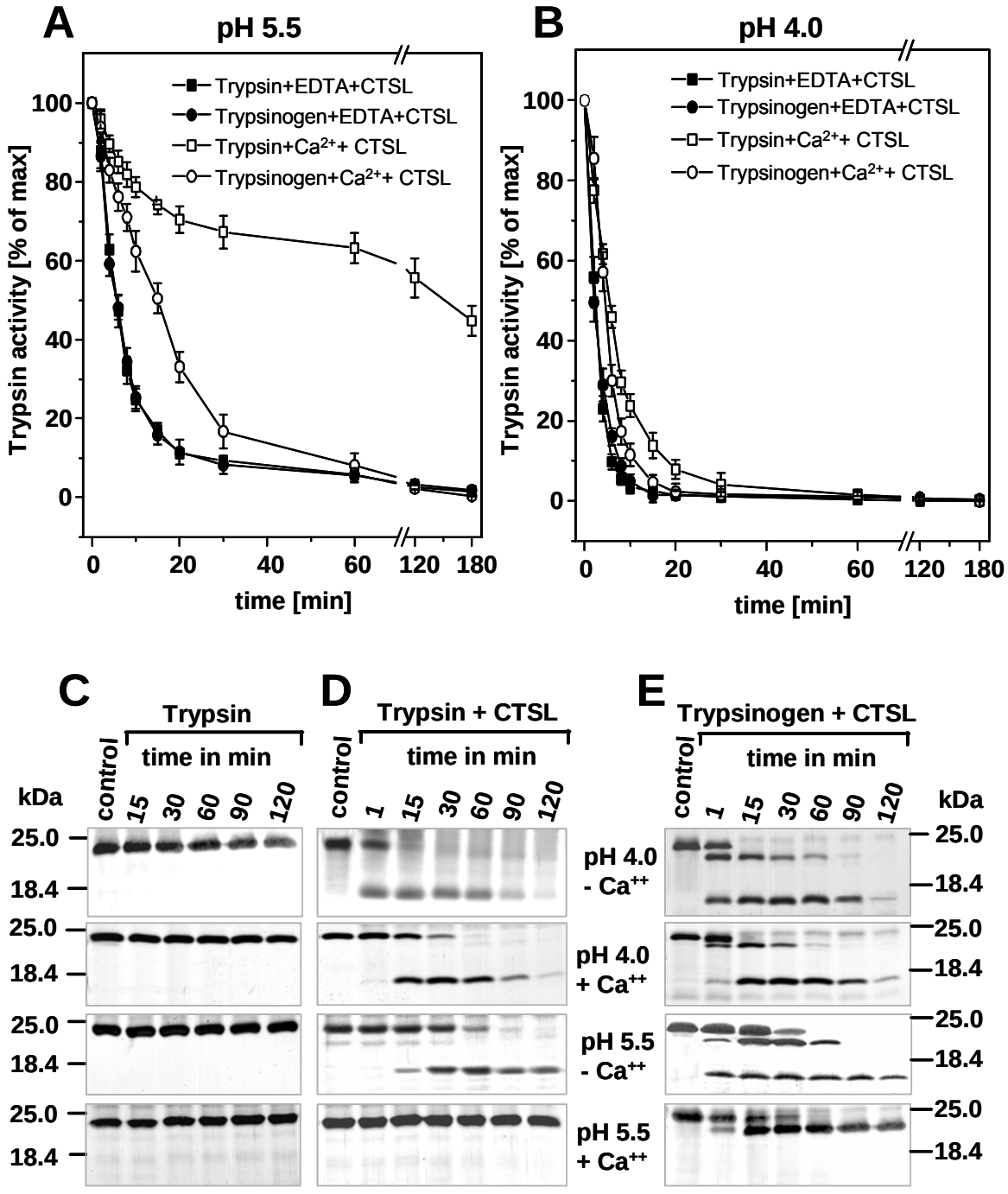
Results shown in supplemental figures 1 and 2

The role of pH and Ca^{++} on proteolytic cleavage

We then studied the inactivation and protein processing via CTSL for up to three hours. Data in supplemental fig.1A demonstrate that at pH 5.5 and in the presence of 10mM $CaCl_2$ trypsin is less susceptible to proteolytic inactivation by CTSL than trypsinogen, and that trypsinogen is rapidly processed to products no longer convertible to activate trypsin by enterokinase. EDTA-removal of calcium greatly increased trypsinogen and trypsin-degradation to similar levels. A pH-decrease to 4.0 accelerated the degradation of trypsinogen and trypsin even further (suppl. fig.1B). This suggests that the stabilizing effect of Ca^{++} is reduced when the pH is lowered to 4.0 This most likely can be accounted for cleavage of trypsin(ogen) by CTSL being dependend on the capability of negatively charged residues to bind Ca^{++}, a highly pH-dependent process. The results for trypsin(ogen) inactivation were confirmed by SDS-PAGE (suppl. fig.1C–E). Panel C demonstrates that trypsin is not auto-degraded over two hours of incubation, nor does CTSL cleave trypsin at pH5.5 and in the presence of Ca^{++} (panel D). Either removal of Ca^{++} or lowering the pH to 4.0 results in rapid degradation of trypsin and the appearance of a 17 kDa protein fragment. This trypsin fragment generated by CTSL at pH 4.0 in the presence of Ca^{++} was sequenced and the resulting N-terminus GNEQFIS(A)(S)K identified the cleavage-site located between E82-G83. SDS-PAGE in panel E demonstrates that trypsinogen is cleaved at the same site when Ca^{++} is removed, which enables CTSL to exert its enzymatic activity. However, primary inactivation occurs via the formation of a truncated trypsin and this process is independent of pH and Ca^{++}. Both trypsinogen cleavage sites were confirmed by mass spectrometry. Supplemental fig.2A demonstrates that the truncated trypsin and the C-terminal 17 kDa fragment are formed at pH4.0, whereas at pH5.5 Ca^{++} protects the CTSL cleavage site at E82-G83, and the overall rate of trypsinogen degradation decreases (suppl. fig.2B).

Supplemental figure 1

Human trypsinogen and trypsin cleavage by Cathepsin L

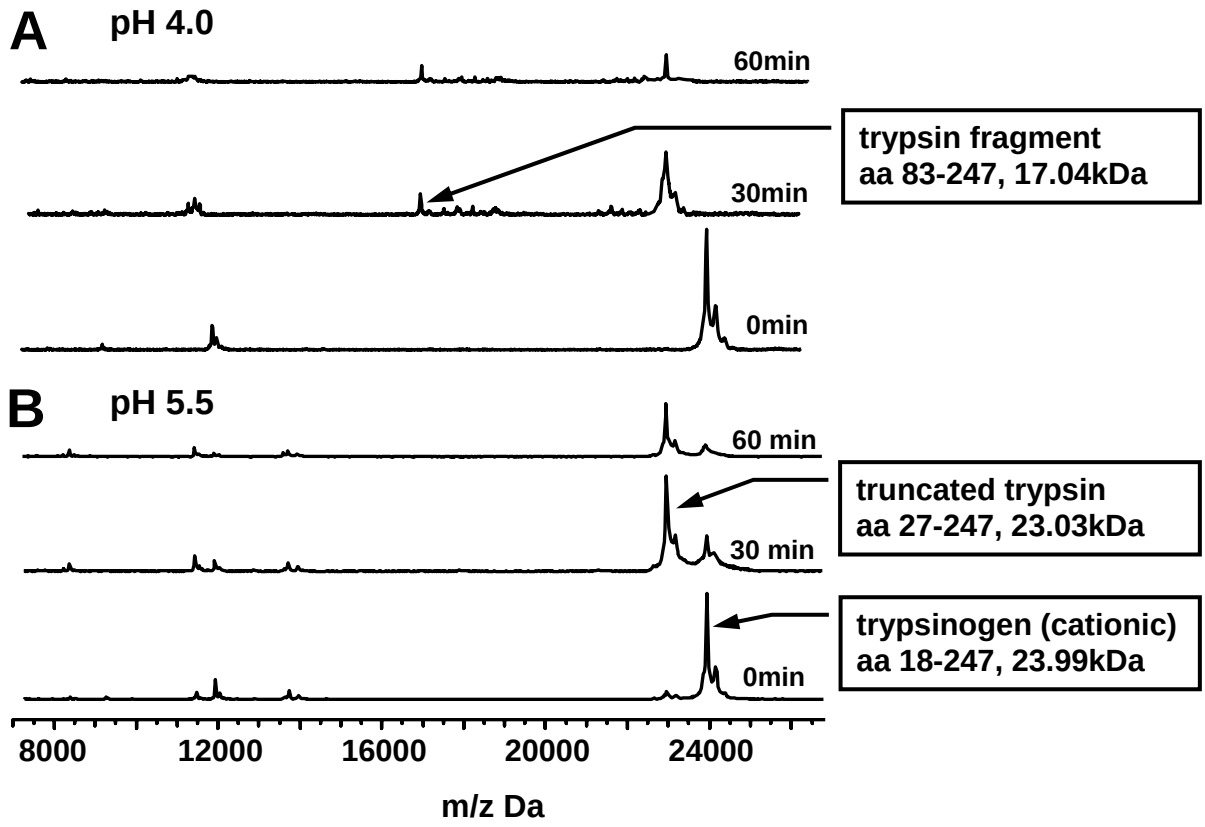


Legend for supplemental figure 1: Effect of Ca^{++} and pH on inactivation of trypsin and trypsinogen.

To study the role of Ca^{++} and pH on the inactivation of trypsin (■) and trypsinogen (●) by CTSL incubations at pH5.5 (A) and 4.0 (B) were performed with 10mM $CaCl_2$ or EDTA (trypsinogen ○, trypsin □). In C-E aliquots of that incubation were used for SDS-PAGE.

Supplemental Figure 2

MALDI -TOF analysis of trypsinogen and trypsin cleavage by Cathepsin L



Legend for supplemental figure 2: Mass spectrometric characterization of CTSL-catalyzed cleavage of trypsinogen and trypsin.

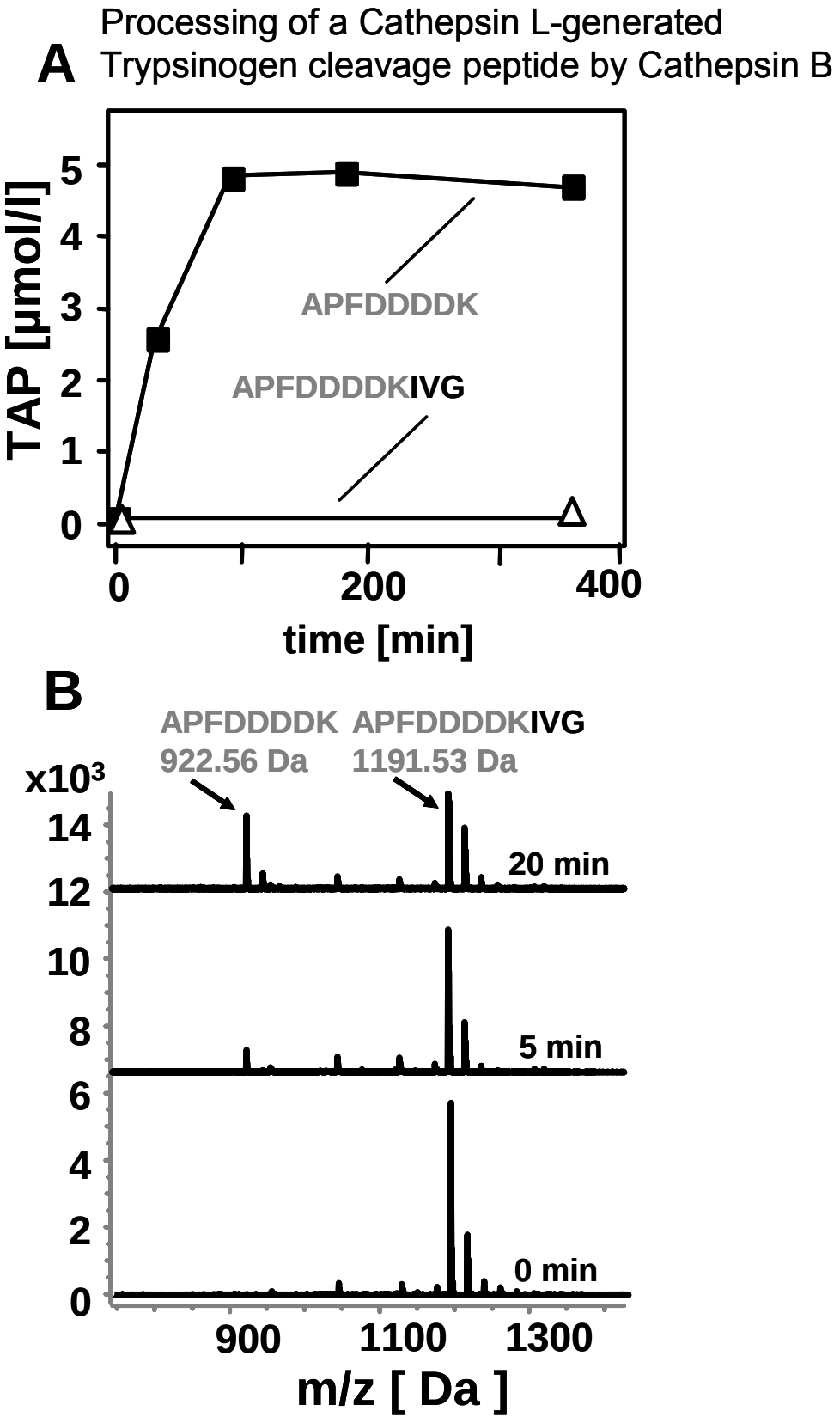
Cleavage of trypsinogen by CTSL as described in supplemental fig. 1 was also directly demonstrated by MALDI-TOF mass spectrometry with 10 mM $CaCl_2$ at pH 4.0 (panel A) and with 10 mM $CaCl_2$ at pH 5.5 (panel B). Mass spectra were obtained at 0 (bottom), 30 and 60 min (top). Amino acid sequences and peptide masses of human cationic trypsinogen were derived from http://www.expasy.ch (Swiss-Prot:P00760). Note that trypsinogen cleavage fragment is generated only at pH 4.0 whereas Ca^{++} protected its generation at pH 5.5. The presence of the fragment confirmed results given in table 1, and fragment mass of 17.04 kDa confirmed N-terminal sequencing described in results.

Results shown in supplemental figure 3

Processing of non-immunoreactive TAP by CTSB

The data in fig.4 and Table 1 show that an elongated non-immunoreactive TAP is generated from trypsinogen by CTSL. The addition of CTSB to trypsinogen plus CTSL resulted in a very rapid generation of TAP immunoreactivity in the TAP-ELISA. This TAP formation exceeded the TAP levels generated by CTSB alone by more than six-fold and amounted to about 50% of the TAP generated by enterokinase (not shown). The combined effect of both cathepsins on trypsinogen did not result in the formation of catalytically active trypsin. The large TAP generation in this experiment must therefore result from a proteolytic or peptidolytic effect of CTSB on the elongated TAP. This hypothesis was confirmed by employing the synthetic peptide APFDDDDKIVG (TAP-IVG), representing the N-terminal sequence of human trypsinogen. Incubation of 5µM of the synthetic peptide with CTSB resulted in TAP-immunoreactivity (supplemental fig.3A). After total conversion this TAP remained stable over 6 hours. Elongated TAP-IVG itself was not detected by the antibody, was not converted to TAP by non-enzymatic reactions, nor was it degraded by CTSL. The conversion of TAP-IVG to TAP was confirmed by mass spectrometry (Suppl. Fig. 3B). The peak representing TAP-IVG disappeared during incubation with CTSB and the peak corresponding to the molecular weight of TAP gradually appeared. No mass-spectrum peaks corresponding to IVG or IV were detected. Therefore CTSB must act via its exoproteolytic activity on TAP-IVG. A conclusion from this experiment is that TAP (trypsin activation peptide) which was previously thought to be generated in a process that results in excess trypsin activity and thus reflects clinical disease severity[23] can also be generated by an alternative pathway in which a sequential processing of trypsinogen by, first CTSL, and subsequently by CTSB neither results in trypsin activity nor does it reflect disease severity.

Supplemental Figure 3



Legend for supplemental figure 3: Processing of a synthetic peptide corresponding to the CTSL-derived trypsinogen cleavage peptide by CTSB

A synthetic peptide corresponding to the one generated by CTSL-cleavage and inactivation of trypsinogen (APFDDDDKIVG, 5 µM in 100 mM sodium acetate buffer, pH 5.5) was assayed with a TAP ELISA and no immunoreactivity was detected (∆). Also addition of CTSL (20µg/ml, not shown) resulted in no immunoreactivity. After exposure to CTSB (20µg/ml), however, large amounts of immunoreactive TAP were generated (■). In panel B the corresponding MALTI-TOF mass spectra are shown that confirm the original and the CTSB-cleavage-induced peptide sequences.

Supplemental Experimental Details

Induction of experimental pancreatitis in cathepsin L-deficient mice

CTSL-deficient ($Ctsl^{-/-}$) and control mice ($Ctsl^{+/+}$) were obtained by breeding heterozygous $Ctsl^{+/-}$ mice generated by Roth et al.[18] using the technique of targeted disruption of the *Ctsl* gene by homologous recombination in E-14-1 embryonic stem (ES) cells. The originally generated 129SV/C57BL6/J mixed genetic background was back-crossed to the FVB/N background for 10 generations, and CTSL-deficient ($Ctsl^{-/-}$) and control mice ($Ctsl^{+/+}$) were obtained by breeding heterozygous $Ctsl^{+/-}$ mice. The CTSL-deficient ($Ctsl^{-/-}$) mice do not show phenotypic alterations of the pancreas (see also figure 1) but have abnormalities in their hair follicle cycle as previously reported [14].

Pancreatitis was induced in 10- to 15-week-old $Ctsl^{-/-}$ and $Ctsl^{+/+}$ mice weighing 20-25g. After fasting for 18 hours with access to water ad libitum, the secretagogue caerulein (Pharmacia Upjohn, Erlangen, Germany) was administered as 7 intraperitoneal injections of 50 µg/kg body weight at hourly intervals[19]. Saline-injected animals served as controls. $Ctsl^{-/-}$ and $Ctsb^{-/-}$ mice, the latter previously described in[7], were used for the preparation of pancreatic acini (see below). For taurocholate-induced pancreatitis mice were anaesthetized, the abdomen was entered through a midline incisions (see Laukkarinen JM, et al. Gut. 2007;56:1590-8), the papilla exposed, and 150µl 2% taurocholate-PBS solution was infused into the pancreatic duct with a pressure of 10 mm Hg. Animals were sacrificed after 6 hours. All animals used in this study were maintained and bred according to our institutional guidelines and animal facility protocols. All subsequent experiments including the induction of acute pancreatitis were performed in accordance with the same guidelines and after prior approval by the institutional animal care committee.

Preparation of serum and tissue samples

Mice were sacrificed at intervals between 1 and 24 hours after the first injection of caerulein, or 6h after the application of taurocholate, and as a reference at 0 hour (untreated controls). After decapitation whole blood was centrifuged at 4°C for 10 minutes at 10,000g, and serum

was stored at -80°C. Pancreatic tissue was removed on ice, weighed, immediately frozen in liquid nitrogen, and stored at -80°C. Tissue for the measurement of pancreatic enzyme activities was thawed and homogenized in iced medium containing 5 mM MOPS, 1 mM $MgSO_4$, and 250 mM sucrose (pH 6.5) with an Ultra-Turrax T25. Samples were sonicated and centrifuged for 5 minutes at 12,000g. Sample preparation for trypsinogen activation peptide (TAP) assay was performed as described previously[7], adding 1 mM EDTA and 0.1% Triton X-100 to tissue homogenates and boiling the samples for 10 minutes. Supernatants generated at 12,000g for 5 minutes were used in the subsequent assays.

Acinar Cell preparation

Acinar cells were prepared from CTSL-/- and CTSL+/+ mice after overnight fasting of animals with access to water ad libitum. Acinar cells were prepared by collagenase digestion (Serva, Germany), maintained and stimulated in DMEM medium containing 2% BSA and 10mM HEPES. After 30 min equilibration in incubation media, acinar cells were stimulated with 0,001mM CCK and incubated for 60 min at 37°C. Acinar cells were washed and transferred to media containing 24.5 mM HEPES, 96 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$ $6xH_2O$, 0.5 mM $CaCl_2$ $2xH_2O$, 2.5 mM NaH_2PO_4 H_2O, 11.5 mM glucose, 5 mM Na fumarate, 5 mM Na pyruvate, 5 mM Na glutamate, 1% DMEM medium (vol/vol) and 1% BSA (pH 7.4) for fluorometric measurement. Trypsin activity in living acini was measured using Ile-Pro-Arg-Rodamin110 as substrate (Invitrogen, Karlsruhe, 10 µM), Caspase-3 activity was determined by PhiPhiLux (Calbiochem, Darmstadt, Cat. No. 235430, 350 mM) with an excitation/emission wavelength of 485 /520 nm [5,15]. Acinar cell necrosis was quantitated using propidium iodide exclusion (ROTH, Aichach, Germany, Ex: 340 nm, Em:.620 nm). Acinar cells were transferred in 96 well plates and enzyme activity was measured over 60 min [5,15].

Biochemical assays

Trypsin in homogenates was measured at 37°C using 64 µM BOC-Gln-Ala-Arg-7-amido-4-methylcoumarin (Bachem, Bubendorf, Switzerland) as a substrate[19,7]. Trypsinogen content was measured as trypsin activity after preincubation with an excess amount of enterokinase (Sigma-Aldrich) over a period of 30 minutes at 37°C. The trypsin activity was corrected for substrate cleavage by enterokinase. Tissue contents of trypsin and trypsinogen were standardized to a purified trypsin preparation (Sigma-Aldrich). Serum amylase and lipase activities were determined enzymatically by commercially available assays (Roche Diagnostics, Mannheim, Germany). TAP was assayed using an enzyme immunoassay (Biotrin, Dublin, Ireland) and protein concentrations were determined according to Bradford10.

Amylase and lipase were measured by photometric assays commercially available (Roche, Hitachi) as kinetic over 30 min with an absorbance at 405nm for amylase and 570nm for lipase. The serum amount of amylase and lipase was standardized using purified enzymes (Sigma, Germany). Cytokines in serum, IL6, IL10, IL12, MCP-1, TNFα and INFγ were measured by FACS analysis with the CBA mouse inflammation Kit according to the manufacturer's instructions (Becton-Dickenson, San Jose, California). Caspase-3 activity was measured by fluorometric enzyme kinetic over 1h at 37°C using Rodamin110-DEVD (Invitrogen, Eugene USA) as substrate (Ex: 485nm, Em: 520nm).

Myeloperoxidase activity in Lung tissue

For myeloperoxidase measurement lung tissue was homogenized on ice in 20 mM potassium phosphate buffer (pH 7.4) and centrifuged for 10 min at 20.000 g at 4°C. The pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% cetyltrimethylammoniumbromide. The suspension was freezed-thawed four times, sonicated twice for 10 seconds, and centrifuged at 20.000 g for 10 min at 4°C. MPO activity was assayed after mixing 50 µl supernatant in 200 µl of 50 mM potassium phosphate buffer (pH 6) containing 0.53 mM O-dianisidine and 0.15 mM H_2O_2. The initial increase in absorbance at

450 nm was measured at room temperature with a Spectramax Spectrophotometer. The results are indicated as units of MPO activity on the basis of 1 unit oxidizing 1 µmol H_2O_2 per minute per mg pancreatic protein. Bars indicate mean values in mU MPO activity per mg pancreatic protein ±SEM from three or more animals per time point.

Human and animal material for morphology and morphometry

Human material from donor pancreas as well as pancreatic juice from controls and pancreatitis patients was obtained as previously reported[20] under an ethics committee approved protocol and with informed consent. For animal models we collected tissue samples at selected time intervals of pancreatitis from both lungs as well as the head, body and tail of the pancreas of $Ctsl^{+/+}$ and $Ctsl^{-/-}$ mice, immediately immersed in iced fixative, and processed for either electron microscopy, paraffin histology or cryosections. For experiments involving the detection of apoptotic cells strips of pancreas were fixed in iced 125 mM phosphate buffer (pH 7.4) containing 4% formaldehyde for 90 min and, after 5µm cryosections were recovered on coverslips, postfixed with iced acetone for 30 sec. Free 3'OH-DNA termini were labeled using the terminal deoxynucleotidyl transferase (TdT) method with fluorescein-labeled digoxigenin nucleotides as previously reported[7],[10]. Fluorescent micrographs were taken at random at 40x magnification (Nikon Diaphot 200, Ex. 485 nm, Em. 530 nm). To quantitate nuclei containing apoptotic bodies in comparison to all nuclei, sections were double labeled with the DNA dye 4,6-diamidino-2-phenylindole (DAPI, Ex. 335 nm, Em. 450 nm). Prints were coded for morphometry and the number of nuclei containing fluorescein-positive apoptotic bodies were counted in a blinded fashion, calculated as percent of all DAPI-positive nuclei and expressed as percent of cells ± SEM.

For resin-embedded thin sections strips of pancreas measuring 1.0 x 0.5 mm were immediately fixed in 125 mM phosphate buffer (pH 7.4) containing 2% glutaraldehyde/2% formaldehyde for 90 min, rinsed extensively in the same buffer, and post-fixed in 2% OsO_4. Tissue blocks were dehydrated in ethanol and embedded in Epon 812. Semi-thin sections were stained with methylene blue and examined by light microscopy. Selected areas, chosen

for detailed study, were thin-sectioned using a LKB Ultratome, picked up on uncoated copper grids, double stained with uranyl acetate and lead citrate, and examined on a Phillips EM10 transmission electron microscope. Pancreatic necrosis was assessed as previously reported and expressed as percentage of area covered by necrotic acinar cells[21,22].

Proteolytic processing of trypsin and trypsinogen by CTSB and CTSL

The human cationic trypsin (PRSSI) expression plasmid was constructed as previously reported[23] and expressed in Escherichia coli Rosetta (DE3) as cytoplasmic inclusion bodies. Refolding and purification of trypsinogen on immobilized ecotin was carried out as reported previously[23,24]. Bovine trypsinogen, enterokinase and CTSB were from Sigma-Aldrich, St. Louis, USA, and bovine trypsin and CTSL were purchased from Calbiochem, San Diego, USA. For the detection of CTSL two different antibodies were used: 33/2 and 3G10 mouse monoclonal anti CTSL were both a kind gift of H. Kirschke and E. Weber (Halle/Saale, Germany). For the detection of trypsin(ogen) we used an anti-bovine trypsin antibody commercially available from Chemicon clone number AB1832A.

The standard incubation mixture (150µl) contained 20µM trypsinogen or trypsin, 1mM Na-EDTA, 2mM DTT, 0.05% BRIJ35 in 100 mM Na-acetate buffer at the indicated pH. Incubations were started by addition of 0.6µM CTSL or CTSB (20mU) in the presence or absence of 10mM $CaCl_2$ at 37°C. After incubation the CTSL activity was inhibited by addition of 0.5 µM E-64 (Sigma-Aldrich, St. Louis, USA). Cleavage sites by CTSL in trypsin(ogen) are numbered in figures according the full length sequences of the pre-pro-proteins.

Trypsin activity was determined using the synthetic chromogenic substrate N-CBZ-Gly-Pro-Arg-p-nitroanilide (200µM) in 100 mM Tris-HCl (pH 8.0), 1mM $CaCl_2$ at 25°C. The release of free p-nitroanilide was recorded at 405nm using the spectrophotometer Varian Cary 1E. Trypsinogen was measured after preincubation with an excess amount of enterokinase for 20 minutes at 37°C and was related to the initial amount of proenzyme.

Sample preparation for TAP assay was performed supplementing the incubation mixture with 1 mM EDTA and 0.1% Triton X-100 and boiling the samples for 10 minutes. Supernatants

generated at 12,000g for 5 minutes were used in the TAP enzyme immunoassay (Biotrin, Dublin, Ireland).

Measurement of CTSL activity

CTSL activity was determined in 10 mM Na-acetate buffer, 1mM Na-EDTA, 2mM DTT, 0.05% BRIJ35 in the pH range between 3.6 and 6.4 using the fluorogenic substrate Z-Phe-Arg-AMC (64µM final concentration, Bachem, Bubendorf, Switzerland) at 37°C. Increase in AMC-fluorescence was recorded at 360nm (excitation)/450nm (emission) wavelength using a Perkin-Elmer LS50 B fluorometer. CTSL activity was calculated as E-64-inhibitor (Calbiochem 0.5 µM) sensitive increase in free AMC and expressed as percent of maximum[10].

SDS-PAGE, Western-blotting and N-terminal protein sequencing

CTSL-treated trypsin and trypsinogen samples were separated using SDS-PAGE. To this end 0.5 - 1µg of each protein sample was boiled in sample buffer (12% glycerol (w/v), 50mM Tris, 2% mercaptoethanol (v/v), 0.01% Serva blue G adjusted with HCl to pH 6.8) for 5 minutes and subsequently separated in a 12% SDS-PAGE at 100 mA for 7 – 9 hours at 4°C. The protein bands were fixed in a solution containing 50% methanol and 10% acetic acid for 30 minutes and stained with 0.025% Serva Blue G in 10% acetic acid for 1 – 2 hours. To increase sensitivity the proteins were additionally stained with silver.

For Western blotting and for N-terminal sequencing the proteins were electrotransferred in Towbin buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol, 3.5 mM SDS, pH 8.3) onto a PVDF membrane (Roth, Karlsruhe, Germany) using a Hoefer miniVE-blot module from Amersham Bioscience, Buckinghamshire, UK. Immunostaining was performed after blocking with milk proteins using an anti-bovine trypsin antibody and a anti-rabbit IgG-secondary antibody coupled to peroxidase (Chemicon, Hofheim, Germany). In order to visualize membrane-bound proteins for Edman-sequencing the protein bands were stained with Coomassie Brillant Blue R-250. N-terminal protein sequencing was performed by Edman

degradation standard protocol[25] using a Procise-c-LC from Applied Biosystems, Foster City, USA.

CTSL detection in pancreatic juice

Pancreatic juice samples were diluted with ice-cold buffer (50 mM HEPES (pH 7.4), 5 mM EDTA, 150 mM NaCl, protease-inhibitor-cocktail (1 ml/mg tissue, containing 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.01 M Na-pyrophosphate, 0.1 M NaF, 1 mM, L-phenyl-methyl-sulfonyl-fluoride and 0.02 % soybean-trypsin-inhibitor)) and adjusted to the same volume. Pancreatic tissue was homogenized by ten strokes in lysis buffer containing 0.25 M Na-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 (BioRad) and equal amounts of protein were used in subsequent experiments. Samples were boiled for 5 min in 4x SDS sample buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol, 0.2 % bromophenol blue, 12 % SDS, 5 % mercaptoethanol], electrophoresed on 12 % or 4-15% SDS polyacrylamide gels (15 µg protein/lane, BioRad, Criterion precast gel, Buchheim, Germany), and proteins were blotted onto nitrocellulose membranes (Hybond C, Amersham Pharmacia, Erlangen, Germany). After overnight blocking in 0.2 % NET-gelatine (1.5 M NaCl, 0.05 M EDTA, 0.5 M Tris-HCl (pH 7.5), 0.5 % Triton X-100, 0.2 % gelatine) immunoblot analysis was performed with a monoclonal anti-cathepsin L antibody (3G10, 1:1000 dilution) followed by horseradish peroxidase-coupled goat anti-rabbit IgG (1:15,000 dilution) and enhanced chemiluminescence detection (Amersham Pharmacia).

Mass spectrometry

Sample preparation was performed by co-crystallization of incubation samples (0.5µl) of trypsinogen with CTSL or of the synthetic peptide APFDDDDKIVG (obtained from Pepscan, Berlin, Germany) with CTSB with 0.5 µl sinapinic acid (20 mg/ml 70% acetonitrile) on a Scout 384-MALDI target. Mass spectra were obtained using a Matrix-Assisted-Laser-Desorption/Ionisation-Time-Of-Flight-Mass-Spectrometer MALDI-TOF-MS Reflex III from

Bruker Daltonics, Germany, in linear mode with external calibration. The annotation of the proteolytic fragments was done with the XMASS and BioTools 2.0 software.

Data presentation and statistical analysis

Data in graphs are expressed as means ± SEM from 5 or more experiments per group. Statistical comparison between the $Ctsl^{-/-}$ and the $Ctsl^{+/+}$ group at various time intervals was done by STUDENT'S t-test for independent samples using SPSS for Windows (SPSS Inc., Chicago, Illinois, USA, version 10.0). Differences were considered significant at a level of $p<0.05$. Data presentation was performed with Origin for Windows (Microcal Software, Northampton, USA, version 7.0).