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Two Different Proteases Are Involved in the Proteolysis of Lamin during Apoptosis[1]

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To investigate the involvement of different proteases in the execution step of apoptosis and to determine their intracellular location, isolated rat thymocyte nuclei were incubated either in the presence of Ca^{2+} and Mg^{2+} or with cytosolic extract from Jurkat T lymphocytes treated with anti-Fas (APO-1, CD-95) antibody. Inhibitors of caspases, VADcmk and DEVDcho, were not effective in hindering Ca^{2+}-induced apoptotic changes in isolated nuclei, but did prevent similar changes in nuclei treated with the cytosolic extract from apoptotic Jurkat cells. In contrast, the inhibitor of the Ca^{2+}-regulated, nuclear scaffold-associated serine protease, AAPFcmk, was able to inhibit lamin B_1 breakdown, as well as chromatin cleavage in nuclei incubated in the presence of Ca^{2+} and Mg^{2+}, but only partially prevented the same changes induced with cytosolic extract. Our findings provide evidence for the involvement of at least two proteases in lamin cleavage. One belongs to the caspase family and to cleave lamins this enzyme must be translocated from the cytoplasm into the nucleus. The second protease has a nuclear location and is activated by Ca^{2+}. Finally, neither of these two lamin-cleaving proteases is responsible for the cleavage of another nuclear target protein, poly(ADP-ribose)polymerase (PARP), during apoptosis. © 1997 Academic Press

Apoptosis is a special form of cell death with characteristic morphological features, which include cell shrinkage, membrane blebbing, chromatin condensation and cell fragmentation with the formation of apoptotic bodies (1). The biochemical machinery involved in the killing and degradation of the cell is expressed constitutively and is accessible for activation by various signals. It appears that although distinct pathways leading to apoptosis are induced by different signals, they are merged to the same "cytoplasmic regulator" of this multistep process. It has been suggested that any or all caspases (ICE-related proteases) are likely candidates for being this "regulator"(2).

Several proteins are known to be cleaved during apoptosis, but the significance of these cleavages to the cell death process is still unclear. Surprisingly, the majority of "early" cleaved proteins have a nuclear location, while a set of "later" cleaved proteins have a cytoplasmic location (3, 4). Using cell-free systems, several groups recently attempted to dissect this complex of cellular processes and to investigate how cytoplasmic protease(s) can affect isolated nuclei (5-8). When added to isolated nuclei, cytoplasmic extracts from cells treated with different apoptotic stimuli are capable of reproducing the nuclear changes typical of apoptosis. This cytoplasmic extract activity can be blocked by selective inhibitors of caspases. However, it is still unclear whether the activated caspase(s) translocates from the cytoplasm to the nucleus as active multimeric peptides which target nuclear proteins directly, or whether it activates some nuclear protease(s) which in turn cleaves the proposed nuclear targets.

Several investigators have demonstrated that both apoptotic morphological alterations and chromatin cleavage can be reproduced by the addition of certain divalent cations (Ca^{2+} *plus* Mg^{2+} or Mn^{2+}) to isolated nuclear preparations (9, 10). However, it is unknown if these effects are mediated by protease(s) present in the nuclear fraction and, if so, whether the same protease(s) is involved in the nuclear degradation in the apoptotic cell. To address these questions isolated thymocyte nuclei were incubated either in the presence of

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Abbreviations: AAPFcmk, Suc-Ala-Ala-Pro-Phe-chloromethylketone; CRP, Ca^{2+}-regulated serine protease; DEVDcho, Ac-Asp-Glu-Val-Asp-aldehyde; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FIGE, field-inversion gel electrophoresis; HMW, high molecular weight; ICE, interleukin-1β-converting enzyme; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose)polymerase; PMSF, phenylmethylsulfonyl fluoride; VADcmk, Z-Val-Ala-Asp-chloromethylketone.

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Ca^{2+} and Mg^{2+} or co-incubated with cytoplasmic extract from Jurkat cells undergoing apoptosis following treatment with anti-Fas antibody. A few selective protease inhibitors were employed in comparative experiments performed with thymocyte nuclear preparations to learn more about the target specificity of proteases in apoptosis. Analysis of the cleavage of nuclear proteins, multistep chromatin degradation, and nuclear morphological changes characteristic of apoptosis were used as criteria in the present study. Our results demonstrate the involvement of at least two proteases in lamin cleavage. One belongs to the caspase family, and to cleave lamins this enzyme must be translocated into the nucleus. The second protease has a nuclear location and is activated by Ca^{2+}. Neither of these proteases cleaves PARP, so we conclude that lamin and PARP cleavage during apoptosis are distinct processes, which are mediated by different proteases.

MATERIALS AND METHODS

Reagents. AAPFcmk and VADcmk were from Enzyme System Products (Dublin, CA, USA). Anti-lamin B_1 antibodies were generously provided by Dr. Scott H. Kaufmann (Mayo Clinic, Rochester, MN, USA), Anti-PARP antibodies were kindly provided by Dr. Guy G. Poirier (Laval University, Quebec, Canada) and by Dr. Antony Rosen (Johns Hopkins University, Baltimore, MD, USA). Rabbit anti-p17 and anti-p12 antibodies and DEVDcho were kindly donated by Dr. Donald W. Nicholson (Merck Frosst Center for Therapeutic Research, Pointe-Claire - Dorval, Quebec, Canada). Goat anti-mouse IgG-HRP was from Pierce (Rockford, IL, USA), goat anti-chicken IgG-HRP was from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA) and rabbit anti-human IgG-HRP was from Dakopatts A/S (Glostrup, Denmark). Human anti-Fas antibodies (clone CH-11) were purchased from AMS Biotechnology (Täby, Sweden). ECL was from Amersham Corp. (Buckinghamshire, UK).

Isolation of nuclei and incubation assay. Thymocyte nuclei were isolated as previously described (10). The purified nuclei were resuspended in an incubation buffer (120 mM KCl, 2 mM K_2HPO_4, 25 mM HEPES, pH 7.0, 0.3 M sucrose) at 4°C. All incubations were conducted at 37°C.

Preparation of cytoplasmic extracts from Jurkat T lymphocytes and incubation assay. Jurkat cells (20×10^6 cells/ml) were incubated with anti-Fas antibody in medium under conditions designed to induce apoptosis (6). Cells were washed with extraction buffer (10 mM HEPES, pH 7.0, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 40 mM β-glycerophosphate, 1 mM DTT, 1 mM PMSF), supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml antipain and 1 μg/ml chymopain, and resuspended in an identical buffer (500 μl/300 $\times$ 10^6cells). After four cycles of freezing and thawing, the cell lysates were centrifuged for 30 min at 120,000 $\times$ g. The clear cytosol was diluted to 10-15 mg protein/ml. A cell-free system (25-30 μl) comprised 10 μl of the cytosolic extract, 3-5 μl isolated nuclei (5×10^6 nuclei) and 10 μl of an ATP-regeneration system, containing final concentration of 2 mM ATP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase. The rest of the volume was extraction buffer without the 'supplemention'. Extracts were then incubated at 37°C for various time periods without or with 1 μl of inhibitors.

Field-inversion (FIGE) and conventional agarose gel electrophoresis. Following treatment, nuclear pellets were prepared for either FIGE or conventional gel electrophoresis as previously described (10). DNA was stained with ethidium bromide, visualized using a 305 nm UV light source, and photographed using Polaroid 665 positive/negative film.

Immunoblot analysis. For protein detection, nuclei were homogenized in high salt buffer (0.4 M NaCl, 20 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 1 mM 1,10-phenantroline, 20 μg/ml leupeptin, 5 μg/ml pepstatin and 0.1% NP-40), incubated for 40 min on ice, and centrifuged at 12,000 $\times$ g for 20 min at 4°C. Extracted polypeptides were resolved at 130 V on 12% or 15% gels and electrophoretically transferred to nitrocellulose (0.2 μm) for 2 h at 100 V. Membranes were blocked overnight in a buffer (50 mM Tris, pH 7.5, 500 mM NaCl) containing 1% bovine serum albumin and 5% non-fat dried milk. Then they were probed with anti-lamin B_1 Ab (1:500 in blocking solution without milk), anti-PARP Ab (1:5000), anti-p17 (1:5000), or anti-p12 (1:1000) Ab, followed by 1 h with goat anti-chicken, goat anti-mouse, or rabbit anti-human IgG (1:10,000 in an identical solution), and then visualized by ECL according to manufacturer's instructions.

RESULTS

To investigate the involvement of proteases in apoptosis-like nuclear degradation and the mechanism(s) of protection by protease inhibitors, experiments were carried out using the nuclear fraction isolated from rat thymocytes. Fig. 1A shows that incubation of thymocyte nuclei in a medium containing Ca^{2+} and Mg^{2+} for 15 min (lane 2) resulted in the formation of HMW DNA fragments varying in size from 700 kbp to less than 50 kbp. The formation of 50 kbp fragments was completely prevented when EGTA was added to the incubation buffer that included both Ca^{2+} and Mg^{2+}, or when nuclei were incubated in Ca^{2+}-free medium, whereas the formation of 700 kbp and 300 kbp DNA fragments was only delayed (10).

Since both the general inhibitor of caspases - VADcmk - and the inhibitor of Ca^{2+}-regulated nuclear scaffold-associated serine protease (CRP) - AAPFcmk - have been found to inhibit glucocorticoid-induced DNA fragmentation in thymocytes in a dose-dependent manner (11), and in view of the known calcium dependency of glucocorticoid-induced apoptosis (12), it seemed reasonable to assume that both proteolytic activities may be activated through Ca^{2+} signaling. To investigate this possibility, isolated nuclei were incubated in the presence of VADcmk or AAPFcmk in buffer containing Ca^{2+} and Mg^{2+}. As shown in Fig. 1A (lanes 3 and 4), AAPFcmk reduced the formation of HMW DNA fragments in a dose-dependent manner during the early phase (15 min) of incubation. An inhibitory effect was also observed at 30 min but gradually vanished (Fig. 1A, lanes 10 and 11). AAPFcmk in identical concentrations was also able to prevent internucleosomal DNA fragmentation (DNA "laddering") in Ca^{2+}/Mg^{2+}-treated nuclei (Fig. 1B, lanes 3 and 4). In contrast, up to 20 μM VADcmk failed to delay the formation of 50 kbp DNA fragments in treated nuclei. This inhibitor at concentrations above 5 μM actually potentiated the HMW DNA fragmentation induced by divalent cations (Fig.

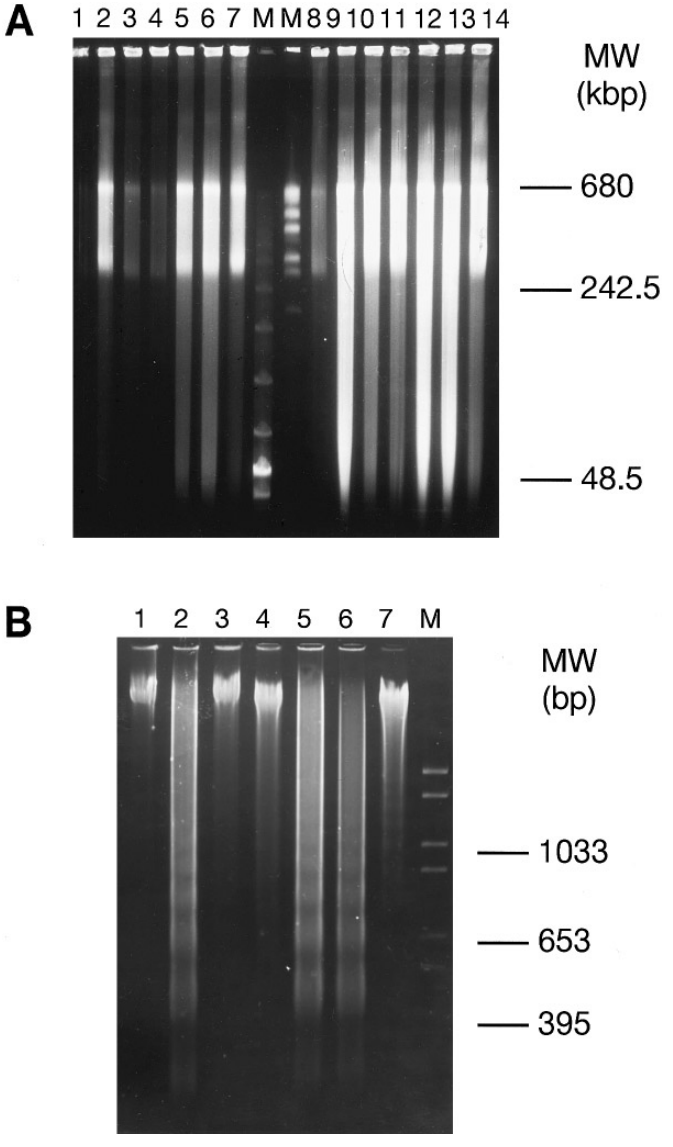

FIG. 1. Effects of protease inhibitors on Ca^{2+}/Mg^{2+}-induced formation of high and low molecular weight DNA fragments in isolated rat thymocyte nuclei. A: Nuclei were incubated without (lanes 1 and 8) or with (lanes 2–7 and 9–14) 5 mM Mg^{2+} and 10 μM Ca^{2+} at 37°C for 15 min (lanes 1–7) or 30 min (lanes 8–14) and examined for HMW DNA fragments by FIGE. Protease inhibitors were added as follows: no inhibitor (lanes 2 and 9); 5 μM AAPFcmk (lanes 3 and 10); 20 μM AAPFcmk (lanes 4 and 11); 5 μM VADcmk (lanes 5 and 12); 20 μM VADcmk (lanes 6 and 13); 50 μM DCI (lanes 7 and 14); lanes MM contained the markers (see Materials and methods). B: Nuclei were incubated for 2 h at 37°C and examined for DNA laddering by conventional gel electrophoresis. Lanes 1–7 correspond to lanes 1–7 in A. M, markers (see Materials and Methods).

1A, lanes 5, 6, 12 and 13). Neither was VADcmk effective in preventing DNA "laddering" in isolated nuclei incubated with Ca^{2+} (Fig. 1B, lanes 5 and 6). Co-incubation of isolated nuclei with 3,4-dichloroisocoumarin (DCI), the inhibitor of a large number of serine proteases, prevented both steps of chromatin cleavage induced by divalent cations; i.e. the formation of HMW DNA fragments and DNA "laddering" (Fig. 1A, lanes 7 and 14; Fig. 1B, lane 7).

The next experiments were designed to investigate the contribution of both caspases and CRP in the nuclear protein cleavage associated with apoptosis. PARP and lamins are among nuclear proteins targeted by proteases at different stages of apoptosis (5,9,11,13). As shown in Fig. 2A, incubation of thymocyte nuclei in a medium containing Ca^{2+} and Mg^{2+} for 15 min resulted in the breakdown of lamin B_1 with the formation of 46 kDa cleavage product (lane 2) which continued during incubation of the nuclei for up 90 min (lanes 3-5). Lamin B_1 breakdown was accompanied by the formation of HMW DNA fragments and preceded DNA "laddering" (see Figs. 1 and 2A). The formation of 46 kDa fragments was completely prevented by Ca^{2+} chelators, showing that in these experimental conditions, lamin B_1 breakdown is a Ca^{2+}-regulated process (Fig. 2A, lane 6). Co-incubation of isolated nuclei with AAPFcmk or DCI equally prevented lamin B_1 degradation (Fig. 2A, lanes 8 and 9). The caspase inhibitors, VADcmk and DEVDcho, the latter being a highly selective inhibitor of caspase-3 (CPP32/apopain), both failed to prevent lamin B_1 breakdown triggered by Ca^{2+} and Mg^{2+} in isolated nuclei (Fig. 2A, lanes 7 and 10). These data were confirmed by confocal microscopy analysis,

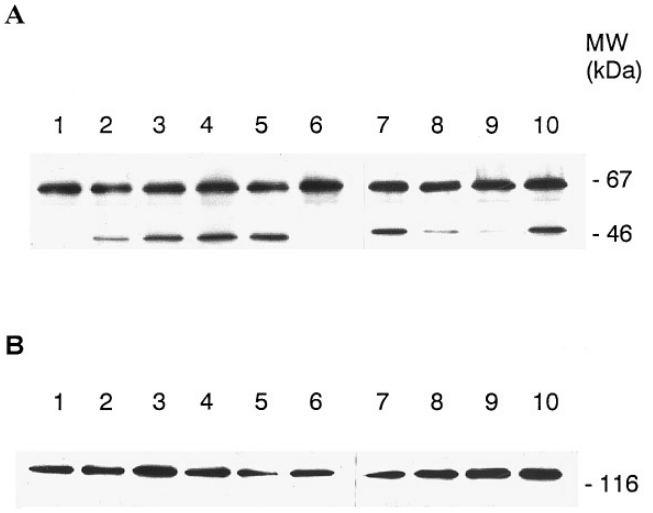

FIG. 2. Effect of protease inhibitors on lamin B_1 (A) and PARP (B) breakdown induced by divalent cations in isolated rat thymocyte nuclei. Nuclei were incubated with (lanes 1–5 and 7–10) or without (lane 6) 10 μM Ca^{2+} and 5 mM Mg^{2+} at 37°C for 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 60 min (lane 4), and 90 min (lanes 5–10) and examined for lamin degradation by Western blot, using chicken anti-lamin B_1 antibody (A), or for PARP, using anti-PARP antibody (B). Protease inhibitors were added as follows: no inhibitors (lanes 1–6), VADcmk (lane 7), DCI (lane 8), AAPFcmk (lane 9), and DEVDcho (lane 10).

which showed that incubation of isolated nuclei in the presence of Ca^{2+} and Mg^{2+} led to changes in the nuclear morphology typically seen in apoptosis, while both AAPFcmk and DCI were able to delay these morphological perturbations (data not shown).

Surprisingly, incubation of isolated nuclei in the presence of divalent cations did not lead to the cleavage of PARP (Fig. 2B). There are at least two possible explanations for this observation: 1) PARP cleavage is independent of Ca^{2+}-activated protease(s), or 2) the enzyme that cleaves PARP is not constitutively present within the nucleus. To analyze these possibilities, a partially purified extract from anti-Fas-treated Jurkat cells was co-incubated with the isolated thymocyte nuclear fraction.

Previous results from our and other laboratories showed that one of the proteases from anti-Fas-treated Jurkat cells (caspase-3) promotes cleavage of PARP and chromatin fragmentation in isolated nuclei (6, 14, 15). Immunoblots using antibodies specific for the p17 and p12 subunits of caspase-3 did not show the presence of either the dormant 32 kDa precursor or the p17 and p12 subunits in isolated nuclei incubated with Ca^{2+} and Mg^{2+} (data not shown). However, using cytosolic extract from apoptotic Jurkat cells, which contains mature p17 and p12, we found that this extract induced both PARP and lamin B_1 degradation in isolated nuclei (Fig. 3A and B, lane 2). PARP cleavage appeared within 5-10 min of incubation, and preceded both lamin B_1 breakdown (Fig. 4A and B) and chromatin fragmentation (data not shown). Extracts from untreated Jurkat cells failed to induce PARP cleavage, lamin breakdown, and chromatin fragmentation in the tested nuclei (data not shown). Both VADcmk and DEVDcho prevented PARP cleavage (Fig. 3A, lanes 3 and 4). It is important to note, that DEVDcho gave a total inhibition at a concentration of 100 nM, whereas VADcmk was effective at concentrations higher than 10 μM. In any concentration used, AAPFcmk was unable to prevent PARP cleavage under these experimental conditions (Fig. 3A, lane 5). The removal of Ca^{2+} from the incubation medium did not influence PARP cleavage induced by cytosolic extracts in isolated nuclei (data not shown).

In addition to PARP cleavage, the cytosolic extract from anti-Fas-treated Jurkat cells induced lamin B_1 breakdown in isolated nuclei (Fig. 3B, lane 2). However, this process was only apparent after 15-20 min of incubation (Fig. 4B). Lamin B_1 breakdown was blocked completely by the addition of VADcmk but only partially by DEVDcho (up to 5 μM) (Fig. 3B, lanes 3 and 4). A small inhibitory effect was also observed using AAPFcmk (Fig. 3B, lane 5).

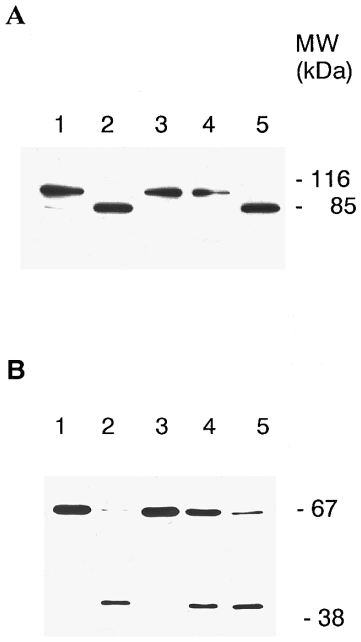

FIG. 3. Effect of protease inhibitors on PARP (A) and lamin B_1 (B) cleavage in isolated rat thymocyte nuclei incubated with cytosolic extracts from anti-Fas-treated Jurkat cells. Nuclei were incubated without (lane 1) or with (lanes 2–5) cytosolic extracts from anti-Fas-treated Jurkat cells for 30 min and examined for PARP (A) or lamin B_1 (B) cleavage by Western blot, using anti-PARP or anti-lamin B_1 antibody. Protease inhibitors were added as follows: no inhibitors (lanes 1–2), VADcmk (lane 3), DEVDcho (lane 4), and AAPFcmk (lane 5).

DISCUSSION

Nuclear disintegration involving HMW and oligonucleosomal chromatin fragmentation is a hallmark of apoptosis. In several experimental models proteolysis of nuclear lamin either precedes or appears concomitantly with HMW chromatin fragments. It has been suggested that chromatin fragmentation in apoptotic nuclei may be triggered by lamin proteolysis (9, 11). However, the proteases involved in this process have not yet been characterized in any detail.

Treatment of isolated thymocyte nuclei with Ca^{2+} and Mg^{2+} results in chromatin fragmentation and nuclear degradation, which are indistinguishable from the nuclear alterations seen in apoptotic thymocytes (9, 10). In Ca^{2+}- and Mg^{2+}-treated nuclei, chromatin fragmentation was preceded by lamin proteolysis, and both processes were blocked by the AAPFcmk peptide inhibitor of CRP but not by the caspase inhibitor, VADcmk. AAPFcmk has previously been found to inhibit apoptosis in thymocytes treated with either glucocorticoids or the Ca^{2+}-mobilizing agent thapsigargin (11, 16, Jiang, S., Zhivotovsky, B. and Orrenius, S. unpublished). Thus, it appears that Ca^{2+} activation of the nuclear scaffold-associated protease can trigger lamin proteolysis and chromatin fragmentation in both isolated nuclei and apoptotic thymocytes. In Ca^{2+}-treated nuclei, but not in apoptotic thymocytes, nuclear disintegration occurs in the absence of PARP proteolysis,

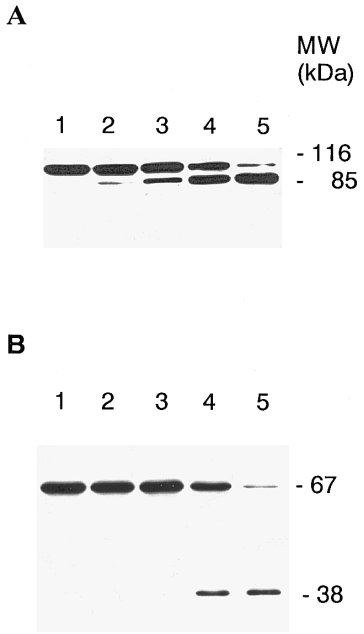

FIG. 4. Time course of PARP and lamin B_1 cleavage in isolated rat thymocyte nuclei incubated with cytosolic extracts from anti-Fas-treated Jurkat cells. Nuclei were incubated with cytosolic extracts from anti-Fas-treated Jurkat cells for 0, 5, 10, 15 and 30 min (lanes 1–5, respectively) and examined for PARP (A) or lamin B_1 (B) cleavage by Western blot, using anti-PARP or anti-lamin B_1 antibody.

indicating that the latter is not required for "nuclear apoptosis" and that additional proteases acting on nuclear target proteins are activated during "cellular apoptosis".

The reconstituted system consisting of isolated thymocyte nuclei and S-100 supernatant from anti-Fas-treated Jurkat cells also reproduces the nuclear disintegration seen in apoptotic cells. However, in this model the AAPFcmk peptide was without effect, whereas both lamin proteolysis and chromatin fragmentation were blocked by caspase inhibitors, particularly by VADcmk. Our finding that the caspase-3 inhibitor, DEVDcho, was a less efficient inhibitor of lamin proteolysis than of accompanying PARP cleavage suggests that multiple caspases contribute to the cleavage of nuclear target proteins in this experimental system and that lamin is not a preferred substrate for caspase-3. This is in accordance with a previous report by Lazebnik and colleagues (13) and with a recent study by Earnshaw and associates (17). Our results further suggest that these caspase activities are derived from the S-100 component of the system, although it is difficult to completely exclude the possibility that caspases constitutively present in the nuclear fraction are also activated. However, we could not detect the presence of caspase-3 or its p17 and p12 subunits in nuclei treated with Ca^{2+} and Mg^{2+}. This confirms the observation by Nicholson and colleagues (15) that caspase-3 was ineffective when added to isolated nuclei.

Thus, we conclude that lamin proteolysis appears to play a critical role in nuclear degradation during apoptosis, and that it can be triggered by distinct proteases which are either present constitutively in the nuclear scaffold or enter the nucleus after having been activated in the cytoplasmic compartment. PARP cleavage may or may not be part of the apoptotic nuclear disintegration but serves as a sensitive indicator of the penetration of activated apoptotic proteases (caspases or granzyme B (18, 19)) into the nuclear matrix. Our observation that PARP proteolysis is not a prerequisite for "nuclear apoptosis" is well in accordance with the previous finding that the ability of cells to undergo apoptosis is not affected in the PARP knock-out mouse (20).

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