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MISSORTING OF CATHEPSIN B INTO THE SECRETORY COMPARTMENT OF CI-MPR/IGFII-DEFICIENT MICE DOES NOT INDUCE SPONTANEOUS TRYPSINOGEN ACTIVATION BUT LEADS TO ENHANCED TRYPSIN ACTIVITY DURING EXPERIMENTAL PANCREATITIS - WITHOUT AFFECTING DISEASE SEVERITY

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The lysosomal protease cathepsin B is thought to play a crucial role in the intracellular activation cascade of digestive proteases and in the initiation of acute pancreatitis. Although cathepsin B has been shown to be physiologically present in the secretory pathway of pancreatic acinar cells it has been suggested that premature activation of zymogens requires an additional redistribution of cathepsin B into the secretory compartment. Here, we studied the role of cathepsin B targeting during caerulein-induced pancreatitis in mouse mutants lacking the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (CI-MPR) which normally mediates the trafficking of cathepsin B to lysosomes. Absence of the CI-MPR led to redistribution of cathepsin B to the zymogen granule enriched subcellular fraction and to a substantial formation of large cytoplasmic vacuoles that contained both, trypsinogen and cathepsin B. However, this did not cause premature intracellular trypsin activation in saline-treated control animals lacking the CI-MPR. During caerulein-induced pancreatitis, trypsinogen activation in the pancreas of CI-MPR-deficient animals was about 40% higher than in wild-type animals but serum amylase levels were reduced and lung damage was unchanged. These data suggest that subcellular redistribution of cathepsin B, in itself, induces neither spontaneous trypsinogen activation nor pancreatitis. Furthermore, we clearly show that a marked increase in intracellular trypsinogen activation is not necessarily associated with greater disease severity.

Key words: *cathepsin B, experimental pancreatitis, M6PR300/IGFII ä-deficient mice, mannose-6-phosphate, missorting, trypsinogen activation*

INTRODUCTION

The premature activation of digestive enzymes in pancreatic acinar cells is one of the earliest events in the onset of acute pancreatitis (1). A key player in this process is the serine protease trypsin, which is a potent activator of other digestive pro-enzymes. The crucial role of trypsin in the onset of acute pancreatitis is further supported by the association of mutations of the cationic trypsinogen gene with hereditary pancreatitis (2-4). The physiological conversion of inactive trypsinogen to trypsin is catalyzed by the intestinal enzyme enterokinase in the gut (1). Caerulein pancreatitis in rodents is the most widely used model for the study of intrinsic and exogenous factors influencing the initiation and course of acute pancreatitis (5, 6).

The mechanisms of premature intracellular trypsinogen activation in pancreatic acinar cells, where no enterokinase is expressed, may involve trypsin autoactivation or trypsin activation *via* lysosomal proteases. Animal and *in vitro* models have shown an involvement of the lysosomal hydrolase cathepsin B in triggering the intra-acinar digestive cascade (7-9). The activation of trypsinogen begins in subcellular organelles which also contain cathepsin B (10, 11) and an *in vitro* activation of trypsinogen by cathepsin B has been known since 1959 (12). However, the intra-acinar activation process remains poorly understood. It is well established that cathepsin B is redistributed from lysosomal to zymogen-containing granules in animal models of acute pancreatitis (13, 14). On the other hand, colocalization of cathepsin B and trypsinogen do not seem to be sufficient to induce acute pancreatitis since cathepsin B was detected as a physiological component of the secretory pathway in human and rodent pancreatic acinar cells (15-17).

The main function of the cysteine protease cathepsin B is the degradation of proteins in lysosomes (18). The proper sorting of cathepsin B to lysosomal compartments is mediated by the sorting signal mannose 6-phosphate. Lysosomal enzymes containing a mannose 6-phosphate group are recognized by mannose 6-phosphate receptors (MPRs) in the Golgi complex and transported to an acidic prelysosomal compartment. At low pH, the MPRs are released from lysosomal enzymes and recycle back to the Golgi complex (19). There are two known MPRs, a 300 kDa cation-independent receptor (CI-MPR) and a 46 kDa cation-dependent receptor (CD-MPR). CI-MPR is also a receptor

for the insulin like growth factor II (IGF II) (20). The targeted disruption of MPRs in mice results in altered phenotypes of different severity. CD-MPR deficient mice have a normal phenotype but show a misrouting and increased serum level of lysosomal enzymes (21, 22). Deficiency of CI-MPR is perinatally lethal due to an accumulation of IGFII in the serum (23, 24). This phenotype can be rescued by an additional deficiency of IGFII (24-26). Triple-deficient mutants lacking both mannose 6-phosphate receptors in addition to the IGFII display a phenotype similar to human I-cell disease, with dwarfism, facial dysmorphism, elevated activities of lysosomal enzymes in serum and lysosomal storage in connective tissue cells (27).

Here, we have analyzed the role of cathepsin B targeting and localization for the initiation of acute pancreatitis in mouse mutants lacking the CI-MPR in an IGFII- deficient background. Double knockout mice are viable but have a dwarf phenotype similar to that of the single IGFII deficient mutants. Cathepsin B activities in epithelial tissues and serum levels of lysosomal enzymes were found to be increased (24-26).

In our experiments, deficiency of the CI-MPR led to redistribution of cathepsin B to the secretory pathway, to colocalization of trypsinogen and cathepsin B in large cytoplasmic vesicles but not to spontaneous intracellular trypsinogen activation. On the other hand, when pancreatitis was induced in these animals premature trypsinogen activation was increased by approx. 40% compared to supramaximally caerulein-stimulated wild-type mice. However, the elevated trypsin activity did not lead to an aggravated disease and resulted in reduced hyperamylasemia suggesting that the extent of acinar cell injury is not determined by intracellular trypsin levels.

MATERIAL AND METHODS

Materials

Caerulein was obtained from Pharmacia (Freiburg, Germany). Human myeloperoxidase was from Calbiochem (San Diego, CA, USA). All other chemicals were of highest purity and were obtained either from Sigma-Aldrich Chemie GmbH (Munich, Germany) or Merck (Darmstadt, Germany), Amersham Pharmacia Biotech (Buckinghamshire, UK), or Bio-Rad (Hercules, CA, USA). Animals were bred at Charles River Breeding Laboratories (Sulzbach, Germany).

Induction of experimental pancreatitis

CI-MPR/IGFII deficient animals were a kind gift of the group of Erwin Wagner, IMP Vienna. Acute pancreatitis was induced in 20-30 week-old *Igf2r*$^{-/-}$/*Igf2*$^{-/-}$ (CI-MPR-deficient) mice (24) as well as in *Igf2r*$^{+/+}$/*Igf2*$^{+/+}$ (wild-type) mice weighing 25-30 g. After fasting for 18 hours with access to water *ad libitum*, the secretagogue caerulein was administered in 7 intraperitoneal injections of 50 µg/kg body weight at hourly intervals (28). Saline-injected animals served as controls. All animal experiments were approved by and conducted under the guidelines of the "Animal Use and Welfare Committee" of the Universities of Muenster and Greifswald.

Surgical procedure and preparation of serum and tissue samples

Adult male black C57Bl6-mice weighing between 25 and 30 g were kept in Nalgene shoebox cages in a 12 h/12 h light/dark cycle with unlimited access to standard chow and water. All animals were adjusted to laboratory conditions over the course of one week prior to the experiments. Ten hours after the onset of intraperitoneal injections of caerulein, mice were anaesthetized with sodium-pentobarbital (72 mg/kg body weight) and sacrificed by drawing whole blood samples from the right ventricle. Blood samples were centrifuged at 4°C, and serum was stored at -80°C for further studies. The pancreas was rapidly removed, trimmed of fat and either fixed in 2% paraformaldehyde/2% glutaraldehyde for electron microscopy or embedded in OCT (Sakura, Zoeterwoude, The Netherlands) for kryo-labellings. The main part of the tissue was frozen in liquid nitrogen and stored at –80°C for later protein analysis. Tissue for the measurement of pancreatic enzyme activities was thawed and homogenized in iced medium containing phosphate buffered saline (PBS). Samples were sonicated and centrifuged for 5 min at 16,000 x*g*. For myeloperoxidase measurements, pancreatic or lung tissue was homogenized in 20 mM potassium phosphate buffer at pH 7.4 and centrifuged for 10 min at 10,000 x*g*. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% cetyltrimethylammonium bromide. The suspension was freeze-thawed for four times, sonicated twice for 10 s and centrifuged at 10,000 x*g* for 5 min.

Preparation of subcellular fractions

For subcellular fractioning, the pancreas was minced on ice with sharp scissors and subsequently transferred to a glass tube containing 4 ml of 5 mM MOPS, 1 mM $MgSO_4$, 250 mM sucrose at pH 6.5. The pancreas was then dounced with five strokes of a soft fitting glass douncer. Tissue homogenization was followed by three steps of density gradient centrifugation with the same sucrose buffer. After a 15 min spin with 500 x*g*, the pellet (mostly containing cell debris and nuclei) was discarded and the supernatant centrifuged with 1300 x*g* for 15 minutes. The resulting pellet represented a zymogen granule enriched fraction. The supernatant was centrifuged with 12,000 x*g* for 15 minutes, resulting in a pellet yielding a lysosomal-enriched fraction and the cytosol in the supernatant (10). Pellets and cytosol were frozen in liquid nitrogen.

Biochemical assays

Trypsin was measured using the fluorogenic substrate R110-Ile-Pro-Arg (Rhodamin 110, bis-CBZ-L-isolecyl-L-prolyl-L-arginine amide dihydrochloride, Molecular Probes, Eugene, OR, USA) in a Fluostar Optima fluorometer (BMG, Offenbach, Germany) at 37°C (29). Trypsinogen content was measured as trypsin activity after preincubation with an excess amount of enteropeptidase over 30 min. The trypsin activity was corrected for substrate cleavage by enteropeptidase. Tissue contents of trypsin and trypsinogen were standardized to a purified trypsin preparation (Sigma, Taufkirchen, Germany) whose activity was determined by active site titration and expressed per mg protein. Parallel titrations of the standard trypsin and mouse trypsin activity from control pancreas homogenates with soy bean trypsin inhibitor showed that the specific activities of both were comparable. Amylase activity was determined by commercially available assays (Boehringer, Ingelheim, Germany).

For the measurement of myeloperoxidase (MPO), tissue was thawed and homogenized on ice in 20 mM potassium phosphate buffer (pH 7.4) and centrifuged for 10 min at 20,000 x*g* at 4°C. The pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexacetyltrimethylammoniumbromid. The suspension was freeze-thawed for four times, sonicated twice for 10 seconds each at 30% power setting, and centrifuged at 20,000 x*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 6.0) 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 Dynatech MR 5000 Elisa reader. The results are expressed in units of MPO on the basis of 1 unit to oxidize 1 μmol H_2O_2 per minute per mg pancreatic protein. Mean values in mU MPO-activity per mg pancreatic protein ±SEM were obtained from three or more animal experiments per time point.

Cathepsin B proteolytic activity was determined in pancreas fractions employing the fluorogenic substrate Z-Arg-Arg-4-methyl-coumarin-7-amide (20μM, Bachem, Weil am Rhein, Germany) as described by Barrett and Kirschke (30). Assays were performed in 50 mM phosphate buffer (pH 5.0) containing 2.5 mM EDTA and 2.5 mM dithiothreitol. Reaction mixtures were preincubated for 10 min at 37°C and incubated in the presence of substrate for 5 min. The release of 7-amino-4-methylcoumarin was monitored by spectrofluorometry over a time course of 15 minutes. Results were expressed as U/l after correction of the protein content in the suspensions.

Western blot analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a discontinuous buffer system and gels were blotted on nitrocellulose membranes (Hybond C, Amersham Pharmacia, Freiburg, Germany). After overnight blocking in NET-gelatine (10 mM Tris/HCl pH 8.0, 0.15 mM NaCl, 0.05% TWEEN 20, 0.2% gelatine) immunoblot analysis was performed followed by enhanced chemoluminescence detection (Amersham Pharmacia, Freiburg, Germany) using horseradish peroxidase coupled sheep anti-mouse IgG or goat anti-rabbit IgG (Amersham Pharmacia, Freiburg, Germany).

Chicken polyclonal peptide antibody against cathepsin B was generated using a synthetic peptide corresponding to amino acid 192-205 of the murine prepro cathepsin B (accession AAH06656), (31). The epitope is part of mature cathepsin B and cathepsin B proforms. Monoclonal HSP70 antibody as a control was obtained from Stressgen (Assay designs Ann Arbor, MI, USA). To test the antibody specificity, tissue from cathepsin B knockout animals was used as previously reported (8).

Morphology

At selected time intervals of pancreatitis, tissue samples were collected from lungs as well as from the pancreas of CI-MPR-deficient and wild-type mice, immediately immersed in iced fixative, and processed for either electron microscopy, paraffin histology or cryosections. Sections were double labeled with the DNA dye 4,6-diamidino-2-phenylindole (DAPI, excitation 335 nm, emission 450 nm).

Immunofluorescence staining

Immunofluorescence staining was performed as described previously (32). Paraffin sections immunoreacted overnight at 4°C with 1:200 diluted rabbit antibody against bovine trypsin (Chemicon, Hofheim, Germany), chicken polyclonal antibody against murine cathepsin B diluted to 1:200. Bound primary antibodies were detected by species-specific secondary antibodies conjugated with Cy3 and diluted 1:500 (Dianova, Hamburg, Germany). After counterstaining the nuclei with DAPI (Sigma, Germany) for 15 sec, samples were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA).

Microscopy and image processing

Immunostained preparations were examined on a Leica DM LB fluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland) equipped with appropriate filters. Separate images for DAPI, Cy3, and FITC staining were captured digitally from triple-stained specimens into colour-separated components using a Leica DC 300F digital camera (Leica Microsystems, Heerbrugg, Switzerland) and Leica DC Twain multi-channel image processing. The red (for Cy3), blue (for DAPI), and green (for FITC) components were merged, and composite images were imported as JPEG files into Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA, USA) for further analysis. Omission of incubation with primary antibodies served as control for specifity and did not lead to specific immunosignals. Haematoxylin and eosin stained sections were examined on a Leica DM LB light microscope and the images were captured digitally as described above.

Electron microscopy

For resin-embedded thin sections, strips of pancreas measuring 1.0x0.5 mm were immediately fixed in 125 mM phosphate buffer (pH 7.4) containing 2% glutaraldehyde/2% formaldehyde for 90 minutes, rinsed extensively in the same buffer, and post-fixed in 2% OsO4. Tissue blocks were dehydrated in ethanol and embedded in Epon 812. Semithin sections were stained with methylene blue and examined by light microscopy. Selected areas, chosen for detailed study, were thin-sectioned using an Ultracut E ultramicrotome (Reichert-Jung, Leica Microsystems, Heerbrugg, Switzerland), picked up on uncoated copper grids, double stained with uranyl acetate and lead citrate, and examined on a Philips EM10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Data presentation and statistical analysis

Data in graphs are expressed as means ±SEM. Statistical comparison between CI-MPR-deficient and wild-type groups at various time intervals was done by Student's *t*-test for independent samples using Sigma Plot for Windows. Differences were considered significant at a level of $p<0.05$.

RESULTS

Characterization of caerulein-induced pancreatitis in wild-type and MPR-deficient mice

Serum amylase activity after caerulein-induced experimental pancreatitis

The measurement of serum amylase activity is a well-established standard parameter to assess the degree of pancreatic injury in experimental pancreatitis. In this study, we have quantified the serum amylase activity in wild-type and CI-MPR-deficient mice with either saline or caerulein treatment. As expected, serum amylase of caerulein treated wild-type mice was approx. 8-fold higher than in saline-treated wild-type mice. In contrast to wild-type mice, caerulein treated CI-MPR-deficient mice showed a significantly lower increase in serum amylase (*Fig. 1*) suggesting a lesser severity of pancreatitis in the knockout mice. The pancreatic content of amylase was identical in saline-treated CI-MPR-deficient and wild-type mice (data not shown), and the decreased hyperamylasaemia can therefore not be explained by a decrease in available secretory vesicles or by a decrease in amylase expression.

Morphological pancreatic changes in cerulein-induced experimental pancreatitis

The morphology of the pancreas was examined in formalin-fixed, paraffin-embedded sections of pancreatic tissue which

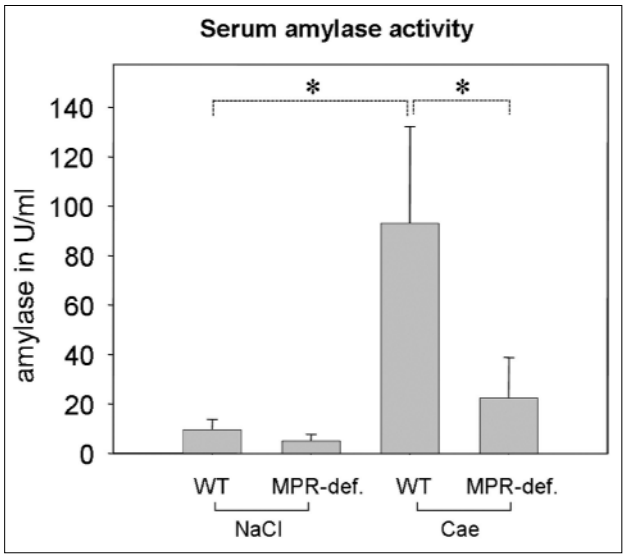

Fig. 1. Serum amylase activity in CI-MPR-deficient (MPR-def.) and wild-type (WT) mice after either saline (NaCl) treatment or after administration of supramaximal concentrations of caerulein (Cae). Ten hours after the first injection, mice were sacrificed, blood samples were taken and amylase levels were measured. Caerulein treatment led to an approx. 8-fold elevated amylase activity in the serum of wild-type mice. In contrast, caerulein-treated CI-MPR-deficient mice showed a significantly lower serum amylase activity. Results are means ±S.E. obtained from four or more mice in each group. Asterisks denote a significant difference at the 5% level when compared to the respective wild-type group or the groups indicated with brackets.

were stained with hematoxylin and eosin. The degree of pancreatic damage is based on cellular vacuolization (in wild-type animals), interstitial edema, granulocyte infiltration and tissue necrosis. In the saline-treated group of wild-type mice, the pancreatic tissue did not display any signs of inflammation. Caerulein-treated WT mice showed clear signs of pancreatic damage which was characterized by interstitial edema, vacuolization in acinar cells and perivascular leukocyte infiltration (*Fig. 2A*). In saline-treated CI-MPR-deficient mice, the pancreatic tissue was morphologically different from wild-type pancreas with large cytoplasmic vacuoles visible in the pancreatic acinar cells (*Fig. 2A*). During caerulein-induced pancreatitis, signs of pancreatic damage including increased interstitial edema, tissue necrosis and leukocyte infiltration could be observed. A direct comparison of the pancreatic damage in caerulein-treated wild-type and CI-MPR-deficient mice, particularly the degree of vacuolization, is not feasible because cytoplasmic vacuoles are already present in the unstimulated pancreas of mice lacking the CI-MPR.

Electron micrographs of pancreata from CI-MPR-deficient and wild-type mice

We further characterized pancreatic acinar cells from CI-MPR-deficient mice by electron microscopic analysis. As shown in *Fig. 2B*, the general architecture of CI-MPR-deficient cells differed significantly from wild-type cells. While ER, Golgi complex and zymogen granules were still present as in the acinar cells of wild-type mice, CI-MPR-deficient cells additionally contained large cytoplasmic vacuoles. As mannose-6-phosphate receptors are absent and therefore lysosomal enzymes cannot be targeted to lysosomes, these vacuoles can be interpreted as a result of missorted lysosomal hydrolases that accumulate in cytosolic vacuoles. During pancreatitis, pancreatic acinar cells of CI-MPR-deficient and wild-type mice displayed similar changes with disruption of the ER and the Golgi complex and a persistence of cytosolic vesicles (*Fig. 2B*).

Immunofluorescence localization of cathepsin B and trypsinogen in the mouse pancreas

For immunofluorescence labelling of cathepsin B, a new polyclonal anti-cathepsin B peptide antibody from chicken was generated. The epitope was chosen to bind cathepsin B from human, rat and mice. The specificity of the antibody was tested using liver tissue of control mice and mice with a targeted disruption of the cathepsin B gene (8). Liver lysates were prepared and aliquots containing 30 µg protein were separated by SDS PAGE, blotted and labelled with the anti-cathepsin B antibody as well as with a constitutive anti-HSP70 antibody as a loading control. The newly established cathepsin B antibody specifically detects cathepsin B with a molecular weight of 25 kDa (*Fig. 3A*).

The newly generated anti-cathepsin B antibody in addition to an anti-trypsinogen antibody was used in immunofluorescence labelling experiments with tissues of wild-type and CI-MPR-deficient mice infused with either saline or caerulein, respectively. In saline-treated wild-type mice, cathepsin B was found in small deposits within pancreatic acinar cells representing lysosomes as well as in secretory vesicles clustered around the acinar lumen. A similar distribution pattern was found for trypsinogen, although this was much more confined to the secretory pole of the acinar cells (*Fig. 3B*). During caerulein-induced pancreatitis, either the cathepsin B-containing compartment or the trypsinogen-containing vesicle area in acinar cells from wild-type mice was enlarged and occupied a much greater area due to the known blockage of zymogen secretion. Additional small vesicles containing lysosomal and secretory enzymes were also visible (*Fig. 3B*). CI-MPR-deficient animals treated with saline displayed very large clusters of cytoplasmic vesicles that clearly contained both, cathepsin B and trpysinogen thus representing a compartment in which both classes of enzymes are physiologically colocalized in the absence of an exogenous stimulus (*Fig. 3B*). This indicates that in the absence of a functional mannose-6-phospate-300 receptor, a massive accumulation of cytoplamic vacuoles containing redistributed cathepsin B and trypsinogen occurs without necessarily leading to pancreatitis. During experimental pancreatitis in CI-MPR-deficient mice, no significant alterations in cathepsin B and trypsinogen labelling are visible in comparison to saline-treated animals lacking the CI-MPR (*Fig. 3B*).

Systemic damage during experimental pancreatitis

The extrapancreatic damage occuring during caerulein-induced pancreatitis was determined by measurement of the myeloperoxidase activity in lung homogenates. In caerulein-treated wild-type and CI-MPR-deficient mice, the lung MPO activity - a quantitative measure of neutrophil infiltration - was significantly elevated compared to saline-treated mice. However, no significant changes in lung myeloperoxidase activity could be observed between wild-type animals and mice lacking the CI-MPR (*Fig. 4*).

Intrapancreatic trypsin activity and trypsinogen activation during caerulein-induced experimental pancreatitis

Since a premature activation of trypsinogen as an initiating event of pancreatitis depends on the amount of trypsinogen

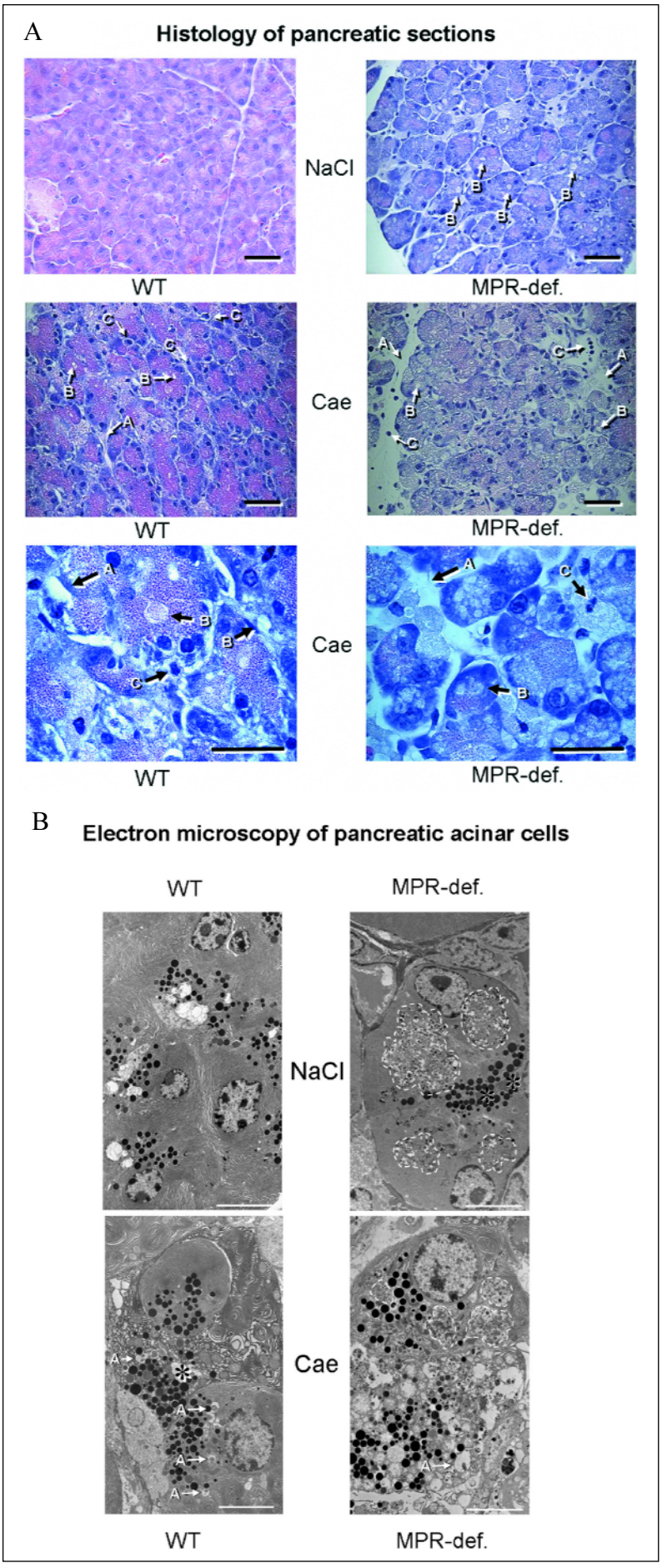

Fig. 2. **A**: Histology of pancreatic sections of saline-treated (NaCl) or caerulein treated (Cae) wild-type (WT) and CI-MPR-deficient (MPR-def.) mice. Pancreatic sections of caerulein-treated mice from both groups showed interstitial edema and infiltration of granulocytes. Acinar cells from CI-MPR-deficient animals contained large cytoplasmic vacuoles already under resting conditions. Interstitial edema (A), pancreatitis-associated vacuolization in acinar cells (B) and perivascular leukocyte infiltration (C) are indicated by arrows. All experiments were repeated at least five times and images were recorded at x200 magnification (x400 for the bottom panels). Calibration bars indicate 200 µm. **B**: Electron microscopy of pancreatic acinar cells from wild-type (WT) and CI-MPR-deficient (MPR-def.) mice. After treatment with saline, the general pancreatic architecture remained intact in pancreatic acinar cells from both, wild-type and CI-MPR-deficient animals. In CI-MPR-deficient cells, irregularly shaped vacuoles (encircled with broken lines) were detectable in either the presence or absence of pancreatitis. During pancreatitis, cytoplasmic vacuoles accumulated in both, wild-type as well as CI-MPR-deficient acinar cells. Pancreatitis associated autophagosomes (A) are indicated by arrows. Astersiks denote the acinar lumen and calibration bars indicate 7.5 µm.

available in the pancreas, the pancreatic total trypsinogen content was determined in CI-MPR-deficient and wild-type mice. Resting levels of pancreatic trypsinogen did not differ significantly between CI-MPR-deficient and wild-type mice. After induction of pancreatitis, the pancreatic trypsinogen content was only increased in wild-type mice but not in animals lacking the CI-MPR (*Fig. 5A*). In wild-type mice, this effect is due to a blockage of enzyme secretion in the presence of ongoing protein synthesis. Apparently, this accumulation of zymogen granules is less prominent in acinar cells of CI-MPR-deficient mice possibly due to the increased amount of cytoplasmic vacuoles present in these cells, in which trypsinogen may be degraded by lysosomal enzymes such as cathepsin L (25). When trypsin activity rather than trypsinogen content was determined, only background activity was found in the pancreas of saline-treated wild-type or CI-MPR-deficient mice. This indicates that the missorting of lysosomal enzymes to cytosolic vacuoles does not, in itself, induce spontaneous intracellular trypsinogen activation. The intraperitoneal injection of caerulein lead to a significant activation of trypsinogen in the pancreas of wild-type and CI-MPR-deficient mice. However, trypsin activity in caerulein-treated mice lacking the CI-MPR was about 40% higher compared to that in caerulein-treated wild-type mice (*Fig. 5B*), in spite of the significant lower trypsinogen content at that stage of the disease (*Fig. 5A*). In all experimental groups, less than one percent of the total

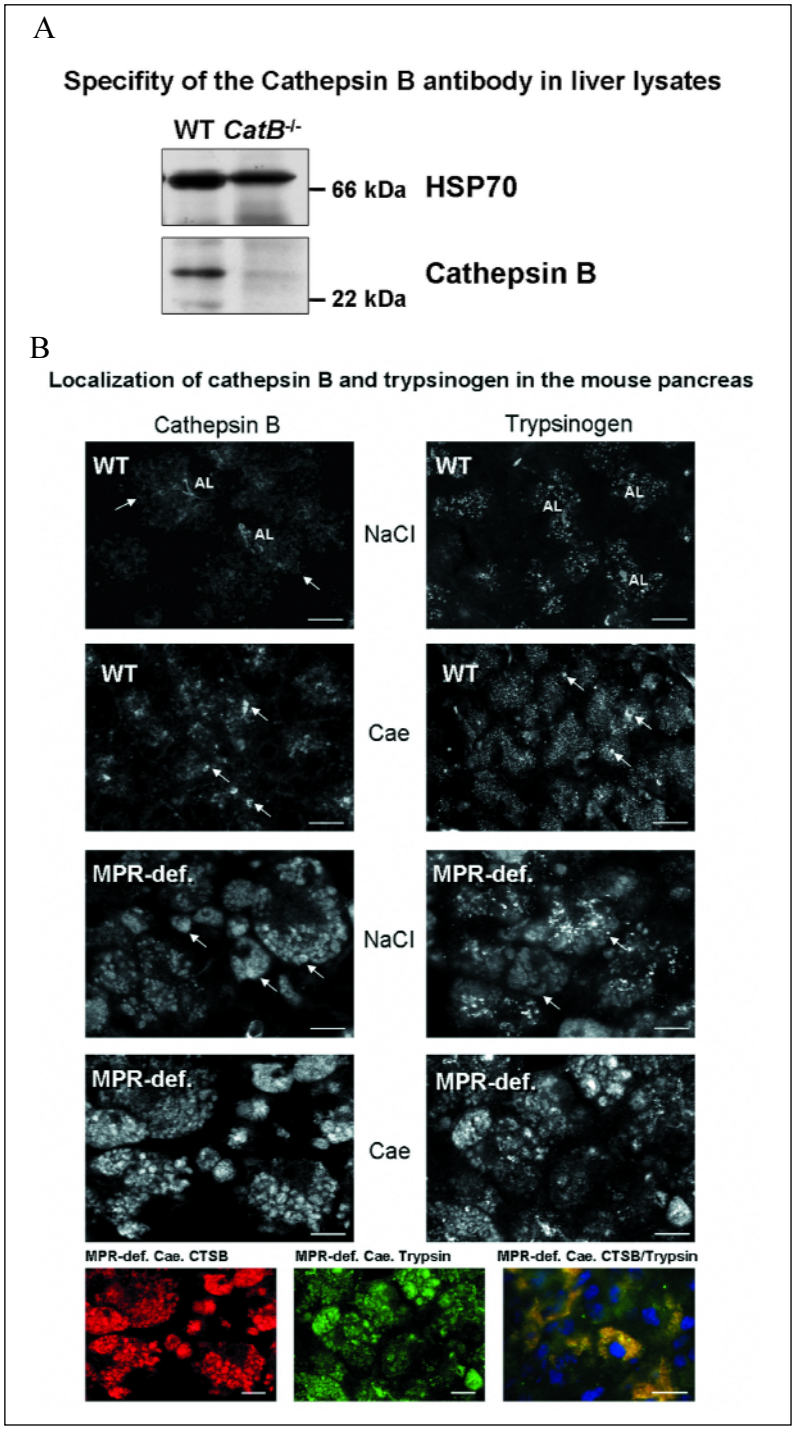

Fig. 3. **A**: The specificity of the polyclonal anti-cathepsin B antibody was analysed in liver lysates of wild-type (WT) and cathepsin B-deficient mice (*CatB*-/-). Liver lysates with a protein content of 30 µg were separated by SDS-PAGE, blotted and labelled with anti-cathepsin B antibodies. Anti-HSP70 antibodies were used as a loading control. The newly established anti-cathepsin B antibody specifically detects cathepsin B with a molecular weight of 25 kDa. Numbers indicate molecular weight markers in kDa. **B**: Immunofluorescence labelling of cathepsin B and trypsinogen in the exocrine pancreas of wild-type (WT) and CI-MPR-deficient (MPR-def.) mice. Mice were infused with either saline (NaCl) or caerulein (Cae). Fluorescence microscopy of paraffin embedded pancreatic sections was carried out with purified anti-cathepsin B or anti-trypsinogen antibodies in addition to Cy3-conjugated anti-chicken IgG or FITC-conjugated anti-rat IgG secondary antibodies. In pancreatic acinar cells from saline-treated wild-type mice, intracellular cathepsin B was present in small deposits representing lysosomes (arrows) and, to a lesser degree, clustered around the apical pole (AL for lumen). Trypsinogen labellings were similarly distributed but more prominent around the acinar cell lumen (AL). After supramaximal caerulein stimulation, both the cathepsin B and the trypsinogen labelled area were enlarged in acinar cells from wild-type animals and additional small vesicles containing lysosomal and secretory enzymes were visible (arrows). In CI-MPR-deficient cells, large clusters of cytoplasmic vesicles containing both cathepsin B and trypsinogen were already visible in saline-treated acinar cells (arrows). During experimental pancreatitis, cathepsin B and trypsinogen labellings are still found in the same cytoplasmic vesicles. All images were recorded at the same magnification, the bottom panels are pseudocolor renditions of the panels above and the bottom right panel is an example for the colocalization of cathepsin B and trypsinogen indicated by the pseuocolour yellow. Calibration bar 10 µm.

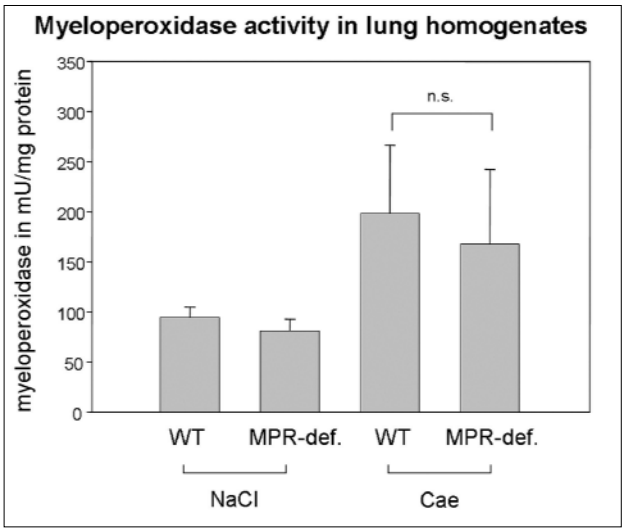

Fig. 4. Quantification of myeloperoxidase activity in lung homogenates of wild-type (WT) and CI-MPR-deficient (MPR-def.) mice with either saline (NaCl) or caerulein treatment (Cae). Myeloperoxidase activity increased during pancreatitis without significant changes between wild-type and CI-MPR-deficient mice. Results are means ±S.E. obtained from four to ten different mice per group.

trypsinogen content in the pancreas participated in intrapancreatic conversion to active trypsin.

Intrapancreatic cathepsin B activity after caerulein-induced experimental pancreatitis

Since lysosomal enzymes are known be elevated in the pancreas of triple-deficient mutants lacking both MPRs in addition to the IGFII (27) and because we could confirm this accumulation in our study with CI-MPR/IGFII double mutants (*Fig. 3B*) we investigated the subcellular compartment in which cathepsin B accumulated by density gradient subcellular fractionation. In saline-treated wild-type animals, cathepsin B was mostly found in lysosomes with only small amounts being detectable in the secretory compartment. Although cathepsin B is capable of activating trypsinogen *in vitro,* it does not seem to do so under physiological conditions and in the absence of pancreatitis (33). We found that in pancreatic acinar cells from both wild-type and CI-MPR-deficient mice, cathepsin B activity was present in the zymogen granule fraction as well as the lysosomal compartments. Our experiments confirmed the expected redistribution of cathepsin B into the zymogen granule-containing fraction with a twofold increase of the zymogen/lysosome ratio occuring in caerulein-treated wild-type mice. (*Fig. 6A*). However, in saline-treated CI-MPR-deficient animals, the content of cathepsin B in the zymogen granule fraction of pancreatic acinar cells was increased nearly to the level found in wild-type mice during experimental pancreatitis, indicating that the CI-MPR deficiency leads to a redistribution of cathepsin B to the secretory compartment already in the resting pancreas. During pancreatitis, no further increase in cathepsin B content in the zymogen granule fraction was observed in the CI-MPR-deficient animals (*Fig. 6A*). When 12,000 x*g* cytosolic fractions were studied, cathepsin B activity was five to six-fold higher in CI-MPR-deficient than in wild-type animals. This difference was irrespective of whether these animals were treated with saline or with supramaximal concentrations of caerulein (*Fig. 6B*). From the immunolabelling studies (*Fig. 3B*)

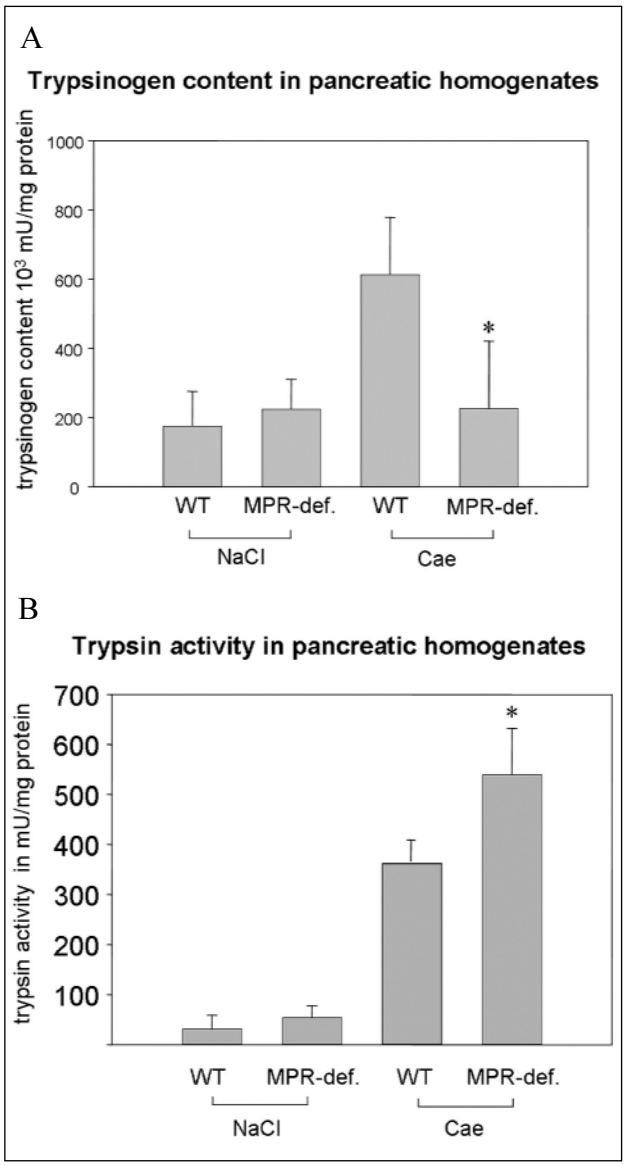

Fig. 5. **A**: Trypsinogen content as determined by quantification of trypsin activity after stimulation with enterokinase (200 U/ml) in pancreatic homogenates of wild-type (WT) or CI-MPR-deficient (MPR-def.) mice with either saline (NaCl) or caerulein treatment (Cae). Trypsin activity after stimulation with enterokinase reflects the overall trypsinogen content of homogenates. Trypsinogen activity increased significantly in pancreatic homogenates of caerulein treated wild-type mice. No significant change of trypsinogen activity could be found in CI-MPR-deficient mice. The overall basal level of trypsinogen content in pancreatic homogenates after saline treatment does not differ significantly between wild-type and CI-MPR-deficient mice. Results are means ±S.E. obtained from four to ten different mice per group. Asterisks denote a significant difference at the 5% level when compared to the respective wild-type group. **B**: Quantification of free trypsin activity in pancreatic homogenates of wild-type (WT) and MPR -deficient (MPR-def.) mice with either saline (NaCl) or caerulein treatment (Cae). Trypsin activity was measured without prior enteropeptidase treatment. In caerulein-treated CI-MPR-deficient mice activity was about 40% higher than that in caerulein treated wild-type animals. Results are means ±S.E. obtained from four to ten different mice per group. Asterisks denote a significant difference at the 5% level when compared to the respective wild-type group.

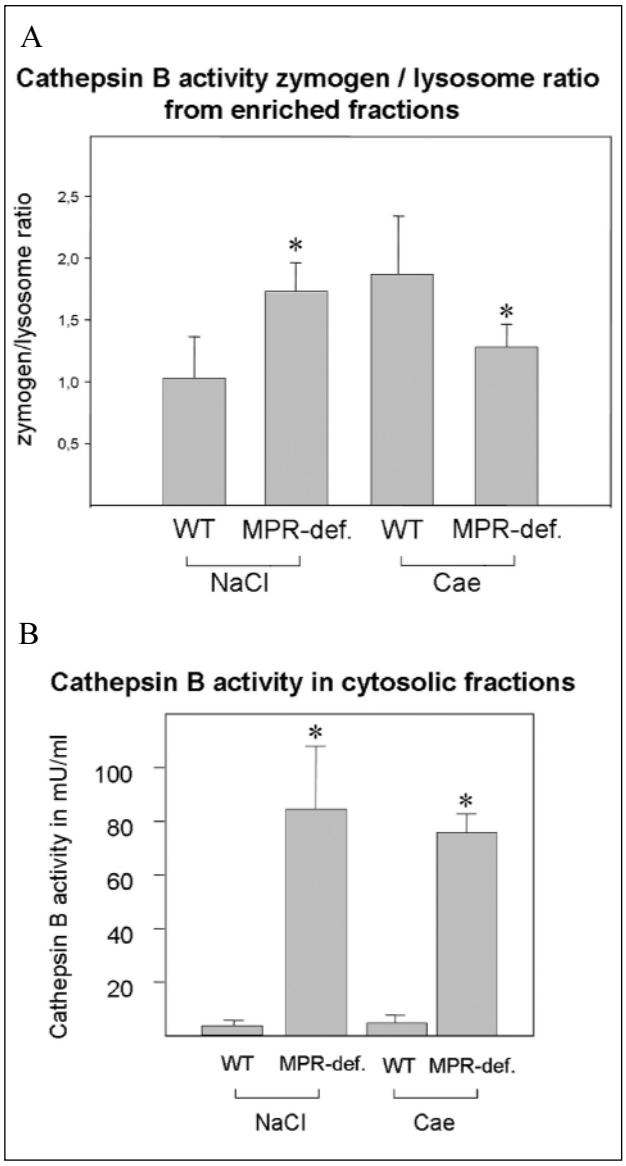

Fig. 6. **A**: Cathepsin B activity in subcellular fractions of wild-type (WT) and CI-MPR-deficient (MPR-def.) mice treated with saline (NaCl) or caerulein (Cae). Pancreatic tissue was homogenized with a glass douncer and subsequently centrifuged using a density gradient to yield a zymogen-enriched fraction, a lysosome-enriched fraction and a cytosolic fraction. An equivalent amount of each fraction corrected for protein content was analyzed for cathepsin B activity using the specific fluorescent substrate Z-Arg-Arg. The distribution in zymogen and lysosome enriched fractions was expressed as zymogen/lysosome ratio. Under resting conditions, the zymogen granule-enriched fraction contained more cathepsin B in CI-MPR-deficient animals than in the wild type controls. In pancreatitis, on the other hand, the CI-MPR-deficient animals carried a lower proportion of their cathepsin B in the zymogen granule fraction. Asterisks denote a significant difference at the 5% level when compared to the respective wild-type group. **B**: The reason for this seemingly reduced cathepsin B content in the secretory pathway was found when cytoslolic fractions were investigated. Under control conditions, as well as during pancreatitis, the vast majority of cathepsin B was recovered from the cytosole, indicating that cathepsin-containing vacuoles of the CI-MPR-deficient mice are fragile and rupture during subcellular fractionation. Asterisks denote a significant difference at the 5% level when compared to the respective wild-type group.

it seems likely that this cytoplasmic activity results from a redistribution of cathepsin B to the cytoplasmic vacuoles rather than to the cytosol. These vacuoles are highly irregular in shape (*Fig. 3B*) and might thus be fragile compartments. We therefore believe the marked increase in the cytosolic fraction to originate from disrupted cytoplasmic vacuoles (during the subcellular fractionation procedure) in the CI-MPR-deficient animals.

DISCUSSION

The mechanism of trypsinogen activation in the initiation of an acute pancreatitis is still being debated. Data published so far provide growing evidence for a crucial role of cathepsins in the activation of trypsinogen. The inhibition of cathepsin B prevents secretagogue-induced trypsinogen activation in isolated acini (7) and in mice (9), whereas cathepsin L is directly involved in the degradation of trypsinogen and trypsin (25). Cathepsin B deficiency almost completely inhibits caerulein-induced trypsinogen activation and reduces pancreatic damage in mice (8) while cathepsin L deficiency increases trypsinogen activation markedly (25). For the activation of trypsinogen by cathepsin B, both enzymes have to be present in the same subcellular compartment. Animal experiments demonstrated a redistribution of cathepsin B after supramaximal secretagogue stimulation from a lysosomal to a zymogen containing granule enriched subcellular compartment (13). In addition, a colocalization of lysosomal proteases with zymogens was identified in pancreatic acinar cells during pancreatitis (34, 35) and shown to be the site where zymogen activation begins (10, 36). Other reports, however, demonstrated the presence of cathepsin B in the secretory pathway of rodents and humans in the absence of pancreatitis (15, 16). Under physiological conditions, cathepsin B is sorted into the secretory compartment and secreted as an active enzyme (16). While it is generally agreed that trypsinogen activation induced by cathepsin B is required for the initiation of the premature protease activation cascade, it remains unclear whether this activation occurs in a compartment where both classes of enzymes are already colocalized or whether it requires an active redistribution of cathepsin B at the beginning of pancreatitis. The mechanisms whereby large amounts of cathepsin B reach the secretory pathway are not well understood but thought to represent a default event in the absence of mannose-6-phosphate receptors that mediate sorting into lysosomes. In the present study, the role of cathepsin B localization during acute experimental pancreatitis was investigated in CI-MPR and IGF II-double deficient mice. Similar to human pancreatitis, caerulein-induced pancreatitis in rodents also leads to a significant intrapancreatic trypsinogen activation that precedes acinar cell injury (37).

Pancreatic acinar cells of CI-MPR-deficient animals displayed a striking morphology with large cytoplasmic vacuoles highly irregular in shape and size in which most of the cathepsin B was detected. Previous studies with triple-deficient mutants lacking both MPRs have demonstrated the presence of these vacuoles in various tissues (38). Our data indicate that these vacuoles do not only contain cathepsin B but also trypsinogen and are likely to represent a compartment for constitutive colocalization of zymogens and lysosomal enzymes. Furthermore, this supports the notion that an impairment of cathepsin B sorting into lysosomes is followed by a default sorting into the secretory pathway. In line with this, Tooze *et al*. found that even in the presence of an intact CI-MPR sorting mechanism, the majority of cathepsin B is already directed towards secretory vesicles (15). The formation of large cytoplasmic vacuoles in CI-MPR-deficient

animals containing cathepsin B and trypsinogen indicates that this lysosomal missorting into the secretory compartment is not only upregulated to capacity but apparently overwhelmed. The inability of acinar cells to handle such large amounts of lysosomal enzymes in the secretory pathway is highlighted by the observation that the cytoplasmic storage vacuoles have a very irregular morphology and do not seem to survive subcellular fractionation indicating their inherent fragility. An increased acinar cell content of cathepsin B has previously been observed in earlier studies with CI-MPR-deficient mice (26). Since it is mostly recovered from the cytosolic fraction following subcellular fractionation, cathepsin B is likely to be present in these fragile vacuoles that have ruptured during fractionation. The fact that cathepsin B could be found in intact lysosomes in the pancreas of CI-MPR-deficient animals is not easily explained since the proper sorting to this compartment is defective. One mechanism whereby cathepsin B might be targeted to lysosomes in CI-MPR-deficient mice is the use of the still intact cation-dependent mannose-6-phosphate receptor (the 46kDa CD-MPR). A further alternative are cell-type specific (38) and unspecific mechanisms found in hepatocytes or colon cancer cells (39-41) that are presently little understood but have been shown to mediate hydrolase sorting to lysosomes in the complete absence of CI-MPR or of CD-MPR.

Even more interesting is the sorting of cathepsin B into intact zymogen granules on subcellular fractionation experiments. During experimental pancreatitis, we found a significant redistribution of cathepsin B to the zymogen granule subcellular compartment in pancreatic acinar cells from wild-type mice (*Fig. 6A*). This redistribution has been previously observed in several pancreatitis models and is regarded as the most crucial event leading to intracellular protease activation (34, 42). However, in saline treated CI-MPR-deficient animals the same extents of redistribution of cathepsin B to the zymogen granule fraction as in wild-type animals after caerulein administration was found. This implies that the absence of CI-MPR leads to a redistribution of cathepsin B to mature secretory vesicles similar to that observed in the early phase of caerulein-induced pancreatitis. However, the activation of trypsinogen - a crucial early event in experimental pancreatitis- does not occur in saline-treated CI-MPR-deficient animals despite the colocalization of trypsinogen and cathepsin B in cytoplasmic vacuoles. Taken together, these observations suggest that neither a maximal subcellular redistribution of cathepsin B into zymogen granules nor a massive colocalization of zymogens with lysosomal enzymes in cytoplasmic vacuoles is, sufficient to induce spontaneous intracellular trypsinogen activation or pancreatitis. One explanation may be that not only trypsinogen activating cathepsin B but also trypsinogen inactivating cathepsin L is sorted into the secretory pathway (25).

Interestingly, intracellular trypsinogen activation during pancreatitis of CI-MPR-deficient animals was found to be significantly higher than in wild-type mice. This markedly increased activation of trypsinogen can be most easily explained by the parallel increase in cathepsin B subcellular redistribution to zymogen granules and the much greater colocalization of both classes of enzymes in animals lacking the CI-MPR. However, as the latter two conditions are already present in saline-treated CI-MPR-deficient animals, additional events induced by supramaximal caerulein stimulation seem to be required for the activation of zymogens. These events may include changes in the biophysical properties within cytoplasmic vacuoles, such as changes in pH and ion concentration, that permit or induce an activation of trypsinogen by cathepsin B.

What is even more striking is that this marked increase in trypsinogen activation within acinar cells of CI-MPR-deficient mice seems to be without any effect for the integrity of the pancreas or for the course of pancreatitis. Systemic consequences of pancreatitis such as the rise in lung myeloperoxidase, which indicates pulmonary damage, are similar in wild-type and CI-MPR-deficient mice. Serum amylase activities indicating local pancreatic damage and acinar cell injury in experimental pancreatitis were even lower in animals lacking the CI-MPR. This suggests that an elevated intracellular trypsin level is not necessarily paralleled by increased pancreatic injury. It has even be suggested that trypsin rapidly autodegrades (30) or is primarily involved in the degradation of other, potentially more damaging proteases as has been suggested for two rare loss-of-function mutations in cationic trypsinogen that are associated with hereditary pancreatitis (3, 43).

Which of these alternative hypotheses accurately describes the events in acinar cells during acute pancreatitis cannot be answered at this time since little is known about the signalling events and additional factors required for the intracellular compartment in which cathepsin B-induced trypsinogen activation occurs. Nevertheless, from the data of our present study we can conclude that the deficiency of the CI-MPR-dependent targeting mechanism leads to a significantly increased redistribution of cathepsin B to the secretory pathway and to the formation of irregular cytoplasmic vacuoles in which cathepsin B and trypsinogen are colocalized. Strikingly, the colocalization of lysosomal enzymes with zymogens alone does not lead to intracellular trypsinogen activation or pancreatitis. After supramaximal caerulein stimulation, on the other hand, the colocalization of cathepsin B with trypsinogen in CI-MPR-deficient mutants results in a marked increase in trypsinogen activation that is not accompanied by an increase in acinar cell injury nor by an aggravated disease.

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REFERENCES

1. Rinderknecht H. Activation of pancreatic zymogens. Normal activation, premature intrapancreatic activation, protective mechanisms against inappropriate activation. *Dig Dis Sci* 1986; 31: 314-321.
2. Whitcomb DC, Gorry MC, Preston RA, *et al*. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* 1996; 14: 141-145.
3. Simon P, Weiss FU, Sahin-Toth M, *et al*. Hereditary pancreatitis caused by a novel PRSS1 mutation (Arg-122—>Cys) that alters autoactivation and autodegradation of cationic trypsinogen. *J Biol Chem* 2002; 277: 5404-5410.
4. Felderbauer P, Stricker I, Schnekenburger J, *et al*. Histopathological features of patients with chronic pancreatitis due to mutations in the PRSS1 gene: evaluation of BRAF and KRAS2 mutations. *Digestion* 2008; 78: 60-65.
5. Warzecha Z, Ceranowicz P, Dembinski A, *et al*. Therapeutic effect of ghrelin in the course of cerulein-induced acute pancreatitis in rats. *J Physiol Pharmacol* 2010; 61: 419-427.

6. Ceranowicz P, Dembinski A, Warzecha Z, *et al*. Protective and therapeutic effect of heparin in acute pancreatitis. *J Physiol Pharmacol* 2008; 59(Suppl 4): 103-125.
7. Saluja AK, Donovan EA, Yamanaka K, Yamaguchi Y, Hofbauer B, Steer ML. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 1997; 113: 304-310.
8. Halangk W, Lerch MM, Brandt-Nedelev B, *et al*. Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J Clin Invest* 2000; 106: 773-781.
9. Van Acker GJ, Weiss E, Steer ML, Perides G. Cause-effect relationships between zymogen activation and other early events in secretagogue-induced acute pancreatitis. *Am J Physiol* 2007; 292: G1738-G1746.
10. Hofbauer B, Saluja AK, Lerch MM, *et al*. Intra-acinar cell activation of trypsinogen during caerulein-induced pancreatitis in rats. *Am J Physiol* 1998; 275: G352-G362.
11. Sherwood MW, Prior IA, Voronina SG, *et al*. Activation of trypsinogen in large endocytic vacuoles of pancreatic acinar cells. *Proc Natl Acad Sci USA* 2007; 104: 5674-5679.
12. Greenbaum LM, Hirshkowitz A, Shoichet I. The activation of trypsinogen by cathepsin B. *J Biol Chem* 1959; 234: 2885-2890.
13. Saluja A, Hashimoto S, Saluja M, Powers RE, Meldolesi J, Steer ML. Subcellular redistribution of lysosomal enzymes during caerulein-induced pancreatitis. *Am J Physiol* 1987; 253: G508-G516.
14. van Acker GJ, Perides G, Steer ML. Co-localization hypothesis: a mechanism for the intrapancreatic activation of digestive enzymes during the early phases of acute pancreatitis. *World J Gastroenterol* 2006; 12: 1985-1990.
15. Tooze J, Hollinshead M, Hensel G, Kern HF, Hoflack B. Regulated secretion of mature cathepsin B from rat exocrine pancreatic cells. *Eur J Cell Biol* 1991; 56: 187-200.
16. Kukor Z, Mayerle J, Kruger B, *et al*. Presence of cathepsin B in the human pancreatic secretory pathway and its role in trypsinogen activation during hereditary pancreatitis. *J Biol Chem* 2002; 277: 21389-21396.
17. Hirano T, Saluja A, Ramarao P, Lerch MM, Saluja M, Steer ML. Apical secretion of lysosomal enzymes in rabbit pancreas occurs via a secretagogue regulated pathway and is increased after pancreatic duct obstruction. *J Clin Invest* 1991; 87: 865-869.
18. Mort JS, Buttle DJ. Cathepsin B. *Int J Biochem Cell Biol* 1997; 29: 715-720.
19. Hille-Rehfeld A. Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. *Biochim Biophys Acta* 1995; 1241: 177-194.
20. Kornfeld S. Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem* 1992; 61: 307-330.
21. Koster A, von Figura K, Pohlmann R. Mistargeting of lysosomal enzymes in M(r) 46,000 mannose 6-phosphate receptor-deficient mice is compensated by carbohydrate-specific endocytotic receptors. *Eur J Biochem* 1994; 224: 685-689.
22. Ludwig T, Munier-Lehmann H, Bauer U, *et al*. Differential sorting of lysosomal enzymes in mannose 6-phosphate receptor-deficient fibroblasts. *EMBO J* 1994; 13: 3430-3437.
23. Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P, Stewart CL. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev* 1994; 8: 2953-2963.
24. Wang ZQ, Fung MR, Barlow DP, Wagner EF. Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature* 1994; 372(6505): 464-467.
25. Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, Efstratiadis A. Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Dev Biol* 1996; 177: 517-535.
26. Sohar I, Sleat D, Gong Liu C, Ludwig T, Lobel P. Mouse mutants lacking the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor are impaired in lysosomal enzyme transport: comparison of cation-independent and cation-dependent mannose 6-phosphate receptor-deficient mice. *Biochem J* 1998; 330: 903-908.
27. Dittmer F, Hafner A, Ulbrich EJ, Moritz JD, Schmidt P, Schmahl W, *et al*. I-cell disease-like phenotype in mice deficient in mannose 6-phosphate receptors. *Transgenic Res* 1998; 7: 473-483.
28. Niederau C, Ferrell LD, Grendell JH. Caerulein-induced acute necrotizing pancreatitis in mice: protective effects of proglumide, benzotript, and secretin. *Gastroenterology* 1985; 88: 1192-1204.
29. Kruger B, Weber IA, Albrecht E, Mooren FC, Lerch MM. Effect of hyperthermia on premature intracellular trypsinogen activation in the exocrine pancreas. *Biochem Biophys Res Commun* 2001; 282: 159-165.
30. Barrett AJ, Kirschke H. Cathepsin B, Cathepsin H, and cathepsin L. *Methods Enzymol* 1981; 80: 535-561.
31. Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, *et al*. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc Natl Acad Sci USA* 2002; 99: 16899-16903.
32. Schnekenburger J, Schick V, Kruger B, *et al*. The calcium binding protein S100A9 is essential for pancreatic leukocyte infiltration and induces disruption of cell-cell contacts. *J Cell Physiol* 2008; 216: 558-567.
33. Halangk W, Kruger B, Ruthenburger M, *et al*. Trypsin activity is not involved in premature, intrapancreatic trypsinogen activation. *Am J Physiol* 2002; 282: G367-G374.
34. Saito I, Hashimoto S, Saluja A, Steer ML, Meldolesi J. Intracellular transport of pancreatic zymogens during caerulein supramaximal stimulation. *Am J Physiol* 1987; 253: G517-G526.
35. Van Acker GJ, Saluja AK, Bhagat L, Singh VP, Song AM, Steer ML. Cathepsin B inhibition prevents trypsinogen activation and reduces pancreatitis severity. *Am J Physiol* 2002; 283: G794-G800.
36. Kruger B, Lerch MM, Tessenow W. Direct detection of premature protease activation in living pancreatic acinar cells. *Lab Invest* 1998; 78: 763-764.
37. Lerch MM, Adler G. Experimental animal models of acute pancreatitis. *Int J Pancreatol* 1994; 15: 159-170.
38. Dittmer F, Ulbrich EJ, Hafner A, *et al*. Alternative mechanisms for trafficking of lysosomal enzymes in mannose 6-phosphate receptor-deficient mice are cell type-specific. *J Cell Sci* 1999; 112: 1591-1597.
39. Gabel CA, Goldberg DE, Kornfeld S. Identification and characterization of cells deficient in the mannose 6-phosphate receptor: evidence for an alternate pathway for lysosomal enzyme targeting. *Proc Natl Acad Sci USA* 1983; 80: 775-779.
40. De Stefanis D, Demoz M, Dragonetti A, *et al*. Differentiation-induced changes in the content, secretion, and subcellular distribution of lysosomal cathepsins in the human colon cancer HT-29 cell line. *Cell Tissue Res* 1997; 289: 109-117.
41. Santama N, Krijnse-Locker J, Griffiths G, Noda Y, Hirokawa N, Dotti CG. KIF2beta, a new kinesin superfamily protein in

non-neuronal cells, is associated with lysosomes and may be implicated in their centrifugal translocation. *EMBO J* 1998; 17: 5855-5867.
42. Saluja AK, Lerch MM, Phillips PA, Dudeja V. Why does pancreatic overstimulation cause pancreatitis? *Annu Rev Physiol* 2007; 69: 249-269.
43. Teich N, Le Marechal C, Kukor Z, *et al.* Interaction between trypsinogen isoforms in genetically determined pancreatitis: mutation E79K in cationic trypsin (PRSS1) causes increased transactivation of anionic trypsinogen (PRSS2). *Hum Mutat* 2004; 23: 22-31.

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