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Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity

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Abstract Recent data show that a strong relation exists in certain cells between mitochondria and caspase activation in apoptosis. We further investigated this relation and tested whether treatment with the permeability transition (PT)-inducing agent atractyloside of Percoll-purified mitochondria released a caspase-processing activity. Following detection of procaspase-11 processing, we further purified this caspase-processing protease and identified it as cathepsin B. The purified cathepsin B, however, was found to be derived from lysosomes which were present as minor contaminants in the mitochondrial preparation. Besides procaspase-11, caspase-1 is also readily processed by cathepsin B. Procaspase-2, -6, -7, -14 are weak substrates and procaspase-3 is a very poor substrate, while procaspase-12 is no substrate at all for cathepsin B. In addition, cathepsin B induces nuclear apoptosis in digitonin-permeabilized cells as well as in isolated nuclei. All newly described activities of cathepsin B, namely processing of caspase zymogens and induction of nuclear apoptosis, are inhibited by the synthetic peptide caspase inhibitors z-VAD.fmk, z-DEVD.fmk and to a lesser extent by Ac-YVAD.cmk.

Key words: Caspase; Cathepsin B; Atractyloside; Mitochondrion

1. Introduction

Apoptosis is a physiological, morphologically distinct form of programmed cell death that occurs in all multicellular organisms and plays a crucial role during embryonic development, in immune cell responses and in tissue homeostasis. Inappropriate apoptosis is associated with serious diseases and disorders including neurodegenerative disorders, AIDS and cancer [1]. Two phenomena that have emerged in apoptosis research in the most recent years are the activation of caspases, a class of intracellular aspartate-specific cysteinyl proteases (for reviews see [2] and [3]), and the involvement of mitochondria in the signaling pathway leading to apoptosis (for reviews see [4] and [5]).

Caspases have been recognized as central executors of apoptosis. They are currently divided into initiator and executioner caspases. Initiator caspases (e.g. caspase-8) are activated as a consequence of cell membrane signaling events, while executioner or effector caspases (e.g. caspase-3) are responsible for the cleavage of crucial substrates involved in the apoptotic degradation phase. Some caspases, such as caspase-1 (ICE), which processes pro-interleukin-1β and pro-interleukin-18 into their mature form, and murine caspase-11, required for the activation of caspase-1 [6], also play an important role in inflammatory responses.

Caspases are synthesized as inactive zymogens, known as procaspases, with a tripartite structure consisting of an N-terminal prodomain of variable length, and a large and small subunit, called the p20 and p10 subunits, respectively. Processing of procaspases into the mature heterotetramer product $(p20p10)_2$ requires cleavage after aspartic acid (Asp) residues located in the interdomain linkers of the protein. This proteolytic activation may occur through autoprocessing, as is the case after oligomerization of procaspase-8 [7,8], or through cleavage by other caspases in a hierarchical manner and thus creating a protease cascade. Such a caspase cascade is found in the Fas pathway where it starts with recruitment of procaspase-8 to the death-inducing signaling complex (DISC) [9] and propagates into activation of caspase-3 [10]. However, other proteases can also activate caspase zymogens. Granzyme B, which is the only other mammalian protease that shares the caspase primary specificity for Asp, cleaves procaspase-3 to active caspase-3 [11,12], and the non-Asp-specific protease cathepsin G can generate active caspase-7 [13]. Therefore, it seems that the proteolytic-sensitive caspase interdomain linkers may allow non-Asp-specific proteases such as those from lysosomes to engage the apoptotic apparatus under pathological conditions.

The first direct evidence for involvement of mitochondria in apoptosis came from a cell free system based on *Xenopus laevis* egg extracts, where it was shown that nuclear apoptotic events occurred only in the presence of the mitochondrial fraction [14]. Release of cytochrome *c* [15,16] and other pro-apoptogenic components – such as the as yet unidentified 50-kDa apoptosis inducing factor (AIF) [17] – are induced by the opening of pores in the inner membrane of mitochondria, a condition known as permeability transition (PT). The opening of these pores allows the equilibrium of ions and respiratory substrate between cytosol and mitochondrial matrix leading to a decrease in the mitochondrial membrane potential and arrest of ATP synthesis [18]. Cytochrome *c* release does not always require induction of PT [19] and is prevented by overexpression of Bcl-2 [20]. Translocation of cytochrome *c* from mitochondria to the cytosol is a crucial step in apoptosis induced by Fas [10,21,22]. Once released, cytochrome *c* in

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interaction with the Apaf1/caspase-9 complex initiates an apoptotic protease cascade [23].

These data clearly indicate that in many cells a strong relation exists between mitochondria and caspase activation in apoptosis. To further investigate this relation, we tested whether Percoll purified mitochondria treated with the PT-inducing agent atractyloside release a caspase-processing activity. Following detection of procaspase-11 processing, we further purified this caspase-processing protease and characterized it as cathepsin B.

2. Materials and methods

2.1. Reagents

Ac-YVAD.cmk was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). z-VAD.fmk, z-FA.fmk and z-DEVD.fmk were supplied by Enzyme Systems Products (Dublin, CA, USA). Atractyloside was purchased from Sigma (St. Louis, MO, USA) and digitonin from Merck (Darmstadt, Germany). Bongkrekic acid (BA) was kindly provided by Dr. Duine (Delft University, Delft, The Netherlands). Anti-rat cathepsin B antibody was from Upstate Biotechnology (Lake Placid, NY, USA) and anti-cytochrome *c* antibody from PharMingen (San Diego, CA, USA).

2.2. Plasmids

The cloning of full-length murine CASP-1, CASP-2, CASP-3, CASP-6, CASP-7, CASP-11, CASP-12 and CASP-14 and their subcloning into pGEM11zf(+) (Promega Biotec, Madison, WI, USA) and the preparation of the in vitro transcribed/translated proteins have been described previously [24,25].

2.3. Cell lines and culture conditions

HeLa and L929 cells were grown in Dulbecco's minimum essential medium supplemented with 5% FCS and 5% NCS, at 37°C under a 5% CO_2 atmosphere.

2.4. Preparation of organelles

Mitochondria were isolated from Wistar rat hepatocytes (8 weeks, male) as described in [26]. They were then further purified on a Percoll gradient (Pharmacia Biotech, Uppsala, Sweden) as described in [27], and were finally washed and resuspended in cell free system (CFS) buffer [17] and used immediately. Rat liver lysosomes were prepared as described in [28]. Nuclei from HeLa cells were purified on a sucrose gradient as described in [29] and conserved in 50% glycerol in NB buffer at −20°C for maximally 2 weeks.

2.5. Purification of the caspase-processing protease

All purification steps were carried out at 4°C to avoid loss of biological activity. Percoll purified rat hepatocyte mitochondria were treated with 5 mM atractyloside (Atr) to induce permeability transition. After centrifugation (100 000 × *g*), supernatant from these mitochondria was first separated by size exclusion chromatography on a Superdex 75 FPLC column (Pharmacia Biotech) in CFS buffer. Active fractions were pooled and loaded on a Mini Q Äkta purifier column (Pharmacia Biotech) that was pre-equilibrated with CFS buffer. Elution was performed with a linear gradient from 0 to 250 mM NaCl in CFS buffer over 15 column volumes at 0.6 ml/min, followed by elu-

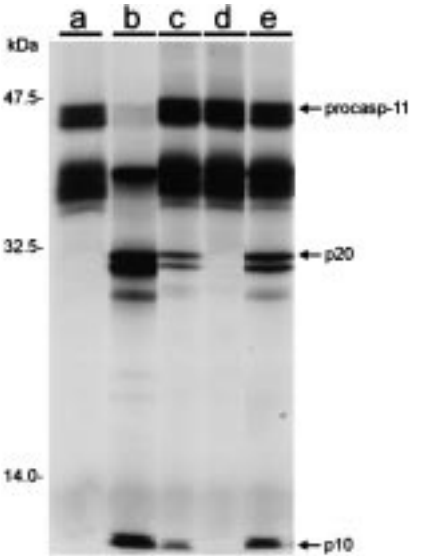

Fig. 1. Cleavage of procaspase-11 by crude supernatant of Atr-treated mitochondria. ^{35}S-labeled in vitro transcribed/translated procaspase-11 (indicated as procasp-11) was incubated with the respective samples as described in Section 2. Lower molecular weight bands of procaspase-11 are presumably due to internal initiation and/or premature termination. Processing was analyzed by 14% SDS-PAGE. Processed products are indicated as the p20 and p10 subunits. Incubation time of the experiment shown was 30 min. Longer incubation times of up to 1.5 h did not alter the cleavage pattern. Lane a: Procaspase-11; lane b: procaspase-11+supernatant of Atr-treated mitochondria; lane c: procaspase-11+supernatant of non-treated mitochondria (note that some activity is observed here as well, which is probably due to spontaneous leakage of the organelles after isolation); lane d: procaspase-11+supernatant of Atr-treated mitochondria pre-incubated for 15 min with z-VAD.fmk (2 μM); lane e: procaspase-11+caspase-8.

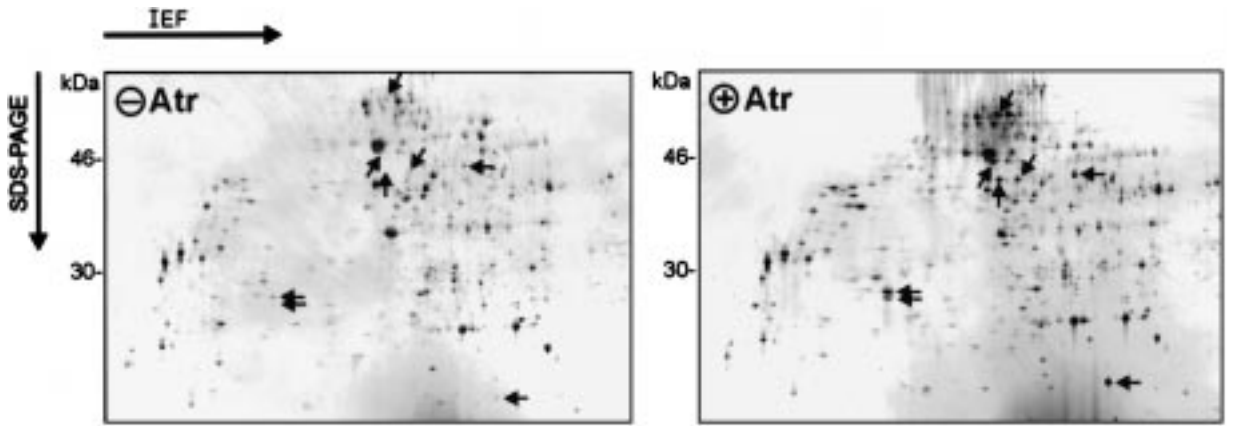

Fig. 2. Comparative 2-dimensional gel electrophoresis of the supernatant of Atr-treated and non-treated mitochondria reveals several differential protein spots. The crude supernatant was first separated by anion exchange on a Mono Q column (FPLC system, Pharmacia); proteins were eluted with a linear gradient from 0 to 250 mM NaCl in CFS buffer over 15 column volumes. Fractions that contained caspase-processing activity were pooled and compared with identical fractions from the supernatant of non-treated mitochondria. Protein spots were revealed by high-sensitivity silver staining. Major differential protein spots are indicated by an arrow.

tion of 2 column volumes of 1 M NaCl in CFS buffer. Fraction collection was started after 3 column volumes and fractions of 200 μl were collected. Most of the proteins eluted in the first 20 fractions of the gradient and the rest of the proteins eluted with 1 M NaCl. All fractions were tested for their ability to process procaspase-11. Activity was present from fractions 7–15 with peak activity in fractions 11–14 which corresponds to an NaCl concentration of 90–107 mM. Analysis of these fractions by 2-dimensional gel electrophoresis and silver staining showed that fractions 9–11 contained only the 25–27-kDa doublet, which was later identified as cathepsin B (fractions 9 and 10 contained only a very small amount of protein which was hardly detectable by silver staining). Fractions 12–15 contained also a more acidic isoform of the 25–27-kDa doublet and starting from fraction 13 other contaminating proteins became detectable.

To study the different activities of the caspase-processing protease, fractions 11–13 were used. Identical fractions of a run with buffer only were used as control.

2.6. Determination of the caspase-processing activity of the protease

^{35}S-labeled caspases were prepared with an in vitro coupled transcription/translation TNT kit from Promega Biotech, using SP6 or T7 RNA polymerase to transcribe genes cloned in pGEM11zf(+) and pLT10TH vector, respectively [24]. The reaction products were analyzed by SDS-PAGE and stored at −70°C until needed. Two μl of this material was incubated for 30 min at 37°C with 2 μl of the crude supernatant or 2 μl of the purified protease in a total volume of 20 μl CFS buffer. Cleavage products were analyzed by SDS-PAGE followed by autoradiography or fluorography of the dried gels. In the case of inhibitor studies, the purified protease was preincubated with inhibitor for 15 min at 37°C.

2.7. Two-dimensional gel electrophoresis (2-DE)

2.7.1. Isoelectro-focusing. Isoelectro-focusing was carried out on 18-cm IPG strips, pH 3–10 (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Samples in CFS buffer were precipitated with acetone and redissolved in lysis buffer.

2.7.2. SDS-PAGE. The second dimension (SDS-PAGE) was run on large horizontal Excell gradient gels 12–14% acrylamide (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer.

2.8. Amino acid sequence analysis

Coomassie blue-stained 2-DE spots were digested in situ with modified trypsin (Promega) overnight at 37°C. Peptides were recovered by sonication in the presence of 0.1% SDS to facilitate efficient peptide extraction from the gel pieces. Peptides were separated by use of a weak anion exchange pre-column (AX-300) in series with a reversed phase column (ODS-300) using an ABI 173 HPLC system. Fractions containing the resolved peptides were collected manually prior to Edman sequencing on an ABI Procise high sensitivity system employing capillary HPLC.

2.9. Western blotting

Proteins were separated by SDS-PAGE (14%) and transferred to nitrocellulose. The blots were incubated with the desired antibody and followed by ECL-based detection (Amersham Life Science, Amersham, UK).

2.10. Determination of apoptogenic activity

L929 cells were plated on coverslips 2 days before the experiment. Cells were permeabilized with 0.03% digitonin in CFS buffer for 3 min. The leaked out cytosol was removed by aspiration and cells were washed with PBS buffer (37°C). Freshly purified cathepsin B in CFS buffer, pH 7.4, supplemented with 2 mM ATP was added on top of the coverslip. After the appropriate incubation time at 37°C, the cells were stained with propidium iodide (3 μM) and analyzed by confocal laser scanning microscopy (CLSM). Apoptogenic activity on isolated nuclei and ladder formation were performed as described in [30].

3. Results

3.1. Purification of a caspase-processing protease.

It is well documented that mitochondria that undergo permeability transition (PT) release apoptogenic factors into the cytosol [15–17]. Accordingly, we tested whether treatment of Percoll purified rat liver mitochondria with the PT-inducing agent atractyloside (Atr) resulted in release of a caspase-processing activity. We tested the processing of in vitro transcribed/translated murine procaspase-1, -2, -3, -6, -7, -11, -12 and -14 [24,25]. Since procaspase-11 was found to be the best substrate for this caspase-processing protease, we have further used its processing as activity assay to purify and identify this

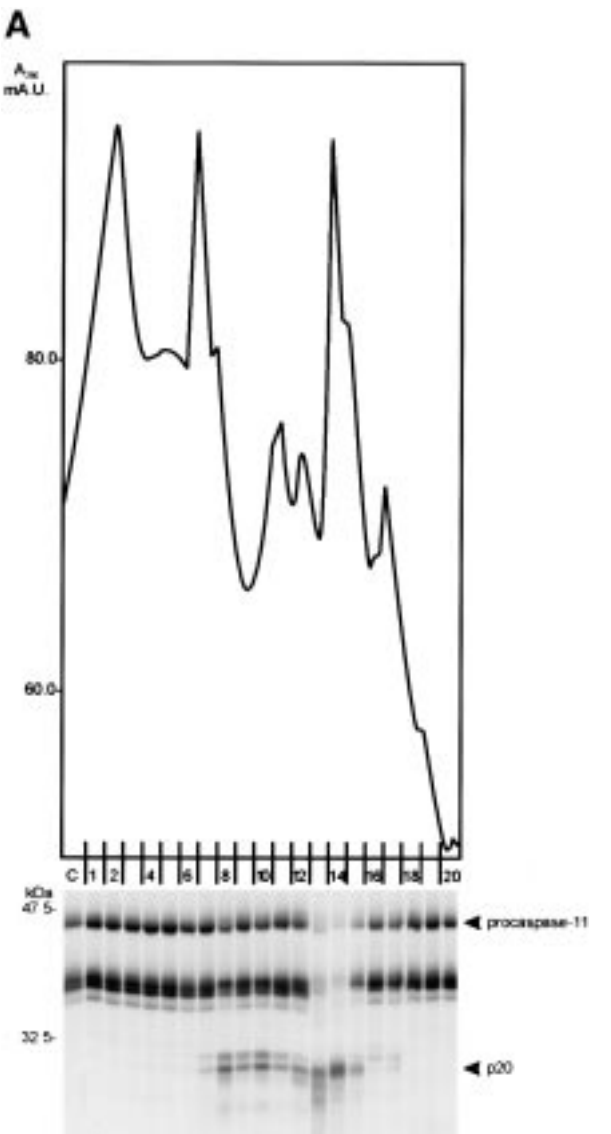

Fig. 3. Purification of the caspase-processing protease. A: The relevant part of the elution profile of the Mini Q column (Äkta purifier, Pharmacia) (mA.U stands for milli absorbance units). The separation was carried out as described in Section 2. The lower part of the figure shows the SDS-PAGE analysis of processing of procaspase-11 by the corresponding mini Q fractions. C is unprocessed procaspase-11. B: The 2-dimensional gel electrophoresis protein pattern of the most active fractions revealed by high-sensitivity silver staining. The 25-kDa doublet that was identified by amino acid sequence analysis as cathepsin B, and the lower MW protein, presumably the light chain of cathepsin B, are indicated by an arrow. To study the different activities of the caspase-processing protease, fractions 11–13 were used.

caspase-processing protease. Fig. 1 shows the proteolytic activity of the supernatant of the Atr-treated and non-treated mitochondria on in vitro transcribed/translated procaspase-11. As shown, in the presence of the Atr-treated sample procaspase-11 disappears (lane b), and this is accompanied by the appearance of the p20 and p10 subunits, thus suggesting a proteolytic maturation similar to the one generated by caspase-8 treatment (lane e). The caspase-processing activity present in the supernatant of Atr-treated mitochondria could be completely inhibited by the broad-spectrum caspase inhibitor z-VAD.fmk (lane d) and iodoacetamide (not shown) suggesting that it is a cysteine protease presumably belonging to the caspase family.

To isolate and further characterize the protease responsible for this activity, we employed high-resolution 2-dimensional gel electrophoresis (2-DE). Comparison of the protein pattern of the supernatant of the Atr-treated and non-treated mitochondria revealed high complexity and thus rendered interpretation difficult. Fractionation of the crude supernatant of Atr-treated mitochondria by anion exchange chromatography on a Mono Q column, followed by comparison of the protein pattern of the fractions that contained procaspase-11 processing activity with the corresponding fractions of the supernatant of non-treated mitochondria, revealed some major differential spots present in the Atr-treated sample (indicated by arrows in Fig. 2). Among these, a double protein spot of approximately 25 kDa was prominent. Further purification of the proteolytic activity through size exclusion chromatography on a Superdex 12 column, followed by anion exchange chromatography on a high-resolution Mini Q column, showed that proteolytic activity was present in 3 consecutive peaks (Fig. 3A). Analysis of the most active fractions (9–15) by high-resolution 2-DE and high-sensitivity silver staining revealed that the 25-kDa doublet was the major component present in these fractions. Fractions 9 and 10 contained only the 25-kDa doublet but in very low amounts. Fig. 3B shows the 2-DE pattern of fractions 11–14. Besides the 25-kDa doublet, a more acidic isoform of the 25-kDa doublet could be detected in fractions 12–15. Starting from fraction 13, more contaminating proteins were present. The high activity present in fractions 13 and 14 correlated with the higher concentration of the 25-kDa doublet. The presence of several isoforms of the 25-kDa doublet could explain why the activity is distributed over several peaks. The more acidic isoform may be generated either by phosphorylation or by proteolytic removal of a small basic fragment. However, anti-phospho-tyrosine, -serine or -threonine antibodies failed to react with the protein, thus rendering phosphorylation unlikely. The observation that the more acidic isoform is present when size exclusion chromatography is combined with anion exchange chromatography but is absent when only anion exchange chromatography is carried out, suggests that the acidic iso-

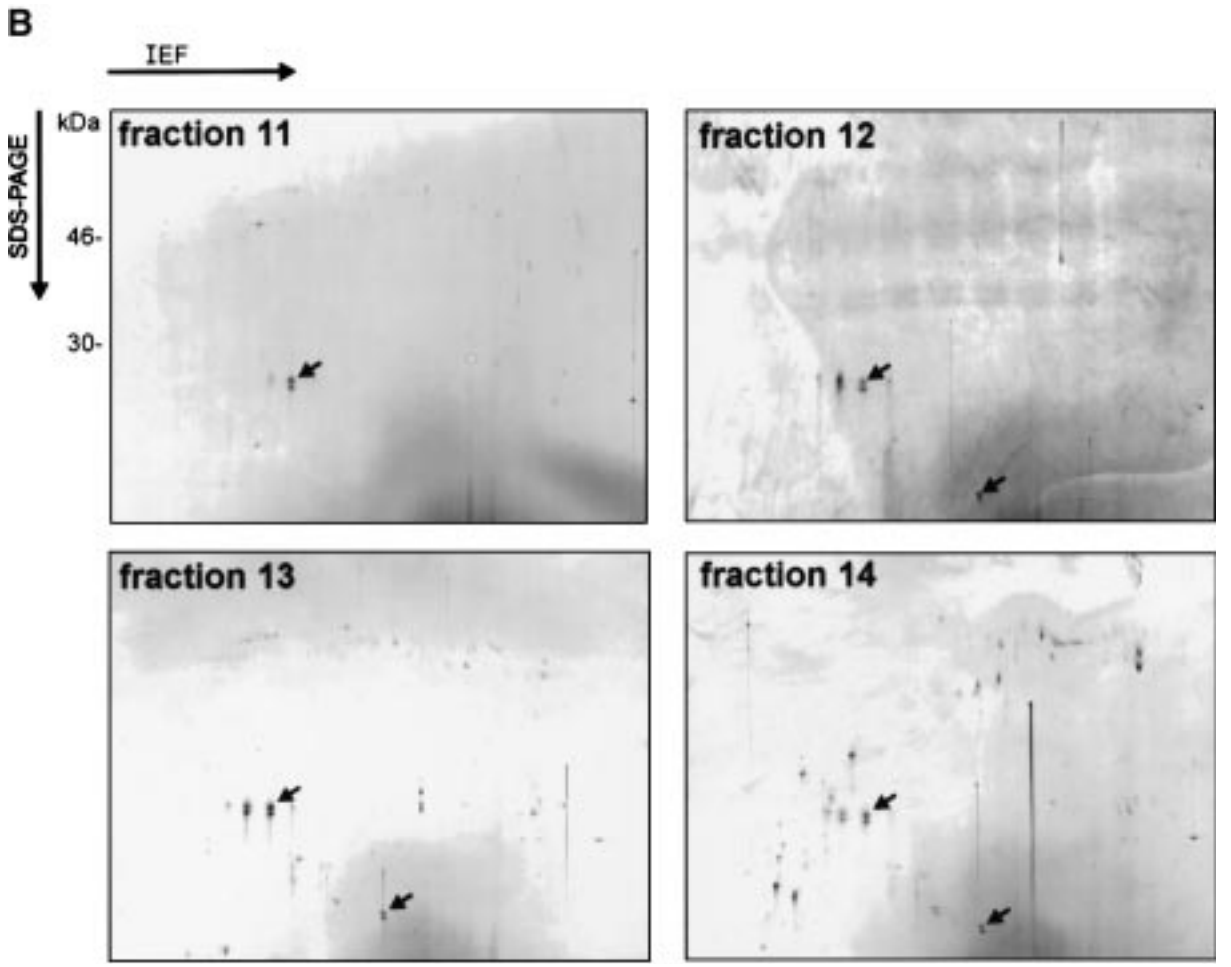

Fig. 3 (*continued*).

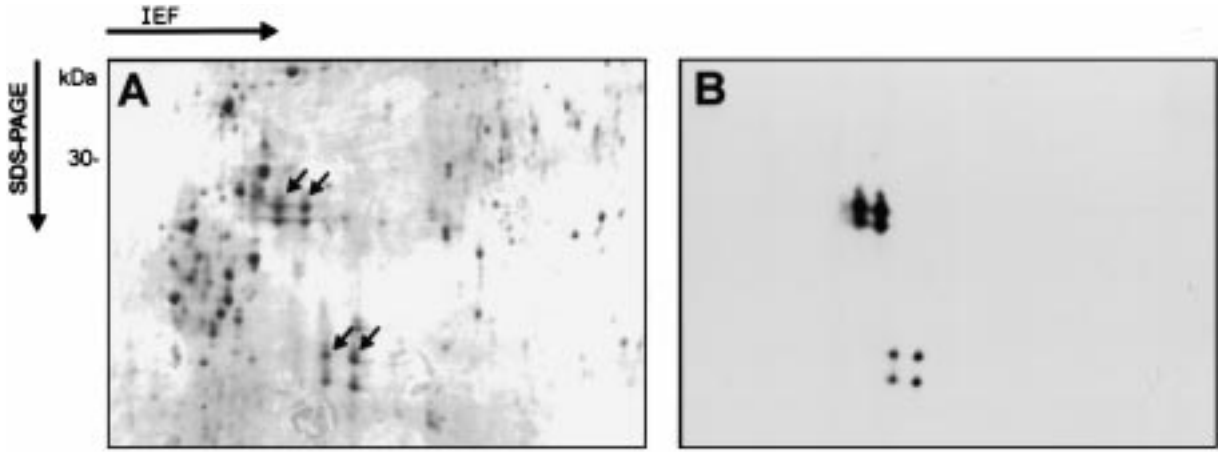

Fig. 4. Immunostaining of cathepsin B on a partially purified fraction of the caspase-processing protease. To obtain a partially purified fraction of the protease, crude supernatant of Atr-treated mitochondria was separated by anion exchange chromatography on a Mini Q column (Pharmacia). Active fractions were pooled and 75% of the material was used for silver staining and the other 25% for the immunostaining. A: Silver staining of a 2-dimensional gel of the partially purified caspase-processing protease (indicated by an arrow). B: The corresponding immunostaining with anti-rat cathepsin B. Note that the horizontal distance between the cathepsin B spots is smaller in the immunostaining; this is due to the different processing of the gels. The lower molecular weight protein spots that are also recognized by the anti-cathepsin B antibody are presumably degradation products of cathepsin B.

form is generated during gel filtration presumably through autoprocessing.

3.2. The caspase-processing protease is identical to cathepsin B

Amino acid analysis of peptides generated by in situ gel trypsin digest on Coomassie blue-stained protein spots, identified the protease as cathepsin B (a list of peptides sequenced is given in Table 1). This was confirmed by Western blotting using a polyclonal antibody against rat cathepsin B. Fig. 4 shows a silver staining of the partially purified protease (A) and the corresponding Western blot (B). Cathepsin B exists as a 40-kDa proform and a 30-, 27- and 25-kDa mature protein. Our purified cathepsin B represents the two-chain form which is composed of the 25/27-kDa chain and a smaller 5000-Da chain (presumably corresponding to the low molecular weight protein spot indicated by an arrow in fractions 12, 13 and 14 of Fig. 3B) that are held together by a disulfide bond [31]. The two-chain form is equally active as the single-chain 30-kDa form. The presence of the two isoforms could be due to a difference in the dipeptide which is normally lost during proteolytic processing into the two-chain form [31].

Commercially available cathepsin B (Sigma) processed procaspase-11 (in CFS buffer at pH 7.4) exactly as our purified cathepsin B did. The p20 and p10 subunits generated by the former also had an identical molecular weight, as judged from their electrophoretic mobility, as those generated by caspase-8 (data not shown).

Since cathepsin B is known to be an abundant lysosomal protein and since Atr, which releases cathepsin B from the mitochondrial preparations, is known as a specific ligand of the mitochondrial adenine nucleotide translocator, we further investigated whether cathepsin B was derived from the mitochondria or from a minor lysosomal contamination present in mitochondrial preparations. Confocal laser scanning microscopy using the anti-rat cathepsin B antibody on a mouse hepatoma cell line failed to detect any cathepsin B in mitochondria while a clear lysosomal staining was visible (data not shown). Furthermore, bongkrekic acid which acts as a stable inhibitor of PT, prevented the Atr-induced release of cytochrome *c* from the intermembrane space of the mitochondria, but did not inhibit the release of cathepsin B (data not shown), indicating that cathepsin B is derived from a lysosomal contamination. This contamination is, however, very minor since we could not detect cathepsin B, one of the most abundant lysosomal proteins, by Western blotting using 200 μg protein of the mitochondrial preparation. In addition, atractyloside also induced the release of cathepsin B from purified lysosomes (Fig. 5).

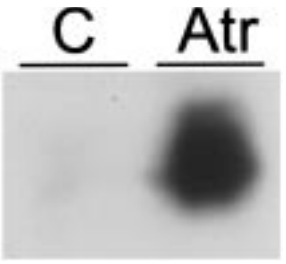

Fig. 5. Atractyloside induces the release of cathepsin B from purified lysosomes. Supernatant (16 μg of protein) from Atr- and control-treated lysosomes was analyzed for the presence of cathepsin B by Western blot analysis.

3.3. Cleavage of caspases by cathepsin B

Since we previously observed cleavage of several caspases with the crude supernatant of Atr-treated mitochondria, we

Table 1
Amino acid sequence analysis of peptides derived from the caspase-processing protease show that it is identical to cathepsin B

224-HYGYTSYSV-232
238-EIMAEIYK-245
269-HEAGDVMG-276
315-GENHCGIESEIVAG-328

The numbers indicate the positions of the amino acid residues in the sequence of rat cathepsin B.

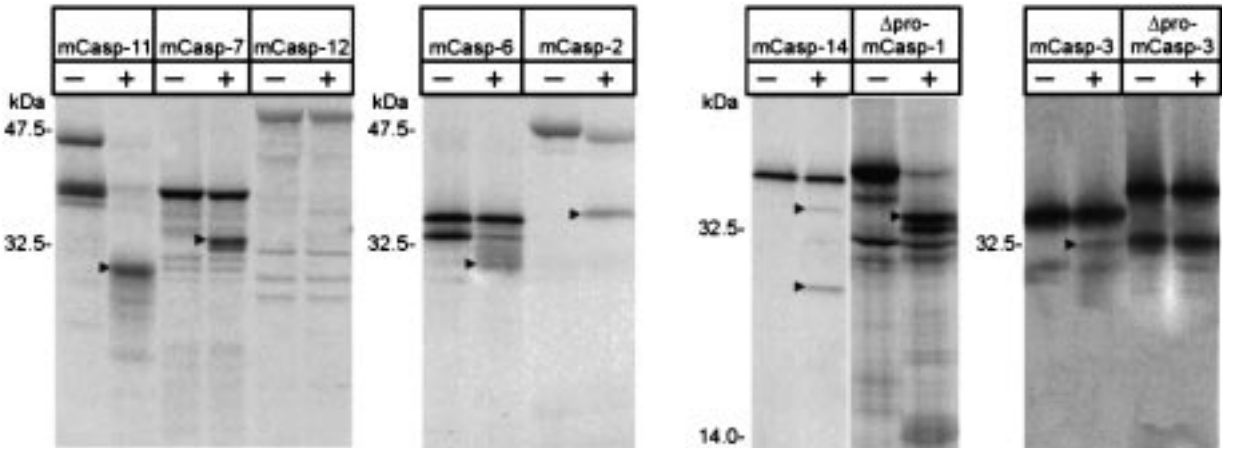

Fig. 6. Processing of caspases by purified cathepsin B. [^{35}S]Methionine-labeled in vitro transcribed/translated caspases (2 μl) were incubated (30 min, 37°C) either with CFS buffer (−) or with 10 μl of purified cathepsin B (+) in a total volume of 20 μl. Processed products were analyzed by SDS-PAGE (14% acrylamide) and are indicated by arrowheads in the figure. The higher molecular weight of caspase-3 without prodomain is due to the presence of an N-terminal His_6-tag and a C-terminal Strep-tag.

tested whether this was also the case for purified cathepsin B. All experiments were done with freshly purified cathepsin B, since it was found to be considerably more biologically active than the commercial preparation. For example, we obtained complete processing of procaspase-11 with freshly purified cathepsin B at concentrations of 2–5 μg/ml (the concentration was estimated from the UV absorbance on the Mini Q column using commercial cathepsin B as a reference), while we could not obtain complete processing with the commercial preparation at a concentration of 30 μg/ml. This means that our purified cathepsin B is at least 6-fold more biologically active than the commercial preparation.

In agreement with our previous results using crude supernatant, we found that procaspase-11 is most efficiently processed into its p20 and p10 subunits by cathepsin B (Fig. 5). Furthermore, the processed form generated by cathepsin B is biologically active (Schotte et al., submitted). Also caspase-1 (without prodomain) was readily cleaved by cathepsin B, which is not surprising since caspase-1 is highly homologous to caspase-11. To the contrary, procaspase-2, -6, -7 and -14 were only weakly processed while procaspase-3 was found to be a very poor substrate (Fig. 5). The processing of procaspase-3 corresponds with the removal of the prodomain since caspase-3 without prodomain was not cleaved by cathepsin B (Fig. 5). Finally, procaspase-12 was not processed by cathepsin B, indicating that it is not a substrate for the protease.

3.4. Cathepsin B is inhibited by synthetic peptide caspase inhibitors

Since cathepsin B has caspase-processing activity and since the broad spectrum caspase inhibitor z-VAD.fmk could inhibit the caspase-processing activity present in the crude supernatant of Atr-treated mitochondria, we tested whether purified cathepsin B was sensitive to the synthetic caspase inhibitors. As shown in Fig. 6, the proteolytic processing of procaspase-11 by cathepsin B was completely inhibited by the broad spectrum caspase inhibitor z-VAD.fmk and the caspase-3 inhibitor z-DEVD.fmk at concentrations of 2 μM, but not by the caspase-1 inhibitor Ac-YVAD.cmk (2 μM). Ac-YVAD.cmk inhibited only partially at a concentration of 50 μM. Also, purified cathepsin B is inhibited by the specific cathepsin B inhibitor z-FA.fmk and leupeptin.

3.5. Cathepsin B has apoptogenic activity

Nuclear chromatin condensation and DNA fragmentation are considered to be the hallmark of apoptosis. Therefore, we tested whether our purified cathepsin B had apoptogenic activity on nuclei. Digitonin-permeabilized cells from which the cytosol was removed were incubated with freshly purified cathepsin B (5 μg/ml, estimated as described above), stained with propidium iodide and analyzed by CLSM for occurrence of nuclear changes indicative of nuclear apoptosis. Already after 15 min of incubation cathepsin B induced chromatin condensation in all nuclei (Fig. 7). After 30 min of incubation most of the nuclei had an irregular shape (shrinkage) and their DNA content was drastically reduced as judged from fluorescence intensity. Both features of nuclear apoptosis – chromatin condensation and DNA fragmentation – were abolished when cathepsin B was preincubated with z-VAD-fmk. Cathepsin B-induced DNA degradation in propidium

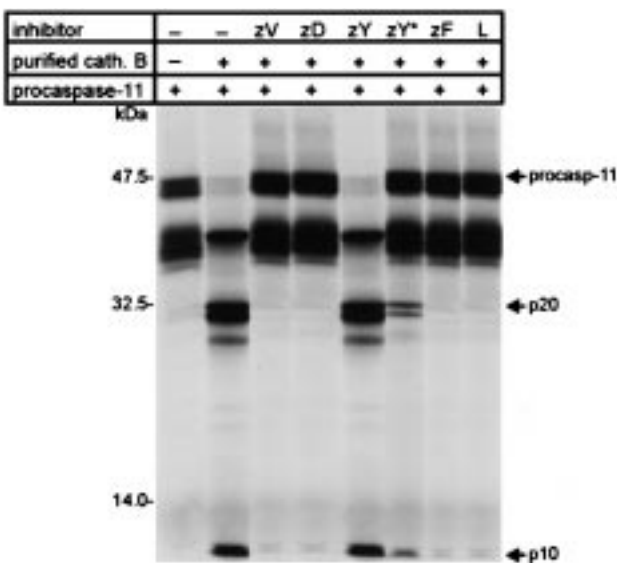

Fig. 7. Inhibition by synthetic peptide caspase inhibitors of procaspase-11 processing by purified cathepsin B. Purified cathepsin B was preincubated with the following inhibitors (2 μM; * 50 μM) for 15 min at 37°C. zV, z-VAD.fmk; zD, z-DEVD.fmk; zY, Ac-YVAD.cmk; zF, z-FA.fmk; L, leupeptin.

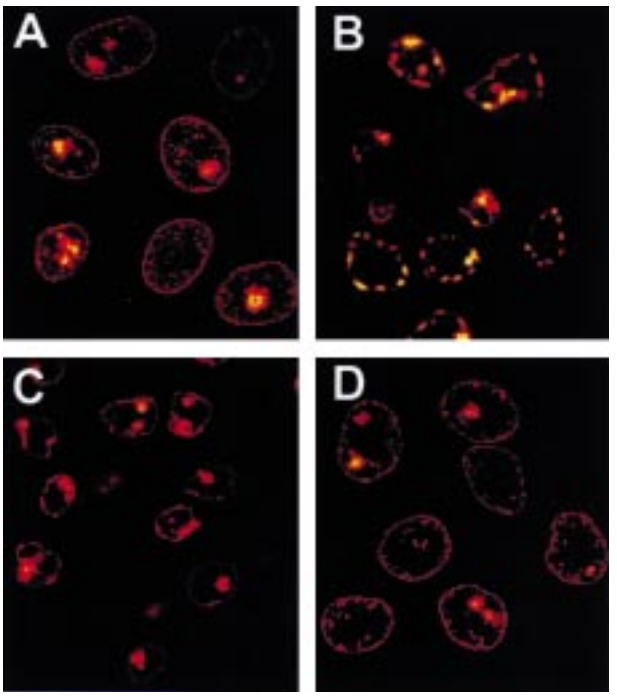

Fig. 8. Induction of nuclear apoptosis by purified cathepsin B. Digitonin-permeabilized L929 cells were incubated at 37°C with CFS buffer (A), purified cathepsin B for 15 min (B) and 30 min (C) or purified cathepsin B preincubated with 2 μM z-VAD.fmk (D).

iodide-stained digitonin-permeabilized cells was also observed by flow cytometry (data not shown). Cathepsin B also induced chromatin condensation and DNA degradation on isolated nuclei from HeLa cells; agarose gel electrophoresis followed by ethidium bromide staining showed a typical DNA ladder (data not shown).

4. Discussion

In this study, we identified the lysosomal protease cathepsin B as an activator of caspase zymogens in vitro. Cathepsin B was purified from the supernatant of a Percoll purified rat liver mitochondrial preparation that was treated with the PT inducing agent atractyloside which is known as a ligand for the mitochondrial adenine nucleotide translocator (ANT). The purified protease corresponded to the 25–27-kDa form which represents the heavy chain of the two-chain lysosomal form of the enzyme [32]. The 25–27-kDa doublet is most likely due to differences in carbohydrate modification [32]. Also the light chain of approximately 5 kDa was present in the preparations. All activities observed with the crude supernatant, namely selective cleavage of caspases and induction of nuclear apoptosis, were similarly observed with purified cathepsin B (Fig. 8). Apparently, no additional caspase-processing activity was present in the supernatant. Since the cathepsin B released was found to be of lysosomal origin, these results indicate that mitochondria which undergo PT do not release proteases that directly activate caspases. These results also indicate that either Atr is not specific for the adenine nucleotide translocator in the mitochondria or that maybe similar proteins are located in the lysosomal membrane.

Murine procaspase-11 was found to be the best substrate for cathepsin B. The p20 and p10 subunits from caspase-11 generated by cathepsin B were identical to those generated by caspase-8. Processed caspase-11 was biologically active as measured by activation of procaspase-3, followed by measurement of the latter by cleavage of the fluorogenic substrate Ac-DEVD.amc (Schotte et al., submitted). Cathepsin B generated only the specific p20 and p10 subunits at neutral pH (pH 7.4), while more acidic or more basic pH resulted in an almost complete degradation of procaspase-11. Caspase-1 was also processed, though to a lesser extent than procaspase-11. Procaspase-2, -6, -7 and -14 were weak substrates for cathepsin B and procaspase-3 was a very poor substrate.

Cathepsin B has exo- as well as endo-peptidase activity. The endopeptidase activity of cathepsin B prefers, but is not limited to, a basic and hydrophobic amino acid at the P1 and P2 substrate sites, respectively. Such a preferred site is present in murine procaspase-11 (residues 264-IR-265) and in murine as well as in human caspase-1 (residues 295 and 296, respectively). These residues are conserved and are just N-terminal of the preferred Asp-Xaa cleavage site between the p20 and p10 subunit [33]. The region N-terminal of the p20 in murine procaspase-11 also contains a preferred site (61-MK-62) for cathepsin B and is located within the predicted Asp-Xaa residues that separate the prodomain from the p20. Caspase-11 and caspase-1 belong to the class of initiator caspases, while caspase-3 is an executioner. Thus, given the specificity of cathepsin B for caspase-11 and caspase-1 and not for caspase-3, cathepsin B may play a regulatory role in the activation of initiator caspases rather than executioner caspases.

Questions remain open as to how cathepsin B itself is activated during apoptosis and other caspase-dependent processes such as the maturation of pro-IL 1β and whether caspase processing occurs in the lysosomes or in the cytosol. However, our observation that the specific p20 and p10 subunits of procaspase-11 are generated only at neutral pH indicates that activation most likely occurs in the cytosol. The mechanism through which cathepsin B is released from the lysosomes also remains an open question. It has been reported that photo-oxidative damage of the lysosomal membrane initially results in disruption of the lysosomal proton-gradient and later in disruption of the lysosomal membrane [34]. Furthermore, severe photo-oxidation, which results in severe lysosomal damage, causes necrosis, whereas moderate stress results only in partial lysosomal leakiness with release of apoptosis-inducing proteases which then leads to apoptosis. Therefore, lysosomes could function as a sensor/effector of oxidative stress induced by certain stimuli and might fulfill a similar role to that of mitochondria in apoptosis.

Our results obtained with an open cell system (namely digitonin-permeabilized cells from which the cytosol was removed) but also with isolated nuclei indicate that cathepsin B also induces nuclear apoptosis. Accordingly, no additional cytoplasmic factors are required for this activity. Cathepsin B has no nuclease activity by itself and therefore must directly or indirectly activate a nuclease through proteolytic cleavage. A plausible candidate is the recently described 'inhibitor of caspase activated deoxyribonuclease' (mouse ICAD, which is homologous to human DFF45) which is cleaved by caspase-3 [35]. This cleavage results in activation and translocation of CAD to the nucleus. However, the complex ICAD-CAD is cytoplasmic, while cathepsin B induces nuclear apoptosis in isolated nuclei. Therefore, the ICAD-CAD complex and possibly other proteins should remain associated with the isolated

nuclei in order to mediate nuclear apoptosis under this condition, a feature that is not supported by the available data. Also, analysis by 2-dimensional gel electrophoresis of cathepsin B-treated (3 min) nuclei from L929 cells revealed a protein spot in the basic region of the gel that specifically disappeared in the cathepsin B-treated sample. The estimated p*I* value of this protein spot is 8.0, while mouse ICAD has a p*I* of 4.5, thus indicating that cathepsin B-induced nuclear apoptosis is not mediated through cleavage of ICAD.

Translocation of cathepsin B from the cytoplasm to the nucleus has been shown during bile-salt induced hepatocyte apoptosis [36]. This indicates the possibility for a direct role of cathepsin B in the nucleus in at least some forms of apoptotic cell death. The reason why several proteases such as caspases, but also other cystein proteases, such as cathepsin B and D and some serine proteases (our own unpublished observation) can induce nuclear apoptosis could be ascribed to the fact that protease-sensitive regions in the nucleases or in their activators or inhibitors that need to be cleaved during apoptosis are susceptible to various kinds of proteases.

Several lines of research pinpoint a role for cathepsin B as intracellular protease in active cell death or apoptosis. It has been shown that cathepsin B plays a role in apoptosis of luminal epithelial cells of the prostate and mammary gland that occurs after hormone ablation [37]. An increased expression of cathepsin B is observed in apoptotic cells and the protein was subsequently localized in the apoptotic bodies. Moreover, a recent immunohistochemical study in infiltrative breast carcinomas of the natural cysteine protease inhibitor cystatin A, which inhibits cathepsin B and also cathepsin H, L and S, suggests a role for cathepsins in breast cancer regression: tumors positive for cystatin A are of larger size and have higher mitotic activity than cystatin A-negative tumors [38]. Cystatin A expression also correlates with negative staining for the anti-apoptotic protein Bcl-2. These findings suggest that the decreased apoptosis observed in tumors that express cystatin A could be due to cystatin A-mediated inhibition of cathepsin B and/or other cathepsins. Still another line of research points to a role for cathepsin B in 1,25-dihydroxyvitamin D_3 (1,25(OH)2D_3)-induced apoptosis. 1,25(OH)2D_3 inhibits breast cancer cell growth both in vivo and in vitro. MCF7 cells treated with 1,25(OH)2D_3 exhibit upregulation of proteins associated with mammary gland regression, namely clusterin and cathepsin B, and downregulation of Bcl-2 [39], thus indicating that increased sensitivity to apoptosis might be correlated with the presence of cathepsin B. In conclusion, our results provide a molecular basis for the apparent link between apoptosis and cathepsin B, namely through a cathepsin B-initiated activation of the caspase cascade resulting in activation of executioner caspases.

Both newly described activities, namely cleavage of caspase zymogens and induction of nuclear apoptosis by cathepsin B, are inhibited by the broad spectrum caspase inhibitor z-VAD.fmk (2 μM), the caspase-3 inhibitor z-DEVD.fmk (2 μM) and, albeit to a lesser extent, by the caspase-1 inhibitor Ac-YVAD.cmk (partial inhibition at 50 μM). This means that the latter inhibitors can no longer be classified as specific caspase inhibitors. As a consequence, in vivo results obtained with these inhibitors have to be interpreted with caution.

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References

[1] Nicholson, D.W. (1996) Nat. Biotechnol. 14, 297–301.
[2] Salvesen, G.S. and Dixit, V.M. (1997) Cell 91, 443–446.
[3] Nicholson, D.W. and Thornberry, N.A. (1997) Trends Biochem. Sci. 22, 299–306.
[4] Kroemer, G., Zamzami, N. and Susin, S.A. (1997) Immunol. Today 18, 44–51.
[5] Mignotte, B. and Vayssière, J.-L. (1998) Eur. J. Biochem. 252, 1–15.
[6] Wang, S., Miura, M., Jung, Y., Zhu, H., Li, E. and Yuan, J. (1998) Cell 92, 501–509.
[7] Martin, D.A., Siegel, R.M., Zheng, L. and Lenardo, M.J. (1998) J. Biol. Chem. 273, 4345–4349.
[8] Yang, X., Chang, H.Y. and Baltimore, D. (1998) Mol. Cell 1, 319–325.
[9] Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H. and Peter, M.E. (1997) EMBO J. 16, 2794–2804.
[10] Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.-M., Krammer, P.H. and Peter, M.E. (1998) EMBO J. 17, 1675–1687.
[11] Van de Craen, M., Van den brande, I., Declercq, W., Irmler, M., Beyaert, R., Tschopp, J., Fiers, W. and Vandenabeele, P. (1997) Eur. J. Immunol. 27, 1296–1299.
[12] Talanian, R.V., Yang, X., Turbov, J., Seth, P., Chayur, T., Casiano, C.A., Orth, K. and Froelich, C.J. (1997) J. Exp. Med. 186, 1323–1331.
[13] Zhou, Q. and Salvesen, G.S. (1997) Biochem. J. 324, 361–364.
[14] Newmeyer, D.D., Farschon, D.M. and Reed, J.C. (1994) Cell 79, 353–364.
[15] Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell 86, 147–157.
[16] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) Science 275, 1132–1136.
[17] Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M. and Kroemer, G. (1996) J. Exp. Med. 184, 1331–1341.
[18] Bernardi, P., Vassanelli, S., Veronese, P., Colonna, R., Szabó, I. and Zoratti, M. (1992) J. Biol. Chem. 267, 2934–2939.
[19] Bossy-Wetzel, E., Newmeyer, D.D. and Green, D.R. (1998) EMBO J. 17, 37–49.
[20] Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.-I., Jones, D.P. and Wang, X. (1997) Science 275, 1129–1132.
[21] Adachi, S., Cross, A.R., Babior, B.M. and Gottlieb, R.A. (1997) J. Biol. Chem. 272, 21878–21882.
[22] Krippner, A., Matsuno-Yagi, A., Gottlieb, R.A. and Babior, B.M. (1996) J. Biol. Chem. 271, 21629–21636.
[23] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cell 91, 479–489.
[24] Van de Craen, M., Vandenabeele, P., Declercq, W., Van den brande, I., Van Loo, G., Molemans, F., Schotte, P., Van Criekinge, W., Beyaert, R. and Fiers, W. (1997) FEBS Lett. 403, 61–69.
[25] Van de Craen, M., Van Loo, G., Pype, S., Van Criekinge, W., Van den brande, I., Molemans, F., Fiers, W., Declercq, W. and Vandenabeele, P. (1998) Cell Death Differ. 5, in press.
[26] Boutry, M. and Briquet, M. (1982) Eur. J. Biochem. 127, 129–135.
[27] Reinhart, P.H., Taylor, W.M. and Bygrave, F.L. (1982) Biochem. J. 204, 731–735.
[28] Lardeux, B., Gouhot, B. and Forestier, M. (1983) Anal. Biochem. 131, 160–165.
[29] Lazebnik, Y.A., Cole, S., Cooke, C.A., Nelson, W.G. and Earnshaw, W.C. (1993) J. Cell Biol. 123, 7–22.
[30] Zamzami, N., Susin, S.A., Marchetti, P., Hirsch, T., Gómez-

Monterrey, I., Castedo, M. and Kroemer, G. (1996) J. Exp. Med. 183, 1533–1544.
[31] Mort, J.S. and Buttle, D.J. (1997) Int. J. Biochem. Cell Biol. 29, 715–720.
[32] Mach, L., Stüwe, K., Hagen, A., Ballaun, C. and Glössl, J. (1992) Biochem. J. 282, 577–582.
[33] Wang, S., Miura, M., Jung, Y., Zhu, H., Gagliardini, V., Shi, L., Greenberg, A.H. and Yuan, J. (1996) J. Biol. Chem. 271, 20580–20587.
[34] Brunk, U.T., Dalen, H., Roberg, K. and Hellquist, H.B. (1997) Free Radic. Biol. Med. 23, 616–626.
[35] Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) Nature 391, 43–50.
[36] Roberts, L.R., Kurosawa, H., Bronk, S.F., Fesmier, P.J., Agellon, L.B., Leung, W.Y., Mao, F. and Gores, G.J. (1997) Gastroenterology 113, 1714–1726.
[37] Guenette, R.S., Mooibroek, M., Wong, K., Wong, P. and Tenniswood, M. (1994) Eur. J. Biochem. 226, 311–321.
[38] Kuopio, T., Kankaanranta, A., Jalava, P., Kronqvist, P., Kotkansalo, T., Weber, E. and Collan, Y. (1998) Cancer Res. 58, 432–436.
[39] Simboli-Campbell, M., Narvaez, C.J., van Weelden, K., Tenniswood, M. and Welsh, J. (1997) Breast Cancer Res. Treat. 42, 31–41.