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Chymotrypsin B Cached in Rat Liver Lysosomes and Involved in Apoptotic Regulation through a Mitochondrial Pathway*[S]

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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 can 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 tumor necrosis factor-α, and intracellular delivery of chymotrypsin B protein induced apoptotic cell death with a potency comparable with 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 RNA interference or pretreatment with chymotrypsin B inhibitor *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone significantly reduced tumor necrosis factor-α-induce 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.

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*, Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI, and endonuclease G following mitochondrial membrane permeabilization (MMP)[3] (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 mitochondrial 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 relocalization 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 extralysosomal 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 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 this study, we report the purification and characterization of one of the proteases termed lysosomal 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

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[3] The abbreviations used are: MMP, mitochondrial membrane permeabilization; LBCP, lysosomal Bid cleavage protease; TLCK, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone; PVDF, polyvinylidene difluoride; RT, reverse transcription; FITC, fluorescein isothiocyanate; RNAi, RNA interference; TNF, tumor necrosis factor; SB-TI, soybean trypsin inhibitor; Ctrb, chymotrypsin B; TPCK, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone; CFP, cyan fluorescent protein; GFP, green fluorescent protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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.

MATERIALS AND 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 this 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). The recombinant proteins glutathione *S*-transferase (GST)-SERPINB3 and -SERPINB4 were batch-purified using glutathione-Sepharose columns (Amersham Biosciences). The c-IAP-1 expression plasmid constructed in pGEX4T was a gift from Dr. Xiaodong Wang (University of Texas Southwestern Medical Center). 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 isopropyl 1-thio-β-D-galactopyranoside for 3 h, 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 $\times$ *g* for 30 min. The pellet was resuspended in ~30 ml of 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 $\times$ *g* for 10 min. This wash was repeated four 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 guanidine-HCl, 0.1 M Tris-HCl, pH 8.0, 100 mM dithiothreitol. 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 guanidine-HCl with its concentration continually decreasing from 3 to 1 M. The renaturation process was conducted at 4 °C for 24 h. The renatured protein solution was then dialyzed against 2 mM HCl, 10 mM $CaCl_2$ and ultracentrifuged (78,100 $\times$ *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 SB-TI-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 (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 $CaCl_2$ (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 $\times$ *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.* (10). After dialysis against buffer A (25 mM Tris-HCl, 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 Biosciences) and was recirculated 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 h at 37 °C and centrifugation for 10 min at 15,000 $\times$ *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 were loaded onto an MP7 HIC column (50 $\times$ 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 was collected and dialyzed against 25 mM ammonium formate, pH 7.4, containing 5 mM NaCl. The solution was lyophilized, redissolved 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 Biosciences), equilibrated, and eluted with buffer A containing 150 mM NaCl. The active fractions were stored at 4 °C until 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 Biosciences) 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 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, redissolved in 120 μl of 1× SDS-PAGE loading buffer, and subjected to 15% SDS-PAGE. After staining with colloidal blue (Invitrogen), the ~25-kDa band 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 liquid chromatography-tandem mass spectrometry using a nanoscale C18 column coupled in-line with an ion trap mass spectrometer (LCQ Deca, Thermo Finnigan). The instrument was run in a 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 nonredundant NCBI protein data base 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, 0.1 mg/ml SB-TI, 0.25 mM chymostatin, 5 mM *N*-ethylmaleimide, 20 μM E-64, 0.2 mM 3,4-dichloroisocoumarin (ICN Biomedicals), 0.5 mM L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone (TLCK), 0.5 mM *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 5 mM iodoacetamide, 10 μM pepstatin A, 10 mM EDTA, 10 mM *o*-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 h 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 K_m—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 Me_2SO. The reaction cuvette contained 100 μl of the reaction buffer (100 mM Tris-HCl buffer, pH 8.0, 10 mM $CaCl_2$, 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 λ_{em} = 460 nm with fluorescence excitation at λ_{ex} = 380 nm. The detected signals were converted into moles of substrate hydrolyzed per s. Initial velocities and substrate concentrations were fit by nonlinear regression to the Michaelis-Menten 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 supplemented with 10% fetal calf serum (Invitrogen) at 37 °C under 5% CO_2. For the RT-PCR analysis of Ctrb mRNA, total RNA was isolated by using an 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 CATCGACGTTGGGTAGACAC; PCR product 133 bp; the primer sequences crosses an intron that 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 $MgCl_2$, 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 aprotinin and disrupted by sonication on ice. After ultracentrifugation at 10,000 × *g* for 30 min at 4 °C, the supernatants containing solubilized proteins were separated on a 12% SDS-PAGE and then electroblotted onto PVDF membrane. The membrane was probed with homemade 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′-GCCAGCCGCTCCTTCAACCAAGGAAGAATA-3′ (sense) and 5′-TATTCTTCCTTGGTTGAAGGAGCGGCTGGC-3′ (antisense).

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 of MT buffer (400 mM mannitol, 10 mM KH_2PO_4, 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 was analyzed 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, permeabilized with methanol at −20 °C for 5 min, blocked with 5% newborn 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 Biotechnology), 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 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 the N-terminal signal peptide of the chymotrypsinogen B precursor, the coding sequence was amplified using the following two primers: 5′-TTTAAGCTTATGGCATTCCTTTGGCTCGTGTCCTGCTTTGCCCTTGTGGGGGCCACCTTTGGCTGTGGAGTCCCTACCATCC-3′ (sense) and 5′-AAAGGATCCTCAGTTGGCTTCCAAGATCTG-3′ (antisense). The PCR product and pEGFP-N1 (Clontech) were cut with HindIII and BamHI (Takara), followed by purification and ligation. Transformed competent cells (TianWei, China) were plated on selective agar plates, and colonies were selected and grown in LB media containing 50 μg/ml kanamycin. The sequences were verified by DNA sequencing. To check whether the new construct generates functional proteins, the proteins were expressed *in vitro* using the Promega TNT-coupled transcription-translation system (Promega) and radiolabeled with [^{35}S]methionine (Amersham Biosciences). 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. G. J. Gores (Mayo Medical School). 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 Dulbecco's modified Eagle's medium without antibiotics. To obtain stable transfected cell lines, the cells were cultured in Dulbecco's modified Eagle's medium 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) 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 phosphate-buffered saline was 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 h, 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 short interfering RNA 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.neovector might form hairpin oligonucleotide(s) that could interfere with the stability of Ctrb mRNA and consequently induce nonspecific down-regulation of Ctrb, a control vector (pSUPER-LacZ) with no significant homology to the *ctrb* gene was used as a nonsilencing 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 and compared with actin as internal control. To determine the effect of RNAi-mediated down-regulation 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 phosphate-buffered saline, 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 (BD Biosciences).

RESULTS

Purification of LBCP

The impetus for this study was our previous observation of an unknown caspase 8-like lysosomal protease capable of converting Bid into truncated Bid at neutral pH. We took painstaking effort to purify this protease. Fig. 1*a* 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. 1*b*) after incubation of the lentil lectin flow-through fraction at pH 5.0 (intralysosomal pH) and 37 °C. This enzymatic activity was designated as LBCP. We believe that it is the incubation that leads to the activation of the LBCP from its premature LBCP zymogen, although the reason so far is not understood. Next, the pH of the active supernatant was changed to 7.0 by dialysis and subjected to anion exchange chromatography. As shown in Fig. 1, *b* and *c*, the eluate fractions (fractions 26–32) at ~270 mM NaCl (Fig. 1*c*) showed the apparent Bid cleavage activity at pH 7.6 and corresponded with the appearance of an ~25-kDa protein observed on silver-stained SDS-polyacrylamide gels (Fig. 1*d*). This band was excised and subjected to liquid chromatography-tandem mass spectrometry analysis (see under "Mass Spectrometric Identification of LBCP"). To the end, it was estimated that 2–6 μg of the protein (~0.3–1 ng/μl) from liver lysosomes of 100 rats could be obtained. The purified LBCP shows a potent Bid cleavage activity because a 10,000-fold molar ratio excess of Bid could be cleaved by LBCP within minutes.

LBCP Is Identified as Chymotrypsin B

Biochemical Characterization of LBCP—To biochemically characterize the LBCP, a wide range of peptidase inhibitors was assayed for their possible potential on the Bid cleavage activity of the purified LBCP. It can be seen that the proteolytic cleavage of Bid was abolished by phenylmethylsulfonyl fluoride, 3,4-di-

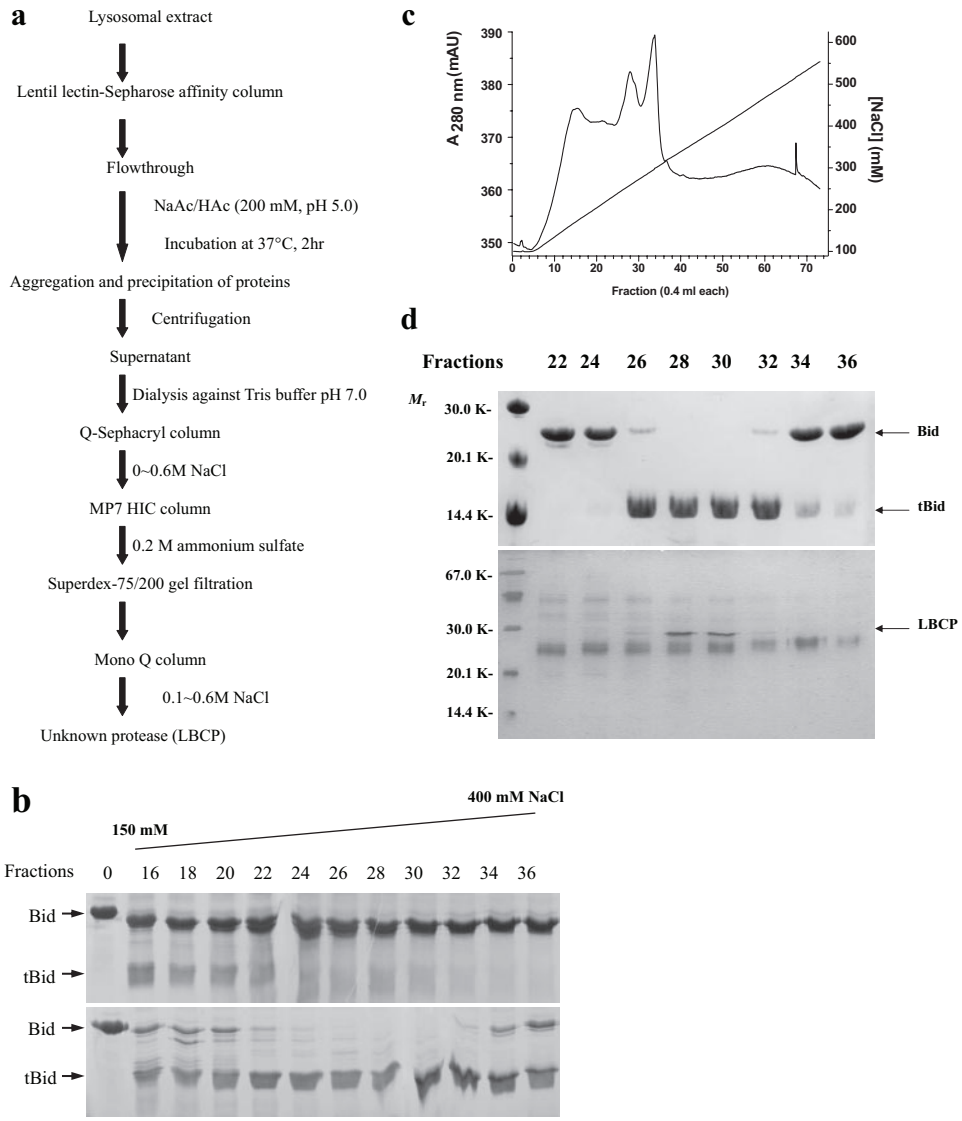

FIGURE 1. **Purification of the LBCP from rat liver lysosomes.** *a,* diagram of the purification scheme. *b,* activation of LBCP. Lysosomal extract from 10 rat livers was prepared. Half of the extract was subjected to dialysis against buffer A and subsequent Bio-Scale Q5 chromatography (*upper panel*). The other half was treated following the purification procedures as described under "Materials and 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 h 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 h, 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 LBCP.

chloroisocoumarin, SB-TI, and chymostatin but not by inhibitors of other classes (Fig. 2*a* 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, whereas 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. 2*b*) was analyzed by electroblotting and N-terminal sequencing, which revealed that the cleavage occurred at Phe-67. This site is unique to the action of LBCP (Fig. 2*c*). To verify this result, a Bid mutant F67A was constructed and processed as described above. As shown in Fig. 2*d*, the mutant Bid was degraded at a slower rate and an ~17-kDa cleavage product of Bid (F67A) with the N-terminal sequence WEADLE appeared instead of the normal ~14-kDa fragment. The ~17-kDa part was identified to be cleaved at Tyr-47. 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-Menten kinetics with a K_m value of 23.7 ± 2.6 μM (Fig. 2*e*). This value is shared by rat Ctrb (12).

Because 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. 2*f*, the apparent release of cytochrome *c* from mitochondria was observed in the presence of Bid and LBCP.

Mass Spectrometric Identification of LBCP—The band of LBCP resolved by SDS-PAGE (Fig. 1*b*) was subjected to trypsin digestion and liquid chromatography-tandem mass spectrometry analysis (see supplemental 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.

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 have 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.

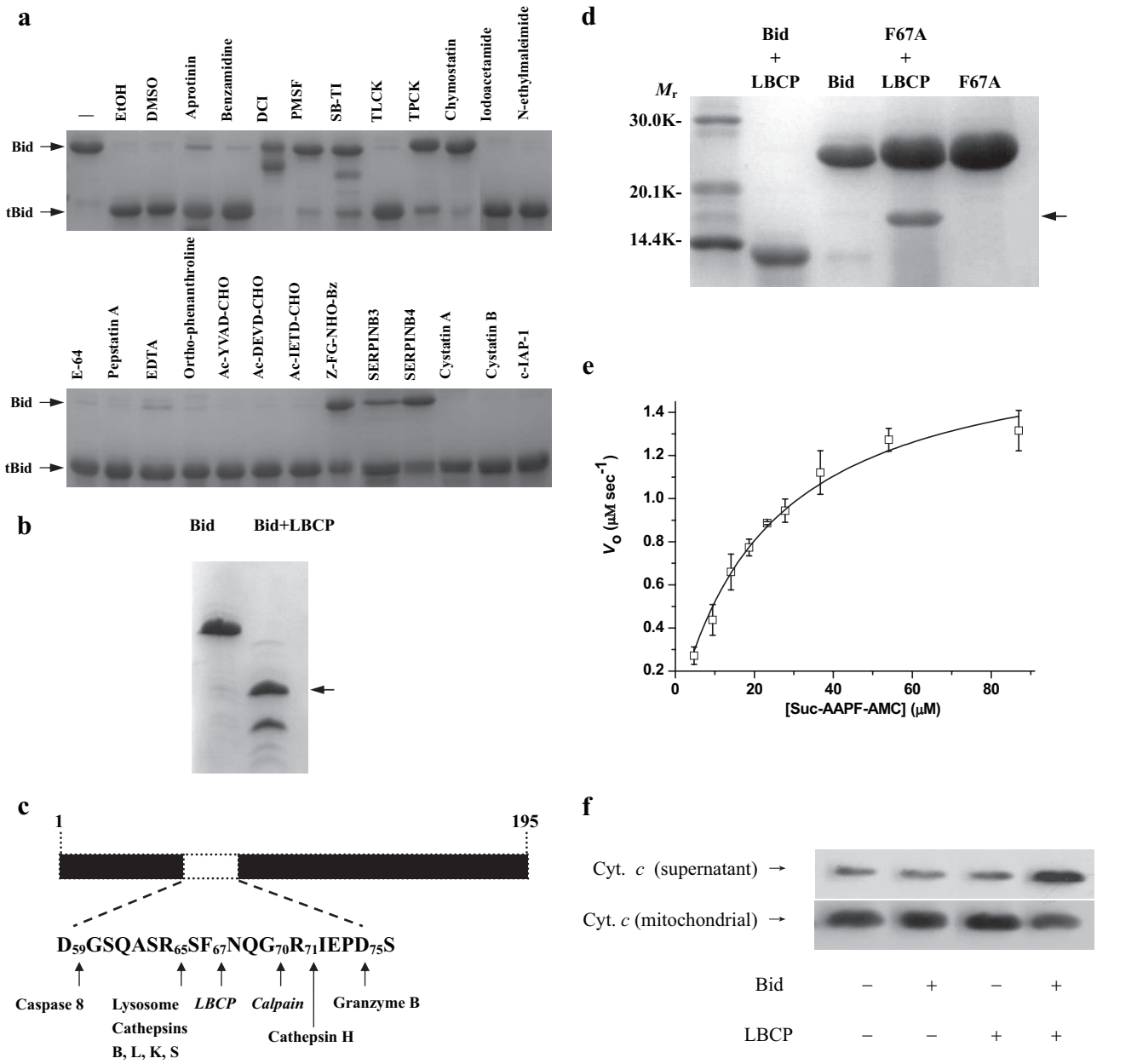

FIGURE 2. **Biochemical assays for LBCP.** *a,* effect of inhibitors on the Bid cleavage activity. Aliquots from the Mono Q fraction at the peak of Bid cleavage activity were preincubated for 30 min at 37 °C in the presence of 0.1 mg/ml aprotinin, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml SB-TI, 0.25 mM chymostatin, 5 mM *N*-ethylmaleimide, 20 μM E-64, 0.2 mM 3,4-dichloroisocoumarin, 0.5 mM TLCK, 0.5 mM TPCK, 5 mM iodoacetamide, 10 μM pepstatin A, 10 mM EDTA, 10 mM *o*-phenanthroline, 1 μM caspase-1 inhibitor I (*Ac-YVAD-CHO*), 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). Bid was then introduced into each incubation mixture. The reaction was stopped after incubation for 3 h at 37 °C. Appropriate solvent controls were run in parallel. The cleavage of Bid was detected by SDS-PAGE. *b,* cleavage of Bid by LBCP. 10 μg of Bid was cleaved by LBCP for 1 h at 37 °C, separated by Tricine/SDS-PAGE on 15% gel, transferred to a PVDF membrane, and stained with Coomassie Blue. The appropriate band was excised, and the N-terminal sequence of the Bid cleavage product was determined with an ABI-491 amino acid sequencer to yield the sequence NQGRIE. The result revealed that the cleavage of Bid by LBCP occurred at Phe-67. *c,* location of the cleavage site, with the locations of the caspase 8, lysosomal cathepsin B, cathepsin L, cathepsin K, cathepsin S, and granzyme B sites shown for comparison. *d,* cleavage of Bid mutant F67A by LBCP compared with that of wild-type Bid. The cleaved product was analyzed by N-terminal sequencing, which revealed that the cleavage occurred after Y47. *e,* Michaelis-Menten nonlinear regression analysis of K_m of LBCP for Suc-AAPF-AMC. This value was determined using real time kinetic initial rates by assembling 100 μl of reaction mixture containing increasing amounts of the substrate and a 5-μl aliquot from the Mono Q fraction 29. By plotting V (μM s^{-1}) *versus* [Suc-AAPF-AMC] and nonlinear regression analysis using the Michaelis-Menten equation, the K_m was found to be ~23.7 ± 2.6 μM. *f,* proteolytic cleavage of Bid by LBCP could induce the release of cytochrome *c* (*Cyt. c*) from isolated mitochondria. 1 μg of 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 product of Bid at a final concentration of 10 μg/ml in a final volume of 50-μl MT buffer (400 mM mannitol, 10 mM KH_2PO_4, 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 supernatants and the pellets were subjected to 15% SDS-PAGE, transferred to a PVDF membrane, and probed with a mouse monoclonal anti-cytochrome *c* primary antibody and a horseradish peroxidase-conjugated rabbit-anti mouse IgG secondary antibody. The target proteins were detected with an ECL kit.

3*a*). 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. 3*b*). Also, a bioinformatics study performed by searching against the gene expression data bases UniGene,

TABLE 1

Specificity of various class inhibitors and their effects on the Bid cleavage activity of LBCP

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, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml SB-TI, 0.25 mM chymostatin, 5 mM *N*-ethylmaleimide, 20 μM E-64, 0.2 mM 3,4-dichloroisocoumarin, 0.5 mM TLCK, 0.5 mM TPCK, 5 mM iodoacetamide, 10 μM pepstatin A, 10 mM EDTA, 10 mM *o*-phenanthroline, 1 μM caspase-1 inhibitor I (Ac-YVAD-CHO), 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). Bid was then introduced into each incubation mixture. The reaction was stopped after incubation for 3 h at 37 °C. Appropriate solvent controls were run in parallel. The cleavage of Bid was detected by SDS-PAGE.

Inhibitors specific for	Inhibitory effect
Serine peptidases	
General	
PMSF, DCI, SB-TI	Complete
Chymotrypsin-like	
TPCK	Complete
Chymostatin	
Undefined	
Aprotinin	Partial
SERPINB3, 4	
Trypsin-like	
TLCK	No
Benzamidine	
Cysteine peptidases	
General	
Iodoacetamide	
N-Ethylmaleimide	
E-64	
Caspase	
Ac-YVAD-CHO	No
Ac-DEVD-CHO	
Ac-IETD-CHO	
c-IAP1	
Lysosomal cysteine proteases	
Cystatin A, B	
Z-FG-NHO-Bz	Partial
Aspartate peptidases	
Pepstatin A	No
Metallopeptidases	
EDTA	No
o-Phenanthroline	

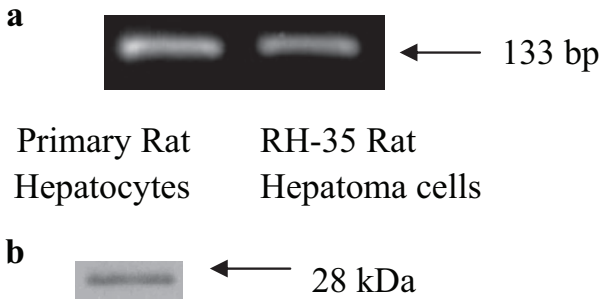

FIGURE 3. **Expression of chymotrypsin B mRNA and protein in RH-35 rat hepatoma cells.** *a,* RT-PCR analysis of chymotrypsin B mRNA expression in primary rat hepatocytes and RH-35 rat hepatoma cells. RNA was isolated from the primary rat hepatocytes and RH-35 rat hepatoma cells and analyzed by RT-PCR with intron-crossing primers specific for rat chymotrypsin B. The sequence of the PCR product was 100% identical to the cDNA sequence of rat chymotrypsin B. *b,* Western blot analysis of chymotrypsin B protein expression in RH-35 rat hepatoma cells. Whole cell lysates of RH-35 hepatoma cells were subjected to 12% SDS-PAGE, transferred to a PVDF membrane, and probed with a homemade rabbit anti-chymotrypsin B primary antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. The target proteins were detected with an ECL kit.

GeneNote, and GeneCard for the human, mouse, and rat genomes also implied that Ctrb expression may not be restricted to the pancreas.

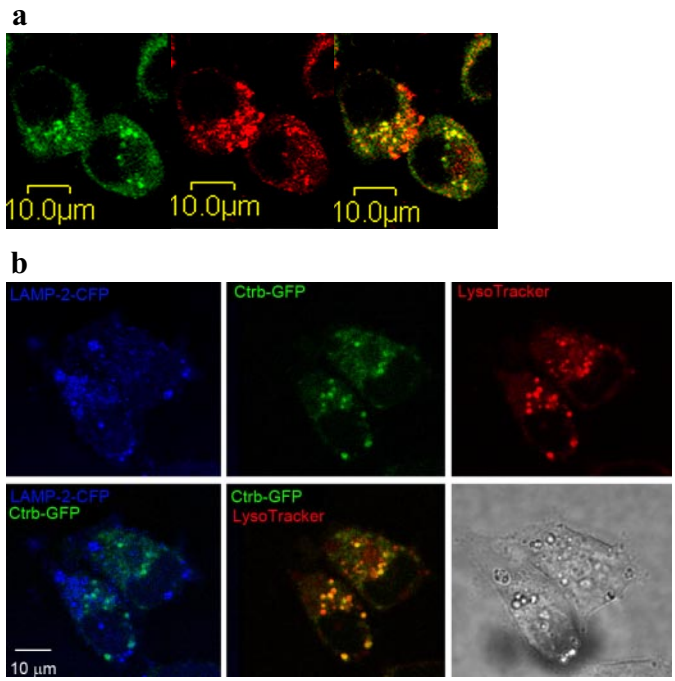

FIGURE 4. **Intralysosomal localization of chymotrypsin B in RH-35 rat hepatoma cells.** *a,* co-localization of chymotrypsin B (*red fluorescence*) and cathepsin D (*green fluorescence*) in lysosomes of RH-35 rat hepatoma cells. RH-35 rat hepatoma cells were fixed, permeabilized, incubated with anti-chymotrypsin 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 lysosome-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*) co-localized with LAMP-2-CFP (*blue*) and LysoTracker Red (*red*) as shown in the overlay image, confirming its lysosomal distribution. *Scale bar,* 10 μm.

Lysosomal Localization and Release of Chymotrypsin B

Like other members of the chymotrypsin family, Ctrb is a well known secretory protein. However, 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, permeabilized, 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 cathepsin D (Fig. 4*a*) verified their co-localization 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 punctate appearance when viewed by con-

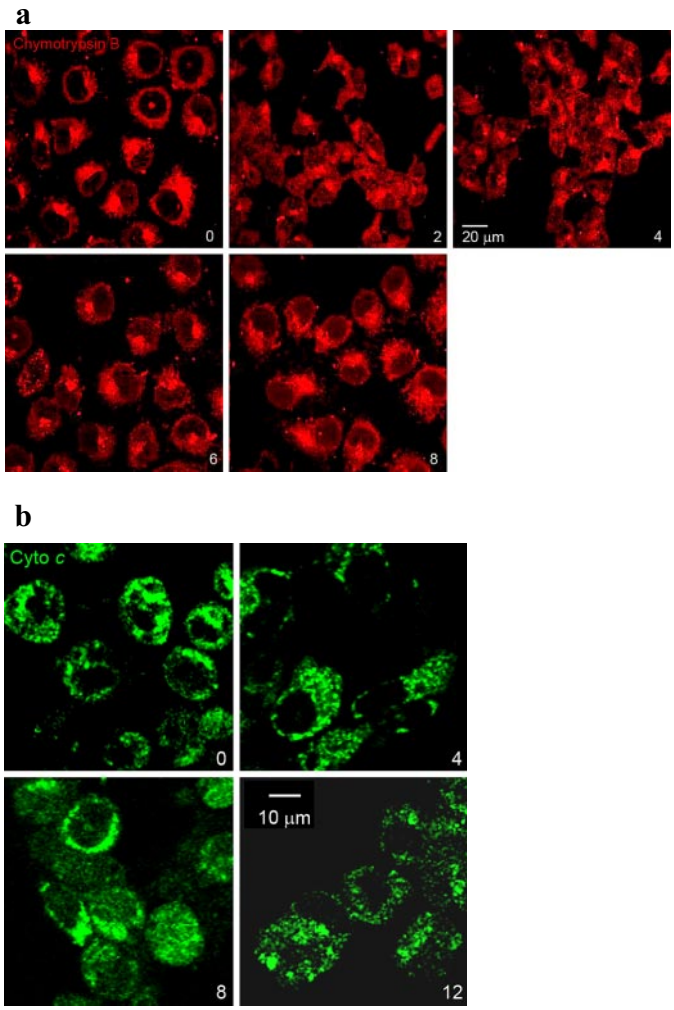

FIGURE 5. **Redistribution of chymotrypsin B and cytochrome *c* induced by TNF-α.** *a,* redistribution of chymotrypsin B induced by TNF-α. RH-35 rat hepatoma cells were treated with 100 ng/ml TNF-α for indicated intervals, fixed, permeabilized, incubated with anti-chymotrypsin B antiserum, and labeled 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 100 ng/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 h of TNF-α treatment, later than the lysosomal release of Ctrb. *Scale bar,* 10 μm.

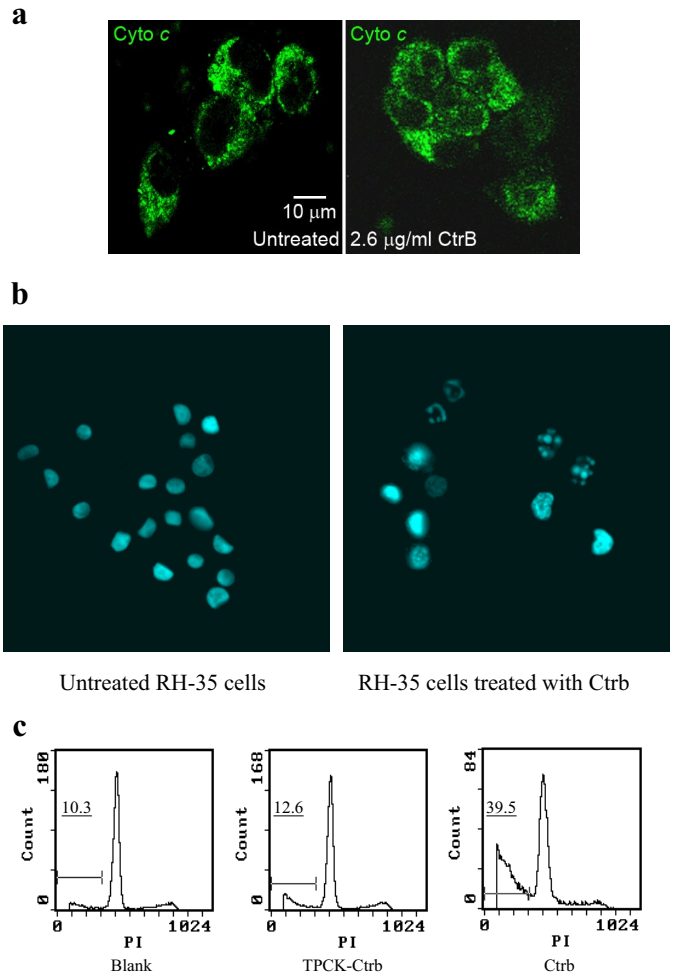

FIGURE 6. **Induction of apoptotic events by intracellular delivery of Ctrb.** *a,* intracellular delivery of Ctrb triggered the rapid release of mitochondrial cytochrome *c* (*Cyto c*) (*green fluorescence*) in RH-35 cells. Recombinant chymotrypsin B (2.6 μg/ml) was delivered to RH-35 rat hepatoma cells 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, and cells were fixed and stained with 4′,6-diamidino-2-phenylindole 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 propidium iodide (*PI*) by cytofluorometric analysis in fixed permeabilized cells. *Numbers* indicate the percentage of cells exhibiting a subdiploid (apoptotic) DNA content.

focal microscopy, indicative of a vesicular compartmentation of the tagged proteins. Overlay images demonstrated virtually complete co-localization of the two fluorescent proteins (Fig. 4*b*). 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 co-localization to the same vesicular compartment. Thus, these observations suggest Ctrb is targeted to lysosomes in the RH-35 cells.

Because 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. 5*a*, 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 h of TNF-α treatment, later than the lysosomal release of Ctrb (Fig. 5*b*).

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. 6*a*),

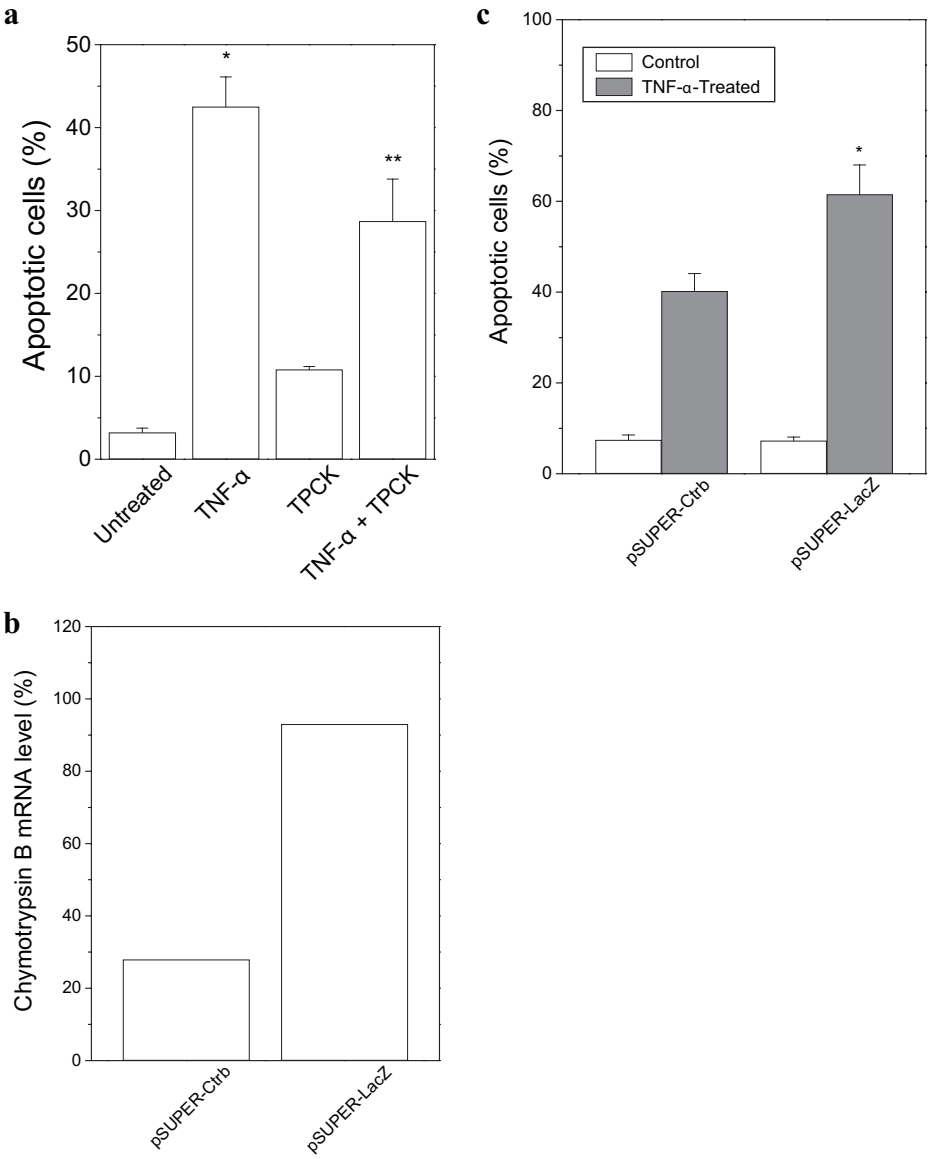

FIGURE 7. **Effects of inhibition of chymotrypsin B activity or knockdown of chymotrypsin B expression on TNF-α induce apoptosis in RH-35 cells.** *a,* pretreatment of RH-35 cells with TPCK inhibited the apoptosis induced by TNF-α. RH-35 hepatoma cells were preincubated with chymotrypsin B inhibitor 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.

chromatin condensation, and nuclear fragmentation (Fig. 6*b*). Moreover, the delivered Ctrb augmented the apoptosis rate to 3.9 times that by the reagent control (Fig. 6*c*), comparable with 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 Attenuates TNF-α-induced Apoptosis

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

Chymotrypsin B Inhibitor TPCK Treatment—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. 7*a*). It can be seen that 28.7% of cells pretreated with TPCK underwent apoptosis compared with 42.5% of cells not pretreated.

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. 7*b*). 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. 7*c*, 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 regulates 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 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 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 the specific inhibitor of Ctrb, TPCK, could significantly but partly attenuate 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 with 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) deserve 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 that lysosomal Ctrb underwent a drastic increase of Ctrb activity following *in vitro* incubation (see Fig. 1*b*), 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 *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 by 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, although they are found exclusively in the cytoplasmic granules within cytotoxic T cells and natural killer cells, and following their secretion they 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 multitasking 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. Ctrb might be an important mediator in cells.

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