CrmA/SPI-2 Inhibition of an Endogenous ICE-related Protease Responsible for Lamin A Cleavage and Apoptotic Nuclear Fragmentation*

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CrmA, a poxvirus gene product with a serpin-like structure, blocks a variety of apoptotic death events in cultured cells. Based on the ability of CrmA to inhibit the interleukin-1β converting enzyme in vitro, it has been speculated that interleukin-1β converting enzyme-related proteases (caspases) essential for apoptosis are the cellular targets of CrmA. Here we found that rabit-pox virus CrmA/SPI-2 inhibits the cleavage of lamin A mediated by a caspase in our cell-free system of apoptosis. In the presence of CrmA/SPI-2, nuclear apoptosis in vitro was blocked at an intermediate stage after collapse of the chromatin against the nuclear periphery and before nuclear shrinkage and disintegration into apoptotic body-like fragments. Using N-(acetyltyrosinylvalinyl-N*-biotinyllysyl) aspartic acid [(2,6-dimethylbenzoyl)-oxy] methyl ketone, which derivatizes the active forms of caspases, we could show that one of five caspases active in the extracts is inhibited both by CrmA/SPI-2 and by a peptide spanning the lamin A apoptotic cleavage site. These results reveal that CrmA/SPI-2 can inhibit a caspase responsible both for lamin A cleavage and for the nuclear disintegration characteristic of apoptosis.

Apoptotic cell death, which is defined by characteristic morphological changes such as chromatin condensation, cell shrinkage, and fragmentation of the cell into apoptotic bodies (1), is the most widely studied form of programmed cell death. Genetic analysis of programmed cell death in the nematode Caenorhabditis elegans identified ced-3 as a master regulatory gene (2) that encodes a cysteine protease homologous to the interleukin-1β converting enzyme (ICE)(3, 4). In mammalian cells, in contrast, multiple cDNAs have been identified that encode ICE-related proteases (now termed caspases, for cysteine aspartate-specific) (5). In addition to caspase-1 (ICE), nine additional human caspases have been described (see Ref. 5 for review). Of these, the enzymes most widely studied with respect with their role in apoptosis include caspase-3 (CPP32/ YAMA/Apopain (7–9)) and caspase-6 (Mch-2 (10)).

CrmA/SPI-2 (11) is a poxvirus gene product with homology to members of the serpin superfamily that appears to assist these viruses in evading the host inflammatory responses (12). Cowpox virus-derived (13–16) and vaccinia virus-derived (17) crmA cDNAs transfected into cells can inhibit apoptosis induced by nerve growth factor depletion (13), serum withdrawal (14), Fas ligation (15–17), and activation of the tumor necrosis factor receptor (15). Based on the ability of purified CrmA protein to inhibit the cleavage of pro-interleukin-1β by ICE (12), it has been assumed that CrmA inhibits apoptosis by blocking the activity of caspases within the transfected cells (16, 17). However, cotransfection of crmA cDNA poorly inhibits cell death induced by overexpression of some caspases such as caspase-2 (ICH-1 (18)) and CED-3 (4, 19). Also, CrmA inhibits the cleavage of poly(ADP-ribose) polymerase (PARP) by purified caspase-3 (CPP32) only poorly (15, 20). Moreover, the recent report that CrmA binds granzyme B (21), a serine protease not structurally related to ICE, reveals that the action of CrmA may not be restricted to caspases. Therefore, it remains to be determined whether the inhibition of apoptosis by CrmA results solely from the blockade of caspase(s).

We have established a cell-free system in which isolated nuclei undergo morphological and biochemical changes characteristic of apoptosis when exposed to cytoplasmic extracts (S/M extracts) from chicken DU249 cells committed to apoptotic cell death (22). Using this in vitro system, PARP was identified as the first confirmed substrate for any caspase in apoptotic cells (23). We subsequently found that lamin cleavage during nuclear apoptosis in vitro requires an enzyme distinct from the PARP-cleaving caspase (24) and showed that Mch2 can act as a lamin protease (20). Labeling of active caspases in S/M extracts using YV(bio)KD-aomk, a biotinylated peptide synthesized based on the ICE cleavage site in pro-interleukin-1β (25), revealed at least five distinct labeled polypeptides, which we termed prICE1–prICE5 (20). Here we show that rabitpox virus (RPV) CrmA/SPI-2 inhibits the caspase(s) catalyzing the cleavage of lamin A and the disintegration of nuclei into apoptotic body-like fragments in the cell-free system.

EXPERIMENTAL PROCEDURES
Reagents—Peptides RL1VE1DNGRQR (D peptide) and RL1VE1A-NGKQR (A peptide) were synthesized based on the apoptosis-specific

1 The abbreviations used are: ICE, interleukin-1β converting enzyme; MeSO·, dimethyl sulfide; MDB, mitotic dilution buffer; PARP, poly-(ADP-ribose) polymerase; RPV, rabitpox virus; YV(bio)KD-aomk, N-(acetyltyrosinylvalinyl-N*-biotinyllysyl) aspartic acid [2,6-dimethylbenzoyloxy] methyl ketone; VV, vaccinia virus; TLCK, Tos-LysCH2Cl)-1-chloro-3-methylamido-7-amin-2-heptanone.

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cleavage site within human lamin A (20), dissolved in phosphate-buffered saline containing 10% dimethyl sulfoxide (Me2SO) at 11 mg/ml, and stored at −80 °C. Other chemicals were obtained from Sigma unless otherwise indicated.

Expression and Purification of His-tagged CrmA/SPI-2 and SPI-7 Proteins—The polyhistidine tract coding region of pET-16b (Novagen) was amplified using primers SM280 (5′-GAAGGAGATATATCGCAGCCATC-3′) and SM291 (5′-GGAAATCTGAGGCTGACGCTTTG-3′), digested with BsiHI and EcoRI, and inserted into pTM1 (26) that had been digested with NcoI and EcoRI. The RPV Spi2 open reading frame was amplified from genomic DNA using primers RM233 (5′-GGCCATGATATCTTGCCAGGAAAT-3′) and SM229 (5′-GAACCAGGTTAACATAATGTGGTGGAGAC-3′) and digested with NcoI and SmaI. The swinepox virus Spi7 open reading frame was amplified from genomic DNA using primers RM231 (5′-CCCATGGATGTTTTTTTGAAACTG-3′) and RM230 (5′-GCGGTACCT-TACATAGGAGGATTTTTGAACTG-3′) and digested with NcoI and BamHI. The fragments were inserted into the multiple cloning site within the pTM1 vector under the control of the T7 promoter (26) and in frame with the polyhistidine tract to construct pTM1-His-Spi2 and pTM1-His-Spi7, respectively.

Recombinant viruses were generated by transfecting pTM1-His-Spi2 and pTM1-His-Spi7 plasmids into sub-confluent CV-1 cells (ATCC) using LipofectACE Reagent (Life Technologies, Inc.). These cells were co-infected with vaccinia virus (VV) strain WR at a multiplicity of infection of 0.05. The resulting progeny virus was plaqued on Rat2 cells in the presence of vTF7–3 (ATCC VR-2153) that expresses the bacteriophage T7 RNA polymerase. At 16–19 h post-infection, the cells were harvested and resuspended in 1 ml of 20 mM Tris, 10 mM NaCl, pH 8.0, at 4 °C. All subsequent steps were performed at 4 °C. The cell suspension was supplemented with 250 μl of 0.5% Triton X-100, 1.5 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5% glycerol, placed on ice for 10 min, and centrifuged at 4800 × g for 5 min. The supernatant was cleared by centrifugation at 40,000 × g for 30 min.

The cleared supernatant from five 150-mm dishes was loaded onto a column packed with 3.5 ml of His-bind resin (Novagen) that had been charged with 50 mM NiSO4 and equilibrated with binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.9). The proteins were eluted with 1 ml of elution buffer (50 mM imidazole, 100 mM NaCl, 200 mM, plus aprotinin (400 mg/ml) and 5% glycerol. This was followed by a wash with stripping buffer (100 mM EDTA, 500 mM NaCl, 20 mM Tris, pH 7.9). Protein purity was monitored by silver staining.

RESULTS AND DISCUSSION

We first examined whether RPV CrmA/SPI-2 affects the apoptotic morphological changes of nuclei in our cell-free system. In the presence of CrmA/SPI-2, the initial condensation of chromatin proceeded uninterrupted. However, overall shrinkage of nuclei and the disintegration of the nuclei into apoptotic body-like fragments were blocked in a dose-dependent manner (Fig. 1, D–G). At 30 μg/ml of CrmA/SPI-2, the nuclear apoptotic changes were halted at an intermediate stage, with the chromatin condensed against the periphery of the nuclei (Fig. 1G). In contrast, SPI-7, another poxvirus serpin predicted to have proapoptotic activity (21), did not affect the internucleosomal fragmentation of nuclear DNA at any concentration tested (Fig. 2, lanes 4–8). SPI-7 also had

3 R. W. Moyer, unpublished observations.
with 10% serpin solvent (lane 3); treatments (nuclei treated as in Fig. 1) were electrophoresed in a 1.5% agarose gel system is not affected by CrmA/SPI-2. YVAD-cmk (lane 1), MDB buffer supplemented with 10% serpin solvent (lane 2), or in S/M extracts pretreated with 10% serpin solvent (lane 3), with CrmA/SPI-2 at indicated concentrations (lanes 4–8), with 20 μg/ml SPI-7 (lane 9), or with 100 μM YVAD-cmk (lane 10). M, molecular mass markers (1 kilobase DNA ladder; Bio-Rad); bp, base pairs.

no significant effect on DNA fragmentation (Fig. 2, lane 9). YVAD-cmk, a broad spectrum caspase inhibitor at the concentration used here, completely blocked both the morphological changes in nuclei (Fig. 1D) and DNA ladder formation (Fig. 2, lane 10), as described previously (23).

To begin to examine the biochemical mechanism of the inhibition of nuclear apoptotic changes by CrmA/SPI-2, we examined the effect of purified CrmA/SPI-2 on the proteolysis of substrates catalyzed by the endogenous caspases in S/M extracts. Cleavage of PARP was not significantly inhibited at any concentration of CrmA/SPI-2 (Fig. 3, upper lanes 4–7), consistent with a recent report that showed a very poor inhibition of PARP cleavage in apoptotic osteosarcoma cell extracts by cowpox virus-derived CrmA/SPI-2 (9). However, the cleavage of nuclear lamin A, another hallmark of apoptosis (28, 29), was inhibited by the CrmA/SPI-2 protein in a dose-dependent manner (Fig. 3, lower lanes 4–7). In contrast, SPI-7 did not inhibit either PARP or lamin A cleavage (data not shown). In control experiments, proteolysis of both PARP and lamin A was blocked by YVAD-cmk, consistent with the cleavage of both substrates by caspases (Fig. 3, lane 8).

Recent studies revealed that the cleavage of both PARP (15) and the 70-kDa protein component of the U1 small ribonucleoprotein particle (30) as well as the morphological changes of apoptosis induced by tumor necrosis factor and Fas (15) are inhibited by transfection of the crmA cDNA into cells. However, those experiments do not necessarily mean that the CrmA-sensitive protease(s) are directly responsible for the observed effects; the results could be explained equally well if CrmA inhibits an upstream protease whose action leads to the activation of downstream proteases that cleave PARP and the U1 70-kDa protein.

Further experiments suggested that the inhibition of lamin A cleavage by CrmA/SPI-2 in our cell-free system is likely to be mediated by direct inhibition of a caspase. This conclusion is based on the use of YV(bio)KD-aomk (25), which covalently derivatizes residues within the active site of active caspases, enabling them to be revealed directly on immunoblots using horseradish peroxidase-conjugated streptavidin and ECL (20). Incubation of S/M extracts with YV(bio)KD-aomk revealed five labeled polypeptides ranging from 19.5 to 17.5 kDa (Fig. 4A, lane 1). We previously termed these species prICE_{1–5} (20).

Labeling with YV(bio)KD-aomk was used to assess the effects of a series of inhibitors on the activities of prICE_{1–5}. These results are most consistent with the conclusion that prICE_{1} is the endogenous lamin protease in S/M extracts. Competition experiments using a peptide spanning the cleavage site within PARP cleavage within the cell-free apoptosis reaction described under “Experimental Procedures” were electrophoresed in a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and stained for PARP and lamin A as described (24). HeLa nuclei were treated in MDB buffer supplemented with 10% serpin solvent (lane 1), in MDB buffer with 30 μg/ml CrmA/SPI-2 (lane 2), or in S/M extracts pretreated with 10% serpin solvent (lane 3), with CrmA/SPI-2 at the indicated concentrations (lanes 4–7), or with 100 μM YVAD-cmk (lane 8).

The data of Fig. 4 show competitions between noncovalent inhibitors (CrmA/SPI-2 and the cleavage site peptides) and the covalent inhibitor YV(bio)KD-aomk. We have been unable to...
observe effective competition at the high concentrations of YV(bio)KD-aomk required to label prICE2 and prICE5, and therefore the effect of lamin cleavage site peptides, CrmA/ SPI-2, or SPI-7 on the activity of these two caspases is not known (data not shown).

Because the cleavage of lamin A in S/M extracts is inhibited by TLCK (24), CrmA/SPI-2 (Fig. 3, lanes 4–7) and the peptide corresponding to the cleavage site in lamin A (20), the caspase that is inactivated by these three inhibitors, prICE2, is most likely a lamin-cleaving enzyme. The fact that TLCK and CrmA/ SPI-2 inhibit both the cleavage of lamin A and the completion of morphological apoptosis in S/M extracts suggests that the activity of the lamin protease (probably prICE2) is required for completion of morphological apoptosis. This makes excellent sense given the structural role of the lamin intermediate filament network in providing a structural support for the nuclear envelope. Lamin cleavage may thus serve not only to release lamin-chromatin interactions as previously suggested (24) but also to render the nuclear envelope malleable so that it can be packaged into condensed apoptotic bodies.

Accumulating evidence suggests that apoptosis is mediated by combinations of caspases acting in concert (20) that may be cell type-specific and stimulus-dependent. Further studies of the effects of inhibitors such as CrmA/SPI-2 on caspases active in apoptosis may ultimately provide us a means of controlling apoptotic cell death in order to suppress unwanted apoptosis in a tissue-specific manner (31) or to induce apoptosis in selected cell populations (32).

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