Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE

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RECENT studies suggest that proteases of the interleukin 1-β-converting enzyme (ICE)/*ced-3* family are involved in initiating the active phase of apoptosis[1–3]. Here we identify a novel protease resembling ICE (prICE) that is active in a cell-free system that reproduces the morphological and biochemical events of apoptosis[4]. prICE cleaves the nuclear enzyme poly(ADP-ribose) polymerase (PARP) at a tetrapeptide sequence identical to one of two ICE sites in pro-interleukin-1-β. However, prICE does not cleave purified pro-interleukin-1-β, and purified ICE does not cleave PARP, indicating that the two activities are distinct. Inhibition of prICE abolishes all manifestations of apoptosis in the extracts including morphological changes, cleavage of PARP and production of an oligonucleosomal ladder. These studies suggest that prICE might be pivotal in initiating the active phase of apoptosis *in vitro* and in intact cells.

To study apoptotic events, we have developed a cell-free system that uses cytoplasmic extracts made from chicken DU249 cells that become committed to apoptosis as a result of an S-phase aphidicolin arrest and are subsequently collected in M phase (S/M extracts[4]). Cleavage of PARP to a fragment of relative molecular mass ~85,000 (M_r ~85K), an early event in apoptosis in intact cells[5], was observed within three minutes after addition of isolated nuclei or purified PARP to S/M extracts (Fig. 1*a*). The PARP fragments produced *in vivo* and *in vitro* were indistinguishable by SDS–PAGE (Fig. 1*b*). Control, real mitotic extracts (RME) prepared without aphidicolin presynchronization did not induce apoptotic changes in added nuclei[4] and did not cleave PARP (Fig. 1*b*).

Proteolysis appeared to be highly selective. Immunoblotting revealed that several other nuclear proteins, including topoisomerase II (170K), ScII (135K[6]), CENP-C (140K[7]) and RCC1 (45K[8], Fig. 1*a*) remained intact for at least 1 h in S/M extracts. In addition, the mobilities of major proteins visible on stained SDS gels were unaltered by incubation of nuclei in S/M extracts (data not shown).

Amino-terminal sequencing of the purified 85K PARP fragment revealed that cleavage occurred between Asp 216 and Gly 217 (data not shown), a site that is conserved in human[9], bovine[10] and chicken[11] PARP. The region immediately surrounding the cleavage site in PARP (Glu-Val-Asp · · / · · Gly) is identical to one of the two ICE cleavage sites in pro-interleukin-1-β. A decamer peptide spanning the putative cleavage site inhibited PARP cleavage, whereas a peptide containing Ala in place of Asp at the P_1 position failed to inhibit PARP cleavage (Fig. 1*e*). Thus Asp in the P_1 position is essential for catalytic recognition by the PARP protease. Only two eukaryotic proteases are known to cleave next to Asp: granzyme B[12], a serine protease that initiates cytotoxic lymphocyte-induced apoptosis[13,14]; and ICE[15,16]. Inhibitor studies (not shown) indicated that the PARP protease was sensitive to iodoacetamide but resistant to the cysteine protease inhibitor E-64 and the serine protease inhibitors PMSF, TLCK or TPCK, a pattern that matches the inhibitor profile of ICE[17]. These results suggest that PARP cleavage involves a protease resembling ICE (prICE).

To explore the possibility that prICE is ICE itself, we examined the stability of human pro-interleukin-1-β in S/M extracts. Pro-interleukin-1-β (31K) was cleaved by purified ICE but not by S/M extracts (Fig. 2*a*, lower). Conversely, purified PARP was cleaved by S/M extracts but not by ICE (Fig. 2*a*, upper).

Because prICE cleaves PARP at an ICE-like cleavage site, we examined the effect of a specific tetrapeptide ICE inhibitor on apoptotic events in S/M extracts. When YVAD chloromethylketone[16] was added to S/M extracts before nuclei, concentrations as low as 4 μM blocked all apoptotic events including PARP cleavage (Fig. 2*b*), morphological changes (Fig. 2*c*) and internucleosomal DNA fragmentation (data not shown). Iodoacetamide had a similar effect. In controls, TPCK, another chloromethylketone, had no effect at concentrations as high as 140 μM (Fig. 2*b*, *c*).

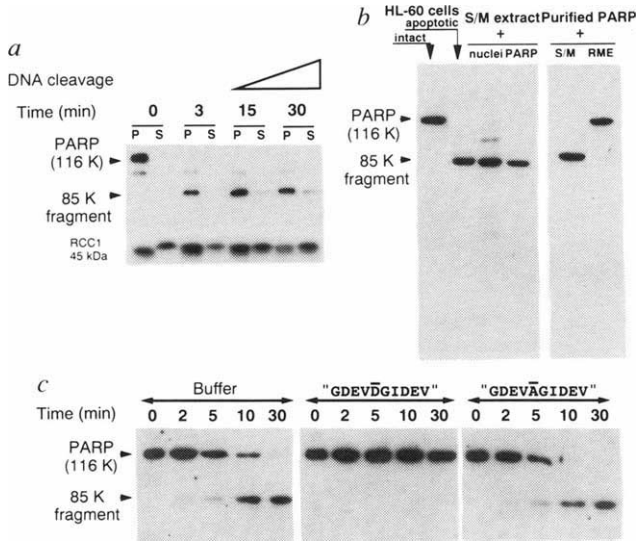

FIG. 1 PARP is cleaved at a site identical to one of the ICE cleavage sites in pro-interleukin-1-β. *a*, Time course of cleavage of PARP during apoptosis *in vitro*. S/M extract was diluted with DB to a final protein concentration of 10 mg ml^{-1}. Isolated HeLa nuclei were added at a concentration of 40,000 per μl. After incubation at 37 °C for the indicated time, the nuclei (pellet, P) were separated from the extract (supernatant, S) by centrifugation at 12,000*g* for 5 min, and both were subjected to electrophoresis followed by immunoblotting with monoclonal antibody C-2-10 as previously described[5]. This antibody recognizes an epitope located between the zinc fingers and automodification domains of PARP[5,21]. The band between the 116K and 85K forms of PARP represents a cross-reacting species present in some preparations of nuclei. *b*, Apoptotic cleavage of PARP produces fragments of the same size in intact HL-60 cells and *in vitro* in S/M extracts and shows that PARP is cleaved only in extracts that have apoptotic activity. HL-60 cells were treated with 17 μM etoposide for 4 h to induce apoptosis[5]. HeLa nuclei and purified bovine PARP[22] were incubated with S/M extract. Right two lanes, 200 ng of purified PARP were incubated with 10 μl of either S/M extracts or RME for 30 min at 37 °C. All samples were subjected to SDS–PAGE followed by immunoblotting with antibody C-2-10. *c*, Inhibition of prICE activity by a decamer peptide spanning the PARP cleavage site: Asp at the P1 position is essential for inhibition of prICE. Peptides GDEVDGIDEV or GDEVAGIDEV were dissolved in 50 mM PIPES, pH 7.4, at a final concentration of 5 mM. Purified bovine PARP was dialysed against DB and diluted with this buffer to 10 μg ml^{-1}. PARP (45 μl) was mixed with 15 μl of either one of the peptides or with 50 mM PIPES. S/M extract (4 μl) was added to each of the samples (final protein concentration 2 mg ml^{-1}), which were then incubated at 37 °C. At the indicated times, an aliquot of each sample was mixed with an aliquot of SDS sample buffer and heated for 3 min at 95 °C. The resulting samples were subjected to SDS–PAGE and immunoblotting with antibody C-2-10. Peptide GDEVDGIDEV inhibited cleavage of PARP by the apoptotic extract under these conditions, whereas peptide GDEVAGIDEV did not. A similar D→A change in pre-IL-1β abolishes cleavage of the precursor by ICE[15].

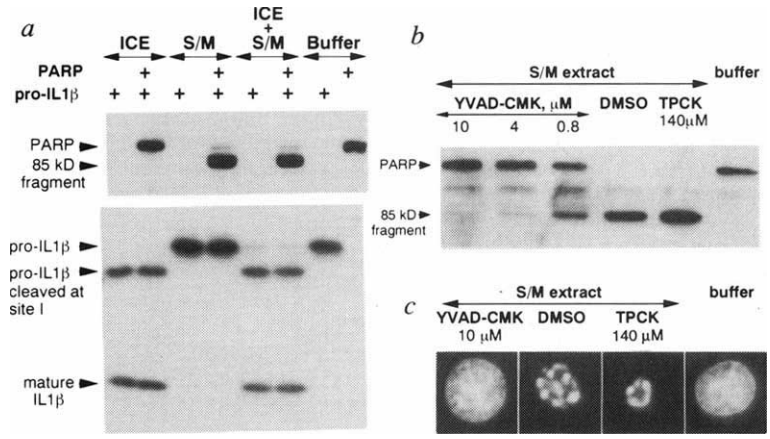

FIG. 2 *a*, prICE does not cleave human pro-interleukin-1-β (pro-IL-1-β), and human ICE does not cleave bovine PARP. Recombinant human pro-IL-1-β was purchased from Cistron Biotechnology (Pine Brook, NJ) as a 10 μg ml^{-1} solution in 10 mM Tris, 0.1% Triton X-100, 0.1 mM EDTA and 10% glycerol. Aliquots (8 μl) of ICE assay buffer (25 mM HEPES, 5 mM DTT, 10% glycerol[15]) were supplemented with 1 μl (10 ng) of pro-IL-1-β solution in DB or in DB plus 1 μl (150 ng) of bovine PARP. In the samples that contained both PARP and pro-IL-1-β, the molar ratio of PARP to pro-IL-1-β was about 4:1. Next, each sample was supplemented with 1 μl (1.5 U) of ICE, 1 μl (45 μg of total protein) of an S/M extract, or both. Samples were then incubated at 37 °C for 30 min, mixed with an aliquot of SDS sample buffer and heated for 3 min at 95 °C. The resulting samples were subjected to electrophoresis in 15% polyacrylamide gels followed by immunoblotting. The blot was probed with a polyclonal antibody that recognizes both the precursor and mature IL-1-β (Cistron Biotechnologies), and then with ^{125}I protein A. After autoradiography, the same blot was probed with murine anti-PARP antibody C-2-10 followed by peroxidase-labelled anti-mouse and detected by ECL. *b, c*, Inhibition of prICE by YVAD · chloromethylketone blocks all apoptotic events in S/M extracts. *b*, YVAD · CMK, (Ac-YVAD · chloromethylketone) a specific inhibitor of ICE, inhibits prICE. A 20 mM stock solution of YVAD · CMK (Bachem), was diluted with 50 mM PIPES, pH 7.4 and then added to aliquots of an S/M extract at the indicated final concentrations. Control aliquots were supplemented with 0.1% DMSO to evaluate the effect of the solvent, or with 140 μM of tosylphenylalanyl-chloromethylketone (TPCK) to exclude possible non-specific effects of the chloromethylketone moiety. After aliquots were preincubated for 15 min at 37 °C, HeLa nuclei were added at a concentration of 100,000 per μl, incubated for 1 h at 37 °C, and the samples were subsequently subjected to SDS–PAGE and immunoblotting for detection of PARP. *c*, YVAD · CMK inhibits apoptotic changes *in vitro*. HeLa nuclei were incubated for 1 h at 37 °C in aliquots of S/M extract that had been preincubated with the indicated concentrations of YVAD · CMK, DMSO or TPCK as described above. An aliquot of nuclei from each sample was stained with DAPI to reveal changes in nuclear morphology.

In summary, we have shown that extracts from cells committed to apoptosis contain a protease activity (prICE) that shares many features with ICE including (1) cleavage at the same tetrapeptide sequence, (2) sensitivity to proteinase inhibitors and (3) inhibition by the specific chloromethylketone YVAD · CMK. prICE is, however, distinguished from ICE by its substrate specificity. Although it is formally possible that chicken ICE does not cleave mammalian pro-interleukin-1-β, we note that purified mammalian ICE failed to cleave purified mammalian PARP under all conditions tested. As PARP is cleaved during apoptosis in mammalian cells[5], this clearly implicates the involvement of a second ICE-like enzyme in apoptosis.

Although PARP cleavage might serve an essential function in apoptosis, by decreasing consumption of NAD[5] and its precursor ATP, an energy source that is required for completion of apoptosis *in vivo*[18,19] and in S/M extracts (Y.L. and W.C.E., unpublished), it is unlikely that PARP cleavage is the only function of prICE. Other substrates cleaved by prICE during the initiation of apoptosis (possibly including nucleases and additional proteases) might be activated and go on to trigger the morphological events of apoptosis. The observation that YVAD · CMK prevents the biochemical and morphological changes of apoptosis in S/M extracts raises the possibility that prICE may lie near the apex of an apoptotic cascade both *in vivo* and *in vitro*. □

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Interleukin-2 transcriptional block by multifunctional Ca^{2+}/calmodulin kinase

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In the presence of costimulation[1], Ca^{2+} influx in T cells leads to activation (transcription of interleukin-2; ref. 2) via calcineurin[3,4]. In the absence of costimulation, Ca^{2+} influx results in anergy (interleukin-2 transcriptional block[5]) through an unknown mechanism. Specific attenuation of interleukin-2 transcriptional induction occurs in Jurkat T cells following pretreatment with a Ca^{2+} ionophore. A >90% block of inducible interleukin-2 reporter gene activity was initiated by transfection of a constitutively active mutant of multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase or CaM kinase II)[6], but not by constitutive mutants

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