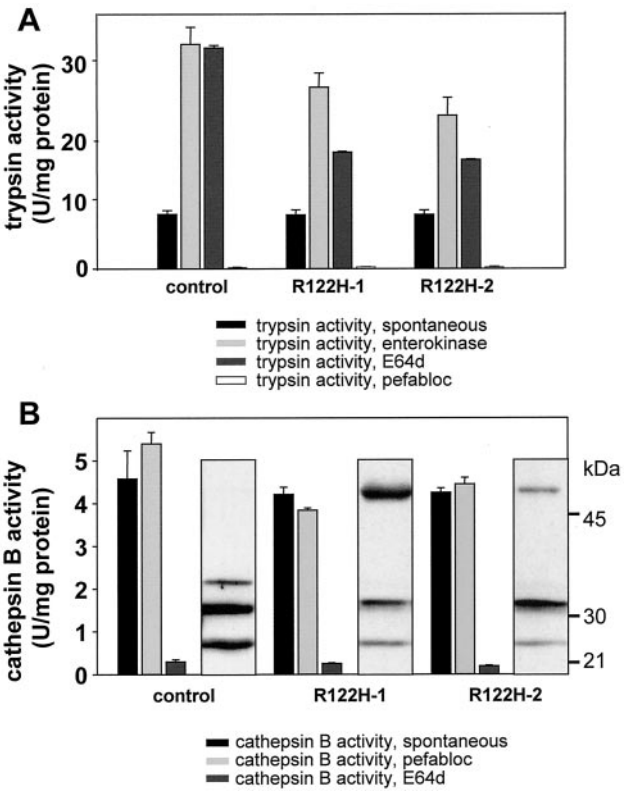

FIG. 3. **Secretion of cathepsin B into the pancreatic ducts.** In pancreatic juice from a patient with sporadic pancreatitis (*control*) and two patients with hereditary pancreatitis (R122H) the activity of cathepsin B and trypsin was determined as described under "Experimental Procedures." In *panel A black bars* indicate spontaneous trypsin activity, *light gray bars* show trypsin activity after enterokinase activation, *dark gray bars* represent trypsin activity after enterokinase activation and the addition of 10 mM E64d, and *white bars* show trypsin activity after inhibition with pefabloc. Data indicate means ± S.E. from three juice samples. In *panel B* the cathepsin B activity in pancreatic juice from the three patients is shown adjacent to Western blots of the respective juice samples. The columns indicate means ± S.E. *Black bars* represent spontaneous cathepsin B activity, whereas light *gray bars* indicate cathepsin B activity after the addition of pefabloc, and *dark gray bars* show cathepsin B activity in the presence of E64d. The Western blots were probed with polyclonal anti-cathepsin B antibody (*AB1* in Fig. 1). Specific signals were detected for pro-cathepsin B (47-kDa band), mature single-chain cathepsin B (32-kDa band), and the heavy chain of mature two-chain cathepsin B (23-kDa band). The ~36-kDa band in the control sample is nonspecific in nature and was not routinely observed in immunoblots of pancreatic juice samples. All samples were loaded at a protein concentration of 15 μg/lane. *U*, unit.

lumen (Fig. 2*E*) or the pancreatic ducts. The density of cathepsin B gold labeling over cross-sections of secretory granules and lysosomes indicated a higher relative concentration in the lysosomal pathway (21.7 ± 3.4 grains/organelle) compared with the secretory pathway (9.4 ± 0.8 grains/organelle). However, in view of the fact that within the pancreatic acinar cell secretory vesicles have a much greater relative volume and are more abundant than lysosomes, this distribution would indicate that at least 20–40% of the cellular cathepsin B is sorted into the secretory pathway under physiological conditions. This distribution would be in accordance with that found in density gradient experiments of rodent pancreas (19, 22) and indicates a highly significant physiological sorting of cathepsin B into the secretory pathway of the healthy human pancreas.

Activity measurements in pancreatic juice samples from patients with chronic pancreatitis demonstrated basal trypsin activity (Fig. 3*A*) and 2–4-fold higher amounts of inactive trypsinogen secreted from the resting pancreas. As expected, trypsin activity in pancreatic juice was inhibited by the serine protease inhibitor pefabloc but not by the cathepsin B inhibitor E64d. In some samples a measurable reduction in trypsin activity was found at high concentrations (10 mM) of E64d for reasons that are unclear at this time. All three juice samples from patients with sporadic or hereditary chronic pancreatitis contained cathepsin B activity at levels between 4.5 and 6 units/mg of protein. Cathepsin B activity was not affected by pefabloc at concentrations that completely inhibited trypsin activity. As expected, the cysteine protease inhibitor E64d (10 mM) inhibited cathepsin B activity completely (Fig. 3*B*). In the corresponding Western blots the presence of cathepsin B in pancreatic juice could be confirmed (Fig. 3*B*). In addition to the 47-kDa pro-cathepsin B and the 32-kDa single-chain mature cathepsin B bands (see also Fig. 1), the heavy chain of the two-chain mature cathepsin B migrating at 23 kDa was also detected. This band was not routinely seen in pancreatic ho-

mogenates (compare with Fig. 1), in all likelihood due to its relatively lower abundance, and was not present in a pancreatic juice sample from a second patient with sporadic chronic pancreatitis (not shown). As shown in Fig. 1, pancreatic homogenates contained both pro-cathepsin B and cathepsin B immunoreactive bands, whereas in Fig. 3 only the hereditary pancreatitis juice samples exhibited pro-cathepsin B bands. However, this banding pattern may not be typical, because a juice sample from another patient with sporadic chronic pancreatitis also exhibited low but detectable amounts of the 47-kDa pro-cathepsin B band (not shown). Clearly, a larger number of independent juice samples need to be studied in the future to determine whether or not pro-cathepsin B is preferentially secreted in certain forms of chronic pancreatitis.

Activation of Recombinant Trypsinogen by Cathepsin B at Acidic pH—The pH optimum of trypsinogen activation by cathepsin B has been reported to be 3.6 for bovine trypsinogen (20), and a similarly acidic milieu, pH 3.8, was used by Figarella *et al.* (21) in the first report on cathepsin B activation of human trypsinogen. On the other hand, Szilágyi *et al.* (15) carried out activation studies at pH 5.0, an environment that appears to mimic the actual lysosomal pH more closely (34–39). We performed our initial activation experiments at pH 4.6 and noticed that increased concentrations of trypsinogen in the Eppendorf tubes used for incubation resulted in higher than expected increases in trypsin activity. A similar effect could be achieved by the addition of BSA or other inert proteins to the reaction buffer. This suggested that trypsinogen and trypsin had bound to the wall of the plastic tubes in a nonspecific manner and that this binding could be prevented by adding excess protein. Whereas this loss of activity due to nonspecific binding was only ~25% at pH 8.0 (not shown), it became much more prominent at acidic pH and, at pH 4.6, amounted to more than 50%. Fig. 4*A* shows how the addition of increasing concentrations of BSA blocked the nonspecific trypsinogen binding and resulted in a maximal trypsin activity at or above 2 mg/ml BSA. On the basis of these results 2 mg/ml BSA was included in all subsequent experiments.

Commercial cathepsin B preparations are inactive and require activation with thiol-containing reagents. Cationic trypsinogen contains five disulfide bridges, and these might undergo dissociation due to the reducing effect of the thiols required for cathepsin B activation. To exclude this potential confounding factor we studied the effect of DTT on cathepsin B activation of cationic trypsinogen. In these experiments the cathepsin B enzyme stock was first activated with 0.2 mM DTT (final concentration), which was then diluted to 0.008 mM in the activation reactions. In addition, the incubation mixtures were supplemented with DTT to the indicated concentrations. As shown in Fig. 4*B*, full cathepsin B activity was reached in 0.008 mM DTT, and full activity was maintained up to at least a 2 mM DTT concentration. Significantly higher DTT concentrations (>10 mM) were required to inhibit the cathepsin B-induced activation of trypsinogen to any detectable extent. In the subsequent experiments we routinely used 1 mM DTT to activate the cathepsin B stock, which was then diluted to a 0.2 mM final DTT concentration in the activation mixtures.

Human cationic trypsinogen readily autoactivates at acidic pH, and all three hereditary pancreatitis mutants used in our study have been shown to exhibit a significantly increased autoactivation rate in comparison to wild-type trypsinogen (12–15). To eliminate trypsin-catalyzed trypsinogen activation (autoactivation), we included the low molecular weight trypsin inhibitor benzamidine at 300 μM final concentration in the cathepsin B activation assays. Benzamidine inhibited cationic trypsin at pH 8.0 with an inhibitory constant (K_i) of ~30 μM

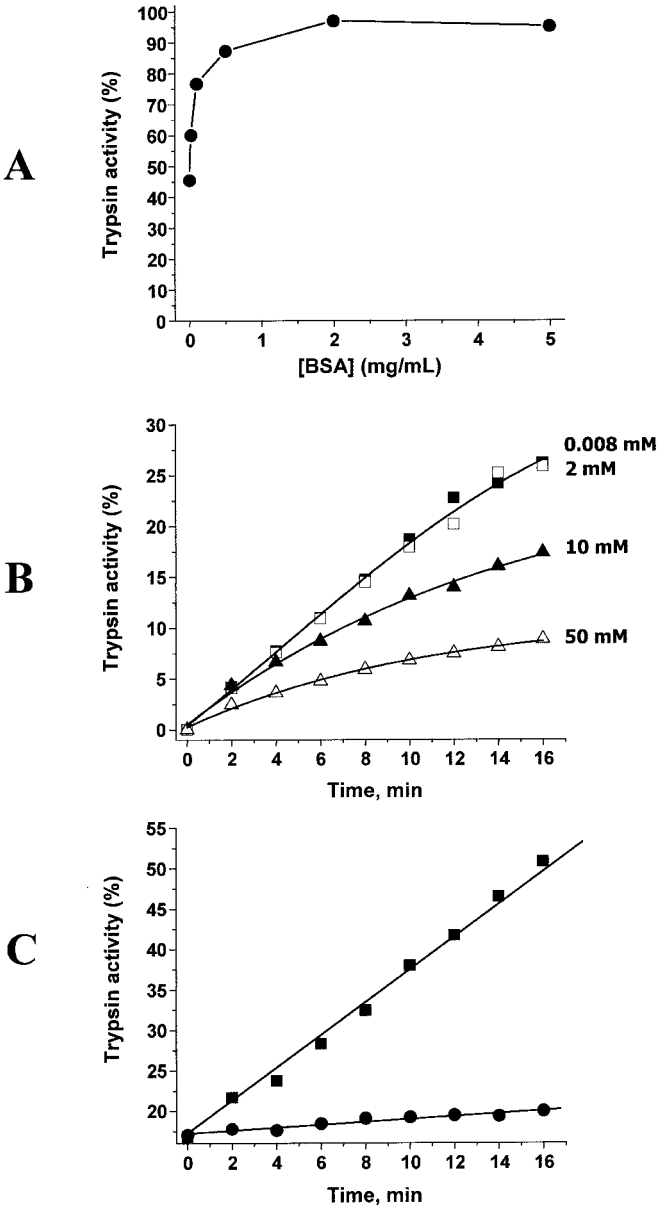

FIG. 4. **Technical aspects of assaying cathepsin B-induced trypsinogen activation at acidic pH.** In these experiments trypsin activity was determined on 2.5-μl aliquots withdrawn from the incubation mixtures at the indicated times using the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide. Trypsin activity was expressed as the percentage of maximal activity obtained by enterokinase activation at pH 8.0, 5 mM $CaCl_2$, 2 mg/ml BSA. *A*, effect of BSA on trypsin activity at pH 4.6. Aliquots of human cationic trypsinogen (2 μM) were activated with 350 ng/ml bovine enterokinase (final concentrations) in 0.1 M sodium acetate buffer (pH 4.6), 5 mM $CaCl_2$, and the indicated BSA concentrations at 22 °C for 20 min. The same results were obtained when BSA was added after enterokinase activation. See details under "Results." *B*, effect of DTT on activation of trypsinogen by cathepsin B at pH 4.6. Human cationic trypsinogen (2 μM) was activated with cathepsin B (45 μg/ml, Calbiochem) at 37 °C in 0.1 M sodium acetate buffer (pH 4.6), 2 mg/ml BSA, 1 mM K-EDTA, 300 μM benzamidine, and the indicated concentrations of DTT in a final volume of 50 μl. First, the cathepsin B stock (1.14 mg/ml) was activated with 0.2 mM DTT (final concentration) on ice for 30 min, and 2 μl of the activated enzyme was added to initiate the activation reactions. Thus, the DTT carryover from the cathepsin B additions was 0.008 mM final concentration in each sample. *C*, inhibition of trypsin-catalyzed trypsinogen activation by benzamidine at pH 4.6. Human cationic trypsinogen (2 μM) was activated with human cationic trypsin (0.2 μM) at 37 °C in 0.1 M sodium acetate buffer (pH 4.6), 2 mg/ml BSA, and 1 mM K-EDTA in the absence (■) or presence (●) of 300 μM benzamidine in a final volume of 50 μl. Note that the trypsin activity observed at 0 min (17%) represents trypsin added to initiate the activation reaction.

(not shown). We chose to use an ~10-fold K_i concentration of the inhibitor because it was expected that at a lower pH the inhibitory capacity might decrease. Fig. 4*C* demonstrates that under these conditions benzamidine almost completely inhibited trypsin-catalyzed trypsinogen activation at pH 4.6. It is important to note that the presence of 300 μM benzamidine in the activation mixtures did not interfere with the trypsin measurements for which benzamidine was diluted to a 3 μM final concentration. Benzamidine at the concentration used in our study had no inhibitory effect on cathepsin B (not shown).

Cathepsin B-induced Trypsinogen Activation Exhibits Sharp pH Dependence between pH 4.0 and 5.2—The initial cathepsin B activation assays of wild-type and mutant trypsinogens were carried out in 0.1 M sodium acetate buffer adjusted to pH 5.0. The experiments resulted in varying activation rates that appeared to depend on the enzyme preparation and the trypsinogen concentration used in individual assays. Interestingly, there appeared to be an inverse correlation between the concentration of the purified trypsinogen stock solutions and the rate of cathepsin B activation, as more dilute trypsinogen preparations resulted in higher final activities. Because purified trypsinogen was routinely stored in 50 mM HCl to prevent autoactivation, we hypothesized that a slight acidification of the assay mixtures caused by the HCl present in the trypsinogen stock could have caused the variations. We therefore determined the pH dependence of cathepsin B activation between pH 3.6 and 5.2 (Fig. 5). In these experiments increasing amounts of HCl or NaOH were added to 0.1 M sodium acetate buffer (pH 5.0), and the final pH values were calculated from the Henderson-Hasselbalch equation. Surprisingly, activation rates increased more than 100-fold when the pH was lowered from 5.2 to 4.0. An apparent pH optimum was reached at pH 4.0, and a further decline was detected at a more acidic pH. Inclusion of 50 mM NaCl in the incubation mixtures at pH 4.6 or 4.0 had no significant effect on cathepsin B activity, excluding the possibility that differences in ionic strength rather than pH caused the drastic changes observed in cathepsin B activity (not shown). Although data are not shown, no cathepsin B-mediated trypsinogen activation was detected over the pH range from 5.2 to 8.0.

Hereditary Pancreatitis-associated Mutations Do Not Affect Activation of Human Cationic Trypsinogen by Cathepsin B—When the pH of the activation assays was adjusted to identical values, no detectable difference was found in the activation rates of wild type and the three mutant trypsinogen preparations at pH 4.0 (Fig. 6*A*) or at pH 4.6 (not shown). Furthermore, when the activation was followed over longer time periods, the final levels of activity were indistinguishable in the assays for wild-type, N29I, N29T, or R122H trypsinogen (Fig. 6*B*). Analysis of progress curves in Fig. 6*B* by the KINSIM and FITSIM programs (40, 41) indicated that the K_m is ~8 μM, and the k_{cat} is ~6×10^{-3} s^{-1}. The maximal activity level approached 100% of the maximal potential trypsin activity, which was determined at pH 8.0 after enterokinase activation of identical amounts of trypsinogen. This observation confirms that the proteolytic cleavage of cationic trypsinogen by cathepsin B is restricted to the Lys-23–Ile-24 peptide bond in the activation peptide and does not involve any significant trypsinogen degradation (21). Additional experiments in which the conditions were changed to omit BSA and/or EDTA from the incubation mixtures or in which 5 mM Ca^{2+} was added also resulted in entirely comparable activation kinetics for the three trypsinogen mutants in comparison to the wild-type zymogen (not shown). Although the absolute activity was lower in the absence of BSA (see Fig. 4*A*) or in the presence of high Ca^{2+} concentrations, the relative activation rates of wild-type and the three mutant trypsinogens remained identical. Finally, activation of wild-type and mutant zymogens was also compared using recombinant human cathepsin B expressed in sf9 insect cells (28). This preparation contained only the single-chain form of mature cathepsin B and was enzymatically deglycosylated. The pH optimum of trypsinogen activation by recombinant human cathepsin B was also at pH 4.0, and the activation profiles of mutant and wild-type trypsinogens at pH 4.0 was essentially identical to the activation data obtained with purified human cathepsin B (Fig. 6) inasmuch as no differences between the different zymogens were found (not shown).

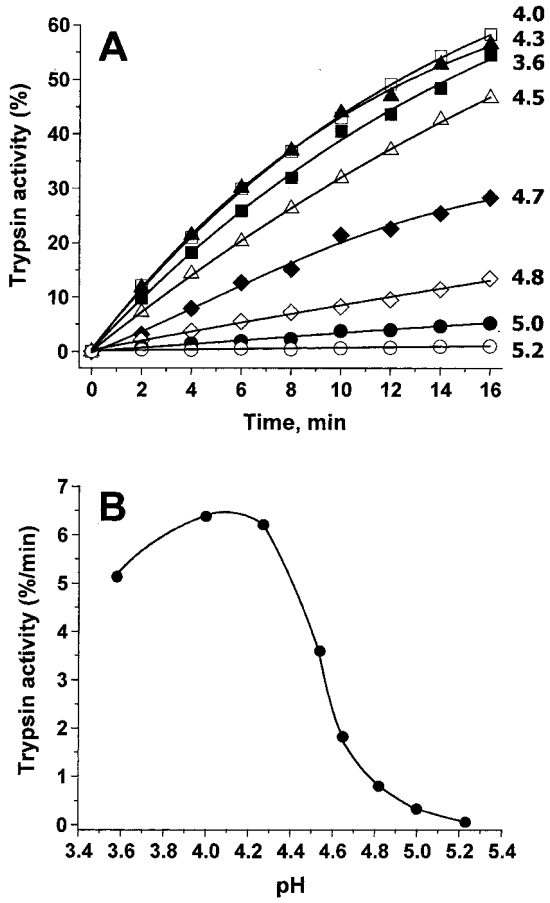

FIG. 5. **Effect of pH on activation of trypsinogen by cathepsin B.** Human cationic trypsinogen (2 μM) was activated with cathepsin B (45 μg/ml, Calbiochem) at 37 °C in 0.1 M sodium acetate buffer at the indicated pH, 2 mg/ml BSA, 1 mM K-EDTA, 300 μM benzamidine in a final volume of 50 μl. The pH of the sodium acetate buffer (originally set to 5.0) was adjusted by adding increasing amounts of HCl or NaOH, and the indicated final pH was calculated from the Henderson-Hasselbalch equation. *A*, aliquots (2.5 μl) were withdrawn at indicated times, and trypsin activity was determined on the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide. Trypsin activity was expressed as the percentage of maximal activity obtained by enterokinase activation at pH 8.0, 5 mM $CaCl_2$, 2 mg/ml BSA. *B*, the initial rates of trypsinogen activation by cathepsin B derived from the time courses (*A*) were plotted as a function of pH. Rates were expressed as percent trypsin generated per min.

In prior experiments that have addressed this issue Szilágyi and coworkers (15) used recombinant trypsinogen with a somewhat different N terminus (Ala-Phe-Pro-Val-$(Asp)_4$-Lys). To exclude that the differences in results between the two studies are caused by a different N-terminal trypsinogen sequence, we expressed and purified wild-type trypsinogen and mutants N29I and N29T with this N terminus. Cathepsin B activated these trypsinogen preparations with comparable efficiency,

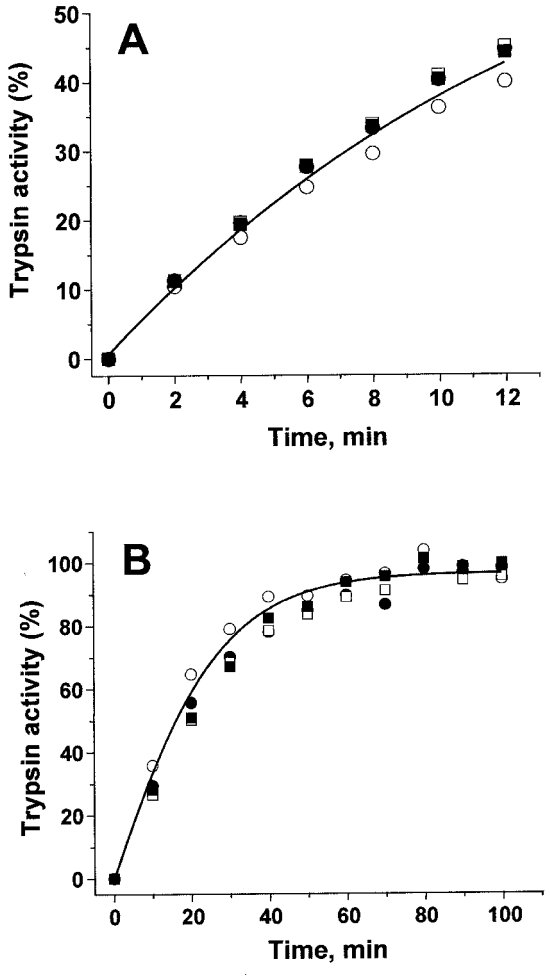

FIG. 6. **Effect of hereditary pancreatitis-associated mutations N29I (□), N29T (○), and R122H (■) on activation of human cationic trypsinogen by cathepsin B at pH 4.0.** Wild-type (●) or mutant zymogens (2 μM) were activated with cathepsin B (45 μg/ml, Calbiochem) at 37 °C in 0.1 M sodium acetate buffer (pH 4.0), 2 mg/ml BSA, 1 mM K-EDTA, 300 μM benzamidine in a final volume of 50 μl. Aliquots (2.5 μl) were withdrawn at the indicated times, and trypsin activity was determined on the synthetic substrate *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide. Trypsin activity was expressed as the percentage of maximal activity obtained by enterokinase activation at pH 8.0, 5 mM $CaCl_2$, 2 mg/ml BSA. In separate experiments, rates (*A*) or full time courses (*B*) of activation were determined.

and again, no significant difference was detected between wild-type and the mutant zymogens (data not shown).

DISCUSSION

Premature intracellular activation of trypsinogen and other digestive proteases is an early event in the course of pancreatitis and is causally related to disease onset. Consequently, if a gain or a loss of trypsin function were involved in the onset of hereditary pancreatitis, either of these mechanisms would require that trypsinogen mutations lead to a significant change in the activation of trypsinogen. Two enzymatic processes could confer such a change in intracellular trypsinogen activation; trypsin-mediated activation (autoactivation) and cathepsin B-induced activation. A pathogenic role for autoactivation is supported by the fact that human cationic trypsinogen has a high propensity for autoactivation and all clinically relevant trypsinogen mutations associated with hereditary pancreatitis (D22G, K23R, N29I, N29T, R122H) have been found to increase the likelihood of autoactivation (3, 9–12). The hypothesis that a cathepsin B-induced activation could be involved in the onset of hereditary pancreatitis is largely based on biochemical and animal studies (42). Cathepsin B, a lysosomal cysteine protease, has been found to activate bovine (20) as well as human (21) trypsinogen *in vitro*. Moreover, cathepsin B was found to be redistributed to a zymogen granule-enriched subcellular compartment during the early course of a variety of animal models of pancreatitis (43). Lysosomal proteins have also been found to co-localize with digestive zymogens during experimental pancreatitis (44), and experiments with either cathepsin B inhibitors (22) or with cathepsin B-deleted knock-out animals (23) have clearly shown that this enzyme is critically involved in intrapancreatic trypsinogen activation. Why cathepsin B activates trypsinogen within the acinar cell is unclear because both classes of enzymes, at least in the rat pancreas, are constitutively co-localized in the secretory compartment under physiological conditions (24), and agents that lead to additional redistribution of cathepsin B into zymogen granules such as chloroquine (45) do not induce pancreatitis. Factors such as stress (32) and changes in intracellular calcium and pH (31, 46) have been shown to greatly affect the cathepsin B-induced trypsinogen activation but may not be sufficient as a final explanation.

Whether cathepsin B-induced activation of trypsinogen is of any relevance in the human pancreas or in the context of hereditary pancreatitis is still uncertain. We have therefore studied whether any of the conditions found in animal experiments that led to the cathepsin B hypothesis are present in the human pancreas. In immunolocalization experiments using electron microscopy we found that cathepsin B is sorted into the secretory compartment of the healthy pancreas under physiological conditions. This indicates that both classes of enzymes are constitutively and abundantly present within the same subcellular compartment of the human pancreas and could potentially interact. It also confirms previous results obtained in the rat and rabbit pancreas (24, 25). Co-localization is possibly due to an insufficient mannose 6-phosphate receptor-mediated sorting of lysosomal enzymes into the lysosomal pathway and their entry into the secretory pathway via a default mechanism (24). When we investigated pure pancreatic juice of patients with either sporadic or hereditary chronic pancreatitis caused by the R122H mutation we found that significant amounts of both trypsin and cathepsin B are secreted as active enzymes. This result suggests that both enzymes were already active within the acinar cells, although we cannot rule out that some trypsinogen activation may have occurred in the pancreatic ducts. Western blots clearly demonstrated that significant amounts of mature, active cathepsin B undergo secretion and must, therefore, have originated from the secretory compartment. All of the above data suggest that cathepsin B has a potential role in the intracellular trypsinogen activation during the course of either sporadic or hereditary chronic pancreatitis.

When, however, we extended our studies to recombinant trypsinogen into which we had introduced three clinically relevant mutations (N29I, N29T, and R122H) we found no difference in the cathepsin B-induced activation of mutant or wild-type zymogens under various experimental conditions. On the basis of the observation that the N29I trypsinogen mutant is activated almost 3-fold faster than the wild-type proenzyme, a recent study by Szilágyi *et al.* (15) suggests that cathepsin B has an important role in the pathogenesis of hereditary pancreatitis. Our results stand in contrast with the published observations and clearly demonstrate that hereditary pancreatitis-associated mutations do not increase or decrease the susceptibility of human cationic trypsinogen to cathepsin B activation. The reasons for the discrepancy are unclear at this time, although it seems of relevance that careful control of pH

is essential to obtain a meaningful comparison between the cathepsin B activation of different trypsinogen preparations.

Given the surprisingly sharp pH dependence and the low pH optimum for trypsinogen activation by cathepsin B (see Fig. 5), it appears questionable whether this enzymic reaction could actually occur in the pancreatic acinar cell secretory pathway, *i.e.* whether pH values in the range of 4.0 would be encountered. Although precise pH determinations along the secretory pathway of the pancreas are lacking, it is well documented that not only lysosomes but also zymogen granules (47) and pancreatitis-associated large cytoplasmic vesicles (48) are actively acidified compartments in rodent acinar cells. Measured pH values for lysosomes from rodent hepatocytes or macrophages usually fall between 4.5 and 5.6 (34–39); however, values as low as 3.6–3.8 have been reported in lysosomes from a human pancreatic adenocarcinoma cell line (49). Under the latter conditions, cathepsin B-catalyzed trypsinogen activation can proceed at full activity. In contrast, further activation would certainly not occur in the slightly alkaline environment of the acinar lumen after secretion.

We conclude that cathepsin B is secreted and active in the pancreatic duct of patients with pancreatitis and constitutively present in the secretory pathway of the normal human pancreas. A co-localization of lysosomal and digestive proteases is therefore a physiological event in humans and not inherently associated with premature zymogen activation or pancreatitis. The factors that determine when and why a transactivation between the two, already co-localized classes of enzymes occurs remain to be determined. These results would suggest a central role for cathepsin B in the initiation of pancreatitis. On the other hand, whereas our experiments do not rule out that cathepsin B-induced trypsinogen activation is involved in premature intrapancreatic zymogen activation, they clearly exclude the possibility that differences in trypsinogen activation by cathepsin B would account for the onset of hereditary pancreatitis.

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