The EMBO Journal vol.15 no.20 pp.5504–5512, 1996

Pivotal role of a DEVD-sensitive step in etoposide-induced and Fas-mediated apoptotic pathways

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We investigated the role of proteases in the pathway that leads from specific DNA damage induced by etoposide (VP-16), a topoisomerase II inhibitor, to apoptotic DNA fragmentation in the U937 human leukemic cell line. In a reconstituted cell-free system, Triton-soluble extracts from VP-16-treated cells induced internucleosomal DNA fragmentation in nuclei from untreated cells. This effect was inhibited by the tetrapeptide Ac-DEVD-CHO, a competitive inhibitor of the interleukin-1β-converting enzyme (ICE)-related protease CPP32, but was not influenced by Ac-YVAD-CHO and Ac-YVAD-CMK, two specific inhibitors of ICE. The three tetrapeptides inhibited Fas-mediated apoptotic DNA fragmentation in the cell-free system. Internucleosomal DNA fragmentation, triggered by either VP-16 or an anti-Fas antibody, was associated with proteolytic cleavage of the poly(ADP-ribose)polymerase (PARP), a decrease in the level of 32 kDa CPP32 proenzyme and the appearance of the CPP32 p17 active subunit. Conversely, the expression of Ich-1L, another ICE-like protease, remained stable in apoptotic U937 cells. Several cysteine and serine protease inhibitors prevented apoptotic DNA fragmentation by acting either upstream or downstream of the DEVD-sensitive protease(s) activation and PARP cleavage. We conclude that a DEVD-sensitive step, which could involve CPP32, plays a central role in the proteolytic pathway that mediates apoptotic DNA fragmentation in VP-16-treated leukemic cells at the crossing with Fas-mediated pathway.

Keywords: apoptosis/CPP32/DNA fragmentation/etoposide/proteases

Introduction

Epipodophyllotoxins such as etoposide (VP-16) and teniposide (VM-26) are topoisomerase II-reactive agents commonly used for the treatment of acute leukemias (Champlin and Gale, 1987). These cytotoxic drugs produce double-strand breaks by stabilizing a transient intermediate of the topoisomerase reaction, in which the enzyme is covalently linked to the 5′ terminus of a DNA duplex (Liu, 1989). This type of DNA damage is thought to be a critical event for epipodophyllotoxin-induced cytotoxicity. Several other mechanisms are involved in cell sensitivity to topoisomerase II inhibitors, and the ability of malignant cells to undergo apoptotic cell death following DNA damage could be the ultimate determinant of their sensitivity to these drugs (Pommier *et al.*, 1994; Dubrez *et al.*, 1995). Overexpression of Bcl-2, a protein that extends cell survival through the inhibition of apoptotic cell death in various cell systems (for a review see Korsmeyer, 1992), inhibits VP-16-induced apoptosis without modifying the formation and repair of DNA strand breaks induced by the drug (Kamesaki *et al.*, 1993). However, the pathway that leads from specific DNA damage induced by epipodophyllotoxins to apoptotic cell death remains poorly understood.

In combination with morphological changes and internucleosomal DNA fragmentation, proteolysis is a hallmark of apoptotic cell death (Gorczyca *et al.*, 1993; Kaufmann *et al.*, 1993; Weaver *et al.*, 1993; Casciola-Rosen *et al.*, 1994; Jensen *et al.*, 1994). In the nematode *Caenorhabditis elegans*, two genes, *ced-3* and *ced-4*, play essential roles in either the initiation or the execution of the cell death program, because recessive mutations in these genes prevent almost all of the cell deaths that normally occur during its development (Hengartner and Horvitz, 1994). *ced-3* encodes a protein highly homologous to the mammalian interleukin (IL)-1β-converting enzyme (ICE; Yuan *et al.*, 1993). Both Ced-3 and ICE induce apoptosis when overexpressed in a variety of host cells, and their pro-apoptotic effect can be prevented by the nematode cell death suppressor gene *ced-9*, whose mammalian counterpart is *bcl-2* (Miura *et al.*, 1993). The balance between the opposite effects of an ICE/*ced-3*-like protease and the Bcl-2/Ced-9 protein family could determine the fate of eukaryotic cells in response to various stimuli, including DNA-damaging agents.

ICE is the first identified member of a new class of cysteine proteases that also includes CPP32/Yama/apopain (Fernandez-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995), Nedd-2 (Kumar *et al.*, 1994), Ich-1L (Wang *et al.*, 1994), Tx/Ich-2/ICE_{rel}II (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995), ICE_{rel}III (Munday *et al.*, 1995), Mch-2 (Fernandez-Alnemri *et al.*, 1995a) and Mch-3 (Fernandez-Alnemri *et al.*, 1995b). Each is capable of inducing an apoptotic cell death when overexpressed in host cells, but this effect could be nonspecific because overexpression of other unrelated proteases has also been shown to induce apoptosis (Williams and Henkart, 1994). ICE was reported to be involved specifically in Fas-mediated apoptosis, based on the inhibitory effects of the cowpox virus CrmA protein and the tetrapeptides YVAD-CHO and YVAD-CMK, three potent inhibitors of ICE (Enari *et al.*, 1995b; Los *et al.*, 1995). However, ICE-deficient mice develop normally, confirming the suspicion that ICE is not the only protease involved in apoptosis *in vivo* (Kuida *et al.*, 1995).

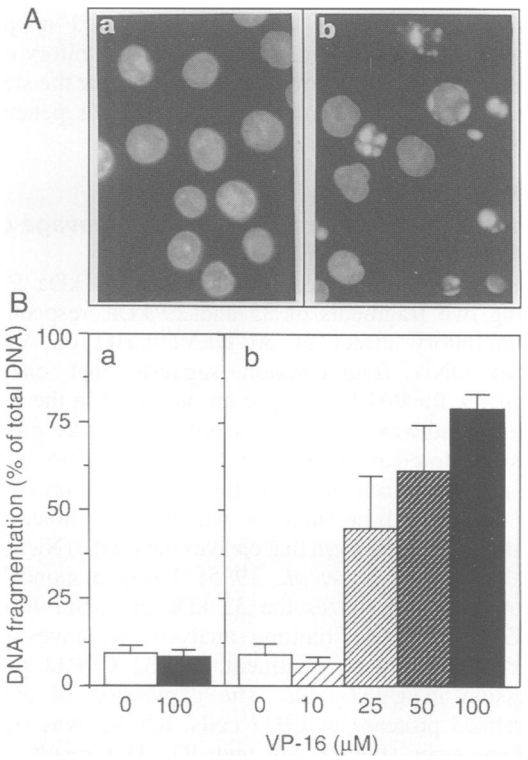

Fig. 1. VP-16-induced apoptosis in U937 cells. (**A**) Morphological aspects of Hoechst 33342-stained U937 cells: (a) untreated cells; (b) cells treated for 3 h with 50 μM VP-16; magnification ×400. (**B**) DNA fragmentation quantified by a filter elution assay in a cell-free system. Isolated nuclei from untreated U937 cells were incubated for 30 min at 37°C with Triton-soluble extracts from U937 cells before performing the filter elution assay. (a) Triton-soluble extracts from untreated cells tested in the absence and presence of 100 μM VP-16. (b) Triton-soluble extracts from cells treated with the indicated concentrations of VP-16 for 3 h. Results are expressed as the means ± SD of three independent experiments.

The exact role of each of the ICE-related cysteine proteases in the apoptotic pathway triggered by a given stimulus is not well defined. Their only common substrate identified so far is poly(ADP-ribose)polymerase (PARP), an enzyme involved in DNA repair and genome maintenance (Kaufmann *et al.*, 1993). However, the relationships between PARP cleavage and apoptotic DNA fragmentation have not been clearly identified. PARP breakdown can be prevented by a tetrapeptide aldehyde, Ac-DEVD-CHO, which contains the P1–P4 amino acid sequence of the PARP cleavage site. The kinetics of inhibition of protease-induced PARP cleavage by Ac-DEVD-CHO suggest that CPP32 and Mch-3 could be the best candidates among ICE-related proteases for PARP cleavage associated with apoptotic cell death (Fernandez-Alnemri *et al.*, 1995b; Nicholson *et al.*, 1995).

In this study we investigated the role of proteases in VP-16-induced apoptosis of leukemic cells by testing the effect of various protease inhibitors in whole cells and in a cell-free system. Our results suggest that a DEVD-sensitive step plays a central role in the proteolytic pathway that leads to PARP cleavage and internucleosomal DNA fragmentation during VP-16-induced apoptosis. The DEVD-sensitive protease(s) could become an important target for strategies aiming to modulate cell sensitivity to epipodophyllotoxins.

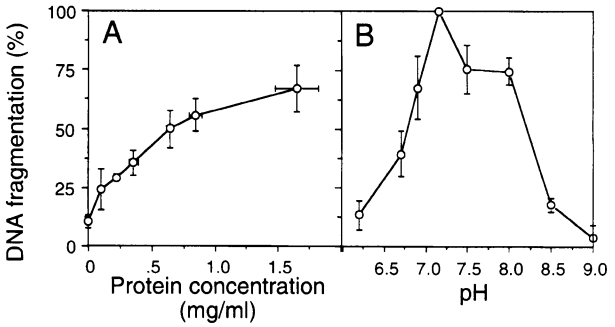

Fig. 2. The role of protein concentration and pH. Isolated nuclei from untreated U937 cells were incubated for 30 min in the presence of Triton-soluble extracts from U937 cells treated with 50 μM VP-16 for 3 h. (**A**) Role of protein concentration in Triton-soluble extracts. Protein concentration was measured using the BCA method. (**B**) Role of lysis buffer pH. Nuclei and Triton-soluble extracts were prepared in lysis buffers at the indicated pH values. Maximal fragmentation, observed at pH 7.2, was standardized as 100%. Results are expressed as means ± SD of three independent experiments performed in triplicate.

Results

The Triton-soluble extracts from VP-16-treated cells induce internucleosomal DNA fragmentation in nuclei isolated from untreated cells

Treatment of U937 cells with VP-16 induces their apoptosis identified by characteristic morphological changes, including chromatin condensation and cytoplasm blebbing (Figure 1A). To investigate the pathway of apoptosis induction in VP-16-treated U937 cells, we used a cell-free system in which nuclei from untreated cells were incubated with Triton-soluble extracts from cells treated for 3 h with various concentrations of VP-16 (Figure 1B). As a control, we checked that the addition of VP-16 to Triton-soluble extracts from untreated U937 cells did not induce DNA fragmentation in nuclei from untreated cells (Figure 1B, panel a). Triton-soluble extracts from treated cells induced DNA fragmentation in nuclei from untreated cells. Using a filter elution assay, DNA fragmentation was observed to be dose dependent (Figure 1B, panel b). The following experiments in the cell-free system were performed using Triton-soluble extracts from U937 cells treated for 3 h with 50 μM VP-16.

Characterization of DNA fragmentation-promoting activity identified in VP-16-treated U937 cells

Using serial dilutions of the Triton-soluble extracts from VP-16-treated U937 cells, we observed that DNA fragmentation in untreated cell nuclei increased with protein concentration (Figure 2A). Subsequent experiments were all performed with extracts in which the protein concentration was 0.8 mg/ml. DNA fragmentation was suppressed when the lysis buffer pH was <6.25 or >9.0 (Figure 2B). The optimal pH was 7.2. Thus, all other reported experiments were performed at pH 7.2. Induction of DNA fragmentation in healthy nuclei by Triton-soluble extracts from treated cells was abolished when $MgCl_2$ was removed from the lysis buffer (Table I). In the absence of EGTA, 5 mM $CaCl_2$ only partially restored DNA fragmentation in this system. Neither $CaCl_2$ nor $MgCl_2$ induced any DNA fragmentation in nuclei from untreated cells. All other experiments were performed in the presence of 5 mM $MgCl_2$ without the addition of Ca^{2+}.

Differential effects of tetrapeptide inhibitors of cysteine proteases in the cell-free system

Ac-YVAD-CHO and Ac-YVAD-CMK, two tetrapeptides reported to specifically inhibit ICE, did not affect DNA fragmentation induced by Triton-soluble extracts from VP-16-treated U937 cells in healthy U937 nuclei (Figure 3B and C). ICE was reported to be involved in Fas-mediated apoptosis. Treatment of U937 cells with an anti-Fas monoclonal antibody (50 ng/ml for 6 h) in the presence of cycloheximide (CHX; 0.8 μg/ml) induced their apoptosis (data not shown). At this concentration, CHX alone did not induce DNA fragmentation in entire U937 cells, nor in the cell-free system. Triton-soluble extracts from anti-Fas antibody-treated U937 cells induced apoptotic DNA fragmentation (~40%) in nuclei from untreated U937 cells (Figure 3A). DNA fragmentation was inhibited when either Ac-YVAD-CHO or Ac-YVAD-CMK was added to the Triton-soluble extracts from anti-Fas antibody-treated U937 cells (Figure 3B and C). The tetrapeptide Ac-DEVD-CHO, which was designed to competitively inhibit PARP cleavage, abolished apoptotic DNA fragmentation induced by Triton-soluble extracts from both VP-16- and anti-Fas antibody-treated U937 cells (Figure 3D). These results suggested that ICE or an ICE-related protease sensitive to Ac-YVAD inhibitors was specifically involved in Fas-mediated apoptosis while another protease inhibited by Ac-DEVD-CHO could be common to both Fas- and VP-16-mediated apoptotic pathways. When used on entire cells, no inhibitory effect of the three tested tetrapeptides was detected at the studied concentrations, suggesting that these peptides penetrated poorly in U937 cells (data not shown).

Table I. Effects of cations on DNA fragmentation in the cell-free system

U937 cell treatment	Buffer	DNA fragmentation (% of total DNA)
0	Control	11.0 ± 4.0
0	$-MgCl_2$, $-EGTA$, $+CaCl_2$ (5 mM)	8.7 ± 1.0
VP-16	Control	57.5 ± 15.5
VP-16	$-MgCl_2$	16.0 ± 9.1
VP-16	$-MgCl_2$, $-EGTA$, $+CaCl_2$ (5 mM)	33.3 ± 11.1

^{14}C-labeled nuclei from untreated cells were incubated for 30 min at 37°C in the presence of Triton-soluble extract from U937 cells. VP-16 treatment was performed with 50 μM for 3 h. Control buffer (5 mM $MgCl_2$) is described in Materials and methods. Each value is the mean ± SD of three independent experiments performed in triplicate.

VP-16- and Fas-induced apoptotic DNA fragmentation are associated with a cleavage of CPP32 immature form

DEVD is the site of cleavage of the 116 kDa PARP, yielding two fragments of 85 and 29 kDa, respectively. The inhibitory effect of Ac-DEVD-CHO on VP-16-induced DNA fragmentation suggests that enzymes responsible for PARP cleavage are involved in the VP-16 apoptotic pathway. CPP32 is a potent ICE-like protease that is able to cleave PARP (Nicholson *et al.*, 1995). Like other proteases from the ICE family, CPP32 proenzyme must be cleaved in subunits which heterodimerize to constitute the active form that cleaves the PARP (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Using a monoclonal antibody that recognizes the 32 kDa precursor form of CPP32 and Western blotting analysis, we investigated the effects of VP-16 treatment on p32 CPP32 protein expression in U937 cells. The expression of another ICE-related protease in U937 cells, Ich-1L, was studied simultaneously (Figure 4A and B). The results were related to PARP cleavage (Figure 4A) and apoptotic DNA fragmentation visualized by agarose gel electrophoresis (Figure 4C), and quantified by a filter elution assay (Figure 4B). When U937 cells were treated with increasing concentrations of VP-16 for 3 h (Figure 4, left panels), both PARP cleavage and apoptotic DNA fragmentation were detected at 50 μM VP-16 and increased with drug concentration. These phenomena were associated with a decrease in p32 CPP32 expression, without any significant change in Ich-1L expression. The time course of apoptosis induction in U937 cells treated with 50 μM of the drug indicated that a p32 CPP32 level decrease, PARP cleavage and apoptotic DNA fragmentation all occurred between 2.5 and 3.0 h of continuous drug exposure. Again, the Ich-1L level did not change after 3 h of treatment. PARP cleavage, apoptotic DNA digestion and p32 CPP32 level decrease, without any changes in Ich-1L level, were also

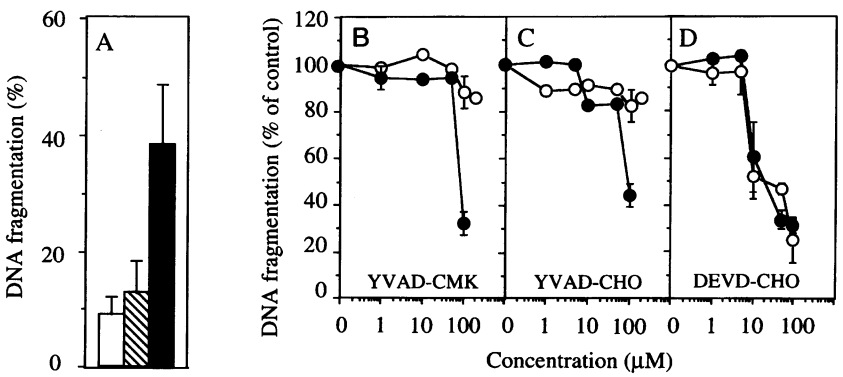

Fig. 3. The role of Ac-YVAD-CMK, Ac-YVAD-CHO and Ac-DEVD-CHO on DNA fragmentation induced by VP-16 or anti-Fas antibody treatment in the cell-free system. (**A**) Fas-mediated DNA fragmentation quantified by a filter elution assay in a cell-free system. Isolated nuclei from untreated U937 cells were incubated for 30 min at 37°C with Triton-soluble extracts from U937 cells before performing the filter elution assay. (□) Extracts from untreated cells. (▨) Extracts from cells treated with CHX (0.8 μg/ml) + control mouse IgM for 6 h. (■) Extracts from cells treated with anti-Fas antibody (50 ng/ml) + CHX (0.8 μg/ml) for 6 h. (**B–D**) Dose–response curves of the effects of the indicated tetrapeptides on VP-16-induced (○) and Fas-mediated (●) apoptotic DNA fragmentation. The DNA fragmentation observed in the control sample was standardized as 100%. Results are the means ± SD of at least three independent experiments performed in triplicate.

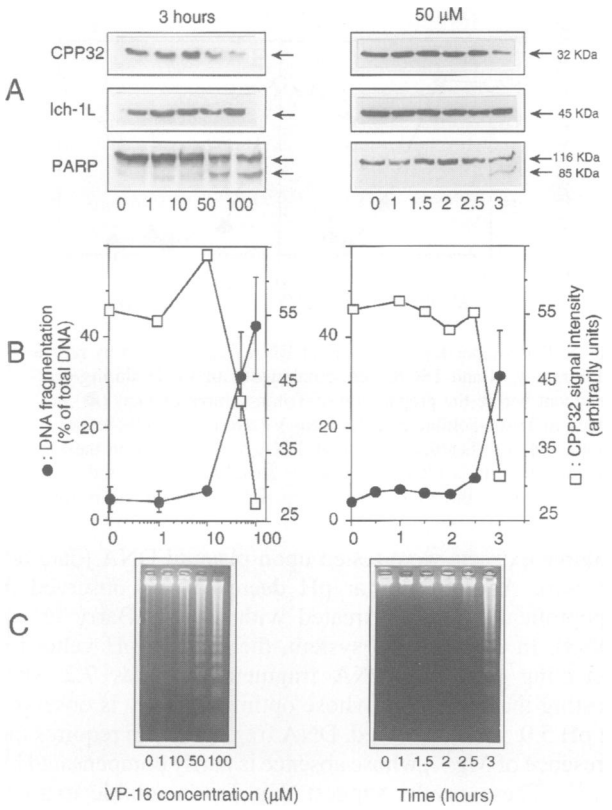

Fig. 4. CPP32 and Ich-1L protein expression, PARP cleavage and internucleosomal DNA fragmentation in VP-16-treated U937 cells. Cells were treated with increasing concentrations of VP-16 for 3 h (left panel) or with 50 μM VP-16 for the indicated time (right panel). (**A**) Western blots of CPP32, Ich-1L and PARP. (**B**) DNA fragmentation (●) was quantified using the filter elution assay in VP-16-treated U937 cells; CPP32 level (□) was measured using densitometric scanning of the Western blot presented in (A). Results of the filter elution assay are expressed as means ± SD of three independent experiments performed in triplicate. (**C**) Internucleosomal DNA fragmentation was visualized after agarose gel electrophoresis.

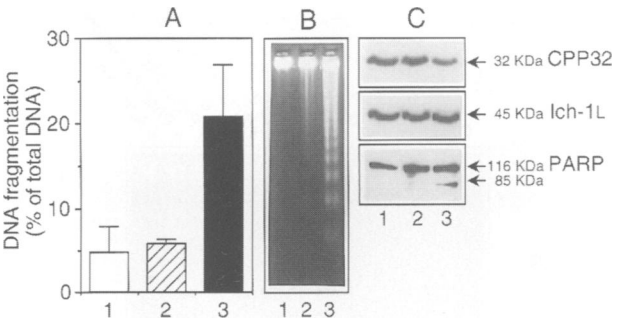

Fig. 5. CPP32 and Ich-1L protein expression, PARP cleavage and internucleosomal DNA fragmentation in anti-Fas antibody-treated U937 cells. (**A**) DNA fragmentation quantified using the filter elution assay. Results are expressed as means ± SD of three independent experiments performed in triplicate. (**B**) Internucleosomal DNA fragmentation visualized by agarose gel electrophoresis. (**C**) Western blots of CPP32, Ich-1L and PARP. Lane 1, untreated cells; lane 2, cells treated for 6 h with 0.8 μg/ml CHX + control IgM; lane 3, cells treated for 6 h with anti-Fas antibody (50 ng/ml) + CHX (0.8 μg/ml).

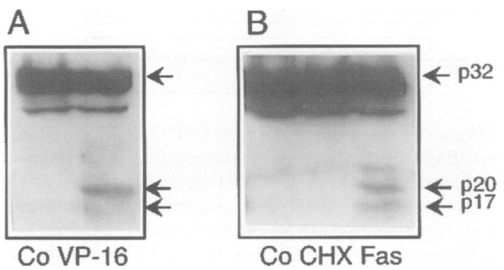

Fig. 6. Cleavage of CPP32 into p17 active subunit in VP-16 and anti-Fas antibody-treated U937 cells. Immunoblot analysis of the degradation of the CPP32 proenzyme (p32) into the p20 and p17 subunits. (**A**) Untreated U937 cells (Co) and cells treated with 50 μM VP-16 for 3 h (VP-16). (**B**) Untreated U937 cells (Co) and cells treated with CHX (0.8 μg/ml) or CHX + anti-Fas antibody (50 ng/ml) (Fas).

observed in U937 cells treated for 6 h with anti-Fas antibody (50 ng/ml; Figure 5). Using a rabbit polyclonal anti-CPP32-p17 antibody, we identified a p20 intermediate cleavage form and the p17 active subunit of CPP32 in apoptotic U937 cells (Figure 6). Overexposure of the blot to identify the cleavage products prevented the observation of CPP32 proform decrease shown in Figure 4. Altogether, these data suggest that CPP32 could be the DEVD-sensitive protease that triggers PARP cleavage and plays a key role in the proteolytic pathway which leads to apoptotic DNA fragmentation in both VP-16- and anti-Fas antibody-treated U937 cells.

Differential effects of various protease inhibitors on VP-16-induced apoptotic DNA fragmentation in U937 cells

To determine whether other proteolytic events could be involved in the VP-16-triggered apoptotic pathway, we investigated the effects of various protease inhibitors on apoptotic DNA fragmentation in both entire cells and the cell-free system. Protease inhibitors were first associated with VP-16 during the 3 h treatment of entire U937 cells, before the preparation of Triton-soluble extracts. DNA fragmentation in healthy nuclei was studied by both agarose gel electrophoresis (Figure 7) and a filter elution assay (Figure 8 and Table II). Simultaneously, we determined the effects of protease inhibitors on cell morphology (data not shown), 32 kDa CPP32 protein level and PARP cleavage (Figure 7). The protease inhibitors were also tested by adding them to Triton-soluble extracts from U937 cells treated with 50 μM VP-16 for 3 h, before incubation with untreated cell nuclei and DNA fragmentation quantification (Table II). All the protease inhibitors tested were checked to be nontoxic for U937 cells at the used concentrations after 3 h of incubation (data not shown). Lack of toxicity was defined by the exclusion of trypan blue and the absence of DNA fragmentation when measured by the filter elution assay.

Calpain inhibitors I and II and the serine protease inhibitor *N*-tosyl phenylalanine chloroketone (TPCK) inhibited DNA fragmentation only when combined early with VP-16 for the treatment of whole cells, not in the cell-free system (Table II and Figures 7 and 8). Phenylmethylsulfonyl fluoride (PMSF), another serine protease inhibitor, was observed to be active in both situations (Table II). All these protease inhibitors also prevented PARP cleavage and CPP32 decrease (Figure 7). The serine protease inhibitor dichloroisocoumarin and the cysteine protease inhibitor thimerosal inhibited DNA fragmentation in both the whole cells and the cell-free system but did not prevent CPP32 expression decrease nor PARP cleavage. All other protease inhibitors tested,

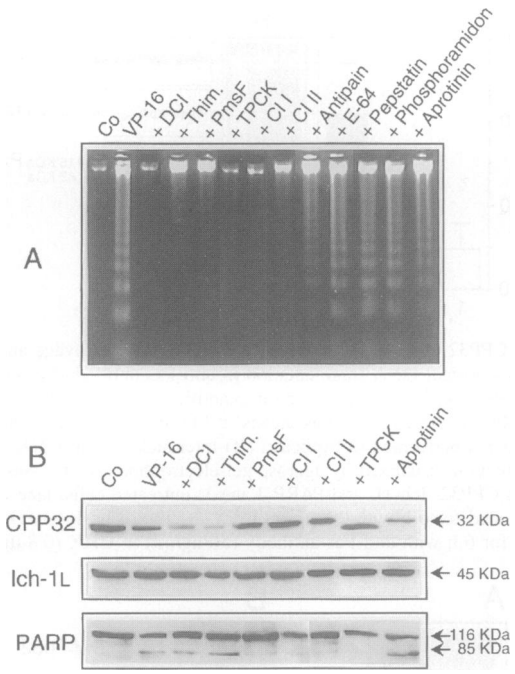

Fig. 7. Effects of protease inhibitors on CPP32 and Ich-1L protein expression, PARP cleavage and internucleosomal DNA fragmentation in VP-16-treated cells. (**A**) Agarose gel electrophoresis of DNA extracted from untreated U937 nuclei incubated for 30 min in the presence of Triton-soluble extracts from untreated (Co) and treated cells. (**B**) Western blot of CPP32, Ich-1L and PARP in untreated (Co) and treated cells. Cells were treated for 3 h with 50 μM VP-16 alone (VP-16) and in the presence of the following protease inhibitors: 50 μg/ml DCI; 15 μM thimerosal (Thim.); 1 mM PMSF; 50 μM CI I; 50 μM CI II; 0.2 mM TPCK; 5 μg/ml antipain; 10 μg/ml E-64; 1 μg/ml pepstatin; 10 μg/ml phosphoramidon; 0.15 IU/ml aprotinin.

including three cysteine protease inhibitors (antipain, aprotinin and E-64), two serine protease inhibitors (α_1-antichymotrypsin and eglin), the aspartyl protease inhibitor pepstatin and the metalloprotease inhibitor phosphoramidon demonstrated no effect on whole cells or in the cell-free system (Table II).

Discussion

Treatment of U937 cells with VP-16 induces morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death. The kinetics of DNA fragmentation are very rapid at VP-16 concentrations >10 μM. They can be reproduced in a reconstituted cell-free system that allows the dissection of some of the biochemical mechanisms of the final phases of induction of apoptotic DNA digestion (Bertrand *et al.*, 1995; Enari *et al.*, 1995a; Martin *et al.*, 1995). Triton-soluble extracts prepared from VP-16-treated cells trigger apoptotic DNA fragmentation in nuclei from untreated U937 cells, suggesting that these extracts contain a factor that is able to activate a nuclear endonuclease. Triton-soluble extracts could also induce changes in the chromatin structure that increase the accessibility of the DNA to nucleases.

Mammalian cells contain a variety of nucleases that could be involved in apoptosis. Most of them were localized in the nucleus, although others were identified in mitochondria and lysosomes (Eastman and Barry, 1992). We did not detect any nuclease activity when Triton-soluble extracts were tested upon plasmid DNA (data not shown). An intracellular pH decrease was observed in apoptotic HL60 cells treated with VP-16 (Barry *et al.*, 1993). In our cell-free system, the optimal pH value for detecting apoptotic DNA fragmentation was 7.2, suggesting that DNase II, whose optimal activity is observed at pH 5.0, is not involved. DNA fragmentation requires the presence of Mg^{2+}, whose absence is partly compensated by Ca^{2+}. These results suggest that Triton-soluble extracts from VP-16-treated U937 cells activate a nuclear endonuclease that triggers apoptotic DNA fragmentation at neutral pH in the presence of Mg^{2+}.

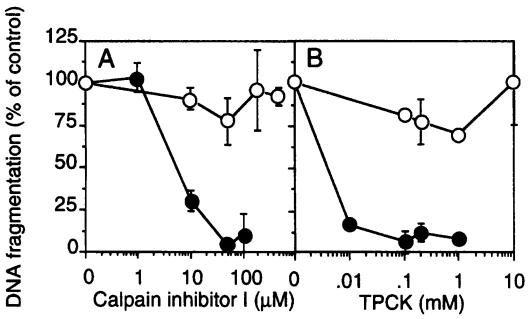

Fig. 8. Dose-dependent inhibition of DNA fragmentation by protease inhibitors. CI I and TPCK were combined with VP-16 during cell treatment before the preparation of Triton-soluble extracts (●) or added to Triton-soluble extracts from VP-16-treated cells before incubation with healthy nuclei (○). DNA fragmentation in these nuclei was measured by a filter elution assay. Results are expressed as means ± SD of three independent experiments performed in triplicate.

ICE and ICE-related proteases have been implicated in the pathway that leads to cell death in both nematodes and mammals. All these proteases cleave their substrate at an unusual site, with Asp at P1, but differ in the peptidic sequences surrounding this cleavage site. Human ICE was first characterized by its ability to process the inactive pro-IL-1β to the proinflammatory cytokine IL-1β. ICE has a unique sequence preference among mammalian proteases, and its minimal substrate is the tetrapeptide YVAD. Tetrapeptide inhibitors Ac-YVAD-CHO and Ac-YVAD-CMK were designed to competitively inhibit pro-IL-1β cleavage by ICE and were reported to be specific ICE inhibitors (Thornberry *et al.*, 1992). These tetrapeptides did not inhibit VP-16-induced DNA fragmentation in our cell-free system. Ac-YVAD-CHO also failed to inhibit apoptosis in VP-16-treated thymocytes (Fearnhead *et al.*, 1995a) and camptothecin-treated osteosarcoma cells (Nicholson *et al.*, 1995). Moreover, thymocytes from ICE–/– knockout mice remained sensitive to radiation-induced apoptosis (Kuida *et al.*, 1995). Altogether, these results suggest that ICE is not involved in apoptosis triggered by DNA damage. Conversely, ICE or an ICE-related protease is probably involved in Fas-mediated apoptosis that is strongly inhibited by Ac-YVAD-CHO and Ac-YVAD-CMK in our and other cell systems (Enari *et al.*, 1995b, 1996; Los *et al.*, 1995) and in thymocytes from ICE–/– mice (Kuida *et al.*, 1995).

Human PARP is one of the specific targets of apoptosis-associated proteolysis (Kaufmann *et al.*, 1993). Most ICE-like proteases have been shown to cleave this 116 kDa protein and to generate two fragments of 85 and 29 kDa. The tetrapeptide aldehyde Ac-DEVD-CHO, which contains the P1–P4 amino acid sequence of the PARP cleavage

Table II. Effect of protease inhibitors on VP-16-induced DNA fragmentation and PARP cleavage

Protease class	Protease inhibitors	Inhibition of DNA fragmentation (%)[a]		PARP cleavage inhibition[d]
		Whole cells[b]	Extracts[c]	
Cysteine	CI I (50 μM)	96.1 ± 2.0[e]	NS	+
Cysteine	CI II (50 μM)	50.8 ± 6.7[f]	NS	+
Serine	TPCK (0.2 mM)	87.0 ± 5.3[e]	NS	+
Serine	PMSF (1 mM)	78.2 ± 8.1[e]	50.0 ± 14.2[f]	+
Cysteine	Thimerosal (15 μM)	91.7 ± 4.7[e]	35.6 ± 1.7[e]	–
Serine	Dichloroisocoumarin (50 μg/ml)	95.0 ± 2.9[e]	47.5 ±14.4[g]	–
Cysteine	Antipain (up to 10 μg/ml)	NS	NS	
Cysteine	Aprotinin (0.15 IU/ml)	NS	NS	–
Cysteine	E-64 (up to 20 μg/ml)	NS	NS	
Serine	α1-Antichymotrypsin (up to 7 μg/ml)	NS	NS	
Serine	Eglin (up to 3.5 μg/ml)	NS	NS	
Aspartyl	Pepstatin (1 μg/ml)	NS	NS	
Metallo	Phosphoramidon (up to 10 μg/ml)	NS	NS	

NS, not significant (Student's *t*-test).
[a]^{14}C-labeled nuclei from untreated U937 cells were incubated for 30 min at 37°C in the presence of Triton-soluble extracts from U937 cells treated for 3 h with 50 μM VP-16. DNA fragmentation was measured by a filter elution assay. Results are expressed as the percentage of inhibition of DNA fragmentation induced by extracts from U937 cells treated with VP-16 alone. Each value is the mean ± SD of three independent experiments performed in triplicate.
[b]Cells were co-treated with VP-16 and protease inhibitors before Triton-soluble extract preparation and incubation with untreated cell nuclei.
[c]Protease inhibitors were added to Triton-soluble extracts from VP-16-treated cells before incubation with nuclei from untreated cells.
[d]Cells were co-treated with VP-16 and protease inhibitors and PARP cleavage was analyzed by Western blotting.
[e]$P < 0.004$.
[f]$P < 0.01$.
[g]$P < 0.04$.

site (DEVD^{216}-G^{217}), is a potent competitive inhibitor of PARP breakdown (Nicholson *et al.*, 1995; Schlegel *et al.*, 1996). In our cell-free system, Ac-DEVD-CHO prevented DNA fragmentation induced in untreated nuclei by Triton-soluble extracts from both VP-16- or anti-Fas antibody-treated U937 cells. ICE and most ICE-related-proteases are able to cleave PARP *in vitro* (Fernandez-Alnemri *et al.*, 1995a,b; Gu *et al.*, 1995; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). However, high intracellular concentrations of ICE, Mch-2, Tx and Nedd-2 were required to cleave the PARP (Gu *et al.*, 1995). CPP32 and Mch-3 kinetic properties suggested that these proteases were the most sensitive to competitive inhibition by Ac-DEVD-CHO (Fernandez-Alnemri *et al.*, 1995b). Inhibition of both PARP cleavage (Nicholson *et al.*, 1995) and apoptotic DNA fragmentation (present study) with the tetrapeptide Ac-DEVD-CHO suggests that the protease(s) sensitive to this inhibitor is necessary for the two events to occur during apoptotic cell death. Hence, we have analyzed the expression of CPP32 protein in U937 cells treated with VP-16 and anti-Fas antibodies.

In VP-16-treated U937 cells, apoptotic DNA fragmentation and PARP cleavage were associated with a decrease in the p32 CPP32 proenzyme level without significant changes in Ich-1L protein expression. Decreased expression of the proenzyme in apoptotic cells was related to its cleavage into p20 and p17 active subunits (Schlegel *et al.*, 1996). Similar observations were made during Fas-mediated apoptosis of U937 cells, suggesting that CPP32 activation is an event common to DNA damage-induced and Fas-mediated apoptosis in these cells.

Although some ICE-related proteases are capable of autoprocessing, the autoprocessing ability of CPP32 appears to be limited (Fernandez-Alnemri *et al.*, 1994). The protease granzyme B mediates T-cell cytotoxic activity by cleaving CPP32 proenzyme (Darmon *et al.*, 1995). ICE is also capable of processing CPP32 to its active form, although the reverse is not true (Tewari *et al.*, 1995). As both ICE and CPP32 seem to be involved in Fas-mediated apoptosis, it is tempting to speculate that ICE is responsible for CPP32 activation in U937 cells treated with anti-Fas antibodies. This hypothesis was confirmed recently in mouse lymphoma cells (Enari *et al.*, 1996). However, in our cell-free system, Ac-YVAD-CHO prevented Fas-mediated apoptotic DNA fragmentation when CPP32 was already activated. Two hypotheses could account for this observation. The half-life of active CPP32 could be very short, and the inhibition of a YVAD-sensitive protease could prevent further activation of the DEVD-sensitive protease. An alternate hypothesis could be that both YVAD- and DEVD-sensitive proteases are necessary for apoptosis to occur in Fas-treated cells, while YVAD-sensitive protease(s) are not required for VP-16-triggered apoptosis.

The effect of various serine and cysteine protease inhibitors on CPP32 expression, PARP cleavage and apoptotic DNA fragmentation in whole cells and the cell-free system allowed us to propose a model for the pathway that mediates VP-16-induced apoptotic DNA fragmentation in U937 cells (Figure 9). According to this model, the first steps of this pathway are inhibited by TPCK, calpain inhibitors and PMSF, all of which prevent a decrease in the level of CPP32 proenzyme, PARP cleavage and apoptotic DNA fragmentation. Accordingly, TPCK was reported to inhibit early stages of the apoptotic pathway triggered by various stimuli in thymocytes (Fearnhead *et al.*, 1995b), and calpain was involved in tumour necrosis factor-mediated apoptosis of U937 cells (Kikuchi and Imajoh-Ohmi, 1995). Whether TPCK and calpain inhibitors influence DNA–protein crosslink induction by VP-16 or act downstream in the apoptotic pathway remains to be determined. Conversely, dichloroiso-

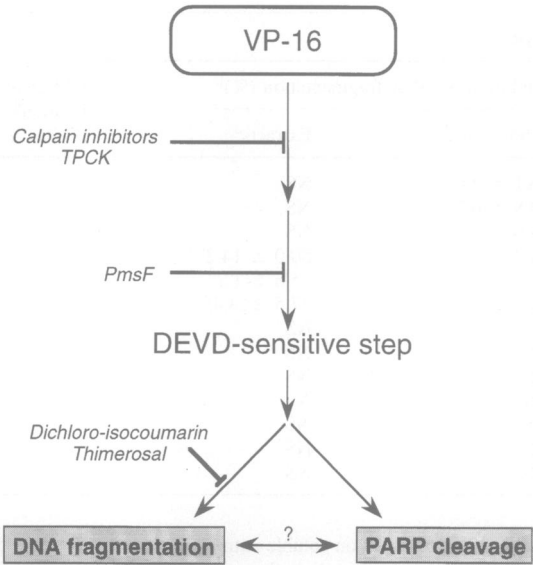

Fig. 9. Proposed model for the proteolytic cascade involved in VP-16-mediated apoptotic DNA fragmentation.

coumarin (DCI) and thimerosal inhibitory effects defined late steps of the apoptotic pathway in VP-16-treated U937 cells because they inhibited apoptotic DNA fragmentation without preventing a decrease in the level of CPP32 proenzyme or PARP cleavage.

In conclusion, our study indicates that the final pathway of VP-16-induced apoptosis in U937 leukemic cells involves a proteolytic cascade which leads to apoptotic DNA digestion at neutral pH in the presence of Mg^{2+}. A DEVD-sensitive protease, which could be CPP32, appears to play a pivotal role in this cascade. Interestingly, the DEVD-sensitive step also appears to be involved in Fas-mediated apoptosis of U937 cells. The sensitivity of malignant cells to cytotoxic agents has been shown recently to be increased by combining a drug and an anti-Fas antibody (Morimoto *et al.*, 1993). The identification of molecules that could modulate DEVD-sensitive protease(s) activity should increase the ability of VP-16 to induce its cytotoxic effect.

Materials and methods

Drug and chemicals

The specific ICE inhibitors Ac–Tyr–Val–Ala–Asp aldehyde (Ac-YVAD-CHO) and Ac–Tyr–Val–Ala–Asp chloromethylketone (Ac-YVAD-CMK) were synthetized by Neosystem Laboratory (Strasbourg, France) and Bachem AG (Bubendorf, Switzerland), respectively. The specific CPP32/apopain inhibitor Ac–Asp–Glu–Val–Asp–CHO (Ac-DEVD-CHO) was obtained from Bachem AG. Calpain inhibitors I (CI I: *N*-acetyl-leu-leu-norleucinal) and II (CI II: *N*-acetyl-leu-leu-methioninal) were purchased from Boehringer (Mannheim, Germany). Other protease inhibitors (PI), $ZnCl_2$, aurintricarboxylic acid, CHX and VP-16 were obtained from Sigma-Aldrich Laboratories (St Quentin Fallavier, France). Anti-human Fas monoclonal antibody (IgM, clone CH-11) was purchased from Biovalley (Medical and Biological Laboratories, Conches, France). 2-[^{14}C]thymidine (50 mCi/mmol) was obtained from Amersham (Les Ulis, France).

Cell culture, morphological studies and drug treatment

The human monocytic leukemic cell line U937 was grown in suspension in RPMI 1640 medium (BioWhittaker, Fontenay-sous-bois, France) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2 mM L-glutamine in an atmosphere of 95% air and 5% CO_2. Cell viability was determined using the trypan blue exclusion assay. For morphological examination, cells were stained with Hoechst 33 342 (5 μg/ml) for 30 min at 37°C, washed twice with PBS, pipetted dropwise onto a glass slide and examined by fluorescence microscopy using a Leitz microscope (Leica, Bron, France) equipped with an epi-illuminator and appropriate filters. VP-16 stock solution (20 mM), prepared by diluting the drug in dimethyl sulfoxide (DMSO), was conserved at –20°C for <1 month. The final concentration of DMSO in the culture medium never exceed 1% (v/v), which was nontoxic to the cells. Cells were resuspended at a density of 1.5×10^6/ml in fresh medium before treatment.

Reconstituted cell-free system

Triton-soluble extracts and nuclear fractions were prepared as described previously (Solary *et al.*, 1993). Cells were washed twice in 10 ml ice-cold PBS without Ca^{2+} and Mg^{2+}, and once in a buffer containing 150 mM NaCl, 1 mM KH_2PO_4, 1 mM EGTA, 1 mM Na_3VO_4, 5 mM $MgCl_2$ and 10% glycerol. The pH of the buffer was adjusted to 7.2 with HEPES buffer. Then the cells were incubated for 10 min on ice at a density of 1.0×10^7 cells/ml in the same buffer containing 0.3% Triton X-100 before centrifugation (2000 *g* for 10 min at 4°C). The supernatants collected were considered as Triton-soluble extracts. Protein concentration in the supernatants was determined using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985). Pellets (nuclei) were washed twice in the lysis buffer without Triton X-100. Triton-soluble extracts (500 μl) from untreated or treated cells were then incubated for 30 min at 37°C in the presence of nuclei from 1.0×10^6 untreated cells. When indicated, either VP-16 or protease and nuclease inhibitors were added to the Triton-soluble extracts before incubation with nuclei. Controls were performed by incubating nuclei from untreated cells with Triton-soluble extracts from untreated cells in the absence or presence of 100 μM VP-16.

Quantification of DNA fragmentation

DNA fragmentation was measured using a previously reported filter elution assay (Bertrand *et al.*, 1995). Exponentially growing cells were prelabeled by adding 0.02 μCi/ml 2-[^{14}C]thymidine in the culture medium for 2 days. Then cells were chased in isotope-free medium overnight. Approximately 1.0×10^6 ^{14}C-labeled cells or ^{14}C-labeled nuclei were loaded onto a protein absorbing filter (polyvinylidene fluorure filters, 0.65 μm pore size, 25 mm diameter; Durapore membrane, Millipore, St Quentin, France). Cells or nuclei were then washed once with 5 ml ice-cold PBS. Lysis was subsequently performed with 5 ml LS10 buffer (0.2% sodium sarkosyl, 2 M NaCl, 0.04 M EDTA, pH 10.0). Filters were washed with 7 ml 0.02 M EDTA, pH 10.0. DNA was depurinated by adding 0.4 ml 1 N HCl at 65°C for 45 min, and then released from the filters by adding 2.5 ml 0.4 N NaOH for 45 min at room temperature. Radioactivity was counted by liquid scintillation spectrometry in each fraction (wash, lysis, EDTA wash and filter). DNA fragmentation was measured as the fraction of d.p.m. in the lysis fraction plus EDTA wash relative to the total intracellular d.p.m.

Analysis of DNA fragmentation by agarose gel electrophoresis

Cellular DNA from whole cells or isolated nuclei was extracted by a salting-out procedure as described previously (Miller *et al.*, 1988). Electrophoresis was performed in a 1.8% agarose gel in Tris–borate–EDTA buffer (pH 8.0) at 20 V for 15 h. After electrophoresis, DNA was visualized by ethidium bromide staining.

Western blot analysis

After treatment, cells were washed twice in PBS, lysed in lysis buffer (150 mM NaCl, 1 mM KH_2PO_4, 1 mM EGTA, 1 mM Na_3VO_4, 5 mM $MgCl_2$ and 10% glycerol containing 0.1 mM PMSF, 0.15 U/ml aprotinin, 1 μg/ml pepstatin), and then centrifuged (15 min, 15 000 *g*). 50 μg protein from the supernatants were separated by SDS–PAGE using a 12% polyacrylamide gel and electroblotted to PVDF membrane (Bio-Rad, Ivry sur Seine, France). After blocking nonspecific binding sites overnight by 5% nonfat milk in TPBS (PBS, Tween 20 0.1%), the membrane was incubated for 2 h at room temperature with anti-human CPP32 or anti-human Ich-1L monoclonal antibody (Transduction Laboratories, Lexington, KY). It was then washed twice with TPBS, incubated further with horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature, and then washed twice with TBPS. The immunoblot was revealed using an enhanced chemiluminescence detection kit (Amersham) by autoradiography.

For the PARP and CPP32 cleavage analyses, cells were lysed in SDS–PAGE sample buffer (125 mM Tris–HCl, pH 6.8, 10% β-mercapto-

ethanol, 4.6% SDS, 20% glycerol and 0.003% bromophenol blue) and cell lysates were subjected to SDS–PAGE on an 8% polyacrylamide gel. The 116 kDa native PARP protein and its 85 kDa cleavage product were detected by immunoblotting with anti-human PARP polyclonal antibody (Vic. 5, kindly given by Dr G.De Murcia) and horseradish peroxidase-conjugated anti-rabbit antibody (Amersham), as described above. A rabbit polyclonal anti-apopain/CPP32-p17 antibody (kindly provided by Dr D.Nicholson), which recognizes both CPP32 proenzyme and its p20 and p17 subunits (Schlegel *et al.*, 1996), was used to detect CPP32 activation in apoptotic U937 cells.

Acknowledgements

The authors thank François Martin and Christopher Goldring for helpful discussions, Gilbert de Murcia for providing the anti-PARP antibody and Donald W.Nicholson (Merck Center for Therapeutic Research) for the anti-CPP32/p17 antibody. This work was supported by grants from the Ligue Bourguignonne Contre le Cancer and the Association pour la Recherche sur le Cancer (ARC # 6808). L.D. received fellowship support from the Ligue Nationale Contre le Cancer.

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Received on February 26, 1996; revised on July 2, 1996