Proteolytic processing and glycosylation of cathepsin B

The role of the primary structure of the latent precursor and of the carbohydrate moiety for cell-type-specific molecular forms of the enzyme

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The lysosomal cysteine proteinase cathepsin B is synthesized in cultured human hepatoma HepG2 cells as an inactive 44 kDa precursor and subsequently processed to the mature single-chain enzyme with a molecular mass of 33 kDa. Intralysosomal conversion into the two-chain form results in subunits of 27 kDa, 24 kDa (heavy chain) and 5 kDa (light chain). Enzymic deglycosylation reveals that the 27 kDa polypeptide is the glycosylated variant of the carbohydrate-free 24 kDa heavy-chain form. The intracellular transport to the lysosomes is dependent upon mannose 6-phosphate-containing *N*-linked oligosaccharides. Receptor-mediated endocytosis of human skin-fibroblast-derived procathepsin B by HepG2 cells resulted in processed molecular forms that are not distinguishable from endogenous cathepsin B, thus favouring rather a cell-type-specific processing than structural differences due to the source of the proenzyme. The conversion step of single-chain cathepsin B into the two-chain enzyme is inhibited *in vivo* by the irreversible cysteine-proteinase inhibitors Z-Phe-Ala-CHN₂ and, albeit weaker, Z-Phe-Phe-CHN₂. Both substances have no effect on the activation of procathepsin B to the mature enzyme. The carbohydrate moiety of cathepsin B exerts no significant influence on the stability and the enzymic activity of the enzyme.

INTRODUCTION

The role of the endogenous cysteine proteinases cathepsins B, H and L in general intracellular protein turnover is well documented (Shaw & Dean, 1980; Grinde, 1983). Immunocytochemical localization demonstrated that these enzymes reside in the lysosomes (Mort *et al.*, 1981b; Li *et al.*, 1985). As is typical for soluble lysosomal enzymes (von Figura & Hasilik, 1986), the intracellular targeting to this organelle is dependent on the creation of the mannose 6-phosphate recognition marker (Hanewinkel *et al.*, 1987; Mason *et al.*, 1987; Nishimura *et al.*, 1988b). As lysosomal proteinases are synthesized as latent procathepsins, proteolytic maturation to the active enzymes occurs either in an acidic prelysosomal compartment, as reported for cathepsin D (Gieselmann *et al.*, 1983), or as an early event in the lysosomes (Nishimura & Kato, 1987). Only a small amount of the proenzymes is secreted by default into the extracellular fluid (Hanewinkel *et al.*, 1987; Reilly *et al.*, 1989). However, the secretion of active and activatable latent high-molecular-mass forms of cathepsins B and L can be elevated as response to viral transformation (Mason *et al.*, 1987; Achkar *et al.*, 1990), malignant dedifferentiation (Mort *et al.*, 1981a; Recklies & Mort, 1982), growth factors (Prenc *et al.*, 1990) or inflammation (Mort *et al.*, 1984; Buttle *et al.*, 1988). Increased transcription of the cathepsin B gene probably accounts for the elevated extracellular concentration of this proteinase in mouse melanomas (Moin *et al.*, 1989; Qian *et al.*, 1989). In the case of Moloney-mouse-sarcoma-virus-transformed Balb/c 3T3 fibroblasts the enhanced secretion of procathepsins B and L as well as other lysosomal enzymes has been correlated to the lack or the malfunction of mannose 6-phosphate receptors on the cell surface (Achkar *et al.*, 1990). Extracellular procathepsin L secreted by Kirsten-virus-transformed mouse NIH 3T3 fibroblasts was reported to be a consequence of a low-affinity interaction with the

endogenous mannose 6-phosphate receptors (Dong & Sahagian, 1990; Lazzarino & Gabel, 1990). In neoplastic tissues the enzymes were reported to occur associated with the plasma membrane (Sloane *et al.*, 1987; Rozhin *et al.*, 1989).

The interrelationship of active and latent high-molecular-mass cathepsin B isoenzymes is not well understood (Mort & Recklies, 1986). On the other hand, the intracellular processes resulting in activation of the proenzyme are also not yet elucidated (Nishimura *et al.*, 1990; Felleisen & Klinkert, 1990). The observed microheterogeneity of endogenous cathepsin B in various tissues was not further characterized, although analyses of cloned cDNAs revealed no striking differences (Fong *et al.*, 1986; Chan *et al.*, 1986). Therefore the role of the endogenous processing machinery and of the mode of glycosylation in the existence of different molecular forms of the enzyme remains to be established.

In the present paper we report on the interrelationship of cathepsin B isoenzymes in human hepatoma HepG2 cells and provide evidence for the existence of cell-type specific processing events. The role of the carbohydrate moiety of cathepsin B (Takahashi *et al.*, 1984) in the enzymic activity of cathepsin B is also addressed.

MATERIALS AND METHODS

Materials

L-[³⁵S]Methionine (specific radioactivity 800 Ci/mmol) and [¹⁴C]methylated molecular-mass standards were purchased from New England Nuclear (Bad Homburg, Germany) or Amersham International (Amersham, Bucks., U.K.). BSA (type V), pepsin (from pig stomach mucosa), phenylmethanesulphonyl fluoride, tunicamycin, pepstatin A and leupeptin were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aprotinin and human serum transferrin were from Serva (Munich, Germany). Protein A-Sepharose, CH-Sepharose 4B and CNBr-activated Sepharose

Abbreviations used: Z-, benzyloxycarbonyl-; -CHN₂, diazomethane; Gly-S-, glycine aldehyde semicarbazone; -NHNap, 2-naphthylamide; CH-, carboxyhexyl-; PNGase F, peptide: *N*-glycosidase F [peptide-*N*⁴-(*N*-acetyl-β-glucosaminyl)asparagine hydrolase], EC 3.5.1.52.

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4B were obtained from Pharmacia-LKB (Uppsala, Sweden). Z-Arg-Arg-NH₂ was either purchased from Bachem (Bubendorf, Switzerland) or prepared by the method of Knight (1980). Z-Phe-Ala-CHN₂ and Z-Phe-Phe-CHN₂ were kindly provided by Dr. E. Shaw (Friedrich-Miescher-Institut, Basel, Switzerland). Gly-Phe-Gly-Sc was prepared and coupled to CH-Sepharose 4B (Pharmacia, Uppsala, Sweden) as described by Rich *et al.* (1986). Peptide: N-glycosidase F (PNGase F) was supplied by Genzyme (Boston, MA, U.S.A.). The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Post-mortem human livers obtained by autopsy within 20 h of death were immediately frozen in liquid N₂ and stored at -70 °C until use. Human liver cathepsin B was purified to homogeneity by the method of Rich *et al.* (1986) and coupled to CNBr-activated Sepharose 4B (Pharmacia) at a density of 2.6 mg/ml of gel. The preparation of an antiserum in rabbits against human liver cathepsin B was reported previously (Hanewinkel *et al.*, 1987). Goat anti-(rabbit IgG) immunoglobulins conjugated to horseradish peroxidase were obtained from Accurate (Westbury, NY, U.S.A.). All other reagents were of the best quality grade available.

Cultivation and labelling of cells

Human diploid skin fibroblasts were maintained in Eagle's minimal essential medium as described by Cantz *et al.* (1972). HepG2 cells were grown analogously. Metabolic labelling of confluent cell layers was done according to the procedure of Hanewinkel *et al.* (1987).

Preparation of cell and medium extracts and immune precipitation of cathepsin B

Non-labelled cells were extracted for analysis by enzymic deglycosylation and subsequent immunoblotting as described previously (Hanewinkel *et al.*, 1987).

Conditioned media were obtained by culturing the respective cell layers for 24 h in serum-free medium (Waymouth MAB 87/3; composition as outlined in the Gibco catalogue) supplemented with 4 µg of human serum transferrin/ml and 30 nM-selenium in the presence of 10 mM-NH₄Cl. Each medium was centrifuged at 400 g for 5 min to remove cell debris, and the supernatant was freeze-dried. The residue was solubilized in 20 mM-sodium phosphate buffer, pH 6.5 (one-tenth of the original volume), and dialysed twice for 2 h against 2 litres of the same buffer. The non-diffusible material was freeze-dried, dissolved in 20 mM-sodium phosphate buffer, pH 6.5 (one-thirtieth of the original volume), dialysed as described above and stored frozen at -20 °C. NH₄Cl-induced secretions after incubation in the presence of 9 µg of tunicamycin/ml were treated analogously.

The extraction and immune precipitation of cathepsin B from labelled cell layers was done as reported previously (Hanewinkel *et al.*, 1987), with the following modifications. After labelling, the cell layer of a 25 cm² flask was washed twice with Hanks balanced salt solution and extracted with 200 µl of SDS/PAGE sample buffer (Laemmli, 1970) without glycerol, containing proteinase inhibitors (2 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride, 1 µM-leupeptin and 10 µg of aprotinin/ml), for 10 min at room temperature. An extract of the medium was prepared by precipitation with 70%-saturated (NH₄)₂SO₄ and solubilization of the precipitate in 200 µl of the buffer used for cell extraction. The extracts were heated at 95 °C for 5 min and then treated with 26 mM-iodoacetamide for 30 min at room temperature. Before immune precipitation, the extracts were diluted 10-fold with buffer A [50 mM-Tris/HCl buffer, pH 8.3, containing 0.15 M-NaCl, 1% (w/v) Triton X-100, 1% (w/v)

sodium deoxycholate, 0.25% (w/v) SDS, 2 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride, 10 µg of aprotinin/ml and 5 mg of BSA/ml] and centrifuged at 10000 g for 10 min at room temperature. The supernatant was mixed end-over-end with pre-immune-IgG-coated Protein A-Sepharose (4 mg/25 cm² flask) for 2 h at 4 °C. After centrifugation (10000 g for 3 min) the supernatant was transferred to a tube containing 10 mg of Protein A-Sepharose coated with IgG of 50 µl of antiserum. The suspension was shaken overnight at 4 °C and centrifuged as above, and the pellet was washed with three 1 ml portions of buffer A without BSA. After further washing steps with 2 × 1 ml of 50 mM-Tris/HCl buffer, pH 8.3, containing 1.5 M-NaCl and 2 × 1 ml of 10 mM-Tris/HCl buffer, pH 6.8, the immune precipitated polypeptides were solubilized in 70 µl of 1.25-fold-concentrated SDS/PAGE sample buffer, heated at 95 °C for 5 min and analysed by SDS/PAGE and fluorography.

Endocytosis experiments

NH₄Cl-induced [³⁵S]methionine-labelled secretions from human fibroblasts or HepG2 cells were incubated with the respective recipient cell lines as described previously (von Figura *et al.*, 1983). Extracts of the recipient cells were analysed by immune precipitation of cathepsin B and subsequent SDS/PAGE and fluorography.

Enzymic deglycosylation

Unlabelled cell extracts or medium proteins were deglycosylated after denaturation with SDS and 2-mercaptoethanol by treatment with 2 munits of PNGase F for 16 h at 37 °C as described previously (Hanewinkel *et al.*, 1987). Subsequent to analysis by SDS/PAGE, cathepsin B polypeptides were detected by immunoblotting.

For deglycosylation of crude native procatepsin B, 50 µg of total protein from HepG2-conditioned medium obtained after incubation in the presence of 10 mM-NH₄Cl was precipitated with 5 vol. of acetone for 30 min at 0 °C. After centrifugation (13000 g for 10 min), the pellet was dried and solubilized in 100 mM-sodium phosphate buffer, pH 7.2, containing 10 mM-1,10-phenanthroline and 0.2% (w/v) Nonidet P-40. The samples were incubated for 8 h at 37 °C with addition of 1 munit of PNGase F after 0, 2, 4 and 6 h.

Assay of the latent activity of procatepsin B

Activation of latent procatepsin B *in vitro* was achieved by the procedure of Mort *et al.* (1981a) with minor modifications. A 40 µl portion of proenzyme solution (eventually diluted with 0.2 mg of BSA/ml) was incubated with 30 µl of pepsin (250 µg/ml) in 0.1 M-sodium formate buffer, pH 3.5, for 30 min at 37 °C. The pre-activation step was stopped by addition of 230 µl of activation buffer (2.7 mM-cysteine in 88 mM-KH₂PO₄/12 mM-Na₂HPO₄/1.33 mM-EDTA buffer, pH 6.8), and enzymic activity was determined against Z-Arg-Arg-NH₂ as described by Barrett & Kirschke (1981).

Other methods

SDS/PAGE with a total monomer concentration of 12.5% (w/v) was performed according to the procedure of Laemmli (1970) with minor modifications as outlined by Hasilik & Neufeld (1980). Detection of labelled polypeptides was done by fluorography as described by Bonner & Laskey (1974).

Transfer of proteins to nitrocellulose sheets was done by the procedure of Towbin *et al.* (1979). The membranes were incubated with affinity-purified rabbit anti-(human liver cathepsin B) antibodies (3 µg/ml), prepared by immunoaffinity chromatography on cathepsin B-Sepharose as described in Johnstone & Thorpe (1982), with the use of 3 M-KSCN in 0.5 M-ammonium

chloride buffer, pH 10.0, for elution. Subsequent detection of cathepsin B-related polypeptides was performed by the use of goat anti-(rabbit IgG) immunoglobulins conjugated to horseradish peroxidase as described by Theisen *et al.* (1986).

Total protein was determined by the method of Lowry *et al.* (1951), with BSA as a standard.

Fig. 1. Molecular forms of cathepsin B in HepG2 cells

(a) A 100 μ g portion of soluble cellular protein was incubated in the absence (–) or in the presence (+) of PNGase F. Subsequent analysis was performed by SDS/PAGE and immunoblotting as described in the Materials and methods section. Analogously treated homogenates from human skin fibroblasts (Fibr.) served as a control. (b) Immunoblot of HepG2 cellular proteins without subjecting the sample to enzymic deglycosylation. The molecular-mass standards used were BSA (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa).

Fig. 2. Biosynthesis of cathepsin B in HepG2 cells

Confluent cell layers (25 cm² flasks) were incubated for 1 h with [³⁵S]methionine (30 μ Ci/ml) and harvested after chase periods as indicated. Cathepsin B was immune precipitated from cell and medium extracts and further analysed by SDS/PAGE and fluorography as described in the Materials and methods section. [¹⁴C]Methylated molecular-mass standards were BSA (69 kDa), ovalbumin (46 kDa), bovine carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

RESULTS

Molecular forms of cathepsin B in human hepatoma cells

Separation of HepG2 cell lysates by SDS/PAGE and subsequent detection of cathepsin B-cross-reactive material yields predominant polypeptides with molecular masses of 24 and 27 kDa (Fig. 1a). Overdevelopment led to the additional appearance of the 33 kDa single-chain enzyme (Fig. 1b). On omission of reduction before electrophoresis, the 24 kDa and 27 kDa proteins could be identified as heavy-chain variants of two-chain cathepsin B (results not shown). Treatment with PNGase F reveals the different electrophoretic mobilities of the heavy-chain polypeptides to be due to *N*-glycosidically linked oligosaccharides.

Biosynthesis of cathepsin B in human hepatoma cells

Metabolic labelling of HepG2 cells for 1 h identifies a protein with molecular mass 44 kDa to be the first detectable translation product of cathepsin B mRNA (Fig. 2). This inactive precursor is converted during 5 h into the single-chain enzyme (molecular mass 33 kDa) and after prolonged chase for 24–44 h into the two-chain forms with the large subunits of 27 and 24 kDa. The 5 kDa light chain cannot be identified in the gel system used. The ratio between glycosylated and deglycosylated heavy chains does not vary significantly during prolonged incubations, suggesting no fundamental difference of these two polypeptides concerning their half-time. About 5–10% of the newly made procathepsin B is secreted into the culture medium. Addition of the lysosomotropic base NH₄Cl or treatment with the *N*-glycosylation inhibitor tunicamycin resulted in more than 95% secretion (results not shown). As already observed for human skin fibroblasts, the final processing step of the single-chain into the two-chain forms could be inhibited *in vivo* by leupeptin, a reversible serine-proteinase and cysteine-proteinase inhibitor (results not shown). Incubation of the cell layers with the irreversible cysteine-proteinase inhibitor Z-Phe-Ala-CHN₂ could also effectively block this conversion (Fig. 3). Inhibition was in addition observed with the related compound Z-Phe-Phe-CHN₂,

Fig. 3. Inhibition of cathepsin B processing in HepG2 cells

HepG2 cells (25 cm² flasks) were preincubated for 16 h with 1% (v/v) dimethyl sulphoxide (–) or 1 μ M-Z-Phe-Ala-CHN₂ (+), labelled for 3 h with [³⁵S]methionine (20 μ Ci/ml) and chased for 5 h and 24 h in the continuous presence of the drug or the solvent alone. Further analysis was done as outlined in Fig. 2 legend.

Fig. 4. Endocytosis and processing of exogenous procathepsin B by human skin fibroblasts and HepG2 cells

Confluent fibroblast (F) or HepG2 (H) cell layers (25 cm² flasks) were incubated with [³⁵S]methionine-labelled NH₄Cl-induced secretions (4 × 10⁶ d.p.m. per flask) from the donor cells indicated for 22 h in the absence (–) or in the presence (+) of 10 mM-mannose 6-phosphate (Man6P) as described in the Materials and methods section. As a negative control, the incubation was performed at 4 °C. Endocytosed cathepsin B was analysed from cell extracts as outlined in Fig. 2 legend.

Fig. 5. Enzymic deglycosylation of native human hepatoma procathepsin B

(a) Portions (10 µg) of medium protein, obtained after treatment with 10 mM-NH₄Cl, were incubated with (lane B) or without (lane A) PNGase F for 8 h under conditions preventing denaturation of procathepsin B as described in the Materials and methods section. (b) Portions (10 µg) of medium protein, obtained in the presence of either 9 µg of tunicamycin/ml and 10 mM-NH₄Cl (lanes C and D) or 10 mM-NH₄Cl alone (lanes E and F), were incubated with (lanes C and E) or without (lanes D and F) PNGase F after denaturation and analysed by SDS/PAGE and immunoblotting as outlined in Fig. 1 legend.

albeit weaker (results not shown). The proteolytic removal of the pro-region was not affected in either case.

Processing of endocytosed procathepsin B

NH₄Cl-induced secretions were obtained from metabolically labelled skin fibroblasts and offered HepG2 cell layers as recipient cells for receptor-mediated endocytosis. The exogenous procathepsin B was taken up in a mannose 6-phosphate-dependent manner and processed into the mature forms identical with the endogenous enzyme. Analogous incubation of fibroblasts with secretions from labelled HepG2 cells confirmed the independence

of the processing products from the source of the proenzyme (Fig. 4).

Glycosylation and latent activity of procathepsin B

As enzymic deglycosylation of mature cathepsin B in its native state is not feasible, the influence of the carbohydrate moiety on the activation and the stability of procathepsin B *in vitro* was tested. Procathepsin B secreted by HepG2 cells can be artificially activated by treatment with pepsin, yielding a polypeptide of molecular mass 37.5 kDa (results not shown). The latent proenzyme was deglycosylated with PNGase F under conditions avoiding denaturation (Fig. 5a). The resulting 39 kDa protein exhibited after pepsin activation a similar enzymic activity against Z-Arg-Arg-NHnap to that of procathepsin B before treatment with PNGase F, the specific activities being 258 and 272 munits/mg of secreted protein respectively. Comparison by SDS/PAGE with the non-glycosylated procathepsin B obtained after biosynthesis in the presence of tunicamycin (Fig. 5b, lanes C and D) as well as by enzymic deglycosylation after denaturation (Fig. 5b, lanes E and F) indicated the complete removal of all N-linked oligosaccharides. In addition, the sensitivity against proteolytic degradation as tested by prolonged incubation with pepsin was not significantly changed (results not shown).

DISCUSSION

The molecular forms of cathepsin B in human skin fibroblasts (Hanewinkel *et al.*, 1987) exhibit differences as compared with the isoenzymes detectable in human liver (Hanewinkel *et al.*, 1987), rat liver (Nishimura & Kato, 1987) and rat hepatocytes (Nishimura *et al.*, 1988b). Cloned cDNAs from various human tissues revealed no striking differences (Chan *et al.*, 1986; Fong *et al.*, 1986), although the existence as single-chain as well as two-chain enzyme is well established (Takio *et al.*, 1983). The processes involved in this conversion, including a potential role of glycosylation for proper conformation, have not yet been addressed.

Using human hepatoma cells, we could confirm the results obtained for cathepsin B from human liver (Hanewinkel *et al.*, 1987). Human hepatoma procathepsin B appears to behave identically with the precursor from human skin fibroblasts (Hanewinkel *et al.*, 1987). The intracellular sorting as well as the uptake of exogenous proenzyme by receptor-mediated endocytosis is dependent on the creation of the mannose 6-phosphate recognition marker. The final processing product in HepG2 cells is mainly the two-chain enzyme. We could demonstrate that the heavy-chain subunit exists glycosylated as well as devoid of any N-linked oligosaccharides, probably as a consequence of the action of lysosomal endoglycosidases. Removal of the carbohydrate moiety obviously does not affect the stability against proteolytic degradation *in vivo*. In addition, these results explain the reported carbohydrate structures for mature cathepsin B from rat liver and pig spleen (Takahashi *et al.*, 1984; Taniguchi *et al.*, 1985). The conversion into the two-chain form occurs in the lysosomes and seems to be a cell-type-specific event. Receptor-mediated endocytosis of human skin-fibroblast-derived procathepsin B by HepG2 cells or vice versa yields processed mature forms indistinguishable from the endogenous enzyme, suggesting the existence of specific endogenous processing machineries rather than structural differences due to the source of the proenzyme. These results are in good agreement with the processing of exogenous mouse procathepsin L by Chinese-hamster ovary cells (Salminen & Gottesman, 1990). In addition, these authors suggested autocatalytic processing events, as the specific cysteine-proteinase inhibitors Z-Phe-Ala-CHN₂ and Z-Phe-Phe-CHN₂ (Shaw *et al.*, 1983) could inhibit the conversion

of single-chain cathepsin L into the two-chain form. In human hepatoma cells Z-Phe-Ala-CHN₂ effectively blocks the conversion of single-chain cathepsin B into the two-chain enzyme, whereas Z-Phe-Phe-CHN₂ exerts less influence on cathepsin B processing. However, to identify the cysteine proteinase(s) involved, highly specific inhibitors would be required. The proteolytic maturation of the latent proenzyme was affected neither by Z-Phe-Ala-CHN₂ nor by Z-Phe-Phe-CHN₂.

Heterogeneity of glycoproteins obtained from different tissues has been attributed frequently to cell-type-specific variations in glycosylation (e.g. Rademacher *et al.*, 1988). As loss of the catalytic properties and enhanced susceptibility to general proteolytic attack could be consequences of removal of the carbohydrate moiety, the influence of *N*-linked oligosaccharides on the stability and the activity of cathepsin B was assessed. For experimental reasons, we had to use native latent procathepsin B for these studies and subsequently activate the proenzyme *in vitro* by digestion with pepsin (Mort *et al.*, 1983). Removal of *N*-linked oligosaccharides results neither in loss of enzymic activity, as already shown for cathepsin L (Smith *et al.*, 1989), nor in a diminished stability against proteolytic degradation. Therefore the existence of cell-type-specific lysosomal endoglycosidases may explain the variation in the ratio between glycosylated and carbohydrate-free two-chain enzyme as observed in various human tissues and cell lines (L. Mach, C. Ballaun & J. Glössl, unpublished work), but does not influence the process of conversion of single-chain cathepsin B into the two chains. In addition, lack of glycosylation therefore cannot explain how expression of cathepsin B cDNAs in *Escherichia coli* does not yield active enzyme (Mort *et al.*, 1988). Similar results have been reported for the production of recombinant cathepsin L and cathepsin D in *E. coli*, where only excessive denaturation and renaturation procedures gave small amounts of enzymically active proteins (Smith & Gottesman, 1989; Conner & Udey, 1990).

Besides the insight we obtained into the molecular processes involved in the late maturation steps of lysosomal cathepsin B, we have no information on the activating enzyme *in vivo*. Various low-molecular mass inhibitors already used successfully for the analysis of processing *in vivo* (Nishimura *et al.*, 1988a; Hara *et al.*, 1988) either proved to have toxic effects on human hepatoma cells or failed to penetrate these cells (K. Stüwe & J. Glössl, unpublished work). Studies performed *in vitro* indicated either the dependence of activation on the action of an aspartic proteinase (Nishimura *et al.*, 1990) or autoactivation of cathepsin B itself (Felleisen & Klinkert, 1990).

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