Activation of procathepsin B in human hepatoma cells: the conversion into the mature enzyme relies on the action of cathepsin B itself

Lukas MACH,* Herwig SCHWIHLA,* Klaus STÜWE,* Andrew D. ROWAN,† John S. MORT†‡ and Josef GLÖSSL*§

*Zentrum für Angewandte Genetik, Universität für Bodenkultur, Gregor-Mendelstrasse 33, A-1180 Wien, Austria,
†Joint Diseases Laboratory, Shriners Hospital for Crippled Children, 1529 Cedar Avenue, Montreal, Quebec H3G 1A6, Canada,
and ‡Department of Surgery, McGill University, Montreal, Quebec H3A 2T6, Canada

In order to elucidate the processing mechanism of the lysosomal cysteine proteinase, cathepsin B, in mammalian cells, recombinant rat and human cathepsin B precursors were expressed in *Saccharomyces cerevisiae*. The active-site cysteine residue was changed to serine to prevent autoprocessing. When the purified proenzymes were incubated with the soluble fraction of post-nuclear organelles obtained from human hepatoma HepG2 cells, processing to a 33 kDa form corresponding to the mature endogenous single-chain enzyme was observed. Inhibitors of metallo-, serine and aspartic proteinases exerted no significant effect on procathepsin B processing *in vitro*. However, the processing activity was effectively blocked by cysteine proteinase inhibitors, in particular E-64 and its cathepsin-B-selective derivative CA-074. Processing positions were identified by using anti-peptide antibodies specific for epitopes in the N- and C-terminal cleavage regions. The single-chain form produced *in vitro* was thus shown to contain an N-terminal extension of at least four residues relative to the mature lysosomal enzyme, as well as a C-terminal extension present in the proenzyme but usually absent in fully processed cathepsin B. On expression of the wild-type proenzyme in yeast, procathepsin B undergoes autoprocessing, yielding a single-chain form of the active enzyme, which contains similar N- and C-terminal extensions. These results indicate that maturation of procathepsin B *in vivo* in mammalian tissues relies on the proteolytic activity of cathepsin B itself.

INTRODUCTION

Cathepsin B is the most abundant lysosomal cysteine proteinase in mammalian tissues [1]. The enzyme is synthesized as a latent precursor, which is subsequently converted into the mature single- and two-chain forms by limited proteolysis (see Scheme 1) [2]. Proteinases involved therein have not yet been unambiguously identified, and their subcellular localization remains to be established. In rat hepatocyte microsomes, activation of endogenous procathepsin B *in vitro* was prevented by addition of pepstatin A, a potent inhibitor of the lysosomal aspartic proteinase cathepsin D [3]. On the other hand, the conversion of the rat macrophage cathepsin B precursor *in vivo* into the lysosomal isoenzymes was not sensitive to pepstatin A, but could be blocked by a metalloproteinase inhibitor [4].

When the cDNA for rat procathepsin B was expressed in the yeast *Saccharomyces cerevisiae*, cysteine-proteinase-dependent processing of the cathepsin B precursor into the mature enzyme was observed [5]. Since endogenous cysteine proteinases have not been identified in yeast [6], an autocatalytic mode of activation in at least an intermolecular manner has been proposed. In addition, a non-activatable recombinant variant of rat procathepsin B, where the active-site cysteine residue (Cys^{29}) was changed to serine, could be converted *in vitro* by purified cathepsins B or L into a processed form resembling the single-chain enzyme [5]. Similarly, a cathepsin B-like proteinase from *Schistosoma mansoni* was reported to undergo autocatalytic maturation *in vitro* [7]. *In vivo*, however, various cysteine-proteinase inhibitors have failed to exhibit significant effects on conversion of newly synthesized human hepatoma procathepsin B into the mature single-chain enzyme [8].

In the present study we provide evidence that the processing of procathepsin B in human hepatoma HepG2 cells relies on the action of cathepsin B itself, as shown *in vitro* under conditions mimicking the actual relative amounts of endogenous proteinases in the (pre)lysosomal compartments *in vivo*.

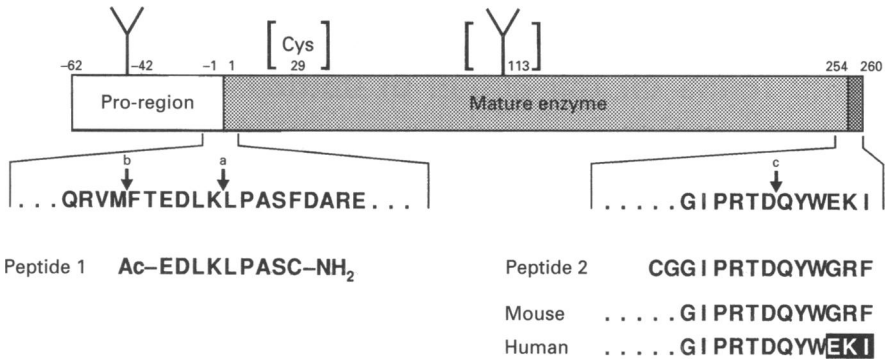
MATERIALS AND METHODS

Materials

A recombinant non-activatable variant of rat procathepsin B with one N-glycosylation site ($Cys^{129} \rightarrow Ser$; $Ser^{115} \rightarrow Ala$) was produced in the yeast *Saccharomyces cerevisiae* as described previously [5]. *S. cerevisiae* strains BJ3501 (*MATα ura3-52 pep4::HIS3 prbΔ1.6R his3-Δ200*) and 409 (*ura3-52 leu2-3 pep4-3 mnn9*) were obtained from the Yeast Genetic Stock Center (Department of Biophysics and Medical Physics, University of California, Berkeley, CA, U.S.A.) and the Biotechnology Research Institute (Montreal, Canada) respectively. *N*-Succinimidyl bromoacetate, Gly-Phe-NHNap, chicken ovalbumin (grade V), pepsin (from pig mucosa), PMSF, E-64 and pepstatin A were obtained from Sigma (St. Louis, MO, U.S.A.). The f.p.l.c. system, concanavalin A–Sepharose, activated CH-Sepharose, Sephadex G-25 (fine grade) and the Mono Q HR (5/5)-column were purchased from Pharmacia (Uppsala, Sweden); DEAE-Trisacryl M and Ultrogel AcA-44 were from LKB (Bromma,

Abbreviations used: CA-074, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; CH-, carboxyhexyl-; -CHN_2, -diazomethane; DEPDA, *NN*-diethylphenylenediamine; E-64, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl-(4-guanidino)-butane; Gly-Sc, glycinaldehyde semicarbazone; KLH, keyhole-limpet haemocyanin; -NHMec, -4-methyl-7-coumarylamide; -NHNap, -2-naphthylamide; PMSF, phenylmethanesulphonyl fluoride; Z-, benzyloxycarbonyl-.

§ To whom correspondence should be addressed.



Scheme 1 Diagram of the human procathepsin B primary structure and location of peptide sequences used for anti-peptide antibody production

The numbering system used is based on the single-chain form of the lysosomal enzyme. The pro-region is assigned negative numbers decreasing towards the N-terminus. The locations of the N-linked oligosaccharide substitution sites (Y) and the active-site cysteine residue are indicated. Elements shown in brackets were altered by site-directed mutagenesis. Arrowed positions indicate: a, N-terminus of the mature lysosomal proteinase; b, N-terminus of the active enzyme resulting from autoprocessing of the recombinant proenzyme; c, C-terminus of the mature lysosomal proteinase. The boxed residues indicate differences between mouse and human procathepsin B sequences in the C-terminal region.

Sweden). Keyhole-limpet haemocyanin (KLH) was obtained from Calbiochem (San Diego, CA, U.S.A.). Z-Arg-Arg-NHNap, Z-Phe-Arg-NHMec and Arg-NHNap were purchased from Bachem (Bubendorf, Switzerland). Endoglucosaminidase H from *Streptomyces plicatus* was from Boehringer Mannheim (Mannheim, Germany). Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were kindly supplied by Dr. E. Shaw (Friedrich-Miescher-Institut, Basel, Switzerland). The E-64 derivative CA-074 was provided by Dr. N. Katunuma (Tokushima, Japan). Rabbit antisera against human cathepsin D and human cathepsin L were supplied by Dr. A. Hasilik (Universität Münster, Germany) and Dr. R. Mason (Virginia Polytechnic and State University, Blacksburg, VA, U.S.A.) respectively. Rabbit polyclonal antibodies against human liver cathepsin B were affinity-purified as reported previously [8]. Goat anti-(rabbit IgG) immunoglobulins conjugated to horseradish peroxidase were purchased from Accurate (Westbury, NY, U.S.A.). Human liver cathepsin B was purified essentially as described [2]. Gly-Phe-GlySc–Sepharose was prepared as previously reported [8]. Human hepatoma HepG2 cells were maintained in culture as described previously [8]. Oligonucleotide *1* (5′-TCCTGTGGCTCTTCCTGGGC-CTTC-3′) was synthesized by Dr. G. Himmler (Institut für Angewandte Mikrobiologie, Universität für Bodenkultur, Vienna, Austria). Peptide *1* (Ac-EDLKLPASC-NH_2) was custom-synthesized by the Armand-Frappier Institute (Montreal, Canada). Complete and incomplete Freund's adjuvants were from Difco (Detroit, MI, U.S.A.). DEPDA was obtained from Aldrich (Steinheim, Germany). All other chemicals were of analytical grade.

Expression of human procathepsin B cDNAs in yeast

Recombinant human procathepsin B ($Ser^{115} \rightarrow Ala$) cDNA was expressed in *S. cerevisiae* BJ3501 as an α-factor fusion construct, leading to secretion of the mature enzyme, as will be reported in detail elsewhere (L. Mach and J. S. Mort, unpublished work; for essential details, please apply to the authors). The codon for the active-site cysteine (Cys^{29}) was subsequently changed by site-directed mutagenesis [9] into serine, by using the Muta-Gene phagemid *in vitro* mutagenesis kit from Bio-Rad (Richmond, CA, U.S.A.) and oligonucleotide *1*, thus creating a diagnostic *Ear*I (New England Biolabs, Schwalbach, Germany) restriction site. The mutant human procathepsin B ($Cys^{29} \rightarrow Ser$; $Ser^{115} \rightarrow Ala$) DNA was sequenced around the region of the mutation by the chain-termination method [10] using the ^{T7}Sequencing kit from Pharmacia (Uppsala, Sweden). The mutant procathepsin B construct was transformed in *S. cerevisiae* strain 409 by the spheroplast method [11], and transformants were screened for secretion of procathepsin B as described [5].

Culture medium containing the non-activatable recombinant mutant form of human procathepsin B was produced essentially as reported for the recombinant rat cathepsin B precursor [5].

Purification of recombinant human procathepsin B

Culture medium (4 litres) was clarified by centrifugation (10 min at 10000 *g*) and concentrated by ultrafiltration using a YM-10 membrane (Amicon, Danvers, MA, U.S.A.). The concentrate (100 ml) was dialysed against 2 × 5 litres of 20 mM Tris/HCl buffer, pH 7.5, and applied to a DEAE-Trisacryl M-column (2.5 cm × 8 cm) equilibrated with the same buffer. After extensive washing of the column, bound proteins were eluted with a linear gradient up to 0.3 M NaCl in equilibration buffer, a total volume of 400 ml being used. Fractions (5 ml) were collected and assessed by SDS/PAGE and immunoblotting for their procathepsin B content, then appropriately combined and applied to a concanavalin A–Sepharose column (1 cm × 10 cm), equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. After washing the column with equilibration buffer, bound glycoproteins were eluted with 0.5 M methyl α-mannoside in equilibration buffer. Those fractions (5 ml) containing procathepsin B were pooled, concentrated by ultrafiltration to 2 ml and applied to an Ultrogel AcA-44 gel-filtration column (1.5 cm × 100 cm) eluted with 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl and 0.1 % (w/v) NaN_3. The procathepsin B-containing fractions (2 ml each) were concentrated, transferred into 20 mM Tris/HCl buffer, pH 7.5, by ultrafiltration, and fractionated by f.p.l.c. on a Mono Q HR (5/5) column by using a linear gradient up to 0.3 M NaCl in 20 mM Tris/HCl buffer, pH 7.5. Procathepsin B, eluted at approx. 200 mM-NaCl, was concentrated, transferred into 20 mM Tris/HCl buffer, pH 7.5, and stored at −20 °C. The final yield was approx. 1 mg of recombinant human mutant proenzyme.

Recombinant rat procathepsin B ($Cys^{29} \rightarrow Ser$; $Ser^{115} \rightarrow Ala$)

was purified by essentially the same procedure but omitting the Mono Q step.

Purification of recombinant human cathepsin B

Culture medium (4 litres) was concentrated as above and dialysed against 2 × 5 litres of 20 mM sodium phosphate buffer, pH 6.0, containing 0.5 M NaCl. The retentate was incubated with 2 mM dithiothreitol for 30 min at room temperature and applied to a Gly-Phe-GlySc–Sepharose column (1 cm × 10 cm) at 4 °C. After washing with 50 mM sodium formate buffer, pH 4.0, the adsorbed cathepsin B was eluted with 1.5 mM dipyridyl disulphide in formate buffer, as reported for the enzyme from human liver [12], yielding approx. 1 mg of recombinant human cathepsin B.

Characterization of recombinant procathepsin B

For enzymic deglycosylation, 2 μg of protein was denatured in 10 μl of 0.5 % (w/v) SDS/0.1 M 2-mercaptoethanol for 5 min at 95 °C. Samples were reconstituted to yield 50 mM sodium citrate buffer, pH 5.5, containing 2.5 % (w/v) Nonidet P-40 and 1 mM PMSF, in a total volume of 30 μl, and incubated with 1 m-unit of endoglucosaminidase H for 16 h at 37 °C. The reaction was stopped by addition of 4 vol. of cold methanol.

The effect of Hg^{2+} ions on the stability of recombinant procathepsin B was assessed by incubation of 1 μg of protein in 10 μl of 20 mM Tris/HCl buffer, pH 7.5, containing 0.5 mM $HgCl_2$ for 15 min at room temperature before addition of 5 μl of 0.3 mg/ml pepsin in 0.5 M sodium formate buffer, pH 3.0. After further incubation for 10 min at 40 °C, the reaction was stopped by addition of an equal volume of cold 20 % (w/v) trichloroacetic acid.

Preparation of HepG2-cell microsomal lysate

Human hepatoma HepG2 cells were harvested and disrupted with a Dounce homogenizer essentially as described in [13]. A post-nuclear supernatant was obtained after centrifugation of the homogenate at 600 *g* for 10 min. Total microsomes were isolated from the post-nuclear supernatant by centrifugation at 100000 *g* for 60 min and washed twice with 0.25 M sucrose in 3 mM imidazole buffer, pH 7.4. Further fractionation of the microsomes by Percoll-density-gradient centrifugation as reported [13] resulted in the separation of a dense and more buoyant fraction of organelles containing lysosomal enzymes, including cathepsin B. As cathepsin B was a candidate processing enzyme, the microsomes were not further fractionated for the processing experiments. The microsomal pellet was disrupted by ultrasonication in 10 mM Mes/Pipes buffer, pH 6.0. In some experiments, EDTA, PMSF and pepstatin A (final concns. 2 mM, 1 mM and 10 μM respectively) were added as proteinase inhibitors to the homogenization buffer. The soluble fraction, referred to as microsomal lysate, was recovered after pelleting residual membranes as above. The protein concentration was determined as described by Bradford [14], with BSA (type V; Sigma) as a standard.

Production of polyclonal anti-peptide antibodies specific for cathepsin B

Peptide *2* (CGGIPRTDQYWGRF) was synthesized on an Applied Biosystems 431A peptide synthesizer by using fluorenylmethoxycarbonyl (Fmoc) chemistry, and was purified by preparative reversed-phase chromatography. The correct sequence of the final product was confirmed by N-terminal sequencing on an Applied Biosystems 470A protein sequencer equipped with an on-line operating 120A phenylhydantoin analyser (Biotechnology Core Facility, Shriners Hospital, Montreal, Canada), and by fast-atom-bombardment mass spectrometry (performed at the Biomedical Mass Spectrometry Unit, McGill University, Montreal, Canada). The purified peptide was coupled to bromoacetylated ovalbumin essentially as described [15]. Bromoacetylation of KLH and conjugation with peptide *1* were carried out by the same procedure.

The conjugates (0.5 mg in 0.5 ml of PBS each) were emulsified with an equal volume of complete Freund's adjuvant and injected intramuscularly into female New Zealand white rabbits. Two booster injections (0.5 mg of conjugate each) were done at 2-week intervals as above, but with incomplete Freund's adjuvant. Rabbits were bled from week 4 on, at 2-week intervals, and respective antisera were pooled.

Affinity purification of antibodies against peptide *1* on nitrocellulose-bound recombinant human cathepsin B was performed as described by Smith and Fisher [16]. Antibodies against peptide *2* were purified by affinity chromatography on immobilized peptide, coupled to activated CH-Sepharose (4 mg of peptide/ml of settled gel) as suggested by the manufacturer, essentially as described previously [8]. The anti-peptide *2* antibodies reacted with natural mouse, rat and human procathepsin B, but not with the respective mature forms. Sequence conservation between the cathepsin B precursors from these species indicated that the epitope recognized by this antibody includes the three N-terminal residues of the C-terminal six-residue extension (see Scheme 1).

Processing of procathepsin B by HepG2 microsomal lysate

Recombinant procathepsin B (0.1 μg) was incubated in the absence or presence of 2 mM cysteine and various cysteine proteinase inhibitors as indicated (10 μM each) with crude total microsomal lysate (25 μg of protein), prepared in the absence or presence of proteinase inhibitors as mentioned above, in 50 mM Mes buffer, pH 5.5, in a total volume of 20 μl for 60 min at 37 °C. The reaction was stopped by addition of 2-fold-concentrated SDS/PAGE sample buffer. The samples were analysed by SDS/PAGE under reducing conditions and immunoblotting.

Other methods

SDS/PAGE and immunoblotting were carried out as described previously [8]. The peroxidase reaction was amplified, when indicated, with DEPDA in accordance with [17]. The enzymic activity of cathepsin B was determined with Z-Arg-Arg-NHNap as described by Barrett and Kirschke [1]. Cathepsin H and dipeptidyl peptidase I (cathepsin C) were assayed with Arg-NHNap and Gly-Phe-NHNap respectively [18,19]. The enzymic activity against 10 μM Z-Phe-Arg-NMec sensitive to 0.5 μM Z-Phe-Phe-CHN_2 [20] in the presence of 1 μM CA-074 (to inhibit cathepsin B) was attributed to cathepsin L.

RESULTS

Expression of human procathepsin B cDNAs in *S. cerevisiae*

When the cDNA for human procathepsin B was expressed as an α-factor fusion construct in yeast, active enzyme accumulated in the culture medium. The mutation $Ser^{115} \rightarrow Ala$ was made in order to decrease the heterogeneity of the active recombinant protein by removing the consensus sequence for N-linked oligo-

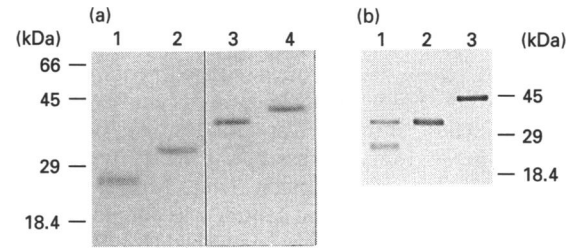


Figure 1 Characterization of recombinant human cathepsin B and its non-activatable precursor

(**a**) Purified cathepsin B from human liver (two-chain form; 2 μg, lane 1), recombinant human cathepsin B (2 μg, lane 2) and the non-activatable recombinant human cathepsin B precursor (2 μg each), which was treated before analysis with (lane 3) or without (lane 4) endoglucosaminidase H as described in the Materials and methods section, were separated by SDS/PAGE under reducing conditions and detected by Coomassie Blue staining. The molecular-mass standards used were BSA (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa) and bovine β-lactoglobulin (18.4 kDa). (**b**) Recombinant human procathepsin B (0.5 μg each) was digested with pepsin after preincubation in the presence (lane 1) or absence (lane 2) of 0.5 mM $HgCl_2$. After separation by SDS/PAGE, cathepsin B-related polypeptides were detected by immunoblotting. Untreated procathepsin B (lane 3) served as a control.

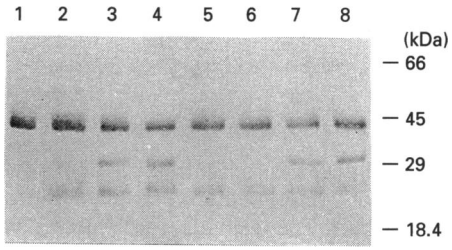


Figure 2 Processing of recombinant rat procathepsin B by HepG2 microsomal lysate

Procathepsin B was incubated with HepG2 lysate in the presence of EDTA, PMSF and pepstatin A and analysed by SDS/PAGE and immunoblotting using anti-(human liver cathepsin B) antibodies as outlined in the Materials and methods section. As a control, procathepsin B was incubated under processing conditions but omitting the microsomal lysate (lane 1). A zero-time point sample (lane 2) served as another control. The immunoreactive 25 kDa polypeptide in lanes 2–8 represents the heavy chain of endogenous two-chain cathepsin B. In lanes 3–8, the following additional substances were present in the reaction mixture: none (lane 3); 2 mM cysteine (lane 4); 10 μM E-64 (lane 5); 10 μM CA-074 (lane 6); 10 μM Z-Phe-Phe-CHN_2 (lane 7); 10 μM Z-Phe-Ala-CHN_2 (lane 8). For molecular-mass standards see legend to Figure 1.

saccharide substitution. This active single-chain form of human cathepsin B, containing an N-terminal extension of six amino acids, was purified to apparent homogeneity by affinity chromatography (Figure 1a, lane 2). Furthermore, the culture medium contained minor amounts of secreted recombinant procathepsin B, which was found to be highly heterogeneous, due to extensive outer-chain elongation on the single N-linked oligosaccharide unit present in the pro-region. To obtain significant amounts of homogeneous recombinant human procathepsin B protein, the active-site cysteine residue (Cys^{29}) was changed by site-directed mutagenesis into serine, thus preventing autoprocessing [5]. In addition, this construct was expressed in a *mnn9* mutant strain of *S. cerevisiae* [21] to inhibit outer-chain elongation of the carbohydrate moiety. Purification of the recombinant human cathepsin B precursor to near homogeneity (Figure 1a, lane 4) was achieved by conventional chromatography.

Characterization of non-activatable human recombinant procathepsin B as an appropriate substrate for analyses of processing *in vitro*

The apparent molecular mass of purified mutant human procathepsin B was determined by SDS/PAGE analysis as 43 kDa. Removal of N-glycosidically linked oligosaccharides with endoglucosaminidase H resulted in a polypeptide of 39 kDa (Figure 1a, lane 3), as also observed for the recombinant rat cathepsin B precursor. These data are in good agreement with the results obtained for human hepatoma procathepsin B [8]. Limited proteolysis of the recombinant human procathepsin B precursor with pepsin yielded a 33 kDa protein (Figure 1b, lane 2), corresponding to single-chain human hepatoma cathepsin B [8], as also observed for the naturally occurring human cathepsin B precursor [22] and the recombinant rat proenzyme. From SDS/PAGE analysis, the molecular mass of the processed recombinant form of rat cathepsin B was previously estimated to be 30 kDa, which compares favourably with the calculated molecular mass of 29.3 kDa [5]. The higher apparent molecular masses obtained in this study, as compared with the previous report [5], have been shown to be due to the anomalously low mobility of cathepsin B in the higher-concentration (12%) acrylamide gels used for SDS/PAGE. Preincubation of recombinant human procathepsin B with Hg^{2+} ions before pepsin treatment destroyed the stability towards extensive proteolysis (Figure 1b, lane 1), thus indicating similar properties of the recombinant and the naturally occurring proenzymes [22].

Processing *in vitro* of recombinant procathepsin B by human hepatoma microsomes

Several lysosomal proteases may be involved in procathepsin B processing *in vivo*, as recently determined by their action on the recombinant rat cathepsin B precursor *in vitro* [5]. Solubilized microsomes from human hepatoma HepG2 cells contain substantial amounts of the apparently relevant enzymes (i.e. cathepsins B, C, D and H) aside from cathepsin L, which was barely detectable, as revealed by both enzymic and immunological methods. Recombinant rat procathepsin B was converted by the HepG2 microsomal lysate into a processed form similar to the product obtained by pepsin treatment of the proenzyme. In addition, a minor degree of non-specific degradation of both the substrate proenzyme and the processed product was observed. However, this could be eliminated by addition of metallo-, serine and aspartic proteinase inhibitors without any significant inhibition of the processing reaction. The general cysteine proteinase inhibitor E-64 effectively blocked proteolytic maturation (Figure 2). The same effect was exerted by its derivative CA-074, which has been shown to be selective for cathepsin B among the lysosomal cysteine proteinases [23]. Omission of EDTA, PMSF and pepstatin A, respectively, one by one in the presence of E-64 did not lead to any significant processing. Significant, albeit weak, inactivation of the processing activity by the peptidyldiazomethyl ketone inhibitors Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 was observed (Figure 2). This is consistent with previously reported results for the interaction of these inhibitors with human liver cathepsin B [24]. Furthermore, it was demonstrated that the processing activity resides in the soluble fraction of the microsomes, as incubation of recombinant rat procathepsin B with microsomal membranes alone did not lead to any detectable processing. Similarly, recombinant human procathepsin B was also converted by the soluble content of HepG2

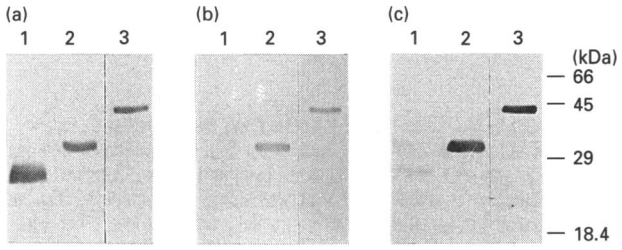


Figure 3 Characterization of two polyclonal anti-peptide antibodies specific for recombinant human cathepsin B

Human liver cathepsin B (1 μg; lane 1), recombinant human cathepsin B (0.5 μg; lane 2) and the recombinant human cathepsin B precursor (0.5 μg; lane 3) were analysed by SDS/PAGE and subsequent immunoblotting using anti-human liver cathepsin B antibodies (panel **a**), anti-peptide *1* antibodies (panel **b**) and anti-peptide *2* antibodies (panel **c**) as described in the Materials and methods section. For molecular-mass standards see legend to Figure 1.

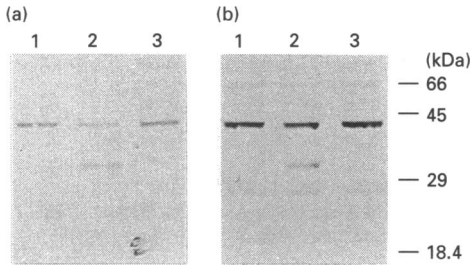


Figure 4 Characterization of the processing product obtained by incubation of recombinant human cathepsin B with HepG2 microsomal lysate

Procathepsin B was incubated with HepG2 microsomal lysate in the presence of EDTA, PMSF, pepstatin A (lane 2) and after addition of 10 μM CA-074 (lane 3) as outlined in the Materials and methods section, and analysed by SDS/PAGE and immunoblotting using anti-peptide *1* antibodies (panel **a**) and anti-peptide *2* antibodies (panel **b**). A zero-time sample (conditions as for lane 2) is shown in lane 1. The peroxidase reaction in panel (**a**) was amplified with DEPDA. For molecular-mass standards see legend to Figure 1.

microsomes into a form corresponding to the endogenous single-chain enzyme (see also Figure 4).

Characterization of the processing product by its reaction with polyclonal anti-peptide antibodies specific for the N-terminal and C-terminal regions of recombinant human cathepsin B

Processing *in vitro* of recombinant rat procathepsin B by purified lysosomal proteases suggested that endoproteolytic cleavage by cathepsins B or L *in vivo* is presumably followed by exopeptidolytic N-terminal trimming, probably due to the repeated action of dipeptidyl peptidase I (cathepsin C) [5]. As this exopeptidase was present in the HepG2 microsomal lysate, albeit at low levels, the processing product was expected to exhibit substantial heterogeneity with respect to its N-terminal region, thus prohibiting the characterization of the cleavage site by microsequencing. Furthermore, the isolation of the processing product in amounts sufficient for detailed analysis did not prove to be feasible. An alternative approach to the characterization of the reaction product was the use of anti-peptide antibodies reacting specifically with an eight-residue region spanning the junction between the pro-region and the fully processed enzyme. A synthetic peptide (peptide *1*), the first eight residues of which represent the four C-terminal amino acids of the pro-region and the four N-terminal amino acids of mature human liver cathepsin B (see Scheme 1), was used as an immunogen to raise an antiserum in a rabbit. The elicited antibodies reacted with recombinant human procathepsin B and the mature recombinant enzyme, where a six-residue N-terminal extension is present, whereas the single- and two-chain forms of human liver cathepsin B failed to exhibit significant reaction (Figure 3, and results not shown). In order to investigate processing at the C-terminus, an antiserum raised against a synthetic peptide (peptide *2*) corresponding to the ultimate 12 amino acids of the C-terminal region of mouse procathepsin B as deduced from the cDNA sequence [25] was used. The reaction with the anti-(peptide *2*) antibodies indicates that recombinant human cathepsin B contains a C-terminal extension as compared with purified mature cathepsin B from human liver (Figure 3 and Scheme 1). These antisera therefore provide a means to characterize the processing product obtained by incubation of the recombinant human cathepsin B precursor with the HepG2 microsomal lysate.

Processing of human recombinant procathepsin B with HepG2 microsomes was completely inhibitable by CA-074 (Figure 4), as observed for the rat cathepsin B precursor (see Figure 2). The single-chain form of the enzyme thus obtained reacted with the antibodies specific for the N-terminal and C-terminal region of recombinant human cathepsin B (Figure 4). These results indicate that the processing activity of the HepG2 lysate acts on the recombinant cathepsin B precursor in a manner identical with purified cathepsin B from human liver [5]. Furthermore, procathepsin B processing by HepG2 lysates *in vitro* yielded the same product as that formed upon autoactivation of the wild-type recombinant human proenzyme in yeast *in vivo*. In mammalian cells, however, the N-terminal and C-terminal extensions are removed from mature cathepsin B, since endogenous cathepsin B from HepG2 cells did not cross-react with anti-(peptide *1*) antibodies and only weakly with anti-(peptide *2*) antibodies (Figure 4).

DISCUSSION

Proteolytic processing of cathepsin B *in vitro* can be mediated by a variety of physiologically relevant and non-relevant proteinases [5]. For this reason it was suggested that activation to the mature forms can occur by cleavage at various sites in an accessible region close to the N-terminus of the mature enzyme. A non-activatable mutant procathepsin B, in which the active-site cysteine residue (Cys^{29}) was changed to serine to prevent autoprocessing, should be an appropriate substrate to identify processing enzyme(s) under conditions reflecting their actual intracellular relative amounts. We provide strong evidence that, at least in human hepatoma cells, the proteolytic maturation of procathepsin B into the single-chain form of the enzyme is mediated by cathepsin B itself. On the other hand, previous studies have shown that various cysteine proteinase inhibitors, namely leupeptin as well as the diazomethanes Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2, did not prevent activation *in vivo* of the cathepsin B precursor in the same cell line [8]. This discrepancy appears to be due rather to the slow inactivation rate of these inhibitors, as observed for cathepsin B *in vitro* [24], than to an inappropriate localization of these substances in the cell, as it was recently shown that Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 readily diffuse across cellular membranes [26].

The processing product obtained by incubation of the recombinant human cathepsin B precursor with HepG2 lysates *in vitro* contained N-terminal and C-terminal extensions as compared

with the enzyme from human liver. These extensions were also observed in recombinant mature human cathepsin B produced in yeast by expression of the cDNA for human procathepsin B. Whereas the N-terminal extension could be removed from the recombinant rat enzyme *in vitro* by dipeptidyl peptidase I [5], the enzyme(s) involved in the C-terminal trimming of the cathepsin B precursors have yet to be identified.

Although purified cathepsins D and L were shown to process the recombinant rat cathepsin B precursor more efficiently than cathepsin B itself [5], these enzymes did not contribute significantly to procathepsin B processing by HepG2 lysates. Besides the complete inhibition of the processing enzyme exhibited by the cathepsin B-selective inhibitor CA-074, any involvement of cathepsin L in procathepsin B processing in HepG2 cells may be ruled out by the finding that cathepsin L occurs at barely detectable levels in this human hepatoma cell line. The lack of an important role for cathepsin D in procathepsin B processing by human hepatoma cell extracts stands in contrast with other studies *in vitro* [3,5]. This discrepancy may be explained by the different assay conditions used. Although processing of recombinant procathepsin B *in vitro* by cathepsin D was determined under close to optimal conditions for the proteolytic activity of this enzyme (i.e. pH 4.5) [5], processing of recombinant cathepsin B precursors by microsomal HepG2 lysates was performed at pH 5.5. The latter pH better reflects the conditions in the endosomal/prelysosomal compartments *in vivo* [27], where proteolytic maturation of lysosomal proteinase precursors probably occurs [28]. Interestingly, the pH optimum for the action of purified cathepsin B on its non-activatable precursor lies in the range pH 4.0–5.0 [5], which is significantly more acidic than for the optimal hydrolysis of synthetic substrates by this enzyme [1]. Therefore it seems likely that the action of cathepsin D on the recombinant cathepsin B precursor is favoured by a lower pH, due to (i) an increase of the enzymic activity of cathepsin D and (ii) a conformational change of procathepsin B rendering it more susceptible to limited proteolysis.

Although the present studies demonstrate that autoprocessing of cathepsin B occurs by an intermolecular mechanism, we cannot provide conclusive information as to how the activation process is initialized. Activation of vacuolar proteinases in the yeast *S. cerevisiae* relies on proteinase A, an aspartic proteinase, which was reported to be autocatalytically activated [29]. Nishimura et al. [30] proposed, by analogy to the yeast system, that the maturation of mammalian lysosomal proteinases may depend on autoactivation of procathepsin D. Our results rather argue against any involvement of cathepsin D in procathepsin B processing, at least in HepG2 cells. Interestingly, the possibility of intramolecular autoactivation of the latent cathepsin B precursor has not yet been investigated. A precedent for intramolecular processing of cysteine proteinases was set recently, when it was reported that a recombinant precursor of the plant cysteine proteinase papain can be activated by such a mechanism [31].

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REFERENCES

1 Barrett, A. J. and Kirschke, H. (1981) Methods Enzymol. **80**, 535–561
2 Hanewinkel, H., Glössl, J. and Kresse, H. (1987) J. Biol. Chem. **262**, 12351–12355
3 Nishimura, Y., Kawabata, T. and Kato, K. (1988) Arch. Biochem. Biophys. **261**, 64–71
4 Hara, K., Kominami, E. and Katunuma, N. (1988) FEBS Lett. **231**, 229–231
5 Rowan, A. D., Mason, P., Mach, L. and Mort, J. S. (1992) J. Biol. Chem. **267**, 15993–15999
6 Achstetter, T. and Wolf, D. H. (1985) Yeast **1**, 139–157
7 Felleisen, R. and Klinkert, M.-Q. (1990) EMBO J. **9**, 371–377
8 Mach, L., Stüwe, K., Hagen, A., Ballaun, C. and Glössl, J. (1992) Biochem. J. **282**, 577–582
9 Clary, J. A., Witney, F. and Geisselsoder, J. (1989) Biotechniques **7**, 282–289
10 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
11 Beggs, J. D. (1978) Nature (London) **275**, 104–109
12 Rich, D. H., Brown, M. A. and Barrett, A. J. (1986) Biochem. J. **235**, 731–734
13 Gieselmann, V., Pohlmann, R., Hasilik, A. and von Figura, K. (1983) J. Cell Biol. **97**, 1–5
14 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
15 Bernatowicz, M. S. and Matsueda, G. R. (1986) Anal. Biochem. **155**, 95–102
16 Smith, D. E. and Fisher, P. A. (1984) J. Cell Biol. **99**, 20–28
17 Conyers, S. M. and Kidwell, D. A. (1991) Anal. Biochem. **192**, 207–211
18 Schwartz, W. N. and Barrett, A. J. (1980) Biochem. J. **191**, 487–497
19 Nikawa, T., Towatari, T. and Katunuma, N. (1992) Eur. J. Biochem. **204**, 381–393
20 Mason, R. W., Green, G. D. J. and Barrett, A. J. (1985) Biochem. J. **226**, 233–241
21 Tsai, P.-K., Frevert, J. and Ballou, C. E. (1984) J. Biol. Chem. **259**, 3805–3811
22 Mort, J. S. and Recklies, A. D. (1986) Biochem. J. **233**, 57–63
23 Towatari, T., Nikawa, T., Murata, M., Yokoo, C., Tamai, M., Hanada, K. and Katunuma, N. (1991) FEBS Lett. **280**, 311–315
24 Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. and Hanada, K. (1982) Biochem. J. **201**, 189–198
25 Chan, S. J., San Segundo, B., McCormick, M. B. and Steiner, D. F. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7721–7725
26 Wilcox, D. and Mason, R. W. (1992) Biochem. J. **285**, 495–502
27 Mellmann, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. **55**, 663–700
28 Rijnboutt, S., Stoorvogel, W., Geuze, H. J. and Strous, G. J. (1992) J. Biol. Chem. **267**, 15665–15672
29 Jones, E. W. (1991) J. Biol. Chem. **266**, 7963–7966
30 Nishimura, Y., Kawabata, T., Yano, S. and Kato, K. (1990) Acta Histochem. Cytochem. **23**, 53–64
31 Vernet, T., Khouri, H. E., Laflamme, P., Tessier, D. C., Musil, R., Gour-Salin, B. J., Storer, A. C. and Thomas, D. Y. (1991) J. Biol. Chem. **266**, 21451–21457

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