

Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution

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Communicated by Thomas D. Pollard, Johns Hopkins University, Baltimore, MD, June 16, 1995

ABSTRACT Although specific proteinases play a critical role in the active phase of apoptosis, their substrates are largely unknown. We previously identified poly(ADP-ribose) polymerase (PARP) as an apoptosis-associated substrate for proteinase(s) related to interleukin 1 β -converting enzyme (ICE). Now we have used a cell-free system to characterize proteinase(s) that cleave the nuclear lamins during apoptosis. Lamin cleavage during apoptosis requires the action of a second ICE-like enzyme, which exhibits kinetics of cleavage and a profile of sensitivity to specific inhibitors that is distinct from the PARP proteinase. Thus, multiple ICE-like enzymes are required for apoptotic events in these cell-free extracts. Inhibition of the lamin proteinase with tosyllysine "chloromethyl ketone" blocks nuclear apoptosis prior to the packaging of condensed chromatin into apoptotic bodies. Under these conditions, the nuclear DNA is fully cleaved to a nucleosomal ladder. Our studies reveal that the lamin proteinase and the fragmentation nuclease function in independent parallel pathways during the final stages of apoptotic execution. Neither pathway alone is sufficient for completion of nuclear apoptosis. Instead, the various activities cooperate to drive the disassembly of the nucleus.

Proteinases of the interleukin 1 β -converting enzyme (ICE)/ced-3 family are essential for the execution of apoptotic cell death (1). These proteinases can induce apoptotic death when overexpressed in cultured mammalian (2–5) or insect (6) cells. Inhibition of ICE family enzymes by CrmA blocks apoptosis induced by factor withdrawal (7) or by the Fas and tumor necrosis factor (TNF) pathways (8). Although gene knockout experiments have revealed that ICE itself is not essential for many types of apoptotic death (9, 10), it does appear to be required for Fas-mediated cell death (10). In addition, the demonstration that apoptosis can be induced by introduction of trypsin, chymotrypsin, or proteinase K into cells (11) raises questions about the interpretation of experiments in which ICE family members are overexpressed in transfected cells. Additional studies using complementary approaches are therefore needed to clarify the role of ICE-like proteinases in apoptosis.

Studies using a cell-free system in which endogenous enzymatic activities drive apoptotic events have provided independent biochemical evidence for the involvement of an ICE-like proteinase in apoptotic cell death (12). This cell-free system uses concentrated extracts from chicken DU249 cells in the condemned (committed) phase of apoptosis (13) to induce a synchronous cascade of apoptotic events in isolated nuclei (14, 15). The DU249 cells become committed to apoptosis as a result of an S-phase aphidocolin block and are subsequently

collected in M phase; hence, the extracts are referred to as S/M extracts. In these extracts, as in apoptotic cells (16), the nuclear enzyme poly(ADP-ribose) polymerase (PARP) is rapidly and selectively cleaved (12). The PARP proteinase does not cleave pro-interleukin-1 β , but otherwise resembles ICE in several respects: it cleaves PARP at the sequence Glu-Val-Asp ↓ Gly, which is similar to a sequence in pro-interleukin-1 β that is cleaved by ICE; and it is selectively inhibited by Tyr-Val-Ala-Asp-CH₂Cl [YVAD "chloromethyl ketone" (YVAD-cmk)], a highly specific inhibitor of ICE family proteinases. These similarities led us to designate the PARP proteinase prICE (proteinase resembling ICE). In further experiments, treatment with YVAD-cmk at concentrations that inhibit prICE abolished all hallmark events of apoptosis in the S/M extracts (12), suggesting that prICE plays a pivotal role in initiating the apoptotic cascade. It now appears that prICE activity is the summation of the activities of multiple ICE family members acting in concert (results described below). We therefore propose to use the term prICE to refer to the constellation of ICE-like proteinases that are active in S/M extracts. Thus, prICE activity might include contributions from Nedd-2 (Ich-1) (3, 4), CPP32 (Yama) (6, 17), TX (5), and other yet-to-be-described ICE family members.

In the present study we have used our cell-free system to characterize the specific cleavage of the nuclear lamins during apoptosis. We and others (15, 18) have previously shown that apoptosis in intact cells is accompanied by disassembly of the nuclear lamina. This intermediate filament meshwork is believed to play a role in nuclear envelope integrity and the organization of interphase chromatin (19). Lamina disassembly during apoptosis is accompanied by proteolytic cleavage of the lamins (18, 20). Using the cell-free system, we now show that all three nuclear lamins are cleaved, that lamin cleavage involves the action of an ICE-like proteinase distinct from the PARP proteinase, and that lamin cleavage is required for packaging of the condensed chromatin into apoptotic bodies.

MATERIALS AND METHODS

Reagents. YVAD-cmk (Bachem) and Ala-Ala-Pro-Phe-CH₂Cl [AAPF "chloromethyl ketone" (AAPF-cmk)] (En-

Abbreviations: ICE, interleukin 1 β -converting enzyme; PARP, poly(ADP-ribose) polymerase; S/M extract, extract from cells committed to apoptosis by an S-phase aphidocolin block and subsequently collected in M phase; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; AAPF-cmk, Ala-Ala-Pro-Phe-CH₂Cl (AAPF "chloromethyl ketone"); YVAD-cmk, Try-Val-Ala-Asp-CH₂Cl; TPCK, Tos-Phe-CH₂Cl; TLCK, Tos-Lys-CH₂Cl.

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zyme Systems Products Livermore, CA) were dissolved as 18.3 mM and 50 mM stock solutions, respectively, in dimethyl sulfoxide (DMSO). Other chemicals were obtained from Sigma and dissolved as follows: phenylmethylsulfonyl fluoride (PMSF), 50 mg/ml (0.29 M) in methanol; 3,4-dichloroisocoumarin, 5 mg/ml in DMSO; aprotinin, chymostatin, leupeptin, antipain, and pepstatin, 10 mg/ml in DMSO; Tos-Lys-CH₂Cl [tosyl-L-lysyl "chloromethyl ketone" (TLCK)] and Tos-Phe-CH₂Cl [tosyl-L-phenylalanine "chloromethyl ketone" (TPCK)], 20 mM in methanol; E-64, 1 mg/ml (2.8 mM) in 10 mM Hepes (pH 7.0).

The following antibodies were used for immunoblotting of PARP and lamins as described (15): C-2-10 monoclonal antibody against PARP (16), an affinity-purified rabbit antibody raised against the carboxyl terminus of lamin A (gift of Brian Burke, University of Calgary), a goat antiserum that recognizes lamins B1 and B2 (gift of Larry Gerace, Scripps Research Foundation, La Jolla, CA), and a rabbit antiserum recognizing lamins A and C (gift of Mervyn Monteiro, University of Maryland). Antibodies were detected with ¹²⁵I-protein A and autoradiography or by ECL (Amersham). In some cases, blots were reprobed with different antibodies after stripping in 60 mM Tris/100 mM 2-mercaptoethanol/2% SDS, pH 6.7.

In Vitro Apoptotic Reaction, Analysis of DNA Fragmentation, and Detection of the Cleavage of PARP and Lamins. S/M extracts, real mitotic extracts, and interphase extracts were prepared from DU249 cells as described (14, 15). Nuclei isolated from HeLa cells were added to these extracts and analyzed for morphologic changes, DNA fragmentation, and protein cleavage as described (12, 15).

Stock solutions of inhibitors were diluted with MDB buffer (15) and added to aliquots of S/M extract. Control aliquots were supplemented with DMSO or methanol to control for solvent effects. After a 15-min preincubation at 37°C, HeLa nuclei or purified PARP and lamins were added, incubated for 1 hr at 37°C, and then subjected to SDS/PAGE and immunoblotting.

Induction of Apoptosis in Cultured Cells. A549 human lung cancer cells (kindly provided by Robert Casero, Johns Hopkins Oncology Center) were cultured in RPMI 1640 medium containing 5% fetal bovine serum penicillin G at 50 units/ml, streptomycin at 50 μg/ml, and 2 mM glutamine. Subconfluent cells were incubated for 48 hr with 20 μM topotecan (20). Apoptotic cells were recovered from the culture supernatant by sedimentation at 200 × g for 10 min and washed once in serum-free medium, and extracts were prepared for SDS/PAGE (20).

Indirect Immunofluorescence of Nuclear Lamins. HeLa nuclei adsorbed to Adhesionslides (Marienfeld, Bad Mergentheim, Germany) were incubated with S/M extracts in the absence or presence of TLCK (100 μM) for 1 hr at 37°C. They were then fixed and stained with 4',6-diamidino-2-phenylindole or antibody against the carboxyl terminus of human lamin A as described (16).

Expression and Purification of Recombinant Human Lamins. The human lamin A cDNA (21) was subcloned into the pET3a vector (22). A sequence coding for the 12-amino acid Myc tag 9E10 (23) was inserted by site-directed mutagenesis at the amino terminus prior to subcloning. This construct was expressed in *Escherichia coli* strain BL21(DE3)/pLysE and protein was purified from inclusion bodies as described (24). Inclusion-body preparations were dissolved in 8 M urea/25 mM Hepes, pH 8/1 mM EDTA/1 mM dithiothreitol and the protein was further purified on the Mono S column of the Pharmacia FPLC system using a 0–1 M NaCl gradient (24). Before use, the recombinant lamins were dialyzed against 20 mM Tris-HCl, pH 8.8/500 mM KCl (150 mM for lamin B)/2 mM EDTA/1 mM dithiothreitol/0.2% Triton X-100.

The human lamin B1 cDNA (25) was subcloned into the pET15b vector so that the lamin B1 was expressed as a fusion

protein with the 20-amino acid histidine tag at the amino terminus (Novagen). The expressed protein was purified with the Ni²⁺ chelation resin (Novagen). The protein eluted from the Ni²⁺ column was concentrated by precipitation with ammonium sulfate at 50% saturation and then dialyzed against 6 M urea/25 mM Tris/1 mM EDTA/2 mM dithiothreitol. The protein was further purified on a Pharmacia Mono Q column with a 0–1 M NaCl gradient.

RESULTS

Cleavage of lamins A, B, and C, a feature of apoptosis in a variety of cell types (18, 20, 26), occurred following addition of HeLa nuclei to S/M extracts (Fig. 1). In control experiments, real mitotic extracts (RME, Fig. 1) or interphase extracts (data not shown) failed to cleave the lamins or induce apoptotic changes in added nuclei (15). Lamin cleavage appears to occur at the same site in the cell-free system and in cells undergoing apoptosis. Cleavage of lamin A resulted in appearance of an ~45-kDa fragment that reacted with an antibody against the carboxyl terminus of the polypeptide (Fig. 1A and B, lane 2). A significant portion of this fragment was released from nuclei in soluble form (Fig. 1A, lanes S). Cleavage of lamin A in the A549 human lung cancer cell line undergoing apoptosis following exposure to etoposide produced a fragment of identical mobility (Fig. 1B, lane 4). Equivalent results were also obtained with the MDA-MB-468 breast cancer cell line (data not shown).

To determine whether the lamin proteinase and the previously described PARP proteinase were identical, we compared several properties of these enzyme activities. In contrast to cleavage of PARP, which is completed within 3 min of addition of nuclei to the extracts (12), lamin cleavage was slower, being noticeable at 15 min and requiring 30–45 min for completion. Moreover, the extent of lamin cleavage was inversely correlated with the number of nuclei added per microliter of extract. When large numbers of nuclei were used, a portion of the lamins remained uncleaved even after a 60-min incubation (Fig. 1B, lane 2). These results suggested that the lamin proteinase might be different from the PARP proteinase.

Studies using a panel of proteinase inhibitors provided additional evidence that the lamin and PARP proteinases are distinct enzymatic activities. YVAD-cmk inhibited both lamin and PARP cleavage, but the former was much more sensitive to the drug. Under the conditions employed (2 × 10⁵ nuclei per μl of extract), 1 or 10 μM YVAD-cmk inhibited PARP cleavage by ~50% (Fig. 2, lanes 4b and 4c). In contrast, cleavage of lamins A and B was completely abolished by 1 μM

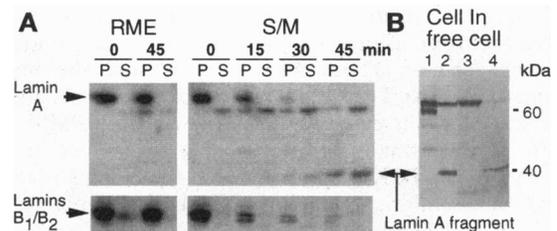


FIG. 1. S/M extracts cleave lamins in isolated nuclei at a site indistinguishable from that cleaved during apoptosis in cultured cells. (A) Nuclei isolated from HeLa cells (2.2×10^6) were incubated in 20 μl of real mitotic extract (RME) (Left) or apoptotic (S/M) extract (Right) for the indicated time at 37°C. After separation of the insoluble nuclear fraction (pellet, P) from the soluble fraction (supernatant, S) by centrifugation at $10,000 \times g$ for 3 min, samples were subjected to SDS/10% PAGE and immunoblotting with antibody against the carboxyl terminus of lamin A (Upper) or an antiserum against lamins B1 and B2 (Lower). (B) HeLa nuclei incubated in buffer (lane 1) or S/M extract (lane 2) were electrophoresed in the same gel as whole cell extracts prepared from A549 human lung cancer cells before (lane 3) or after (lane 4) induction of apoptosis with topotecan.

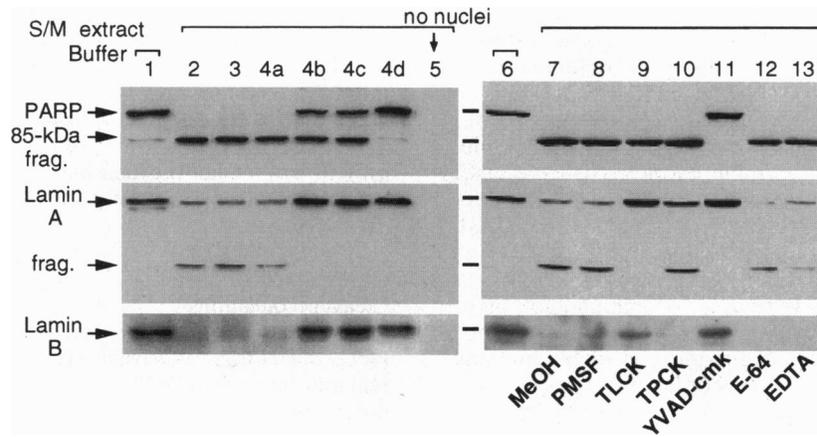


FIG. 2. The PARP and lamin proteinases show a different pattern of sensitivity to proteinase inhibitors. After a 15-min preincubation of S/M extract with inhibitors at 37°C, HeLa nuclei were added (200,000 per μ l), incubated for 1 hr at 37°C, and then subjected to SDS/PAGE and immunoblotting. Lanes 1 and 6, nuclei incubated in buffer alone; lanes 2–4, Preincubation of S/M extract with buffer (lane 2), with 1% DMSO (lane 3), or with 0.1, 1.0, 10, or 100 μ M YVAD-cmk (lanes 4a–4d, respectively); lane 5, S/M extract alone (no nuclei added); lanes 7–13, preincubation of S/M extract with 0.5% methanol (lane 7), 1 mM PMSF (lane 8), 100 μ M TLCK (lane 9), 100 μ M TPCK (lane 10), 100 μ M YVAD-cmk (lane 11), 10 μ M E-64 (lane 12), or 10 mM EDTA (lane 13). After detection of lamin B with 125 I-protein A (Bottom) the same blot was then probed for PARP with monoclonal antibody C-2-10 by ECL (Top), stripped of anti-PARP antibody, and probed for lamin A by ECL (Middle).

YVAD-cmk (lane 4b), suggesting that the lamin proteinase might be a second ICE-like proteinase with a higher affinity for YVAD-cmk than the PARP proteinase. In addition, cleavage of lamin A (Fig. 2 Middle) and lamin B (Fig. 2 Bottom) was abolished by TLCK (lane 9), an inhibitor that had no effect on PARP cleavage (Fig. 2 Top). A number of other proteinase inhibitors including PMSF (1 mM), TPCK (100 μ M), E-64 (10 μ M), EDTA (10 mM), aprotinin (0.097 trypsin inhibitor unit/ml), chymostatin (20 μ g/ml), leupeptin (20 μ g/ml), antipain (20 μ g/ml), pepstatin (10 μ g/ml), 3,4-dichloroisocoumarin (10 μ g/ml), EGTA (10 mM), and AAPF-cmk (100 μ M) had no effect on the cleavage of nuclear lamins or PARP in S/M extract (Fig. 2 and data not shown).

The observation that TLCK inhibits lamin, but not PARP, cleavage allowed us to assess the role of the lamin proteinase in the pathway of apoptotic execution. Nuclei added to S/M extracts ordinarily undergo a sequential series of structural changes that starts with condensation and collapse of the chromatin against the nuclear envelope, proceeds with separation of this rim of condensed chromatin into discrete regions, and concludes with packaging of these regions into compact apoptotic bodies that ultimately bud through the nuclear membrane (15). YVAD-cmk prevents all of these morphological changes (12). In contrast, addition of TLCK to the extracts resulted in arrest after the first stage, with the condensed chromatin forming a continuous rim around the nuclear periphery (Fig. 3A). This arrest was specific for TLCK and was not observed when extracts were treated with PMSF, 3,4-dichloroisocoumarin, aprotinin, chymostatin, leupeptin, antipain, pepstatin, TPCK, E-64, EGTA, or AAPF-cmk (data not shown). Interestingly, treatment with TLCK did not prevent the cleavage of DNA into oligonucleosomal fragments (Fig. 3C).

To determine whether the lamin proteinase is a soluble component in S/M extracts or an endogenous nuclear protein that is activated by an extract component, we examined the ability of S/M extracts to cleave purified recombinant lamins. Purified cloned human lamin A underwent some proteolysis in *E. coli*, and appeared on immunoblots as a series of bands (Fig. 4, lane 3). The major recombinant lamin A species migrated slightly more slowly than mature lamin A in HeLa nuclei (Fig. 4, lanes 8c and 9), possibly as a result of the presence of a Myc tag and the absence of carboxyl-terminal processing of the recombinant protein (27).

Incubation of recombinant lamin A in S/M extracts resulted in cleavage, yielding a major immunoreactive fragment of \approx 52 kDa (Fig. 4, lane 5). No cleavage was seen when recombinant lamin A was incubated in control, interphase extract (Fig. 4, lane 4). Although the fragments differ slightly in their mobility in SDS/PAGE from those seen with intact nuclei, the differences in mobility between the full-length and cleaved forms are consistent with those expected if the recombinant and native lamins are cleaved at the same site.

The cleavage of recombinant lamin A was abolished by addition of 1 μ M YVAD-cmk to S/M extracts (Fig. 4, lane 8a),

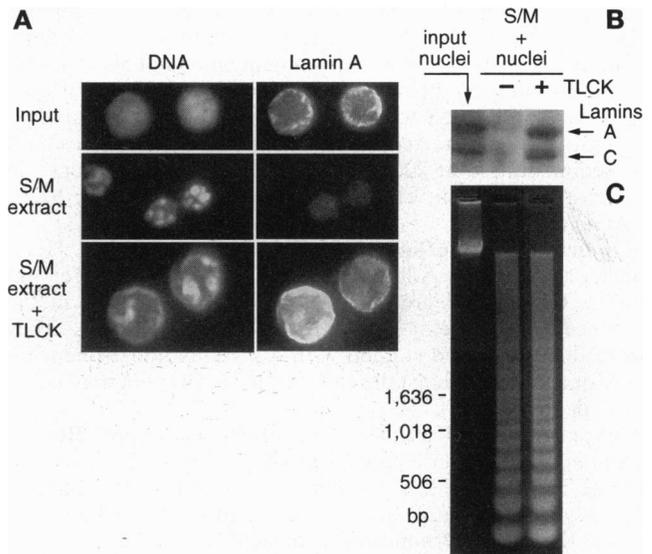


FIG. 3. Inhibition of the lamin proteinase blocks the packaging of condensed chromatin into apoptotic bodies but does not affect internucleosomal DNA cleavage. (A) HeLa nuclei were stained with 4',6-diamidino-2-phenylindole to reveal the intranuclear distribution of DNA (Left) and with anti-lamin A (Right). (B and C) HeLa nuclei (50,000 per μ l) were incubated with S/M extract supplemented either with TLCK (100 μ M) or MDB buffer. (B) Half of each sample was analyzed by immunoblotting with an antibody recognizing lamins A and C. (C) DNA isolated from the other half of the sample was subjected to electrophoresis in a 1% agarose gel and detected as described (15).

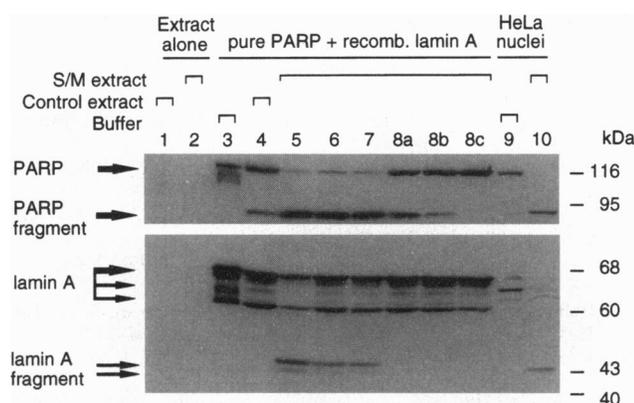


FIG. 4. Purified recombinant lamin A is cleaved in S/M extracts. Lane 1, control interphase extract alone; lane 2, S/M extract alone; lanes 3 and 4: purified PARP (40 ng/ μ l) and lamin A (40 ng/ μ l) in buffer (lane 3) or control interphase extract (lane 4); lanes 5–8, purified PARP and lamin A in S/M extract pretreated with 0.5% methanol (lane 5), 100 μ M TLCK (lane 6), 100 μ M TPCK (lane 7), or 1, 10, or 100 μ M YVAD-cmk (lanes 8a–8c, respectively); lane 9, HeLa nuclei in buffer; lane 10, HeLa nuclei in S/M extract. S/M extracts were pretreated with proteinase inhibitors for 15 min at 37°C. After a further 60 min, the samples were subjected to SDS/PAGE and immunoblotting. Sequential detection of PARP and lamin A by ECL was as described in the legend to Fig. 2.

about 10 times less YVAD-cmk than was required to block PARP cleavage (Fig. 4, lanes 8a–c, compare *Upper* and *Lower*). However, cleavage of recombinant lamin A in S/M extracts was inhibited only \approx 50% by TLCK and was also inhibited by TPCK (Fig. 4, lanes 6 and 7). Similar results were obtained with recombinant human lamin B (data not shown). We do not have a definitive explanation for this difference in the cleavage of recombinant and nuclear lamins. Despite the fact that the lamin protease is a soluble cytoplasmic activity in S/M extracts, cleavage of endogenous nuclear lamins may also involve an additional TLCK-sensitive nuclear factor.

DISCUSSION

The execution phase of apoptosis is mediated, at least in part, by activation of *ced3*/ICE family proteinases (1, 28). One substrate cleaved very early in apoptosis by these enzymes is the nuclear enzyme PARP (12, 16). Here, we have studied a second proteolytic event that is common to apoptosis in a number of cell types: cleavage of the nuclear lamins into discrete fragments. Like the PARP proteinase, the lamin proteinase was inhibited by YVAD-cmk, an inhibitor of ICE-like proteinases. However, the lamin proteinase differs from the PARP proteinase, as shown by differences in the kinetics with which these enzymes cleave their substrates and in the relative sensitivity of these two activities to YVAD-cmk and TLCK. Our experiments provide evidence for the involvement of multiple ICE-like proteinases in a single apoptotic pathway. This represents a fundamental difference from *Caenorhabditis elegans*, where a single ICE-like proteinase (the product of the *ced-3* gene) has been identified (29, 30).

Although the identity of the lamin protease is unknown, one can envision three possibilities. (i) The lamin proteinase might be the so-called nuclear scaffold proteinase that cleaves lamins A and C to produce a fragment with ATPase activity (31). However, the nuclear scaffold proteinase is inhibited by a specific chloromethylketone inhibitor, AAPF-cmk, by leupeptin, and by removal of Ca^{2+} (32), none of which inhibit the lamin proteinase in our cell-free system (data not shown). (ii) The lamin protease could be a TLCK-sensitive serine esterase that is activated by the PARP proteinase or by a second ICE-like enzyme as part of a proteinase cascade. This would

explain the preferential sensitivity of the lamin proteinase to YVAD-cmk and would be consistent with the delayed kinetics of lamin cleavage relative to PARP cleavage. However, the resistance of the lamin protease to a variety of additional serine esterase inhibitors is difficult to explain by this model. (iii) It is possible that the lamin proteinase is itself an ICE family member that is sensitive to both YVAD-cmk and TLCK. This is consistent with the inhibitor studies described above, and also with preliminary results indicating that a cloned human ICE-like enzyme can cleave lamins in a cell-free system (A.T., E. Alnemri, T. Fernandes-Alnemri, G. Litwack, R.D.M., R.D.G., Y.A.L., G.G.P., S.H.K., and W.C.E., unpublished work). CPP32, one candidate for the PARP proteinase (17), does not cleave purified recombinant lamins *in vitro*.

The lamin proteinase appears to play an important role in the later stages of apoptosis. When its activity is selectively inhibited, the pathway of morphological changes is blocked at the stage of chromatin condensation and collapse against the nuclear rim. Subdivision of the characteristic condensed chromatin rim into individual domains and their subsequent condensation into discrete apoptotic bodies do not occur. This phenotype is consistent with current understanding of the role of lamins in nuclear architecture. The lamins bind to DNA in general and to scaffold attachment regions in particular (33, 34). Thus, it has been proposed that the lamins play a major functional role in the organization of the chromatin around the nuclear periphery. Our observations suggest that lamin cleavage is required to disrupt this lamin–chromatin interaction, thereby freeing the chromatin at the nuclear rim to be packaged into multiple apoptotic bodies.

Finally, the present study has demonstrated that multiple biochemical pathways operate in parallel during the execution phase of apoptosis. We have specifically examined three distinct activities: the PARP proteinase, the lamin proteinase, and the fragmentation nuclease. We have found that both the PARP proteinase and the fragmentation nuclease are fully active when the lamin proteinase is inhibited by TLCK. Under these conditions, the apoptotic pathway proceeds only part way, and events are blocked at an early stage with the chromatin collapsed against the nuclear rim. Thus the PARP proteinase and the fragmentation nuclease acting alone or in combination are insufficient to drive the final disassembly of the nucleus into apoptotic bodies. Conversely, the combination of the PARP and lamin proteinases is also not sufficient to drive the apoptotic pathway. We show here that both enzymes are fully active in the presence of EDTA (Fig. 2). We have previously shown that nuclei exhibit no hint of chromatin condensation or fragmentation nuclease activity under these same conditions (15), possibly because of inactivation of the domain nuclease by EDTA (35). What emerges from these observations is a picture of nuclear apoptotic events in vertebrate cells that begins with the activation of multiple ICE-like proteinases and then ramifies into a number of pathways that operate in parallel to effect the rapid disassembly of the entire cell.

We thank Brian Burke, Larry Gerace, and Mervyn Montiero for gifts of antibodies. This work was supported by grants from the Human Frontier Science Program and the National Institutes of Health (W.C.E.), a National Institutes of Health grant and Leukemia Scholar Award (S.H.K.), and grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (G.G.P.). A.T. is a postdoctoral fellow of the Ryoichi Naito Foundation for Medical Research, the Yamanouchi Foundation for Research on Metabolic Disorders, and the Sasakawa Health Science Foundation.

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