Chymotrypsin B Cached in Rat Liver Lysosomes and Involved in Apoptotic Regulation through a Mitochondrial Pathway

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Abstract

Lysosomes can trigger the mitochondrial apoptotic pathway by releasing proteases. Here, we report that a 25-kDa protein purified from rat liver lysosomes possesses a long-standing potent Bid cleavage activity at neutral pH, and the truncated Bid could in turn induce rapid mitochondrial release of cytochrome c. This protease was revealed as chymotrypsin B by biochemical and mass spectrometric analysis.

Although it was long recognized as a digestive protease exclusively secreted by the exocrine pancreas, our data support that it also expresses and intracellularly resides in rat liver lysosomes. Translocation of lysosomal chymotrypsin B into cytosol was triggered by apoptotic stimuli, such as TNF-α; and intracellular delivery of chymotrypsin B protein induced apoptotic cell death with a potency comparable to cathepsin B, suggestive of a lysosomal-mitochondrial pathway to apoptosis regulated by chymotrypsin B following its release. Noteworthily, either knockdown of chymotrypsin B expression by RNAi or pretreatment with chymotrypsin B inhibitor N-p-tosyl-L-phenylalanine chloromethyl ketone significantly reduced TNF-α-induced apoptosis. These results demonstrate for the first time that chymotrypsin B is not only restricted to the pancreas, but can function intracellularly as a pro-apoptotic protease.

Keywords: Lysosome; chymotrypsin B; Bid; apoptosis; mitochondrion

Introduction

Apoptosis is an evolutionarily conserved process critical in various biological events, such as embryonic development, maintenance of tissue homeostasis, and removal of damaged cells. Mitochondria are viewed as one of the most pivotal sensors and amplifiers in an apoptotic process by releasing apoptogenic proteins such as cytochrome c, Smac/DIABLO, and endonuclease G following mitochondrial membrane permeabilization (MMP) [1]. MMP is mainly mediated by Bcl-2 protein family members [2]. Bid (BH3 interacting domain death agonist) is an abundant proapoptotic member of the Bcl-2 family, which following activation through proteolytic cleavage, is involved in various pathways of apoptosis that interplay the activation of caspases with mitochondria dysfunction [3,4].

Recently accumulating evidence has indicated that, in addition to mitochondria, lysosomes also play important roles in the apoptosis [5]. As reported, several lysosomal acid-dependent proteases, known as cathepsins, have been implicated in apoptosis induction following their translocation to the cytosol as a result of moderate lysosomal rupture, probably via activation of the mitochondrial apoptotic pathway [6-8]. Interestingly, proteolytic activation of Bid may present a mechanism through which extra-lysosomal cathepsins can elicit MMP and subsequent caspase activation. Many isoforms of cathepsins (cathepsins B, H, L, S, K, X, C and D) have been identified to be Bid-cleaving proteases. Incubation of full-length Bid with cathepsins B, H, L and S, respectively, resulted in Bid activation and subsequent rapid cytochrome c release from isolated mitochondria. The physiological functions of cathepsins, however, have been controversial because they are usually autolyzed or denatured in the physiological conditions of the cytosol [9].

In the course of our study of mitochondrial membrane permeabilization [10,11], an unknown caspase 8-like activity capable of converting Bid into truncated Bid (tBid) at neutral pH was detected in highly purified rat liver lysosomal extracts. This gave us an impetus to purify the responsible lysosomal
protease(s). In the present study, we report the purification and characterization of one of the proteases termed Lyosomal Bid Cleavage Protease (LBCP). The cleavage site on Bid by LBCP is different from that of caspase 8, lysosomal cathepsins, granzyme B and calpain. By mass spectrometric assays, analysis of inhibitor specificity, and a determination of its Bid cleavage sites, LBCP was found to be identical to chymotrypsin B, a robust and stable serine endopeptidase previously known as an intestinal digestive enzyme expressed and secreted solely by the exocrine pancreas. Upon stimulation with TNF-α, the intralysosomal chymotrypsin B was released into the cytosol, triggered the release of mitochondrial cytochrome c, and resulted in apoptosis through activation of the mitochondrial apoptotic pathway.

Methods
Expression and purification of recombinant proteins
Mouse Bid expression plasmid constructed in pET-23d vector was kindly provided by Dr. Bruno Antonsson (Serono Pharmaceutical Research Institute, Switzerland). The Bid protein was expressed and purified as described previously [10]. Expression and purification of cystatins A and B in pHD389 vector, kindly provided by Dr. Ingemar Björk (Swedish University of Agricultural Sciences, Sweden), essentially followed his method. The SERPINB3 and SERPINB4 expression constructs (the coding sequences inserted into the pGEX-2T vector) were obtained from Dr. Gary A. Silverman (Harvard Medical School, USA). The recombinant proteins glutathione-S-transferase (GST) -SERPINB3 and -SERPINB4 were batch purified using glutathione-Sepharose columns (Amersham). The c-IAP-1 expression plasmid constructed in pGEX4T was a gift from Dr. Xiaodong Wang (University of Texas Southwestern Medical Center, USA). The GST-c-IAP-1 fusion protein was affinity purified on glutathione-Sepharose by standard methods.

The rat chymotrypsinogen B expression vector constructed in pET-17b was a generous gift from Dr. László Gráf (Eötvös Loránd University, Hungary). The vector was transformed into the BL21 (DE3) pLysS Escherichia coli strain. After induction with IPTG for 3 hr, the cells were collected in a 1/10 volume of 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA and frozen. The cells were then thawed and sonicated, and the inclusion body fraction was pelleted at 12,000×g for 30 min. The pellet was resuspended in ~30 ml Triton wash solution (0.5% Triton X-100, 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl) using a homogenizer. The inclusion bodies were pelleted at 25,000×g for 10 min. This wash was repeated 4 times and one final wash was performed to remove any remaining Triton using the homogenizer again in 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl. For renaturation of the expressed protein, the inclusion body fraction was solubilized with 6 M GuHCl, 0.1 M Tris-HCl, pH 8.0, 100 mM DTT. The solubilized protein was dialyzed against the refolding buffer containing 5 mM cysteine, 1 mM cystine, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, and GuHCl with its concentration continually decreasing from 3 M to 1 M. The renaturation process was conducted at 4 °C for 24 hr. The renatured protein solution was then dialyzed against 2 mM HCl, 10 mM CaCl₂ and ultracentrifuged (78,100×g, 30 min). The zymogen was activated by trypsin (Sigma) at a 200:1 (w/w) zymogen/trypsin ratio. After treatment with L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone (TLCK; ICN), the active form was purified by affinity chromatography on an SBTI-Sepharose column (Sigma). Immediately
after chymotrypsin B was eluted from the column with 1 mM HCl, the solution was neutralized with ammonia, and then lyophilized. The purity of the preparation was analyzed by SDS-PAGE. The enzyme concentration was determined by active site titration with 4-methylumbelliferyl p-trimethylammoniocinnamate chloride (MUTMAC, Sigma).

**Preparation of lysosomes and mitochondria**

Lysosomes were purified from the livers of Sprague-Dawley rats according to the method described by Stoka et al. [6] with modifications. We analyzed all purifications with one lysosomal and two mitochondrial markers to minimize any cross-contamination between the two organelles. To eliminate the contamination of mitochondria, the lysosomal fractions were incubated for 3 min at 37 °C in the presence of CaCl₂ (final concentration 1 mM). Soluble lysosomal constituents were released by three freeze-thaw cycles with a 15-s vortex between each cycle. The suspension was centrifuged at 10,000 ×g for 10 min to pellet the lysosomal membranes, and the supernatant was collected. Mitochondria were isolated from Sprague-Dawley rat livers according to the protocol described by Luo et al. [3].

**Purification of lysosomal Bid cleavage protease**

Five batches of lysosomal extracts from 20 rat livers were used as the starting material for the following purification procedures until the last step. All steps were carried at 4 °C unless otherwise noted. The Bid cleavage activity at pH 7.6 was followed after each step (Zhai et al., 2001). After dialysis against buffer A (25 mM Tris-HCl at pH 7.6, 10 mM NaCl, 10 mM 2-mercaptoethanol) containing 0.5 M NaCl, the lysosomal extract was loaded onto a 3-ml lentil lectin column (Amersham) and was re-circulated to provide sufficient interaction with the medium. The flow-through from the affinity column was then dialyzed against buffer A, followed by acidification at a final concentration of 200 mM NaAc/HAc at pH 5.0. Following incubation for 2 hr at 37°C and centrifugation for 10 min at 15,000×g, the supernatants were collected and dialyzed against buffer A.

This solution was then loaded onto a Bio-Scale Q5 column (Bio-Rad) equilibrated with buffer A, and eluted with a linear gradient of 45 ml from buffer A to buffer A containing 600 mM NaCl. Active fractions were pooled and, at a final concentration of 1.0 M ammonium sulfate, loaded onto a MP7 HIC column (50 × 7.8 mm, Bio-Rad) equilibrated with 1.0 M ammonium sulfate in buffer A. 10 ml of buffer A containing 200 mM ammonium sulfate was applied to the column, and the eluate collected and dialyzed against 25 mM ammonium formate at pH 7.4 containing 5 mM NaCl. The solution was lyophilized, re-dissolved in 0.5 ml of buffer A containing 150 mM NaCl, and loaded onto a Superdex 200 10/30 column coupled with a Superdex 75 10/30 column (Amersham), equilibrated, and eluted with buffer A containing 150 mM NaCl. The active fractions were stored at 4 °C till all five batches were processed to this point.

At the last step, all batches of the active fractions from the gel-filtration columns were pooled. After dilution to lower the concentration of NaCl to 100 mM, the solution was loaded onto a Mono Q 5/5 column (Amersham) equilibrated with buffer A containing 100 mM NaCl, and eluted with 30 ml of buffer A containing a linear gradient of NaCl increasing from 100 mM to 600 mM. Fractions of 0.4 ml were collected and assayed for Bid cleavage activity.

**In-gel digestion and mass spectrometric identification**

0.4 ml of the active peak fraction from the last purification step was lyophilized, re-dissolved in
120 µl of 1× SDS-PAGE loading buffer, and subjected to 15% SDS-PAGE. After staining with colloidal blue (Invitrogen), the ~25K bands that corresponded with the activity were subjected to in-gel digestion with trypsin (10 ng/µl). The tryptic peptides were extracted and dried in a vacuum centrifuge, and were analyzed by LC-MS/MS using a nano-scale C18 column coupled in-line with an ion trap mass spectrometer (LCQ Deca, Thermo Finnigan). The instrument was run in data-dependent mode, cycling between one full MS scan and MS/MS scans of the three most abundant ions. The MS and MS/MS data were used to search the non-redundant NCBI protein database using MASCOT software.

**Effect of inhibitors on the Bid cleavage activity**

Aliquots from the Mono Q fraction at the peak of Bid cleavage activity were preincubated for 30 min at 37°C in the presence of 0.1 mg/ml aprotinin (inhibitors were from Sigma unless otherwise indicated), 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml soybean trypsin inhibitor (SB-TI), 0.25 mM chymostatin, 5 mM N-ethylmaleimide, 20 µM E-64, 0.2 mM 3,4-dichloroisocoumarin (DCI; ICN Biomedicals), 0.5 mM L-1-chloro-3-[4-tosylamido]-7-amino-2-heptano ne (TLCK), 0.5 mM N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 5 mM iodoacetamide, 10 µM pepstatin A, 10 mM EDTA, 10 mM ortho-phenanthroline, 1 µM caspase-1 inhibitor I (Ac-YVAD-CHO; Calbiochem), 100 µM caspase-3 inhibitor I (Ac-DEVD-CHO), 1 µM caspase-8 inhibitor I (Ac-IETD-CHO), 0.25 mM cathepsin inhibitor I (Z-FG-NHO-Bz), SERPINB3 (500:1 molar ratio), SERPINB4 (500:1), cystatin A (500:1), cystatin B (500:1), or c-IAP1 (500:1). 10 µg of Bid was then introduced into each incubation mixture, and the final volume adjusted to 20 µl. The reaction was stopped after incubation for 3 hr at 37°C. Appropriate solvent controls were run in parallel.

**N-terminal sequencing of Bid cleaved by lysosomal protease**

Bid cleaved by the lysosomal protease was separated by Tricine-SDS-PAGE on 15% gel and transferred to a PVDF membrane (Gelman). The appropriate band was excised, and the N-terminal sequence of the Bid cleavage product was determined with an ABI-491 amino acid sequencer (Applied Biosystems).

**Determination of Km**

The enzyme assay was performed at 37°C on an F-4500 fluorescence spectrophotometer (Hitachi). A 1 mM stock solution of the substrate Suc-AAPF-AMC was made in DMSO. The reaction cuvette contained 100 µl of the reaction buffer (100 mM Tris-HCl buffer, pH 8.0, 10 mM CaCl2, 0.1 M NaCl) into which various volumes of the substrate solution and 5 µl of the sample to be tested were introduced. The progression of the fluorescence increase was measured at λem = 460 nm with fluorescence excitation at λex = 380 nm. The detected signals were converted into moles of substrate hydrolyzed per second. Initial velocities and substrate concentrations were fit by nonlinear regression to the Michaelis-Menton equation.

**Reverse transcription (RT)-PCR detection of Ctrb mRNA**

Primary rat hepatocytes isolated from Sprague-Dawley rats and rat hepatoma RH-35 cells (Chinese Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal calf serum (GIBICO-BRL) at 37°C under 5% CO2. For the RT-PCR analysis of Ctrb mRNA, total RNA was isolated by using a RNeasy Mini Kit (Qiagen). RT-PCR was carried out by using an Access RT-PCR Kit (Promega), with primers specific for rat Ctrb (GATCGCACAGGTCTTTAAGAA, and...
CATCGACGTGGGTAGACAC; PCR product 133 bp; the primer sequences crosses an intron which could eliminate the disturbance of genomic DNA contaminations.) The PCR products were subjected to 3 % agarose gel electrophoresis followed by DNA sequencing.

**Western blot analysis of Ctrb protein**

RH-35 hepatoma cells were lysed in ice-cold buffer consisting of 10 mM Tris-HCl (pH 7.3), 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, 1 mM ATP, 2 mM sodium orthovanadate, 100 µM phenylarsine oxide, 3 mM diisopropyl fluorophosphate, 10 µg/ml leupeptin, and 10 µg/ml aprotonin, and disrupted by sonication on ice. After ultracentrifugation at 10,000 × g for 30 min at 4 °C, the supernatant containing solubilized proteins were separated on a 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto PVDF membrane. The membrane was probed with home-made antibody against Ctrb, and then incubated with a horseradish peroxidase-conjugated second antibody. After four washes with 0.05% TBST, the target protein was detected by using an enhanced chemiluminescence assay.

**Site-directed mutagenesis of Bid**

The cleavage site mutation of Bid was generated using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions and was confirmed by DNA sequencing. The mutagenic oligonucleotides are: 5′-GCCAGCCGCTCCTCAACCAAGGAAGAATA-3′ (sense), and 5′-TATTCTTCTTGGTTGAAGGAGCGGCTGGC-3′ (anti-sense).

**In vitro assay for cytochrome c release induced by cleaved Bid**

Bid was cleaved by LBCP at 37 °C for 1 h. An aliquot of rat liver mitochondria equal to 50 µg of protein was incubated with cleaved Bid at a final concentration of 10 µg/ml in a final volume of 50 µl MT buffer (400 mM mannitol, 10 mM KH₂PO₄, and 50 mM Tris-HCl, pH 7.2, 5 mM succinate) at 30°C for 30 min. After incubation, the reaction mixture was pelleted by centrifugation for 5 min at 12,000 × g. The cytochrome c in the supernatant and mitochondrial pellet were analysed by Western Blot with an anti-cytochrome c antibody (Pharmingen).

**Immunofluorescence and confocal microscopy**

For observation of the intracellular Ctrb or cytochrome c by immunofluorescence, RH-35 cells were fixed with 4 % paraformaldehyde at room temperature for 1 h, permeablized with methanol at -20 °C for 5 min, blocked with 5% new born calf serum at room temperature for 30 min, incubated with a rabbit-anti Ctrb antiserum (US Biological), a rabbit-anti cytochrome c antibody (Santa Cruz) or a goat-anti cathepsin D antibody at 4 °C overnight, and probed with Cy3-labeled or FITC-labeled secondary antibodies (Sigma) at 37 °C for 1.5 h.

LysoTracker Red DND-99 (Molecular Probes) was loaded into RH-35 cells by incubating the cells in probe-containing media at a final concentration of 50 nM for 1 h at 37°C [28]. Confocal microscopy was performed with an inverted Olympus FluoView FV500 Laser Scanning Confocal Microscope (Osaka) using excitation and emission wavelengths of 488 and 507 nm for GFP and FITC, 433 and 475 nm for CFP, 570 and 590 nm for LysoTracker, and 550 and 570 nm for cy3, respectively.

**Construction of rat pre-chymotrypsinogen B expression vector (Ctrb-GFP) and transfection**

With the rat chymotrypsinogen B expression vector pET-17b lacking 51 nucleotides encoding N-terminal signal peptide of the chymotrypsinogen B precursor, the coding sequence was amplified using the following two
primers:
5′-TTTAAGCTTATGGCATTCCTTTGGCTC
GTGTCCTGCTTTGCCCTTGTGGGGCCAC
CTTTGGCTGTGGAGTCCCTACCATCC-3′
(sense), and
5′-AAAGGATCCTCAGTTGGCTTCCAAGAT
CTG-3′ (anti-sense). The PCR product and
pEGFP-N1 (Clontech) were cut with Hind III
and BamH I (TaKaRa), followed by purification
and ligation. Transformed competent cells
(TianWei, China) were plated on selective agar
plates, and colonies were selected and grown up
in LB media containing 50 µg/ml kanamycin.
The sequences were verified by DNA
sequencing. In order to check whether the new
construct generate functional proteins, the
proteins were expressed in vitro using the
Promega TNT coupled transcription-translation
system (Promega) and radiolabeled with
\[^{35}\text{S}\]-methionine (Amersham). Efficiency of
expression was monitored by analyzing the
translation with SDS-PAGE and
autoradiography. Expression plasmids for
transfection were purified using the Plasmid
Midi kit (Qiagen).
The expression plasmids were transfected into
RH-35 cells using Lipofectamine Plus reagent
(Invitrogen) according to the procedure
recommended by the manufacturer. The expression vector for LAMP-2-CFP was a
generous gift from Dr. GJ Gores (Mayo Medical
School, USA). In brief, RH-35 cells grown in 35
mm dishes at 60 % confluence were transfected
with 4 µg of the plasmid, 6 µl of lipid, and 6 µl
of Plus reagent in 0.8 ml of DMEM without
antibiotics. To obtain stable transfected cell
lines, the cells were cultured in DMEM
containing 600 µg/ml G418 for 3 weeks.

**Delivery of Ctrb into rat RH-35 hepatoma
cells**
Recombinant chymotrypsin B was delivered to
RH-35 rat hepatoma cells by using the
BioPORTER protein transfection reagent (Gene
Therapy Systems, USA) according to the
manufacturer’s instructions. Briefly, 50 µl of
Ctrb solution (1 µM) was incubated in the
presence or absence of 0.2 mM TPCK for 30
min at 37°C. Ctrb solutions or PBS (Blank) were
then used to hydrate the dried BioPORTER. The
solution was pipetted up and down, and
incubated at room temperature for 5 min.
Finally, the volume of the complexes was
brought to 0.5 ml with serum free medium and
then transferred directly onto the RH-35 cells.
After incubation for 5 hr, 2 ml of serum-containing medium was added. The cells
were incubated overnight and then subjected to
apoptosis assays.

**RNAi studies**
The mammalian expression vector,
pSUPER.retro.neo (OligoEngine) was used for
expression of siRNA in RH-35 cells. The
gene-specific insert specifies a 19-nucleotide
sequence corresponding to nucleotides 421-439
of Ctrb. These sequences were inserted into the
pSUPER.retro.neo backbone after digestion
with BglII and HindIII. This vector was referred
to as pSUPER-Ctrb. Considering bioinformatics
studies revealed that the empty
pSUPER.retro.neo vector might form hairpin
oligonucleotide(s) that could interfere the
stability of Ctrb mRNA and consequently
induce non-specific downregulation of Ctrb, a
control vector (pSUPER-LacZ) with no
significant homology to Ctrb gene was serves as
a non-silencing control.
The transient transfections of the RH-35 rat
hepatoma cells with pSUPER-Ctrb or
pSUPER-LacZ (control) vectors were done with
Nucleofector Solution V coupled with program
T-030 (Amaxa). Transfected cells were analyzed
by real-time PCR for actin and Ctrb using the
Bio-Rad MyIQ. Data were calculated by the
relative quantitation method, compared to actin
as internal control.

To determine the effect of RNAi-mediated
downregulation of Ctrb on TNF-α-induced apoptosis, transiently transfected RH-35 cells were treated with 100 ng/ml TNF-α for 6 h and subjected to flow cytometry analysis of apoptosis.

**Apoptosis assays**

Cells were harvested by mild trypsinization, washed with cold PBS, and fixed with 70% ethanol. Cells were stained with propidium iodide and the percentage of hypodiploid (apoptotic) cells with a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

**Results**

**Purification of lysosomal Bid cleavage protease (LBCP)**

The impetus for the current study was our previous observation of an unknown caspase 8-like lysosomal protease capable of converting Bid into truncated Bid (tBid) at neutral pH. We took painstaking effort to purify this protease. Fig. 1a shows the many steps involved in purification as we followed the Bid-cleavage activity at pH 7.6. Starting with highly purified lysosome preparations (mitochondrial contaminations lower than 1%) from rat livers, a robust and stable Bid cleavage activity with maximum activity at pH 7.6 was detected in the supernatant (Fig. 1b) after incubation of the lentil lectin flowthrough fraction at pH 5.0 (intralysosomal pH) and 37°C. This enzymatic activity was designated as lysosomal Bid cleavage protease (LBCP). We believe that it is the incubation that leads to the activation of the LBCP from its premature LBCP zymogen, although the reason is so far not understood. Next, the pH of the active supernatant was changed to 7.0 by dialysis and subjected to anion exchange. As shown in Fig. 1b and 1c, the eluate fractions (fractions 26–32) at ~270mM NaCl (Fig. 1c) showed the apparent Bid cleavage activity at pH7.6, and corresponded with the appearance of a ~25kDa protein observed on silver stained SDS-PAGE gels (Fig. 1d). This band was excised and subjected to LC-MS/MS analysis (see “Mass spectrometric identification of LBCP”). To the end, it was estimated that 2 - 6 µg of the protein (ca. 0.3 - 1 ng/ µl) from liver lysosomes of 100 rats could be obtained. The purified LBCP shows a potent Bid cleavage activity since a 10,000 fold molar ratio excess of Bid could be cleaved by LBCP within minutes.

**LBCP is identified as chymotrypsin B**

1. **Biochemical characterization of LBCP**

To biochemically characterize the LBCP, a wide range of peptidase inhibitors were assayed for their possible potential on the Bid cleavage activity of the purified LBCP (refer to http://merops.sanger.ac.uk). It can be seen that the proteolytic cleavage of Bid was abolished by PMSF, DCI, SB-TI and chymostatin, but not by inhibitors of other classes (Fig. 2a and Table 1). Noticeably, in contrast to benzamidine and TLCK, TPCK, which belongs to the class of chymotrypsin-like peptidase inhibitors, specifically inhibited the activity of LBCP. Of five endogenous protein inhibitors tested, only SERPINB3 and SERPINB4 counteracted LBCP to some extent, while cystatins A and B, and c-IAP-1 had no effect. Thus, the inhibition profile suggested that LBCP is a chymotrypsin-like serine protease.

To identify Bid cleavage sites by the LBCP, the ~14 kDa C-terminal part of Bid resolved by SDS-PAGE (Fig.2b) was analyzed by electroblotting and N-terminal sequencing, which revealed that the cleavage occurred at Phe67. This site is unique to the action of LBCP (Fig. 2c). In order to verify this result, a Bid mutant F67A was constructed and
processed as described above. As shown in Fig. 2d, the mutant Bid was degraded at a slower rate and a ~17 kDa cleavage product of Bid (F67A) with the N-terminal sequence WEADLE appeared instead of the normal ~14 kDa fragment. The ~17kDa part was identified to be cleaved at Tyr47. The specific cleavage site of Bid by the LBCP supports the idea that this enzyme is a chymotrypsin-like protease.

We also used the fluorogenic substrate Suc-AAPF-AMC for a continuous fluorometric assay of LBCP activity. Cleavage of Suc-AAPF-AMC by LBCP showed Michaelis-Menton kinetics with a $K_m$ value of 23.7 ± 2.6 $\mu$M (Fig. 2e). This value is shared by rat chymotrypsin B (Ctrb) [12].

Since proteolytic activation of Bid acutely enhances its pro-apoptotic potential [3, 4], we tested whether LBCP was capable of facilitating the release of cytochrome c from isolated mitochondria through truncation of Bid. As shown in Fig.2f, the apparent release of cytochrome c from mitochondria was observed in the presence of Bid and LBCP.

2. Mass spectrometric identification of LBCP

The band of LBCP resolved by SDS-PAGE (Fig.1b) was subjected to trypsin digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (see Supplementary Information, Fig. S1). All the tryptic peptides showed 100% identity with the reported sequence of a 28 kDa protein, chymotrypsinogen B (Rattus norvegicus, GI: 6978717). The consistency between these data and the biochemical characterization of LBCP justifies its identification as chymotrypsin B (Ctrb).

Expression of chymotrypsin B mRNA and protein in hepatocytes

Ctrb, encoded by a single copy nuclear gene [13] (Locus ID: 24291), has been well known as a protease expressed and secreted by the exocrine pancreas. No other tissues had been demonstrated experimentally to contain this enzyme. However, our above results indicated that this enzyme should reside in rat liver. To address this question, we isolated total RNA from both primary rat hepatocytes and RH-35 rat hepatoma cells, and performed reverse transcription (RT)-PCR assay (Fig. 3a). The sequence of the PCR product was 100% identical to the cDNA sequence of rat Ctrb, supporting the expression of Ctrb in rat liver cells. Furthermore, Western blot analysis of Ctrb protein revealed the existence of Ctrb protein in RH-35 hepatoma cells (Fig. 3b). Also, a bioinformatics study performed by searching against the gene expression databases UniGene, GeneNote, and GeneCard for the human, mouse, and rat genomes (for details, refer to http://www.ncbi.nlm.nih.gov/UniGene and http://bioinfo.weizmann.ac.il) also implied that Ctrb expression may not be restricted to the pancreas.

Lysosomal localization and release of chymotrypsin B

Unfavorable against the current concept that, like other members of the chymotrypsin family, Ctrb is a secretory protein, we obtained Ctrb protein from rat liver lysosomes. To verify that Ctrb is indeed localized to liver lysosomes, the intracellular Ctrb was visualized and identified by immunofluorescence. Cathepsin D, a typical lysosomal protease, was used as a marker of lysosomes. RH-35 rat hepatoma cells were fixed, permeablized, incubated with anti-chymotrypsin B antiserum or anti-cathepsin D antibody, respectively, and labeled with proper fluorescent-labeled secondary antibodies. The
merged red fluorescence of Ctrb and green fluorescence of Cathepin D (Fig. 4a) verified their colocalization to the same vesicular compartment.

To further investigate the intralysosomal localization of Ctrb, Ctrb in its full form pre-chymotrypsinogen B (chymotrypsinogen B precursor containing the N-terminal signal peptide) was tagged with GFP, followed by intracellular delivery of this construct together with another fluorescent reporter plasmid encoding lysosome-associated membrane protein-2-CFP (LAMP-2-CFP), to the RH-35 cells. Both Ctrb-GFP and LAMP-2-CFP displayed a punctuate appearance when viewed by confocal microscopy, indicative of a vesicular compartmentation of the tagged proteins. Overlay images demonstrated virtually complete colocalization of the two fluorescent proteins (Fig. 4b). Moreover, Ctrb-GFP-transfected cells were co-loaded with LysoTracker Red. The merged red fluorescence of LysoTracker Red and green fluorescence of Ctrb-GFP further verified their colocalization to the same vesicular compartment. Thus, these observations suggest Ctrb is targeted to lysosomes in the RH-35 cells.

Since a functional localization of Ctrb in cytosol is a prerequisite to inducing apoptosis, we next studied the release of Ctrb from lysosomes under apoptotic stimuli. RH-35 cells were treated with TNF-α (100 ng/ml). As shown in Fig. 5a, the Ctrb (red fluorescence) underwent redistribution from the vesicular compartments to the cytoplasm in a time-dependent manner, with an onset as early as 2 h after TNF-α administration. Moreover, it is interesting to observe that mitochondrial release of cytochrome c occurred after 8 hrs of TNF-α treatment, later than the lysosomal release of Ctrb (Fig. 5b).

**Verification of involvement of chymotrypsin B in the apoptotic induction**

To verify further the cytosolic Ctrb could induce apoptosis, Ctrb protein was delivered intracellularly to RH-35 cells by BioPORTER. After its administration, we observed a significant release of mitochondrial cytochrome c (Fig. 6a), chromatin condensation and nuclear fragmentation (Fig. 6b). Moreover, the delivered Ctrb augmented the apoptosis rate to 3.9 times that by the reagent control (Fig. 6c), comparable to that induced by granzyme B [14] or cathepsin B (data not shown). Neither the TPCK-treated Ctrb nor the delivery reagent alone produced marked increases in apoptosis rate. These results strongly suggest that Ctrb is capable of triggering apoptosis through a mitochondrial pathway via cleavage of Bid.

**Suppression of endogenous Ctrb activity attenuated TNF-α-induced apoptosis**

To further demonstrate the involvement of Ctrb in the apoptotic regulation, we reduced the endogeneous Ctrb activity by its specific inhibitor TPCK or RNA interference.


RH-35 cells were treated with TNF-α (100 ng/ml), and its apoptosis was quantified by flow cytometry. Pretreatment of RH-35 cells with TPCK (40 µM), significantly attenuated apoptosis induced by TNF-α (Fig. 7a). It can be seen that 28.7 % of cells pretreated with TPCK underwent apoptosis compared with 42.5% of cells not pretreated.

b. Knockdown of Chymotrypsin B expression by RNAi

Furthermore, we have used the pSUPER.retro.neo system to transiently suppress the expression of the Ctrb gene. RH-35 rat hepatoma cells were transfected with the pSUPER-Ctrb or pSUPER-LacZ control vector. When analyzed 72 h after transfection, the pSUPER-Ctrb-transfected cells showed a significant reduction (~70.0%) in Ctrb mRNA compared with the
pSUPER-LacZ cells, as measured by real time RT-PCR (Fig 7b). We then determined the survival of cells transfected with pSUPER-Ctrb or pSUPER-LacZ vectors under the condition of 100 ng/ml TNF-α treatment. As shown in Fig. 7c, cells transfected with pSUPER-Ctrb had a survival rate of 59.8%, significantly higher than that of cells transfected with pSUPER-LacZ vector (39.5%), suggesting that RNAi-mediated knockdown of endogenous Ctrb expression significantly decreased TNF-α-induced apoptosis. Taking the results together, Ctrb is identified as one of the executors and mediators that regulate apoptosis in RH-35 cells induced by TNF-α.

**Discussion**

Caspases are well known to be universal apoptotic executioners. Recently, non-caspase death effectors including calpains, granzymes, Omi/htra2, and lysosomal cathepsins have been reported to be involved in apoptosis [15, 16]. It has been strongly suggested that mild perturbation of lysosomes followed by release of entrapped enzymes can initiate apoptosis via a lysosomal-mitochondrial pathway [5-9]. An attractive candidate bridging the lysosomal proteases and the downstream mitochondrial membrane permeabilization (MMP) is Bid. Bid has been identified as a substrate of caspase 8, calpain, granzyme B, and cathepsins [3, 4, 6, 17]. So far, several orthodox lysosomal cathepsins have been shown to activate Bid and induce apoptosis [9, 18]. Nevertheless, this function of cathepsins remains controversial [8, 19]. It has been suggested that, besides the known papain-like cathepsins, other lysosomal proteases might exist and be involved in apoptotic regulation through Bid cleavage. In this study, we have purified a protease from rat liver lysosomes with strong Bid cleavage activity that is optimal at neutral pH. Via biochemical and mass spectrometric assays, this protease was identified as Chymotrypsin B (Ctrb), a well-known secretory protease synthesized in pancreas. We have now demonstrated for the first time that Ctrb co-localizes with lysosomes of rat hepatocytes. In view of the release of Ctrb from lysosomes during apoptosis and its pro-apoptotic property, Ctrb could be involved in the lysosomal-mitochondrial apoptotic pathway. In addition to cathepsins, which normally function at an acidic pH, Ctrb, which functions optimally at the neutral pH of cytosol, should be recognized as another class of lysosomal proteases potentially involved in the regulation of apoptosis. It is interesting to note that both RNAi studies and pretreatment of RH-35 cells with specific inhibitor of Ctrb, TPCK, could significantly but partly attenuated apoptosis induced by TNF-α. Transfection of pSUPER-Ctrb caused a marked (~70%) decrease in Ctrb mRNA levels, and resulted in a moderate (~20%) decrease in the apoptosis rate upon TNF-α treatment, comparable to that as a result of silencing cathepsins [8]. This suggests that Ctrb contributes to TNF-α-induced apoptosis in RH-35 cells. However, the relative contributions of Ctrb and other pro-apoptotic proteases to apoptosis and their mutual effects (synergetic or simply additive) deserves further investigation.

The manner by which Ctrb is retained in lysosomes and is activated in vivo remains an open question. The luminal pH and redox potential within the late endolysosomal system are subtly designed to denature substrates, allowing for increased hydrolytic efficiency. These unique conditions impose stringent requirements that can be met almost exclusively by cathepsins in their mature forms. In contrast, Ctrb is incompatible with the denaturing luminal environment, with acidification leading to substantial loss of its activity [20]. We would
like to suppose that lysosomal Ctrb must take on a strategy to protect from the deleterious conditions in the lysosomal lumina and sustain its potential as a neutral serine protease. Actually, in our purification procedure, we did observe lysosomal Ctrb underwent a drastic increase of Ctrb activity following \textit{in vitro} incubation (see Fig. 1b), which indicates its inactive but potentially activable status in lysosomes. Presumably, lysosome Ctrb might be stored in its premature form, chymotrypsinogen B, or alternatively, co-exist with an inhibitory factor. This form of Ctrb may then secede from lysosomes to the cytosol under either physiological or pathological conditions in intact cells. The details underlying the release and activation of Ctrb \textit{in vitro} remain unknown.

There is a growing body of evidence in apoptosis research that supports the participation of chymotrypsin-like serine proteases in cell death regulation \[21, 22\]. Most of this evidence was obtained by using specific inhibitors (mainly TPCK) \[21, 23\] and implicates serine proteases in various steps of the apoptotic process, such as being the intermediate linking early lysosomal rupture and MMP \[24\] and having a role in the postmitochondrial stage \[25\], and the terminal stage of internucleosomal DNA fragmentation \[26\]. These versatile actions indicate variability in the abundance, localization and physiological substrates of the putative chymotrypsin-like proteases within individual apoptotic cells. However, little is known about the family members and the targets of their actions during the apoptotic process. Granzymes are serine family proteases involved in apoptosis regulation, whereas they are found exclusively in the cytoplasmic granules within cytotoxic T cells and natural killer cells, and following their secretion enter the target cell via endocytosis and induce apoptosis. In contrast, Ctrb represents the first discovered neutral serine protease that ubiquitously distributes in conventional lysosomes of various cell types, and functions intracellularly as an pro-apoptotic agent. This finding raises many questions including the regulation of Ctrb expression and activity, the trafficking mechanism responsible for its lysosomal delivery, its cellular targets and other novel roles. We anticipate that our findings will stimulate additional interest to serine protease-dependent apoptosis and will gain a deeper insight into the complex nature of apoptosis when the aforementioned questions are answered. Other lysosomal proteases are known to be multi-tasking with extralysosomal roles in the cytosol, nucleus and cell exterior during both normal cell function and pathological conditions such as cancer, inflammation and neurodegenerative disorders \[27\]. Thus, the interrelationship between Ctrb and the other lysosomal proteases is also interesting and deserves further study. The wide tissue expression and lysosomal localization of Ctrb suggest that it might be involved in some important physiological and/or pathological events. It is therefore possible that Ctrb performs other functions that are unrelated to induction of apoptosis. To sum up, our finding encourages a rethinking of the function of Ctrb and further study to elucidate its substrates, and could possibly change the specific function assigned to Ctrb from an intestinal digestive protease to a more important mediator in cells.

\textbf{Acknowledgements}

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School, USA), L. Gráf (Eötvös Loránd University, Hungary) and G. A. Silverman (Harvard Medical School, USA) for kindly providing plasmids and reagents, and Drs. A. Barrett (Wellcome Trust Sanger Institute, UK), P. S. Liu (University of Texas Southwestern Medical Center at Dallas, USA) and Prof. R. S. Chen (Institute of Biophysics, CAS) for valuable suggestions and discussions. We also express our appreciation to Drs. X. D. Wang (University of Texas Southwestern Medical Center at Dallas, USA), Z. C. Cui (Medical University of Dalian, China), Y. P. Tu and D. Wolff (Creighton University, USA) for their helpful comments and suggestions during manuscript preparation. The authors are also indebted to Mrs. W. M. Zhong for her technical assistance.
Figure Legends

Figure 1. Purification of the lysosomal Bid cleavage protease (LBCP) from rat liver lysosomes.
(a) Diagram of the purification scheme. (b) Activation of LBCP. Lysosomal extract from 10 rat livers was prepared. Half of the extract was subjected to dialysis against buffer A and subsequent Bio-Scale Q5 chromatography (upper panel). The other half was treated following the purification procedures as described in Methods (lower panel). Aliquots (20 µl) of the Q5 fractions (1 ml each) were incubated with ~8 µg of Bid at 37°C for 3 hours, and then subjected to 15% SDS-PAGE and Coomassie-staining. Potent Bid cleavage activity appeared only in the latter case (lower panel). (c) Mono Q anion-exchange chromatography. (d) Aliquots of 2 µl from the last purification step, the Mono Q column, were assayed for Bid cleavage activity by incubation with ~8 µg of Bid in a total volume of 12 µl at 37°C for 3 hours, and then subjected to 15% SDS-PAGE and Coomassie-staining (upper panel). Additional 25 µl aliquots from the same samples were subjected to 15% SDS-PAGE followed by silver staining (lower panel). The arrow shows the lysosomal Bid cleavage factor (LBCP).

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**Figure 5. Redistribution of Chymotrypsin B and cytochrome c induced by**
TNF-α. (a). Redistribution of Chymotrypsin B induced by TNF-α. RH-35 rat hepatoma cells were treated with 100 ng/ml TNF-α for indicated intervals, fixed, permeabilized, incubated with anti-chymotrypsin B antiserum, and labeled with cy3-labeled secondary antibody. Immunofluorescence images were recorded by laser scanning confocal microscope. The Ctrb (red fluorescence) underwent redistribution from the vesicular compartments to the cytoplasm in a time-dependent manner, with an onset as early as 2 h after TNF-α administration. Scale Bar: 20 µm. (b). Release of mitochondrial cytochrome c (green fluorescence) in RH-35 cells as observed by immunofluorescence. RH-35 rat hepatoma cells were incubated with 100ng/ml TNF-α for indicated intervals, fixed, permeabilized, incubated with anti-cytochrome c antibody, and labeled with FITC-labeled secondary antibody. Release of mitochondrial cytochrome c occurred after 8 hrs of TNF-α treatment, later than the lysosomal release of Ctrb. Scale Bar: 10 µm.

Figure 6. Induction of apoptotic events by intracellular delivery of Ctrb. (a) Intracellular delivery of Ctrb triggered the rapid release of mitochondrial cytochrome c (green fluorescence) in RH-35 cells. Recombinant chymotrypsin B (2.6 µg/ml) was delivered to RH-35 rat hepatoma cells by using the BioPORTER protein transfection reagent, and the release of mitochondrial cytochrome c (green fluorescence) in RH-35 cells was observed by immunofluorescence. Scale Bar: 10 µm. (b) Intracellular delivery of Ctrb induced nucleic fragmentation. Recombinant chymotrypsin B (2.6 µg/ml) was delivered to RH-35 rat hepatoma cells by BioPORTER, cells were fixed and stained with DAPI and observed under a fluorescence microscope. (c) Intracellular delivery of Ctrb induced DNA hypodiploidy. Recombinant chymotrypsin B (2.6 µg/ml) was delivered to RH-35 rat hepatoma cells by BioPORTER. The nuclear DNA content was determined with PI by cytofluorometric analysis in fixed-permeabilized cells. Numbers indicate the percentage of cells exhibiting a subdiploid (apoptotic) DNA content.

Figure 7. Effects of inhibition of chymotrypsin B activity or knockdown of chymotrypsin B expression on TNF-α induce apoptosis in RH-35 cells. (a) Pretreatment of RH-35 cells with TPCK inhibited the apoptosis induced by TNF-α. RH-35 hepatoma cells were preincubated with chymotrypsin B inhibitor TPCK (40 µM) for 30 min and then treated with 100 ng/ml TNF-α for 6 h. The apoptotic rates were measured by cytofluorometric analysis of the percentage of cells exhibiting a subdiploid (apoptotic) DNA content. *: p < 0.01 in comparison with untreated cells; **: p < 0.05 in comparison with RH-35 cells treated with 100 ng/ml TNF-α. (b) Knockdown of chymotrypsin B expression by RNAi. RH-35 cells were transfected with pSUPER-Ctrb vector, which contains a 19-nucleotide sequence corresponding to nucleotides 421-439 of Ctrb, or pSUPER-LacZ control vector, respectively, and cultured for 48 h. The expression of Ctrb mRNA was analyzed by real-time PCR. The expression of Ctrb mRNA was significantly down-regulated in RH-35 cells transfected with a pSUPER-Ctrb vector. (c) Down-regulation of Ctrb mRNA expression by RNAi partly prevented cells from TNF-α-induced apoptosis. RH-35 cells were transfected with pSUPER-Ctrb or pSUPER-LacZ, respectively, and cultured for 48 h. Then cells were treated with 100 ng/ml TNF-α for 6 h and apoptosis was assessed by flow cytometry. *: p < 0.05 in comparison with RH-35 cells transfected with pSUPER-Ctrb.
References

a

Lysosomal extract

\[ \downarrow \]

Lentil lectin-Sepharose affinity column

\[ \downarrow \]

Flowthrough

\[ \downarrow \]

NaAc/HAc (200 mM, pH 5.0)

Incubation at 37°C, 2hr

Aggregation and precipitation of proteins

\[ \downarrow \]

Centrifugation

\[ \downarrow \]

Supernatant

\[ \downarrow \]

Dialysis against Tris buffer pH 7.0

Q-Sepharl column

\[ \downarrow \]

0~0.6M NaCl

MP7 HIC column

\[ \downarrow \]

0.2 M ammonium sulfate

Superdex-75/200 gel filtration

\[ \downarrow \]

Mono Q column

\[ \downarrow \]

0.1~0.6M NaCl

Unknown protease (LBCP)
b

Fractions 0 16 18 20 22 24 26 28 30 32 34 36

150 mM

400 mM NaCl

Bid

Bid

tBid

tBid

b by guest on September 1, 2017 http://www.jbc.org/ Downloaded from
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Co-localization of Chymotrypsin B (red fluorescence) and Cathepsin D (green fluorescence) in lysosomes of RH-35 rat hepatoma cells. RH-35 rat hepatoma cells were fixed, permeabilized, incubated with anti-chymotrypsin B antiserum and anti-cathepsin D antibody, and labeled with cy3-labeled and FITC-labeled secondary antibodies. Immunofluorescence images were recorded by laser scanning confocal microscope. The merged red fluorescence of chymotrypsin B and the green
fluorescence of cathepsin D (lysosomal marker) yielding a yellowish fluorescence, indicated the lysosomal localization of Ctrb in RH-35 rat hepatoma cells. Scale Bar: 10 µm. (b). Localization of Chymotrypsinogen B-GFP fusion protein in lysosomes of RH-35 rat hepatoma cells. RH-35 cells were stably transfected with the plasmids encoding the fusion proteins Chymotrypsin B-green fluorescence protein (Ctrb-GFP) and lysosome-associated membrane protein-2-cyan fluorescent protein (LAMP-2-CFP), a lysosomal-associated membrane protein. After loading the cells with LysoTracker Red for 1 h to selectively stain the lysosomal compartment, they were imaged alive by laser scanning confocal microscopy. Ctrb-GFP (green) colocalized with LAMP-2-CFP (blue) and LysoTracker Red (red) as shown in the overlay image, confirming its lysosomal distribution. Scale Bar: 10 µm.
Figure 5. Redistribution of Chymotrypsin B and cytochrome c induced by TNF-α. (a). Redistribution of Chymotrypsin B induced by TNF-α. RH-35 rat hepatoma cells were treated with 100 ng/ml TNF-α for indicated intervals, fixed, permeabilized, incubated with anti-chymotrypsin B antiserum, and labeled with cy3-labeled secondary antibody. Immunofluorescence images were recorded by laser scanning confocal microscope. The Ctrb (red fluorescence) underwent redistribution from the vesicular compartments to the cytoplasm in a time-dependent manner, with an onset as early as 2 h after TNF-α administration. Scale Bar: 20 µm. (b). Release of mitochondrial cytochrome c (green fluorescence) in RH-35 cells as observed by immunofluorescence. RH-35 rat hepatoma cells were incubated with 100ng/ml TNF-α for indicated intervals, fixed, permeabilized, incubated with anti-cytochrome c antibody, and labeled with FITC-labeled secondary antibody. Release of mitochondrial cytochrome c occurred after 8 hrs of TNF-α treatment, later than the lysosomal release of Ctrb. Scale Bar: 10 µm.
a

![Untreated RH-35 cells vs RH-35 cells treated with Ctrb](image)

b

Untreated RH-35 cells vs RH-35 cells treated with Ctrb

c

Blank vs TPCK-Ctrb vs Ctrb
Figure 6. Induction of apoptotic events by intracellular delivery of Ctrb. (a) Intracellular delivery of Ctrb triggered the rapid release of mitochondrial cytochrome c (green fluorescence) in RH-35 cells. Recombinant chymotrypsin B (2.6 µg/ml) was delivered to RH-35 rat hepatoma cells by using the BioPORTER protein transfection reagent, and the release of mitochondrial cytochrome c (green fluorescence) in RH-35 cells was observed by immunofluorescence. Scale Bar: 10 µm. (b) Intracellular delivery of Ctrb induced nucleic fragmentation. Recombinant chymotrypsin B (2.6 µg/ml) was delivered to RH-35 rat hepatoma cells by BioPORTER, cells were fixed and stained with DAPI and observed under a fluorescence microscope. (c) Intracellular delivery of Ctrb induced DNA hypodiploidy. Recombinant chymotrypsin B (2.6 µg/ml) was delivered to RH-35 rat hepatoma cells by BioPORTER. The nuclear DNA content was determined with PI by cytofluorometric analysis in fixed-permeabilized cells. Numbers indicate the percentage of cells exhibiting a subdiploid (apoptotic) DNA content.
a

Apoptotic cells (%)

- Untreated
- TNF-α
- TPCK
- TNF-α + TPCK

b

Chymotrypsin B mRNA level (%)

- pSUPER-Cont
- pSUPER-LacZ
Figure 7. Effects of inhibition of chymotrypsin B activity or knockdown of chymotrypsin B expression on TNF-α induce apoptosis in RH-35 cells.

(a) Pretreatment of RH-35 cells with TPCK inhibited the apoptosis induced by TNF-α. RH-35 hepatoma cells were preincubated with chymotrypsin B inhibitor TPCK (40 µM) for 30 min and then treated with 100 ng/ml TNF-α for 6 h. The apoptotic rates were measured by cytofluorometric analysis of the percentage of cells exhibiting a subdiploid (apoptotic) DNA content. *: p < 0.01 in comparison with untreated cells; **: p < 0.05 in comparison with RH-35 cells treated with 100 ng/ml TNF-α. (b) Knockdown of chymotrypsin B expression by RNAi. RH-35 cells were transfected with pSUPER-Ctrb vector, which contains a 19-nucleotide sequence corresponding to nucleotides 421-439 of Ctrb, or pSUPER-LacZ control vector, respectively, and cultured for 48 h. The expression of Ctrb mRNA was analyzed by real-time PCR. The expression of Ctrb mRNA was significantly down-regulated in RH-35 cells transfected with a pSUPER-Ctrb vector. (c) Down-regulation of Ctrb mRNA expression by RNAi partly prevented cells from TNF-α-induced apoptosis. RH-35 cells were transfected with pSUPER-Ctrb or pSUPER-LacZ, respectively, and cultured for 48 h. Then cells were treated with 100 ng/ml TNF-α for 6 h and apoptosis was assessed by flow cytometry. *: p < 0.05 in comparison with RH-35 cells transfected with pSUPER-Ctrb.
Peptide No. 1  (103-108)

IA QV FK

y₁ y₂ y₃ y₄ y₅

b₃ b₄ b₅
Peptide No. 2 (112-118)

Y₆ Y₅ Y₄ Y₃ Y₂ Y₁

F N M F T V R

b₂ b₃ b₄ b₅

Relative Abundance

m/z
Peptide No. 4 (80-102)
Figure S1. Sequences of four tryptic peptides from the unknown lysosomal protease with Bid cleavage activity: comparison with rat prec-hymotrypsinogen B1. Sequences were obtained from LC-MS/MS performed on the tryptic peptides generated from the SDS-gel-purified 25-kDa unknown lysosomal protease with Bid cleavage activity. The sequence of rat chymotrypsinogen B1 was reported by Bell et al. (Bell et al., 1984). Numbers in parentheses denote the amino acid position in the cDNA sequence of rat chymotrypsinogen B1.
Chymotrypsin B cached in rat liver lysosomes and involved in apoptotic regulation through a mitochondrial pathway
Qi Miao, Yang Sun, Taotao Wei, Xingyu Zhao, Kai Zhao, Ling Yan, Xujia Zhang, Hongjun Shu and Fuyu Yang

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