Cathepsin B Activity Initiates Apoptosis via Digestive Protease Activation in Pancreatic Acinar Cells and Experimental Pancreatitis*

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Pancreatitis is associated with premature activation of digestive proteases in the pancreas. The lysosomal hydrolase cathepsin B (CTSB) is a known activator of trypsinogen, and its deletion reduces disease severity in experimental pancreatitis. Here we studied the activation mechanism and subcellular compartment in which CTSB regulates protease activation and cellular injury. Cholecystokinin (CCK) increased the activity of CTSB, and caspase 3 in vivo and in vitro induced redistribution of CTSB to a secretory vesicle-enriched fraction. Neither CTSB protein nor activity redistributed to the cytosol, where the CTSB inhibitors cystatin-B/C were abundantly present. Deletion of CTSB reduced and deletion of cathepsin L increased intracellular trypsin activation. CTSB deletion also abolished CCK-induced caspase 3 activation, apoptosis-inducing factor, as well as X-linked inhibitor of apoptosis protein degradation, but these depended on trypsinogen activation via CTSB. Raising the vesicular pH, but not trypsin inhibition, reduced CTSB activity. Trypsin inhibition did not affect apoptosis in hepatocytes. Deletion of CTSB affected apoptotic but not necrotic acinar cell death. In summary, CTSB in pancreatitis undergoes activation in a secretory, vesicular, and acidic compartment where it activates trypsinogen. Its deletion or inhibition regulates acinar cell apoptosis but not necrosis in two models of pancreatitis. Caspase 3-mediated apoptosis depends on intravesicular trypsinogen activation induced by CTSB, not CTSB activity directly, and this mechanism is pancreas-specific.

Acute pancreatitis has long been regarded as a disease that is characterized by autodigestion of the pancreas by its own proteases (1). This hypothesis appears plausible because no other organ synthesizes and secretes such large amounts of serine and cysteine proteases as the exocrine pancreas (2, 3). However, under physiological conditions, serine proteases are discharged from the pancreas as inactive precursor zymogens, and, most prominently, trypsinogen only undergoes activation when in contact with the intestinal brush border and its enzyme enterokinase. Two discoveries have given new relevance to the autodigestion hypothesis. One is the observation that the autosomal dominant inherited form of pancreatitis is associated with germline mutations in the cationic trypsinogen (PRSS1) gene (4, 5) and that most inherited risk factors for pancreatitis involve alterations in digestive proteases (6, 7). The other is the fact that the mechanism of premature intracellular protease activation and its contributing biochemical and immunological factors are increasingly better understood (8, 9) and have parallels in experimental models that mimic human pancreatitis (10–12), allowing the conclusion that CTSB3 is a critical intracellular player. CTSB is a lysosomal hydrolase that has long been shown to activate trypsinogen in vitro (13) but has also been found to be involved in the pathophysiology of experimental models of pancreatitis (14–16). Ultimate proof for the contribution of CTSB to intrapancreatic trypsinogen activation came from studies employing CTSB-deleted animals (17), which lack digestive protease activation during pancreatitis. Several explanations were offered why a lysosomal enzyme should activate a secretory protease, and these were based on the observations that CTSB activity is shifted from a lysosome-enriched subcellular fraction to a secretory vesicle-containing subcellular compartment (both generated by density gradient centrifugation) (18) and that, on immunogold electron microscopy, the two classes of enzymes co-localize to the same intracellular vacuoles (16, 19, 20). These vacuoles have later been characterized as secretory in nature but also contain markers of crinophagy, autophagy, and endocytosis (21, 22). Technical advances that allowed for direct visualization and quantitation of intracellular protease activity in living acinar cells not only permitted a much more detailed investigation of the underlying mechanisms (23) but also resulted in a number of inconsistencies with the autoactivation hypothesis first addressed 15 years ago (24). One is the observation that most of the intracellular

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trypsin activity assumed to confer tissue damage (25) is actually involved in autodegradation rather than autoactivation (26), at least in experimental models involving rodents. Other authors have deleted-specific trypsin isoforms (T7) and argue that their absence is immaterial for the disease course and that trypsin-independent inflammatory pathways determine disease progression and severity (27) despite the fact that both events clearly interact (8). A third inconsistent observation is that massive missorting and colocalization of trypsinogen and CTSB, when induced by deletion of the relevant mannose-6-phosphate receptor pathway for lysosomal enzyme sorting, induces trypsinogen activation but not pancreatitis (28). The last inconsistency is the observation that the deletion of either CTSB or CTSL reduces the severity of experimental pancreatitis (17, 29) but has opposing roles in trypsinogen activation, and both have been implicated in pro- and anti-apoptotic events. In this study, we have attempted to further define the subcellular compartment in which CTSB activity (rather than trypsin activity) arises following supramaximal caerulein/cholecystokinin stimulation to identify some of the conditions on which it depends and to clarify which mechanism of cell death it affects.

Results

CTSB and Related Protease Activities in Subcellular Fractions after in Vivo Caerulein Stimulation—To determine whether some of the inconsistent observations regarding the role of CTSB in protease activation, pancreatitis severity, and tissue injury are due to different experimental approaches, we tested protease activity in live cell imaging of acini, subcellular fractions, and whole tissue homogenates and compared all of these techniques and materials. C57BL/6 mice were injected with supramaximal concentrations of caerulein, which histologically leads to experimental pancreatitis, and protease activity was studied in subcellular fractions for up to 8 h. Very little active trypsin was recovered in untreated wild-type animals (Fig. 1A), but, as early as 1 h after treatment, trypsin activity rose...
dramatically and specifically within the secretory vesicle-containing fraction. After 8 h, trypsin activity remained high but had shifted to a lighter lysosome- and autophagosome-containing fraction. In mice in which CTSB had been deleted, no trypsin activity developed in any of the fractions, confirming the near-total dependence of trypsinogen activation on the presence of CTSB in the mouse pancreas (Fig. 1E). Chymotrypsin behaved somewhat differently from trypsin in that no basal or spontaneous activity was detectable under resting conditions (Fig. 1C), but a sharp rise was found after 1 h and was completely confined to the secretory vesicle fraction. After 8 h, chymotrypsin activity could no longer be detected, and its much less sustained activity rise (in comparison with trypsin) was most likely due to the more rapid degradation of chymotrypsin. In CTSB−/− mice, no chymotrypsin activation ever developed (Fig. 1D).

As expected, CTSB activity was already present in the pancreas under resting conditions (Fig. 1E), with only minimal substrate cleavage detectable in the cytosolic fraction. 1 h after the start of caerulein stimulation, this activity shifted from the lysosomal to the secretory vesicle-containing fraction, where it has been found previously co-localized with digestive zymogens. After 8 h, this redistribution had almost completely reverted to its prestimulatory state, and only a small activity increase could be detected in the cytosol, which was most probably due to leakage from damaged secretory or other vesicles in the late disease stage. Again, as expected, CTSB activity did not arise, nor did redistribution occur in CTSB-deleted animals (Fig. 1F). The subcellular distribution of CTSL followed the same pattern with a shift to the secretory vesicle-containing compartment after 1 h that was maintained to some degree until 8 h but involved practically no loss of CTSL to the cytosol until the end of the experiment (Fig. 1G). Deletion of CTSB had no effect on the activity or redistribution of CTSL in the subcellular fractions, indicating that CTSL is neither activated nor degraded by CTSB (Fig. 1H). In Fig. 1, the characterization of the subcellular fractions on the protein level under resting conditions is shown. The zymogen granule marker synclinorin predominated in the secretory vesicle fraction, the lysosomal proteins LAMPI2 and LIMP2 in the lysosomal fraction, and GAPDH in the cytosol (Fig. 1I). After 8 h of supramaximal caerulein stimulation, the lysosomal compartment not only contained the zymogen granule marker synclinorin (Fig. 1J) but also the processed form of LC3 (LC3 II), indicating that this lighter fraction had by this late time point assumed a role in acinar cell autophagy (21, 30).

Most of the above data refer to substrate-defined CTSB (or CTSL) activity, which is affected by a variety of factors, including activation, degradation, inhibition, and the biophysical properties of the subcellular environment. To exclude most of these effects, we subjected subcellular fractions to protein analysis by Western blotting with either monospecific anti-CTSB antibody or the biotin-coupled suicide substrate NS-196, an inhibitor that binds the active site of processed/activated CTSB but not its inactive precursor. The gels in the bottom panel of Fig. 1K indicate that the distribution of the pro and processed forms of CTSB under resting conditions (0h) was almost equal between the lysosomal and secretory vesicle fractions. However, CTSB protein massively shifted to the secretory vesicle fraction after 1 h of caerulein pancreatitis, where it was almost exclusively present in the processed 33-kD form, whereas the heavier precursor was absent. The top gel in Fig. 1K and the densitometry indicate the mean of several experiments of NS-169 on Western blots and confirm that the CTSB shift from the lysosomal to the secretory vesicle-containing fraction represents not only a shift in activity (as in Fig. 1E) or in processed CTSB (as in the gels below) but also a true activation of CTSB within the secretory vesicle-containing fraction. This answers the longstanding question of what the CTSB activity shift represents; it can now be identified as an increase in processing of CTSB in that fraction to the active form of the protein. Also of note is the observation that little CTSB protein was present in the cytosol.

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In the next series of experiments, we investigated to what extent live imaging of protease activities corresponds to measurements obtained in homogenates. In isolated acini exposed to supramaximal caerulein, a rapid but ultimately transient increase in intracellular trypsin activity could be documented (Fig. 2A) using a previously established live cell imaging technique (23). This corresponded fairly well to the increase in trypsin activity in homogenates from the same acini (Fig. 2B). A similar rapid and transient increase was found for the activity of CTSL using a fluorogenic substrate following caerulein stimulation of acini, amounting to a 10- to 15-fold activity increase, that was absent in controls (Fig. 2C). On the other hand, CTSL activity measured in the homogenates of the same acini indicated only a 1.5-fold increase (Fig. 2D), although most of the CTSL after caerulein stimulation was present in the processed, i.e. 33-kDa form (Fig. 2E). This discrepancy between the CCK-induced activity increase on live cell imaging and in homogenates could be caused by a number of factors, but the most likely explanation involves the preserved subcellular compartmentalization maintained in the former, which is lost during homogenization in the latter. We therefore studied the active site inhibitor (suicide substrate) NS-196 again in acini. In Fig. 2F, CCK stimulation significantly enhanced the already prominent basal NS-196 band that was totally absent in acini from CTSB−/− mice. The CTSB Western blot below indicates that the CTSB activity corresponds to the processed 33-kD CTSL band. The densitometry in Fig. 2G roughly corresponds to the CTSL activity increase in homogenates (Fig. 2D) and confirms the absence of activated CTSL in the CTSB−/− animals. Changing the pH in the measurement buffer of homogenates from 5.5 to neutral 7.5 greatly reduced the activation of CTSL.

We also tested the effect of pH changes in living acini (Fig. 3) on protease activation and cell damage. When acini were stimulated with supramaximal CCK, a rapid and transient activity increase in CTSL was observed that was dramatically reduced in acini from CTSB−/− animals (Fig. 3A). The activation of chymotrypsin, a zymogen that undergoes trypsin-dependent activation, was similarly reduced (Fig. 3B). On the other hand, necrosis as measured by propidium iodine exclusion remained unaffected by the absence of CTSB in acini (wild-type versus knockout). This indicates that necrosis of acinar cells is independent of the presence of either CTSB or chymotrypsin.
To raise or neutralize the pH in zymogen granules and lysosomes, we used concentrations of chloroquine that are known to dissipate the intracellular pH gradient across vesicular membranes (31). This led to the same reduction in CTSB activity (Fig. 3D) and trypsin activation (Fig. 3E) as the genetic deletion of CTSB. Again, acinar cell necrosis was unaffected by the pH shift (Fig. 3F) or the activity changes of either protease. The manner by which intracellular vesicles maintain their acidic pH is via V-type ATPases (32). When we inhibited V-ATPases with bafilomycin A1 to dissipate the pH gradient, this pH neutralization had the same effect as seen previously with chloroquine: reduction of CTSB activity (Fig. 3G) and trypsin activity (Fig. 3H) but had no effect on necrosis (Fig. 3I). These data suggest that the changes in pancreatic damage previously attributed to CTSB, chymotrypsin, and trypsin activity do not involve acinar cell necrosis and that development of necrosis is independent of the intracellular pH, which, on the other hand, affects the activation and activity of all three proteolytic enzymes.

Caspase 3 Activation and Apoptosis in Acinar Cells Is Dependent on Zymogen Activation—A second form of cellular injury involves apoptosis, a more regulated form of cell death. When we used homogenates of acini treated with supramaximal CCK (Fig. 4) the activity increase of CTSB was completely abolished by the presence of chloroquine (Fig. 4A), as was the conversion to the active form of CTSB, as indicated by the molecular weight change and NS-196 labeling (Fig. 4A, right panel). In parallel with the activity increase in CTSB, CCK induced an increase in caspase 3 activity, which represents a key enzyme in the development of apoptosis (Fig. 4B). This increase was, again, abolished in the presence of chloroquine. When we studied the V-ATPase inhibitor bafilomycin A1, the same reduction in CTSB activity (Fig. 4C), caspase 3 activity (Fig. 4D), trypsin activation (Fig. 4E), and chymotrypsin activity (Fig. 4F) could be demonstrated in the homogenates, indicating that not only digestive and lysosomal protease activation is pH-dependent but also that of caspase 3, a critical component of the apoptosis pathway. Also, the degradation of apoptosis-inducing factor (AIF) and the inhibitor of apoptosis protein family member XIAP is affected by inhibition of V-ATPase (Fig. 4D, right panel).

We next repeated the experiments in acini from CTSB and CTSL knockout animals (Fig. 5). CCK stimulation in CTSB−/− animals induced no trypsinogen activation, whereas trypsin activity in CTSL−/− animals greatly increased in comparison with wild-type mice (Fig. 5A). Similar results were seen for chymotrypsin activation (Fig. 5B), confirming that CTSB is a trypsinogen-activating and CTSL a trypsin-degrading enzyme. The increased CTSB activity in response to CCK was also seen in CTSL knockout animals (Fig. 5C), indicating that CTSL neither activates nor degrades CTSB. Analysis of caspase 3 activities showed (Fig. 5D) that the absence of CTSB prevented the increase in caspase 3 activity and that the absence of CTSL increased the CCK-stimulated caspase 3 activity. In parallel, the CCK-triggered degradation of AIF was abolished in the absence...
of CTSB but increased in CTSL−/− animals (Fig. 5E). Taken together, these data clearly indicate that CTSB has a pro-apoptotic and CTSL an anti-apoptotic effects in pancreatic acinar cells.

**Inhibition of Serine Protease Activity Reduces Apoptosis**—To test whether this cathepsin effect is a direct one or involves the activity of digestive proteases, we used the serine protease inhibitor nafamostat (Fig. 6). Its presence in pancreatic acini completely inhibited trypsin (Fig. 6A) and chymotrypsin (Fig. 6B) but not the CCK-induced activation of CTSB (Fig. 6B). However, nafamostat completely prevented the CCK-induced increase in caspase 3 activity (Fig. 6D) in living acini. Nafamostat also prevented the degradation of AIF and XIAP, as shown by Western blotting (Fig. 6E), with no inhibitory effect on caspase 3 activity in vitro (Fig. 6F). To support these findings, we used a second inhibitor of trypsin, N166, confirming our findings of complete inhibition of trypsin in pancreatic acini (Fig. 6G), without affecting cathepsin B activation (Fig. 6H). As seen with nafamostat, N166 prevented caspase 3 activation (Fig. 6I). For confirmation, we employed N166 in freshly prepared
acini and measured protease activation after supramaximal CCK stimulation. The intracellular trypsin activity was completely blocked by N166 (Fig. 6J), whereas cathepsin B activity and necrosis remained unaffected (Fig. 6, K and L). To test whether these findings are acinar cell-specific, we treated freshly prepared murine hepatocytes with nafamostat and induced apoptosis via two different methods. Treatment of cells with 200 ng/ml Fas ligand induced apoptosis via the death receptor pathway, whereas heat-induced apoptosis (incubation of cells for 1 h at 43 °C) led to death receptor-independent apoptosis. Both methods resulted in increased caspase 3 activity, and treatment of cells with 100 μM nafamostat had no effect on caspase 3 activity (Fig. 6M). Degradation of AIF was only observed upon heat-induced apoptosis, without the protective effect seen in acini with nafamostat treatment (Fig. 6M, right panel). This can be interpreted as direct evidence for a pathway in which the CTSB-dependent activation of caspase 3 requires activation of trypsin by CTSB and is not a direct CTSB effect.

Cathepsin B but Not Trypsin Activity Is Inhibited in the Cytoplasm—Cathepsin B-mediated apoptosis is a well studied mechanism in different cell types in which release of cathepsin B into the cytoplasm results in degradation of anti-apoptotic protein and induction of apoptosis (33). Purified recombinant CTSB displayed relevant residual activity outside of the known pH optimum (Fig. 7) at neutral pH (Fig. 7A). As described previously, a slight increase in cathepsin B activity can be found in cytoplasmic fractions during caerulein pancreatitis at 8 h but not in untreated controls or 1 h after onset of the disease. For this reason, we studied cytoplasmic fractions in detail. CTSB activity was only observed 8 h after induction of pancreatitis. In contrast to our in vitro experiment (Fig. 7A), this activity was completely abolished at pH 7 or higher (Fig. 7B), and fractions of CTSB-deficient animals functioning as controls did not display CTSB activity (Fig. 7C). In contrast to cathepsin B activity, at 8 h after induction of pancreatitis, trypsin activity was only detected in the cytoplasmic fraction at neutral pH (Fig. 7D) and nearly abolished in CTSB-deleted animals (Fig. 7E). Because the...
majority of CTSB activity can be found in the zymogen fraction 1 and 8 h after induction of pancreatitis, we used these fractions and added aliquots of cytoplasmic fraction, which resulted in a significant decrease in cathepsin B activity under neutral pH but not under acidic conditions (Fig. 7F). The two most potent physiological CTSB inhibitors, cystatin B and cystatin C, were found to be abundantly present in the cytosol, and only a small amount of cystatin C could be detected in secretory vesicles (Fig. 7G). Both inhibitors are stably expressed over the time course of pancreatitis within the cytosolic fraction (Fig. 7G, right panel). Taken together, the activity measurements of CTSB and the localization of cystatin B and C suggest that those inhibitors prevent cytosolic cathepsin B but not trypsin activity in a pH-dependent manner and that, therefore, CTSB cannot directly contribute to cellular damage, e.g. apoptosis.

**Discussion**

Pancreatitis has long been regarded as a disease brought about by autodigestion of the pancreas by its own digestive proteases (1). It is also a highly unpredictable disorder that can arise as a single isolated acute attack, develop in recurring disease episodes, or manifest itself as a chronic disease with progressive loss of exocrine and endocrine tissue that is then replaced by extracellular matrix (36). Twenty years ago it was reported that the hereditary variety of pancreatitis can be associated with trypsinogen mutations (4), and this seminal discovery has led to two important insights: that pancreatitis can clinically be a continuum, often beginning as acute attacks that
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Nafamostat inhibits trypsin activity without affecting cathepsin B

serine protease inhibition results in decreased apoptosis

A specific trypsin inhibitor (Novartis) shows a similar effect as nafamostat

F in vitro caspase 3 activity

G, H trypsin activity

cathepsin B activity

caspase 3 activity

J trypsin activity

K cathepsin B activity

L necrosis

M Nafamostat has no effect on hepatocyte apoptosis
caspase 3 activity

control FasL Heat

control 100µM Nafamostat
FIGURE 6. To test whether these effects on apoptosis were cathepsin-dependent or digestive protease-dependent, we used the serine protease inhibitor nafamostat (Naf, 100 μM), which completely inhibited trypsin activation in acini (A) as well as chymotrypsin activation (B) but did not affect CTSB activation (C). D–F, the fact that caspase 3 activation as well as degradation of AIF and XIAP were abolished by nafamostat (D and E) indicates that CTSB-induced trypsinogen activation and not CTSB activation per se mediates caspase 3 activity (F), resembling enzyme activity measurement of recombinant cathepsin B (A). Adding the cytosolic fraction to the zymogen fraction resulted in inhibition of CTSB activity at pH values between 6.5 and 8 but not under acidic conditions. These findings suggest that the cytoplasmic fraction contains endogenous inhibitors for CTSB that are pH-dependent. G, Western blotting of cystatin B and C showed a strong signal in the cytoplasmic fractions (Cyt) but only weak in zymogen (ZG) and lysosomal fraction (Lys), that was stable over the time course of caerulein-induced pancreatitis (1h and 8h). The graphs represents mean ± S.E. from three or more experiments. *, differences significant at the 5% level.

FIGURE 7. A, the optimal catalytic activity of cathepsin B is limited to acidic conditions (pH 5), but also under physiological pH 7.5, as in cytoplasm, CTSB shows a third of maximal catalytic activity, and CTSB activity is presented in relative fluorescence units (RFU). B, in subcellular fractions of pancreatic tissue, increased CTSB activity 8 h after caerulein stimulation can be found in cytoplasmic fractions. This increased activity is only stable under acidic conditions and is completely abolished at pH 7 or higher. C, cytoplasmic fractions from CTSB-deleted animals functioned as negative controls. D and E, in contrast to cathepsin B, trypsin activity in cytoplasmic fractions after 8 h of caerulein stimulation is stable under neutral pH conditions (D), whereas CTSB-deleted animals display markedly decreased trypsin activity (E). F, in the zymogen fraction we detected a stably increased CTSB activity, even at neutral pH, at 8 h of caerulein treatment (F), resembling enzyme activity measurement of recombinant cathepsin B (A). Adding the cytosolic fraction to the zymogen fraction resulted in inhibition of CTSB activity at pH values between 6.5 and 8 but not under acidic conditions. These findings suggest that the cytoplasmic fraction contains endogenous inhibitors for CTSB that are pH-dependent. G, Western blotting of cystatin B and C showed a strong signal in the cytoplasmic fractions (Cyt) but only weak in zymogen (ZG) and lysosomal fraction (Lys), that was stable over the time course of caerulein-induced pancreatitis (1h and 8h). The graphs represents mean ± S.E. from three or more experiments. *, differences significant at the 5% level.
subsequently progresses to chronic disease (5), and that proteases such as trypsin can have a direct and very important role in disease onset. Recent experimental studies in rodents have suggested that the role of trypsin is probably more complex than just being a triggering event when the enzyme is activated or that of a disease driver when its activity persists. They found that activation and inhibition of trypsin does not necessarily affect the acute, recurrent, and chronic phase of the disease in the same manner (26, 37) and even suggested that some injurious effects of trypsin may be extra- rather than intracellular (38).

What has been firmly established and remains undebated is that trypsin activation is an early characteristic of many varieties of experimental acute pancreatitis (11) as well as of human acute pancreatitis, where it correlates with disease severity (39), that injury begins in acinar cells (16, 20, 23), and that the most effective intracellular activator of trypsinogen activation is CTSB (13, 17). Direct evidence that CTSB-induced trypsinogen activation (rather than solely autoactivation of trypsinogen) is also a critical component of human disease onset is still lacking (40) despite the fact that CTSB is clearly present in the human secretory pathway (2). For the lysosomal enzyme CTSB to activate trypsinogen, a digestive protease undergoing regulated exocytosis via secretory vesicles, the two classes of enzymes must interact. Under pathological conditions, they could do so in any intracellular compartment, including the cytosol, provided that the membrane confines of their respective vesicles (lysosomes, secretory vesicles) are lost. A cytosolic role of CTSB has been assumed previously in cancer cells where the intracellular segregation of vesicular compartments is clearly lost (41). Experimental evidence in exocrine acinar cells, however, suggests that CTSB and trypsinogen are both found in colocalized, membrane-confined intracellular vesicles where transactivation can be initiated (16, 23). How they colocalize is another matter. Constitutive missorting of CTSB into the secretory pathway because of incomplete physiological mannose-6-phosphate receptor transport-induced (28) missorting in the initiating phase of pancreatitis (19), fusion of mature secretory vesicles with lysosomes (42), or colocalization via endocytosis from the luminal (22) or basolateral (43) cell surface as well as defective degradation of autophagolysosomes (21, 44, 45) have all been suggested to explain the subcellular redistribution of lysosomal enzyme activity into the secretory compartment and their colocalization with digestivezymogens.

FIGURE 8. A–C, to study whether trypsin-induced caspase 3 activation translates into apoptosis in vivo, we used two models of experimental pancreatitis and measured apoptosis by TUNEL assay in tissue sections. After 8 h of caerulein-induced pancreatitis, caspase 3 activity was greatly reduced in pancreatic homogenates of CTSB−/− pancreatitis animals, and when TUNEL-positive acinar cells were quantitated (examples are presented in B; scale bars represent 50 μm), the reduction in apoptosis was even greater (C). D–F, in the duct ligation-induced pancreatitis model, the deletion of CTSB did not affect pancreatic injury markers such as serum amylase activity (D) and lipase activity (E), but the number of apoptotic cells was greatly reduced (F). The graphs represents mean ± S.E. from three or more experiments. *, differences significant at the 5% level.
Our data confirm that abundant lysosomal CTSB is already present in secretory vesicles under normal, physiological, and resting conditions. The players are thus already in place before pancreatitis is initiated. They further show that CTSS activity dramatically increases following a supramaximal secretory stimulus, that the activity increase is restricted to the heavy granule secretory compartment, and that it represents regular processing of CTSS from its precursor 44-kDa pro-CTSS to the 33-kDa form of CTSS (46). We used Western blotting to quantitate the binding of the novel active site label NS-196 (47) and could thus demonstrate that the 33-kDa form is indeed the active form of CTSS and that activation/processing occurs in the heavy secretory vesicle fraction. CTSS activation also happens without the requirement for additional CTSS being added by either fusion with other types of vesicles (endosomes or lysosomes) or missorting via transport from the Golgi or endocytosis. The critical condition appears to be the pH in the colocalization compartment because its neutralization via different mechanisms, including inhibition of V-type ATPases, not only prevented trypsin activation by CTSS but also the activation, i.e. processing, of CTSS itself to the active 33-kDa form in that compartment. Because acidification inside acinar cell compartments is a process intimately linked to Ca²⁺-dependent stimulation (48), its prevention or pH neutralization in pre-existing acidic organelles is a strategy that cannot only prevent the activation of trypsin but also that of its activator, CTSS.

Another contributing factor in this process could be the role of CTSS inhibitors. We found small amounts of cystatin C (but not cystatin B) in the vesicular activation compartment. Although cystatin B and C act mostly in the cytosol, CTSS activity in pancreatic acinar cells appears to be restricted to its action in membrane-confined vesicles because of the pH-dependent inhibitory capacity of cystatin C (49), which is reduced when acidification induces dimerization of cystatin C. Neutralization of cytoplasmic vesicles therefore not only reduces the activation of CTSS (processing as determined by NS-196 and Western blotting) but may also reduce CTSS activity (as conventionally measured via fluorogenic substrates) by increasing the availability of the CTSS inhibitor cystatin C within that compartment.

These experiments show that colocalization of CTSS with trypsinogen inside secretory vesicles is a physiological process that does not require additional transfer of lysosomal enzymes into this compartment. They also show that the subcellular redistribution of CTSS activity to the heavier subcellular fraction represents pH-dependent processing of CTSS to its active 33-kDa form, but they say little about the consequences of the resulting transactivation of trypsin. This is presently the topic of considerable debate because, aside from the fact that trypsin mutations cause hereditary pancreatitis (4), some experimental observations remain inconsistent. Deleting expression or inhibiting activity of CTSS has repeatedly been found to reduce the severity of pancreatitis (15, 17). On the other hand, increasing CTSS activity redistributed to the secretory pathway, despite increasing trypsinogen activation, does not increase the severity of pancreatitis or lead to spontaneous pancreatitis (28). Overexpression of trypsin(ogen), including but not restricted to hereditary pancreatitis-relevant mutant trypsinogen isoforms, leads to pancreatic injury or more severe pancreatitis (37, 50), but the deletion of other trypsinogen isoforms does not have the expected opposite effect of reducing disease severity (27). Although our experiments do not exclude CTSS effects in non-acinar cells or extrapancreatic effects of CTSS-induced trypsin activation (38), our data regarding the role of CTSS activation inside the vesicular compartment of acinar cells are quite unequivocal. CTSS activation induces apoptosis rather than necrosis of acinar cells in a pancreas-specific mechanism. This role of CTSS, however, involves its activation inside a vesicular compartment rather than CTSS activity in the cytosol where, unlike in tumor cells (41), it does not appear to play a role. CTSS activity also does not mediate its pro-apoptotic effect directly but via the activation of trypsin, and trypsin inhibition prevents the pro-apoptotic actions of CTSS. In this pathway, the antagonist of CTSS is CTSL (Fig. 9), which exerts an anti-apoptotic effect and is known to degrade rather than activate trypsin (29). The trypsin dependence of CTSS-dependent apoptosis is important because it resolves an apparent paradox. We show that transactivation of trypsin by CTSS is strictly a vesicular and not a cytosolic process. However, the activation of caspase 3 and the degradation of apoptosis-inducing factor may not be restricted to membrane-confined compartments and would be inconsistent with the strictly intravesicular role of CTSS. If active trypsin is the mediator, then the pathway becomes much more coherent because trypsin activity has been shown to rapidly progress from its initiating site in vesicles toward the cytoplasm (23). Our data clearly demonstrate the inhibitory capacity of the cytosol on cytoplasmic CTSS activity. Release of CTSS to the cytosol does not necessary lead to apoptosis induction or cellular damage (33) because of the potent inhibition of
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cysteine proteases by cystatin B and C. This mechanism represents an efficient, protective mechanism for the prevention of cellular damage when lysosomal enzymes are released to the cytoplasm. In contrast to CTSB activity, trypsin activity was not blocked in the cytoplasm even when the physiological cytoplasmic pH was within the activity range of trypsin and other pancreatic digestive enzymes. In both pancreatitis models we employed (supramaximal stimulation-induced and duct ligation-induced), the effect of deleting CTSB on apoptosis was rather consistent, and CTSB appears to have a uniform pro-apoptotic action in the pancreas. The overall severity and the role of CTSB in mediating it, on the other hand, may be quite different between pancreatitis models and depend not only on the parameters used to assess this severity but also on the balance between necrosis and apoptosis in the respective disease variety or model.

It has been suggested by other investigators that apoptosis is essentially a beneficial event in the context of pancreatitis because an increase in apoptosis was paralleled by a decrease in necrosis under defined experimental conditions and, on balance, conferred a lesser overall severity of the disease (51). This effect varies greatly between experimental models (52), and it is presently unknown whether this reflects the human condition. A limitation of our study was that we used a highly reductionist model, that of isolated pancreatic acini, to separate necrosis from apoptosis to study the role that either trypsin or cathepsin B play in these respective processes. This appears appropriate because acinar cells have long been considered to be the earliest site in which cell injury arises (35), but more recent studies have shown that infiltrating inflammatory cells and their cytokines have a much earlier and more prominent effect than previously thought possible (8, 9). They also affect the balance between necrosis and apoptosis, and we therefore included experimental pancreatitis in this study. Another limitation of our study is the necessary restriction to a given repertoire of assays for studying necrosis and apoptosis, which included propidium iodine exclusion, caspase 3 activation, and XIAP or AIF degradation, all of which are well established but reflect only individual aspects of the respective cell death mechanism. Other factors, like receptor-interacting protein kinase activation, a potent necrosis regulator in acinar cells (53), still require further investigation in this context. Although we mostly restricted our investigations to acinar cells and the role of trypsin and cathepsin B, we believe that cell death on an extended scale is generally not a beneficial event because it results, at the very least, in atrophy of the organ. In situations where apoptosis and necrosis compete during an inflammatory process, the former is certainly the less injurious event (51).

Our results show that only a small amount of CTSB is released into the acinar cell cytosol during pancreatitis and that its activity is blocked there by the neutral pH and the presence of cystatins. Cathepsin B-mediated degradation of cystosolic proteins is therefore implausible, whereas pancreatic digestive proteases like trypsin are proteolytically active within the cytoplasm. The availability of large amounts of digestive proteases is a distinct feature of pancreatic acinar cells, which may explain why apoptosis and necrosis induction differs from other cells. The ultimate severity of pancreatitis may not depend on primarily intra-acinar cell events but on the action of infiltrating immune cells that use other mechanisms of cell death induction, such as TNFα (8).

We conclude that, in pancreatitis, CTSB undergoes activation in a secretory, vesicular, and acidic compartment where it activates trypsinogen. pH neutralization of this compartment prevents CTSB activation/processing as well as CTSB-induced activation of trypsin. CTSB deletion or inhibition regulates acinar cell apoptosis but not necrosis. In the exocrine pancreas, caspase 3-mediated apoptosis depends on CTSB-induced trypsin activation, not on CTSB activity directly.

Materials and Methods

Materials—Caerulein was obtained from Sigma. Collagenase of Clostridium histolyticum (EC.3.4.24.3) from Serva (lot no. 14007, Heidelberg, Germany) was used for acinar cell isolation. Collagenase type I from C. histolyticum was used for hepatocyte isolation, and recombinant human active cathepsin B was purchased from Sigma. Recombinant mouse Fas ligand was obtained from R&D Systems. Substrates for trypsin R110-(CBZ-IPR)2, cathepsin L R110-(CBZ-FR), and caspase-3 R110-DEVD were obtained from InVitrogen. Substrate for chymotrypsin Suc-AAPF-AMC and cathepsin B AMC-Arg, were obtained from Bachem (Bubendorf, Switzerland). The amylase and lipase quantification kit was obtained from Roche-Hitachi and bafilomycin A1 from InvivoGen (Toulouse, France). Nafamostat, a trypsin inhibitor, recombinant human caspase 3, as well as chloroquine were from Sigma. Z-DEVD-fmk and Z-VAD-fmk, two caspase 3 inhibitors, were obtained from Bachem. NS-196 was provided by N. Schaschke. A specific intracellular trypsin inhibitor (N166) was a gift from Novartis (54).

The following antibodies were used for Western blotting analysis: anti-GAPDH from Meridian, anti-lysosome-associated membrane protein 2 (LAMP-2) from Sigma, anti-syncollin from BD Transduction Laboratories, anti-cathepsin B and cystatin B from R&D Systems, lysosomal integral membrane protein 2 (LIMP-2) and LC3 from Novus Biologicals, AIF and the inhibitor of apoptosis family member XIAP from Cell Signaling Technology, cystatin C from Novus Biologicals, and pancreatic amylase from Santa Cruz Biotechnology.

Induction of Pancreatitis—C57BL/6 mice were obtained from Charles River Laboratories (Sulzdorf, Germany). CTSB−/− mice (17) as well as CTSL−/− mice (29, 55) were maintained in our animal facility, and both had a genetic C57BL/6 background. Pancreatitis was induced by 8-hourly injections of caerulein (50 µg/kg/body weight) (12).

A second model of pancreatitis was employed using pancreatic duct ligation (34). Animals were anesthetized with ketamine/xylasine, and duct ligation was performed by surgical strand. Animals were starved overnight with access to water ad libitum. All animal experiments were performed after prior approval by the institutional animal care committee and as reported previously (56).

Pancreas samples were frozen in liquid nitrogen after removal and stored at −80 °C, except for samples for subcellular fractionation, which were used immediately. Organ samples for histological evaluation were fixed in 4.5% formaldehyde for...
paraffin embedding (57). Serum was sampled and stored at −20 °C.

**Subcellular Fractionation**—Subcellular fractions were performed on pancreatic tissue of caerulein-treated, and untreated animals served as 0-h controls. Subcellular fractionation was made immediately after sacrificing the animals by a modified protocol described by Saluja et al (14, 15). The pancreas was cut in small pieces in ice-cold homogenization buffer containing 240 mM sucrose, 5 mM MOPS, and 1 mM MgSO4 (pH 6.5). Final fractionation was performed directly after cutting by two strokes of Douncer size A followed by two strokes of Douncer size B. The fractions were separated by density centrifugation. The post-nuclear supernatant was separated by 150 × g centrifugation for 10 min at 4 °C. The zymogen granule-enriched fraction was centrifuged at 470 × g for 15 min at 4 °C, followed by a lysosome-enriched fraction at 12,200 × g for 12 min at 4 °C. The remaining supernatant was cleared up by centrifugation at 20,800 × g for 10 min at 4 °C and was used as the cytoplasmic fraction.

The purity of the fractions was demonstrated by Western blotting. Syncollin (a zymogen marker), LAMP-2 (a lysosomal marker), and GAPDH (a cytosolic marker) served as references for successful fractionation.

**Isolation of Acinar Cells**—Acinar cell isolation was performed by collagenase digestion. Cells were maintained and stimulated in Dulbecco’s modified Eagle’s medium containing 10 mM HEPES and 2% BSA. Acinar cells of C5BL/6, CTSB stimulated in Dulbecco’s modified Eagle’s medium containing 240 mM sucrose, 5 mM MOPS, and 1 mM MgSO4 (pH 6.5). Final fractionation was performed directly after cutting by two strokes of Douncer size A followed by two strokes of Douncer size B. The fractions were separated by density centrifugation. The post-nuclear supernatant was separated by 150 × g centrifugation for 10 min at 4 °C. The zymogen granule-enriched fraction was centrifuged at 470 × g for 15 min at 4 °C, followed by a lysosome-enriched fraction at 12,200 × g for 12 min at 4 °C. The remaining supernatant was cleared up by centrifugation at 20,800 × g for 10 min at 4 °C and was used as the cytoplasmic fraction.

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**Isolation of Acinar Cells**—Acinar cell isolation was performed by collagenase digestion. Cells were maintained and stimulated in Dulbecco’s modified Eagle’s medium containing 10 mM HEPES and 2% BSA. Acinar cells of C5BL/6, CTSB−/−, and CTSL−/− mice were stimulated with 0.1 μM CCK and pre-treated with 100 μM chloroquine to neutralize the intravesicular pH (31) or 100 μM bafilomycin A1. Protease activity was measured in living cells over at time period of up to 60 min as well as in acinar cell homogenates 30 min after CCK stimulation (22). Untreated cells served as controls. In vivo measurement of protease activation in living acinar cells was performed in a cell medium system at pH 7.4 containing 24.5 mM HEPES, 96 mM NaCl, 11.5 mM glucose, 6 mM KCl, 1 mM MgCl2, 6H2O, 0.5 mM CaCl2, 2H2O, 2.5 mM NaH2PO4 H2O, 5 mM sodium fumarate, 5 mM sodium glutamate, 5 mM sodium pyruvate, and 1% BSA and DMEM. Trypsin activity was measured by adding 10 μM R110-IPR substrate, and for CTSS 20 μM AMC-Arg2 was used. Necrosis was determined by propidium iodide (58).

**Isolation of Hepatocytes**—Murine hepatocytes were isolated by collagenase digestion as described previously by Kim et al. (59). Initially, the liver was perfused with PBS to remove blood. Solution containing 3 mg/ml collagenase type I, 0.6% NaCl, 0.05% KCl, 1.2% HEPES, and 0.07% CaCl2 was injected into liver tissue to start digestion. The tissue was cut into small pieces. Cells were isolated through a cell strainer (70 μm). Murine hepatocytes were maintained in DMEM containing 10% FCS and penicillin/streptomycin. Apoptosis was induced by high temperature (cells were maintained at 43 °C for 1 h) and by stimulation with 200 ng/ml Fas ligand over 6 h.

**Biochemical Assays**—Serum amylase and lipase were measured as a photometric assay against a purified enzyme reference standard (Sigma). Protease activity was determined in pancreatic homogenate, acinar cell homogenate, and subcellular fractions of pancreatic tissue. Trypsin and chymotrypsin activity was measured as kinetic over 60 min at 37 °C in buffer containing 100 mM Tris and 5 mM CaCl2 (pH 8.0) by fluorometric assay using fluorochrome substrates. CTSS activity was measured in 100 mM sodium acetate and 5 mM CaCl2 (pH 5.5) containing 10 mM DTT and at pH 4.0 for CTSS activity. Cathepsins were measured as kinetic over 1 h at 37 °C by fluorometric assay using specific fluorochrome substrates. Caspase 3 activities were also determined by specific fluorogenic substrates under neutral pH conditions in PBS (29). Trypsin as well as cathepsin B activity under different pH conditions was measured in 100 mM sodium acetate containing 5 mM CaCl2, and 10 mM DTT in a range of pH 3–12.

**Western Blotting and NS-196 Blotting to Determine Active Cathepsin B**—Cells and tissue for Western blotting analysis were lysed in buffer containing 25 mM HEPES, 75 mM NaCl, 0.5% Triton X-100, 5% glycerin, and 1 mM EDTA in the presence of 1 mM PMSF, 5 mM Na3P2O7, 10 mM NaF, and 1 μg/ml aprotinin or in PBS for NS-196 analysis of active CTSS. Protein concentration was determined by Bradford protein assay. 20 μg of protein was loaded on polyacrylamide gel and transferred to a nitrocellulose membrane as described previously (8, 57). To determine the presence of activated CTSS protein rather than substrate-based activity, which is affected by a variety of biophysical factors, we used Western blotting detection of NS-196, a biotinylated CTSS-specific epoxysuccinyl peptide that binds to the catalytic center of CTSS (47). Protein lysates were diluted in 250 mM sodium acetate trihydrate, 10 mM EDTA, and 2.5% Triton X-100 (pH 5.5). For the assay, 200 μg of total protein was incubated with 1 μM NS-196 for 5 min on ice. Lysates were precipitated with TCA (3% final concentration) and the protein pellet was washed with acetone and then diluted in 50 μl of loading buffer containing 10% β-mercaptoethanol. 25 μl of each sample was loaded on 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane. Blocking was achieved in NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, and 0.05% Triton X-100) containing 2% gelatin. Labeling of NS-196 was also done in NET-gelatin containing streptavidin-conjugated peroxidase (1:15,000) for 45 min at room temperature. NET 0.2% Tween was used for washing. Detection of bands was performed with the Fusion-FX system for chemiluminescence detection using ECL substrate obtained from Thermo Scientific.

**Histology and TUNEL Assay**—Paraffin sections were used for hematoxylin and eosin staining as well as fox apoptosis quantification by TUNEL assay. The FragEL™ DNA fragmentation detection kit from Millipore (catalog no. QIA39–1EA) was used to analyze acinar cell apoptosis. Quantification of apoptotic cells was performed by ImageJ, and apoptotic cells were calculated as percent of total cells (56).

**Statistical Analysis**—All Data are expressed as mean ± S.E. of at least four or more animals. Statistical analysis was made by SigmaPlot using Student’s t test for independent samples or analysis of variances for samples without normality.

**Author Contributions**—M. S., D. J., M. P., B. K., S. M., P. W., and T. W. were involved in the acquisition of the data, analysis and interpretation of the data, and drafting of the manuscript. F. U. W., N. S., T. W., and W. H. provided technical support and critical revision of the manuscript for important intellectual content. M. S., M. M. L., and J. M. conceived, designed, and supervised the study, drafted the manuscript, and obtained funding.

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