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Cleavage of lamin A by Mch2α but not CPP32: Multiple interleukin 1β-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis

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ABSTRACT **Although proteases related to the interleukin 1β-converting enzyme (ICE) are known to be essential for apoptotic execution, the number of enzymes involved, their substrate specificities, and their specific roles in the characteristic biochemical and morphological changes of apoptosis are currently unknown. These questions were addressed using cloned recombinant ICE-related proteases (IRPs) and a cell-free model system for apoptosis (S/M extracts). First, we compared the substrate specificities of two recombinant human IRPs, CPP32 and Mch2α. Both enzymes cleaved poly(ADP-ribose) polymerase, albeit with different efficiencies. Mch2α also cleaved recombinant and nuclear lamin A at a conserved VEID↓NG sequence located in the middle of the coiled-coil rod domain, producing a fragment that was indistinguishable from the lamin A fragment observed in S/M extracts and in apoptotic cells. In contrast, CPP32 did not cleave lamin A. The cleavage of lamin A by Mch2α and by S/M extracts was inhibited by millimolar concentrations of Zn^{2+}, which had a minimal effect on cleavage of poly(ADP-ribose) polymerase by CPP32 and by S/M extracts. We also found that *N*-(acetyltyrosinylvalinyl-N^{ϵ}-biotinyllysyl)aspartic acid [(2, 6-dimethylbenzoyl)oxy]methyl ketone, which derivatizes the larger subunit of active ICE, can affinity label up to five active IRPs in S/M extracts. Together, these observations indicate that the processing of nuclear proteins in apoptosis involves multiple IRPs having distinct preferences for their apoptosis-associated substrates.**

A key question in cell death research is whether the apoptotic cascade is driven by the action of a single interleukin 1β-converting enzyme (ICE)-related protease (IRP) (1–7) or by multiple IRPs acting in concert (8). In the nematode *Caenorhabditis elegans*, a single IRP is required for all developmental cell deaths (1, 9). In contrast, cDNA cloning experiments show that at least seven IRP mRNAs are expressed in a single human cell type (4, 7, 10, 11), raising the possibility that multiple IRPs might be required for completion of apoptosis in vertebrates. The individual roles of these multiple IRPs during apoptosis are currently unclear.

To begin to address this question, we have compared proteolytic cleavage of two apoptotic substrates by cloned IRPs expressed in *Escherichia coli* and by cell-free extracts (named S/M extracts, prepared from chicken DU249 hepatoma cells committed to apoptosis by an S-phase aphidocolin block and subsequently collected in M phase) (12, 13). Exogenous nuclei incubated in S/M extracts recapitulate nuclear apoptotic events, including endonucleolytic cleavage of DNA, chromatin condensation, and fragmentation of the nucleus (12). Incubation of nuclei or purified poly(ADP-ribose) polymerase (PARP) in S/M extracts results in rapid, quantitative cleavage of the PARP to a 85-kDa fragment indistinguishable from that observed in a wide variety of apoptotic cells (13–15). This cleavage occurs at a conserved DEVD↓G sequence and is mediated by an enzyme with substrate recognition properties and inhibitor sensitivity similar to ICE. We termed this proteolytic activity prICE [protease(s) resembling ICE (13)]. Subsequent investigations have shown that the cloned human IRPs CPP32, Mch2α, and Mch3α as well as the *C. elegans* IRP CED-3 all cleave PARP (5, 7, 11, 16, 17). ICE itself can also cleave a PARP subfragment when added in considerable excess (18); however, at near physiological levels, it does not cleave full-length native PARP (5, 13).

Although PARP was the first apoptosis-specific IRP substrate to be identified, the physiological significance of PARP cleavage in apoptosis is presently unknown (for review, see ref. 15). In contrast, cleavage of the nuclear lamins is a proteolytic event that appears to be required for completion of nuclear reorganization during apoptosis. Lamin A is cleaved in S/M extracts (8) to fragments that are indistinguishable from those produced in cells undergoing apoptosis (8, 19–21). The inhibitor profile of the lamin protease suggests that lamin cleavage depends upon the activity of an IRP distinct from the PARP-cleaving IRP (8). Inhibition of the lamin protease by Tos-Lys-CH_2Cl (TLCK) (8) or the serpin-like IRP inhibitor CrmA/SPI-2 (A.T., P.-Y. Musy, G.G.P., R. W. Moyer, and W.C.E., unpublished work) abolishes nuclear fragmentation *in vitro*. This has led to the speculation that activity of the lamin protease may be a prerequisite for apoptotic nuclear disassembly (8).

Despite these advances in our understanding of IRP activity during apoptosis, it remains to be determined whether lamin cleavage is directly catalyzed by an IRP and whether multiple

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Abbreviations: ICE, interleukin 1β-converting enzyme; IRP, ICE-related protease; 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; prICE, protease(s) resembling ICE; DMSO, dimethyl sulfoxide; YVAD-cmk, Tyr-Val-Ala-Asp-CH_2Cl.

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IRPs are active in S/M extracts. Here we show that the human IRP Mch2α (7) cleaves human lamin A at a conserved sequence that corresponds to the site of lamin A cleavage in S/M extracts. In contrast, CPP32 does not cleave lamin A under conditions where it efficiently cleaves PARP. We also show by direct affinity labeling that multiple IRPs with distinct substrate recognition properties are active simultaneously in S/M extracts. These experiments suggest that multiple IRPs with different preferences for apoptotic substrates play distinct roles in apoptotic execution.

MATERIALS AND METHODS

Assay of Protease Activity in *E. coli* Lysates Containing Recombinant Human IRPs. Lysates from *E. coli* DH5α expressing CPP32α and Mch2α were prepared as described (7), except that the *E. coli* lysis buffer was supplemented with chymostatin, leupeptin, antipain, pepstatin, phenylmethylsulfonyl fluoride, and aprotinin. Purified recombinant human lamin A (8), purified bovine PARP (13), and nuclei isolated from HeLa S3 cells (12) were incubated with the *E. coli* lysates at 37°C; substrate cleavage was analyzed by immunoblotting as described (8, 13). To control for the effects of endogenous *E. coli* proteases, the substrates were also incubated with lysates from *E. coli* that were not transformed with plasmids or with lysates from *E. coli* expressing Mch2β, a catalytically inactive truncated version of Mch2α (7). Autoradiographic films were scanned using the COLLAGE software (Fotodyne, New Berlin, WI) so that cleavage could be quantitated from the intensity of the signals of uncleaved and cleaved substrates.

Mapping the Lamin A Cleavage Site. Lamin A (amino acids 1–463) tagged with $(His)_6$ at its C terminus was purified using Ni^{2+}-agarose resin (Novagen) according to the manufacturer's instructions. After 200 pmol of this substrate was incubated with 1.25 mg of *E. coli* lysate expressing Mch2α for 120 min, the cleaved C-terminal fragment was repurified on Ni^{2+}-agarose, subjected to SDS/PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (22), and microsequenced. The sequence revealed multiple amino acids at each position, but after subtraction of the sequences of two bacterial proteins that also bound the metal column [*E. coli* 30-kDa protein (23) and *E. coli* curved DNA binding protein (24); both are perfect 12/12 matches], the remaining sequence consisted of N*K*REFESRL, a perfect match to positions 231–241 of human lamin A.

Direct Labeling of Active IRPs in S/M Extracts. S/M extracts were prepared from chicken DU249 cells as described (12). *N*-(acetyltyrosinylvalinyl-N^{ϵ}-biotinyllysyl)aspartic acid [(2,6-dimethylbenzoyl)oxy]methyl ketone (25) [here termed YV(bio)KD-aomk] was kindly provided by Nancy Thornberry (Merck), dissolved in dimethyl sulfoxide (DMSO) at 10 mM, and stored at −80°C. S/M extracts were incubated with YV(bio)KD-aomk for 5 min at 37°C, subjected to SDS/PAGE in 16% gels, transferred to nitrocellulose, probed with peroxidase-labeled streptavidin, and visualized by the Enhanced Chemiluminesence kit (Amersham).

Labeling and Quantitation of Active IRPs in *E. coli* Lysates. Lysates (150 μg) from *E. coli* expressing CPP32α or Mch2α and purified recombinant ICE (1.2 ng) (13) were incubated with 10 μM of YV(bio)KD-aomk for 5 min at 37°C and processed for detection of the bound biotin as described above for S/M extracts. The bands corresponding to the larger subunit of active CPP32α ($\approx$17 kDa), Mch2α ($\approx$19 kDa), and ICE ($\approx$20 kDa) were scanned and their intensity was measured using the COLLAGE software. The quantity of active IRP giving a labeling intensity corresponding to that seen with 1 ng of purified recombinant ICE was defined as "1 IRP unit." One milligram of *E. coli* lysate containing CPP32α and Mch2α had 24 and 19 IRP units of active enzymes, respectively. When defining the total number of IRP units in a given amount of S/M extract, the labeling of all YV(bio)KD-aomk-labeled bands corresponding to $prICE_{1\text{-}5}$ was determined. When defining the number of PARP-cleaving IRP units in a given amount of S/M extract, only the content of active $prICE_1$ was used, because this species appeared to have a much higher affinity for the PARP cleavage site peptide than did $prICE_{2\text{-}5}$.

RESULTS

Cleavage of Lamin A by Mch2α, but Not by CPP32. To assess the possibility that lamins are cleaved by IRP(s) in apoptosis, we examined the ability of cloned human IRPs to cleave lamin A. *E. coli* lysates containing recombinant Mch2α, an IRP resembling CED-3, cleaved purified recombinant human lamin A (Fig. 1*a*, lane 4) to generate a fragment that comigrated with a fragment produced in S/M extracts (Fig. 1*a*, lane 3). In control experiments, lysates from untransformed *E. coli* DH5α (Fig. 1*a*, lane 2) or from *E. coli* expressing the inactive Mch2 splice variant Mch2β (Fig. 1*a*, lane 5) failed to cleave lamin A. Lamin A cleavage by Mch2α was readily inhibited by the IRP inhibitor Tyr-Val-Ala-Asp-CH_2Cl (YVAD-cmk) (Fig. 1*c*), but not by a cocktail of other inhibitors that was routinely included in the *E. coli* lysis buffer. This mixture contained chymostatin, leupeptin, antipain, pepstatin, phenylmethylsulfonyl fluoride, and aprotinin. Mch2α also cleaved endogenous human lamin A in HeLa nuclei (Fig. 1*b*, lane 2). Again, the lamin A fragment comigrated with a fragment observed in apoptosis *in vitro* (Fig. 1*b*, lane 4) and *in vivo* (8).

Not all IRPs involved in apoptosis are active against the lamin substrate. CPP32, a second IRP homologous to CED-3, appears to be incapable of cleaving lamin A (Fig. 1 *a*, lane 6, and *b*, lane 3). After normalization of the amounts of active enzymes in *E. coli* lysates (see *Materials and Methods*), the proteolytic activities of Mch2α and CPP32 against different substrates were compared. The rate of lamin A cleavage by Mch2α was roughly comparable with that of S/M extracts, whereas CPP32 failed to cleave this substrate (Fig. 1*d*). The converse was true for PARP cleavage. Initial rates of PARP cleavage by CPP32 and S/M extract were essentially identical (Fig. 1*e*). Although Mch2α does possess PARP cleavage activity (7), this is considerably lower than the activity of CPP32 against PARP (Fig. 1*e*). These results revealed that Mch2α and CPP32 have distinct preferences for lamin A and PARP, respectively.

Lamin A Cleavage Site. Peptide sequencing of the Mch2α cleavage product from a purified recombinant human lamin A subfragment (see *Materials and Methods*) revealed that cleavage occurred at D_{230} of lamin A (Fig. 2*a*). Alignment of lamin A sequences available in the data bases revealed that this cleavage site, which lies in the midst of the α-helical rod domain, is well-conserved across species from mammals to *Drosophila* (Fig. 2*a*).

We have used synthetic peptides to confirm that the endogenous lamin protease in S/M extracts recognizes the Mch2α cleavage site on lamin A. We synthesized an 11-mer peptide corresponding to the lamin A cleavage site (RLVEIDNGKQR) and a mutant peptide containing alanine in place of aspartate (RLVEIANGKQR). The cleavage site peptide inhibited lamin A cleavage by S/M extracts (Fig. 2*b*, lane 3) and by Mch2α (data not shown), whereas the mutant peptide had no effect (Fig. 2*b*, lane 4). The lamin A peptide did not affect PARP cleavage in S/M extracts (Fig. 2*b*, lane 3). This result strongly suggests that Mch2α and the endogenous lamin protease in S/M extracts cleave lamin A at the same VEID$\Downarrow$NG bond. Moreover, these results demonstrated a requirement for aspartate at the P_1 position of the lamin cleavage site, a characteristic of IRPs (26).

In a control experiment, we confirmed our previous finding that cleavage of PARP in the apoptotic extracts could be specifically inhibited by a peptide (GDEVDGIDEV) spanning

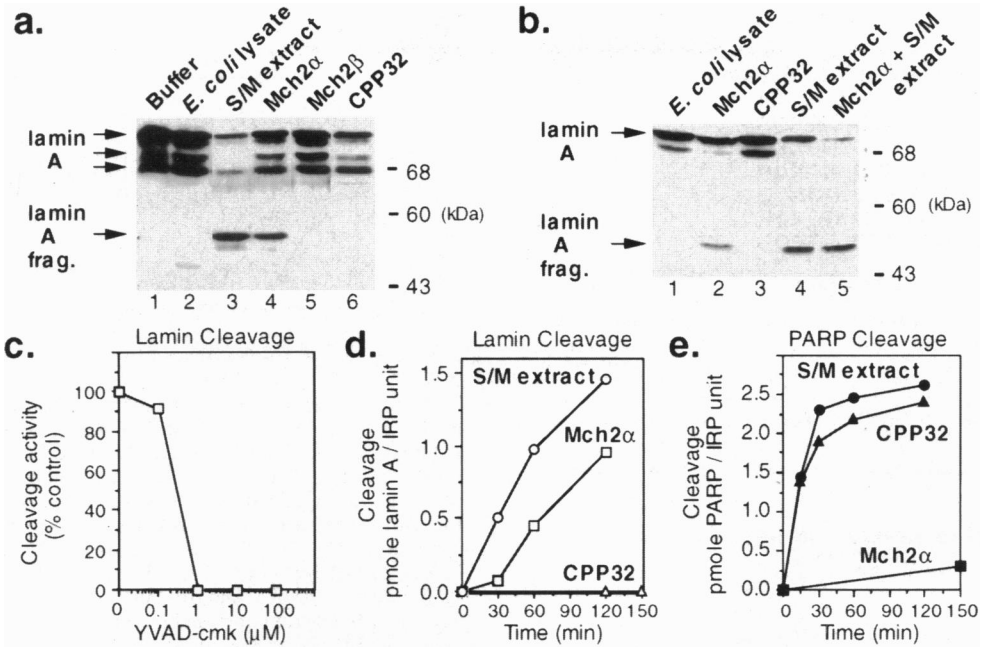

FIG. 1. (*a*) Cleavage of recombinant human lamin A by Mch2α. Purified recombinant human lamin A (400 ng) (8) was incubated for 120 min at 37°C with MDB buffer (10 mM Hepes, pH 7.0/40 mM β-glycerophosphate/50 mM NaCl/2 mM $MgCl_2$/5 mM EGTA/1 mM DTT) (12) (lane 1), with 30 μg of lysate from untransformed *E. coli* DH5α (lane 2), with 70 μg S/M extract (12) (lane 3), or with 30 μg of lysate from *E. coli* expressing cDNAs encoding Mch2α (7) (lane 4) or Mch2β (lane 5). Lane 6, purified recombinant human lamin A (200 ng) was incubated for 150 min at 37°C with 150 μg of *E. coli* lysate containing CPP32 (4, 7). (*b*) Cleavage of endogenous HeLa nuclear lamin A by Mch2α. HeLa nuclei (1×10^6) were incubated at 37°C for 120 min with lysate from untransformed *E. coli* DH5α (60 μg) (lane 1) and from *E. coli* expressing Mch2α (120 μg) (lane 2) or CPP32 (70 μg) (lane 3); with S/M extract (140 μg) (lane 4); or with a combination of Mch2α lysate (120 μg) plus S/M extract (140 μg) (lane 5). (*c*) A specific ICE inhibitor blocks cleavage of recombinant human A by Mch2α. *E. coli* lysates (30 μg) expressing Mch2α were preincubated with 1% DMSO or with 0.1, 1, 10, and 100 μM YVAD-cmk (Bachem) for 15 min at 37°C before addition of recombinant human lamin A and incubation for 120 min. (*d*) Time course of lamin A cleavage by IRPs. Recombinant human lamin A was incubated as described in *a* for the times indicated with lysate from *E. coli* expressing CPP32 (△) or Mch2α (□), or with S/M extracts (○). The amount of cleaved lamin A per IRP unit of each active enzyme was calculated for each time point. (*e*) Time course of PARP cleavage by IRPs. Purified bovine PARP (200 ng) (13) was incubated for the time indicated with 150 μg of lysate from *E. coli* expressing CPP32 (▲) or Mch2α (■), or with 70 μg of S/M extracts (●). The amount of cleaved PARP per IRP unit of each active enzyme was calculated for each time point.

the PARP cleavage site (13). This peptide had no detectable effect on the efficiency of lamin A cleavage at concentrations where it substantially lowered the efficiency of PARP cleavage (Fig. 2*b*, lane 5). PARP cleavage by CPP32 was inhibited by the PARP cleavage site D peptide (Fig. 2*b*, lane 9) but only poorly by the mutant A peptide (Fig. 2*b*, lane 10). Together, these observations confirm that the endogenous lamin and PARP proteases in S/M extracts have distinct substrate recognition properties similar to those of Mch2α and CPP32.

Inhibition of Lamin A but Not PARP Cleavage by Zn^{2+}. Cleavage of purified recombinant human lamin A by Mch2α (Fig. 3*a*) and by S/M extracts (Fig. 3*b*) was inhibited by Zn^{2+} in a dose-dependent manner. Zn^{2+} also blocks the cleavage of endogenous nuclear lamin A in HeLa nuclei induced by S/M extracts (Fig. 3*b*). Under these conditions, completion of morphological apoptosis was also blocked (12).

In contrast, cleavage of purified bovine PARP catalyzed by both recombinant CPP32 (Fig. 3*a*) and S/M extracts (Fig. 3*b*) was relatively insensitive to Zn^{2+}. A similar result was obtained with endogenous nuclear human PARP (Fig. 3*b*).

Multiple Active IRPs in S/M Extracts. Our functional studies reveal that S/M extracts contain at least two distinct IRPs with differing substrate preferences and differing inhibitor profiles (Figs. 2*b* and 3*b*). To directly visualize these enzymes, we set out to establish whether YV(bio)KD-aomk, a probe that was developed to covalently derivatize the larger subunit of active ICE but not its inactive proenzyme (25), would also directly label other IRPs. Control experiments (Fig. 4*b*) indicated that 10 μM YV(bio)KD-aomk labels cloned human CPP32 and Mch2α in *E. coli* extracts, indicating that this reagent will react with the active sites of diverse IRPs in addition to ICE. When this same approach was applied to S/M extracts, up to five polypeptides of ≈19.5, 19, 18.5, 17.8, and 17.6 kDa were reproducibly labeled after reaction with 100 μM YV(bio)KD-aomk (Fig. 4*a*, lane 1). All of these labeled species differed in mobility from the p20 subunit of purified human ICE (Fig. 4*a*, lanes 7 and 9). In keeping with our earlier terminology, we have termed these bands $prICE_{1\text{-}5}$. $prICE_{1\text{-}5}$ exhibit a distinct hierarchy of labeling by YV(bio)KD-aomk. $prICE_4$ is labeled at 0.1 μM YV(bio)KD-aomk (Fig. 4*a*, lane 5), $prICE_1$ and $prICE_5$ are labeled at 1 μM (Fig. 4*a*, lane 4), and $prICE_2$ and $prICE_3$ require 100 μM for labeling (Fig. 4*a*, lane 1). This labeling hierarchy suggests that $prICE_{1\text{-}5}$ exhibit a hierarchy of affinities for YV(bio)KD-aomk.

Several control experiments indicate that the labeled species correspond to active IRPs. First, preincubation of S/M extracts with 100 μM YVAD-cmk, a broad spectrum inhibitor of IRPs at this concentration, abolished the labeling of all five bands (Fig. 4*a*, lane 2). Second, no labeled polypeptides were detected in extracts from nonapoptotic cells (Fig. 4*a*, lane 8), indicating that $prICE_{1\text{-}5}$ are active only in apoptotic cells. Third, labeling required the addition of YV(bio)KD-aomk (Fig. 4*a*, lane 6), thereby ruling out the trivial explanation that the labeled species correspond to endogenous biotinylated proteins. Finally, the labeled species roughly comigrate with the large subunits of known human IRPs [17 kDa for CPP32 (4, 16); 19 kDa for Mch2α (7)]. No labeling was seen of species in the size range expected for IRP precursors (30–50 kDa).

$prICE_1$ can be distinguished from $prICE_{2\text{-}5}$ by its affinity for the PARP cleavage site peptide (13). The addition of this peptide to S/M extracts markedly diminished the labeling of $prICE_1$ whilst leaving the labeling of $prICE_4$ and $prICE_5$

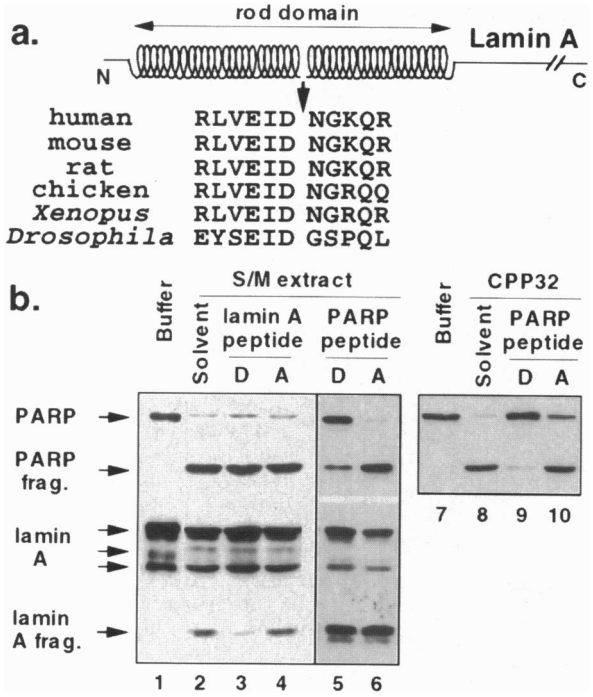

FIG. 2. (*a*) The site of lamin A cleavage in the rod domain is widely conserved across different species. The human sequence shown corresponds to the lamin A peptide used in *b*. (*b*) Use of peptide inhibitors to confirm the PARP and lamin cleavage sites and to demonstrate the differing specificities of the lamin and PARP proteases. Purified bovine PARP (300 ng) alone (lanes 7–10) or in combination with recombinant human lamin A (200 ng) (lanes 1–6) was incubated at 37°C for 15 min (lanes 7–10) or for 120 min (lanes 1–6) in MDB (lane 1 and 7); in S/M extract (75 μg) plus 4% DMSO (lane 2), plus 4 μg/μl of lamin A cleavage site peptide (D peptide = RLVEIDNGKQR, lane 3) or plus 4 μg/μl of mutant peptide (A peptide = RLVEIANGKQR, lane 4); in S/M extract (66 μg) plus 1 μg/μl of PARP cleavage site peptide (D peptide = GDEVDGIDEV, lane 5) or plus 1 μg/μl of mutant peptide (A peptide = GDEVAGIDEV, lane 6); in *E. coli* lysate containing CPP32 (30 μg) plus 2% DMSO (lane 8), plus 2 μg/μl of PARP cleavage site peptide (D peptide, lane 9) or plus 2 μg/μl of mutant peptide (A peptide, lane 10).

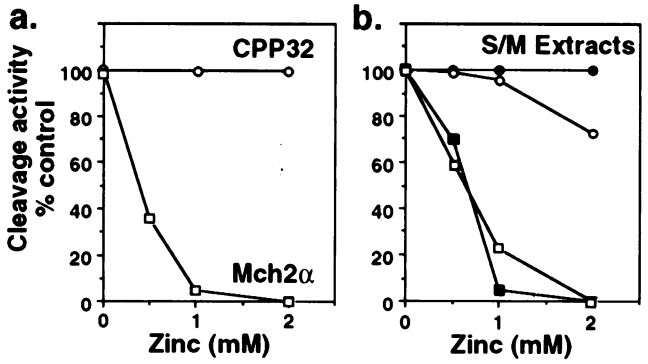

FIG. 3. Effects of Zn^{2+} on the substrate cleavage by IRPs. (*a*) Zn^{2+} inhibits cleavage of purified recombinant human lamin A by Mch2α but not cleavage of purified PARP by cloned human CPP32. Purified PARP (300 ng) (○) and purified recombinant human lamin A (400 ng) (□) were incubated with *E. coli* lysates (30 μg) containing CPP32 or Mch2α, respectively, in the presence of $ZnCl_2$ at the indicated concentrations. The *E. coli* lysates were previously dialyzed against MDB lacking EGTA for 3 hr at 4°C (12). After incubation for 15 min (for PARP cleavage) or 120 min (for lamin A cleavage), substrate cleavage was quantitated from immunoblots similar to those shown in Fig. 1 *d* and *e*. (*b*) Effects of Zn^{2+} on the cleavage of PARP and lamin A by endogenous IRPs in S/M extracts. Dialyzed apoptotic S/M extracts (140 μg) were incubated either with HeLa nuclei (1.5×10^6; solid symbols) for 60 min at 37°C, or with a mixture of purified PARP and lamin A (as in *a*; open symbols). Substrate cleavage was quantitated from immunoblots as in *a*. ○, Purified bovine PARP; ●, human nuclear PARP; □ recombinant human lamin A; ■, human nuclear lamin A.

unchanged (Fig. 4*c*, lane 2). Under the conditions of this experiment, labeling of $prICE_2$ and $prICE_3$ was not readily observed. In a control experiment, the labeling of $prICE_1$ was not inhibited by the D ⇒ A mutant peptide (Fig. 4*c*, lane 3), which does not inhibit PARP cleavage in S/M extracts (13). Together, these results indicate that $prICE_1$ has a greater affinity for the PARP cleavage sequence than $prICE_4$ and $prICE_5$.

DISCUSSION

Accumulating evidence suggests that the cleavage of nuclear lamins is a universal event in apoptosis *in vivo* (8, 19–21) and *in vitro* (8). Here, we have shown that the CED-3-related human IRP Mch2α cleaves lamin A. A second well-characterized CED-3-related human IRP, CPP32, did not cleave lamin A under conditions in which it readily cleaved PARP. Thus, CPP32 alone cannot mediate all of the proteolytic events essential for the apoptotic disintegration of nuclei (8). Recombinant ICE was similarly inactive against lamin A (unpublished data). Peptide sequencing revealed that the cleavage by Mch2α occurs adjacent to a conserved asp residue in the α-helical rod domain of lamin A. This cleavage could potentially disrupt lamin–lamin interactions as well as interactions of lamins with other nuclear components such as matrix attachment regions on the DNA (27), the retinoblastoma gene product (28), the adenovirus E1B 19-kDa product (29), and core histones (30).

The observation that a peptide corresponding to the site of lamin A cleavage by Mch2α also inhibited lamin A cleavage in S/M extracts strongly suggests that recombinant Mch2α and the endogenous lamin protease in S/M extracts cleave lamin A at the same site. Although these data are consistent with the notion that Mch2α is responsible for lamin cleavage in apoptotic cells, we cannot rule out the possibility that other novel CED-3-related IRPs (T.F.-A. & E.S.A., unpublished work) might also cleave the lamins.

Although further study is required to reveal the biological relevance of the inhibition of lamin A cleavage by Zn^{2+}, it is worth noting that Zn^{2+} is known to be a modulator of apoptosis *in vivo*. Zn^{2+} depletion has been associated with enhanced apoptosis in the small intestine (31) and thymus (32) of rodents as well as with induction of apoptosis in cultured cells (33–36). Conversely, Zn^{2+} supplementation inhibits internucleosomal DNA fragmentation and morphological features of apoptosis *in vivo* (34, 37–40) and *in vitro* (12, 41). The targets for this suppression of apoptosis by Zn^{2+} remain unclear, although apoptotic nucleases have been suggested as possible candidates (42, 43). An alternative hypothesis is that some of the effects of Zn^{2+} on apoptosis are exerted, at least in part, through a modulation of the actions of IRPs such as Mch2α. Consistent with this hypothesis, we have observed that lamin cleavage by recombinant Mch2α is inhibited by millimolar concentrations of Zn^{2+}. It has also been reported that ICE activity is inhibited by Zn^{2+} at concentrations higher than 375 μM (44). Conversely, it is worth noting that the effects of Zn^{2+} on apoptosis are unlikely to be mediated by effects on CPP32, which was relatively insensitive to the cation.

Based on the observation that CPP32 and Mch2α can be labeled with the active site titrant YV(bio)KD-aomk (25), we have developed a procedure to directly visualize the spectrum of IRPs that is activated during apoptosis. This procedure labeled at least five distinct polypeptides in S/M extracts. We termed these $prICE_{1\text{-}5}$. These five labeled polypeptides all correspond to active IRPs, because the labeling is abolished by

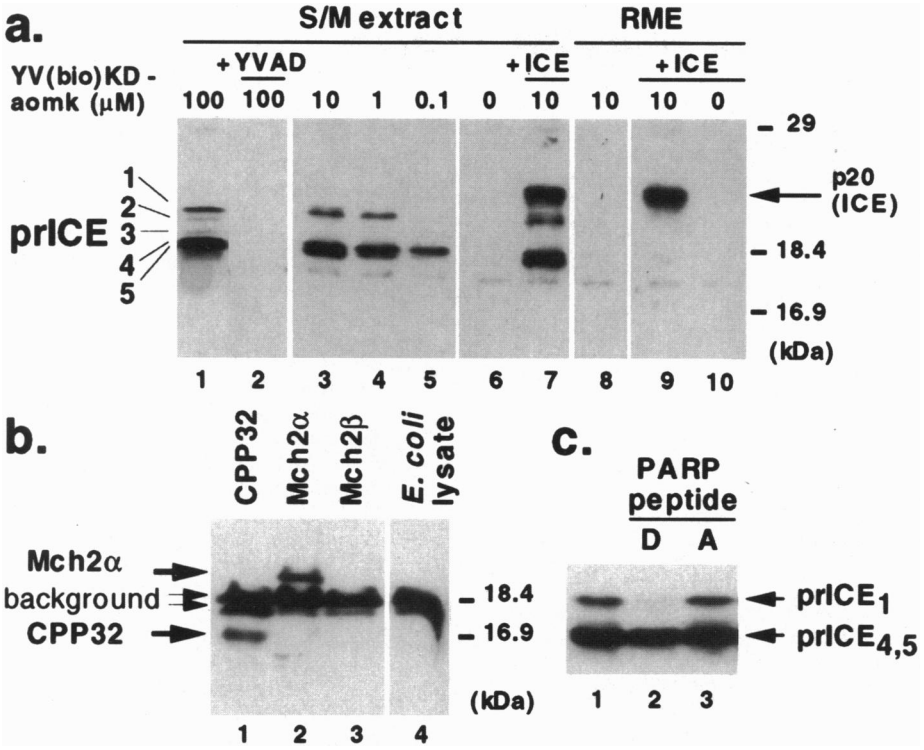

FIG. 4. (*a*) Labeling of multiple active IRPs in apoptotic S/M extracts. YV(bio)KD-aomk (25) was added at the indicated concentration to 30 μg of S/M extract (lanes 1–7) or control real mitotic (i.e., nonapoptotic) extract (12) (lanes 8–10). In lanes 7, 9, and 10, extracts were supplemented with 1.2 ng of purified ICE (13). In lane 2, S/M extract was preincubated with 100 μM YVAD-cmk at 37°C for 15 min to inactivate IRPs before addition of YV(bio)KD-aomk. (*b*) YV(bio)KD-aomk labels active recombinant CPP32 and Mch2α. Lysates (150 μg) from untransformed *E. coli* (lane 4) or from *E. coli* expressing CPP32 (lane 1), Mch2α (lane 2), or Mch2β (lane 3) were incubated with 10 μM of YV(bio)KD-aomk at 37°C for 5 min and electrophoresed in a Tris·tricine gel (22). The strong background bands represent two streptavidin-binding proteins in *E. coli* that are detected without labeling with YV(bio)KD-aomk. (*c*) Competitive inhibition of $prICE_1$ labeling by the PARP cleavage site peptide. Active IRPs in 100 μg of S/M extract were labeled with 10 μM YV(bio)KD-aomk in the presence of 2% DMSO (lane 1), 2 μg/μl of PARP cleavage site D peptide (13) (lane 2), or 2 μg/μl of mutant A peptide (lane 3). $prICE_2$ and $prICE_3$ (that require 100 μM YV(bio)KD-aomk for labeling) were not visualized in this experiment.

pretreatment of extracts with YVAD-cmk. The simplest interpretation of these results is that S/M extracts contain at least five distinct active IRPs. These could be the products of five different genes or certain of the labeled species could be produced by either alternative splicing of a common mRNA transcript or alternative proteolytic processing of a primary translation product. cDNA cloning studies have revealed that Jurkat cells express at least seven different IRP genes (4, 7, 10, 11) and produce at least five alternatively spliced forms of the ICE transcript (10).

Regardless of their origin, our further analysis revealed functional distinctions between several of these enzymes. First, the labeled species show a 100-fold range in apparent affinity for YV(bio)KD-aomk, with $prICE_4$ being labeled at concentrations as low as 0.1 μM and $prICE_2$ and $prICE_3$ being labeled only at 100 μM. Second, preincubation of S/M extracts with the PARP cleavage site peptide resulted in a preferential decrease in the labeling of $prICE_1$. Thus, among the major prICE species, $prICE_1$ appears to have a selective affinity for the PARP cleavage site. It is therefore tempting to suggest that $prICE_1$ might be a PARP protease. In similar experiments, use of the lamin peptide as an inhibitor brought about a selective decrease in the labeling of $prICE_5$, This result, together with the results of IRP inhibition with the serpin-like inhibitor CrmA/SPI2 suggest that $prICE_5$ may have a selective affinity for the lamin cleavage sequence (A.T., P-Y. Musy, G.G.P., R. W. Moyer, and W.C.E., unpublished work). The preferred substrates for $prICE_{2-4}$ remain to be determined.

These results show that the YV(bio)KD-aomk reagent can directly label active apoptosis-associated IRPs in cell-free extracts and that the substrate preferences of these labeled IRPs can be analyzed using cleavage site peptides. This methodology should be of significant utility in the future analysis of apoptotic execution in a wide range of cell death events.

Our observations suggest that apoptotic execution in higher eukaryotic cells involves the coordinated action of multiple IRPs, each with a distinctive menu of preferred targets. To the extent that expression of these IRPs differs between various cell types, the cleavage of various substrates and the sensitivity to various inhibitors might also vary. Instead of a single CED-3-related "death protease" and a universal death pathway, it is possible that different cell types might use a number of subtly different alternative pathways (45). Such a mechanism might provide important flexibility to the range of cellular responses to environmental signals.

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