RESEARCH COMMUNICATION

Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP32

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Interleukin-1β converting enzyme (ICE)-like proteases, which are synthesized as inactive precursors, play a key role in the induction of apoptosis. We now demonstrate that benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK), an ICE-like protease inhibitor, inhibits apoptosis by preventing the processing of CPP32 to its active form. These results suggest that novel inhibitors of apoptosis can be developed which prevent processing of proforms of ICE-like proteases.

INTRODUCTION

Apoptosis is a major form of cell death important for the control of cell populations during normal development and in certain diseases [1,2]. Various aspects of the apoptotic process are conserved between the nematode and man. In *Caenorhabditis elegans* two genes, *ced-3* and *ced-4*, are critical for cell death [3,4]. *Ced-3* encodes a protein related to mammalian interleukin-1β converting enzyme (ICE) [3,4], a member of a new class of cysteine proteases with a marked specificity for aspartic acid in the P_1 position [5,6]. Currently there are seven known members of this family: ICE, prICE/CPP32/Yama/apopain, ICE_{rel}II/Tx/Ich-2, ICE_{rel}-III, Ich-1/Nedd 2, Mch-2 and Mch-3 [3,5,7–17]. Overexpression of these proteases results in apoptosis [7,10–15]. Studies with ICE knockout mice suggest that ICE itself is not apparently required for apoptosis except in Fas-induced apoptosis in thymocytes [18,19]. These results suggest that either there is redundancy amongst the proteases or that an ICE homologue(s) other than ICE is of critical importance in apoptosis [20]. These proteases are synthesized as inactive precursors requiring cleavage at specific Asp residues to two subunits of approximate molecular masses 20 and 10 kDa, which together form the active enzyme [5,6]. As the cleavage occurs at Asp residues, it suggests that these proteases may autoprocess, or alternatively that some ICE-like proteases may activate other family members [6,7,9].

Apoptosis is characterized by a series of distinctive morphological and biochemical changes. For example the chromatin condenses and is cleaved initially into large kilobase pair fragments (200–300 and 30–50 kb pairs) followed by internucleosomal fragmentation [1,21–23]. Proteolysis of poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair and maintenance of the genome, has been proposed as an early biochemical marker of apoptosis [16,24]. PARP is cleaved at an Asp residue (Asp-216–Gly-217) with differing efficiencies by several ICE-like proteases, including one termed prICE/CPP32/Yama/apopain [8,9,16] and also by Mch-2 [7], Mch-3 [17] and TX [25].

We have recently demonstrated that benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK), an inhibitor of ICE-like proteases, inhibits apoptosis in THP.1 cells induced by diverse stimuli [26] and Fas antigen-induced apoptosis in Jurkat T-cells [27]. In this study, we demonstrate that Z-VAD.FMK inhibits apoptosis by blocking the activation of proCPP32 into its active form, rather than by preventing the proteolytic action of CPP32 directly.

MATERIALS AND METHODS

Chemicals

Acetyl Asp-Glu-Val-Asp aldehyde (Ac-DEVD-CHO), [^{35}S]PARP and rabbit polyclonal antibodies to CPP32 were from Merck Frosst, Quebec, Canada. Acetyl Tyr-Val-Ala-Asp aldehyde (Ac-YVAD-CHO) was obtained from Bachem, Switzerland, and Z-VAD.FMK was from Enzyme Systems (Dublin, CA, U.S.A.). 1-Tosylamido-2-phenylethylchloromethyl ketone (TLCK) was purchased from Boehringer-Mannheim U.K. (Lewes, U.K.) and the Fas monoclonal antibody (IgM, clone CH-11) was from TCS Biologicals (Bucks., U.K.).

Cell culture and treatments

The human monocytic tumour cell line, THP.1, was obtained from ECACC (Wiltshire, U.K.) and the leukaemic T-cell line, Jurkat (clone E-6), was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The cell lines were maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 in air at 37 °C. The cells were maintained in logarithmic growth phase by

Abbreviations used: ICE, interleukin-1-converting enzyme; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; Ac-YVAD-CHO, acetyl Tyr-Val-Ala-Asp aldehyde; Ac-DEVD-CHO, acetyl Asp-Glu-Val-Asp aldehyde; PARP, poly(ADP-ribose) polymerase; TLCK, 1-tosylamido-2-phenylethylchloromethyl ketone; DTT, dithiothreitol.

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routine passage every 2–3 days. To induce apoptosis in THP.1 cells, 2×10^6 cells/ml were incubated either alone or in the presence of cycloheximide (25 μM) and TLCK (100 μM) as previously described [26]. In order to assess the possible effects of various ICE-like protease inhibitors, THP.1 cells were also pretreated for 1 h with Z-VAD.FMK (10 μM), Ac-DEVD-CHO (20 μM) and Ac-YVAD-CHO (20 μM) before being exposed to the apoptotic stimulus. To induce apoptosis in Jurkat cells, 2×10^6 cells/ml were stimulated with 200 ng/ml anti-human Fas as described previously [27].

Preparation of cell lysates from THP.1 and Jurkat cells

For preparation of lysates from THP.1 cells, cells were incubated as required and then placed on ice, washed twice with ice-cold RPMI 1640 without serum and resuspended in Pipes buffer [50 mM Pipes/KOH (pH 6.5), 2 mM EDTA, 0.1 % (w/v) 3-[(3-cholamideopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonic acid (CHAPS), 5 mM dithiothreitol (DTT), 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 2 mM PMSF) at a concentration of 6×10^6 cells/10 μl. The cells were frozen and thawed three times in liquid nitrogen and then centrifuged for 30 min at 20000 ***g***. The supernatant fraction was then centrifuged for a further 30 min at 100000 ***g***. Lysates from anti-Fas-stimulated Jurkat cells were prepared as previously described [27]. The protein concentration in the supernatant fractions (the lysate) was determined by the Bradford assay (Bio-Rad).

Apoptosis assessed by flow cytometry

Apoptosis was assessed by flow cytometry using Hoechst 33342 as previously described [26,28]. These cells have been shown to be apoptotic based on a number of criteria including ultrastructure and DNA analysis.

Immunoblot detection of CPP32

THP.1 and Jurkat T-cells were stimulated to undergo apoptosis with apoptotic stimuli and Fas antibodies respectively, and samples of 10^6 cells were prepared for SDS/PAGE as described [29]. Cellular proteins were resolved on an SDS/12 %-polyacrylamide gel under denaturing conditions and blotted onto nitrocellulose membrane. The blotted proteins were probed with rabbit polyclonal antibodies directed to the p17 subunit of CPP32 followed by chemiluminescence detection [30].

[^{35}S]PARP cleavage assay

Proteolysis of PARP in cell lysates was measured by the ability of the lysate to cleave [^{35}S]PARP to a 24 kDa fragment as previously described [8]. Briefly, cell lysates containing 10 μg of protein in 20 μl of Pipes buffer were incubated for 30 min at 37 °C with 5 μl of [^{35}S]PARP (128 Bq/μl) in the presence or absence of inhibitors as indicated. The reactions were stopped by the addition of Laemmli buffer containing 2-mercaptoethanol and SDS. After boiling for 5 min, the samples were separated on an SDS/10 %-polyacrylamide gel. The gel was fixed for 10 min in a mixture of glacial acetic acid/methanol/water (1:2:7, by vol.), followed by soaking for 20 min in Enlightning Fluorographic Enhancer (Dupont). The gel was then dried and exposed to autoradiography film.

RESULTS AND DISCUSSION

Ac-DEVD-CHO but not Z-VAD.FMK is a potent inhibitor of PARP protease activity in cell lysates derived from cells undergoing apoptosis

We have previously shown that the induction of apoptosis in THP.1 cells by various stimuli can be enhanced by co-treatment with the trypsin-like protease inhibitor, TLCK [26]. Induction of apoptosis in these cells, assessed by morphological, flow cytometric and biochemical criteria, including the proteolysis of PARP and cleavage of chromatin into large kilobase pair fragments was completely abrogated by the ICE-like protease inhibitor, Z-VAD-FMK [26]. This compound is also a good inhibitor of Fas-induced apoptosis in Jurkat cells [27] and apoptosis induced by various stimuli in thymocytes and hepatocytes [31,32], suggesting that its target(s) may be a common mediator of apoptosis in diverse systems.

To investigate the nature of this target, we first examined whether cell lysates derived from THP.1 cells treated with cycloheximide and TLCK contained PARP protease or CPP32-like activity, which appears to be necessary for apoptosis [8,16]. Cell lysates derived from treated but not control THP.1 cells readily cleaved [^{35}S]PARP, forming the 24 kDa product [Figure 1 (top), lanes 1 and 2], as previously described [8]. This PARP protease activity was detected within 30 min of exposure to an apoptotic stimulus (results not shown) and was totally inhibited by low concentrations of Ac-DEVD-CHO [Figure 1 (top), lanes 3–6], a specific inhibitor of CPP32, which is in good agreement with previous studies [8]. As this compound is not cell permeable [8], low concentrations had relatively little effect on apoptosis (Table 1) and high concentrations (> 100 μM) were required to

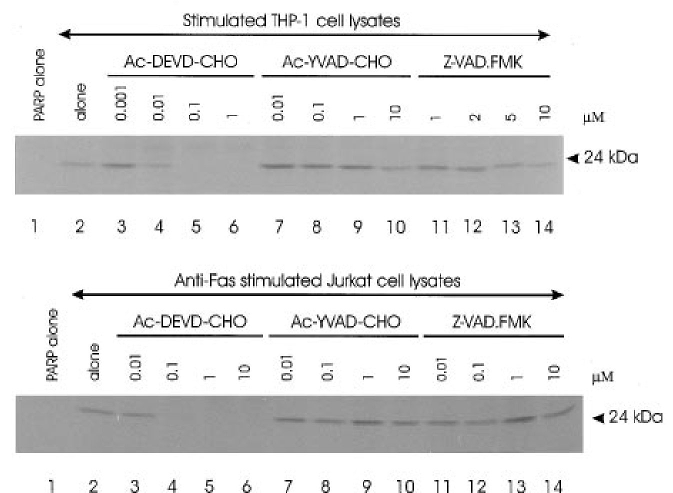

Figure 1 Ac-DEVD-CHO but not Z-VAD.FMK or Ac-YVAD-CHO inhibits [^{35}S]PARP proteolysis by lysates from THP.1 and Jurkat cells

Top: lysates (10 μg of protein) from THP.1 cells incubated for 1 h in the presence of cycloheximide and TLCK were incubated with [^{35}S]PARP at 37 °C for 30 min, either alone (lane 2) or with Ac-DEVD-CHO (lanes 3–6), Ac-YVAD-CHO (lanes 7–10) or Z-VAD.FMK (lanes 11–14). Lane 1 is from an incubation of [^{35}S]PARP alone. Samples were resolved by SDS/PAGE and the formation of the 24 kDa cleavage product was visualized by autoradiography [8]. Bottom: lysates from Jurkat cells treated with Fas antibody were prepared as described in the Materials and methods section and incubated with [^{35}S]PARP at 37 °C for 30 min with inhibitors as in the top panel. PARP protease was inhibited by Ac-DEVD-CHO (lanes 3–6) but not by Ac-YVAD-CHO (lanes 7–10) or Z-VAD.FMK (lanes 11–14).

Table 1 Z-VAD.FMK but not Ac-DEVD-CHO or Ac-YVAD-CHO inhibits apoptosis in THP.1 cells

THP.1 cells (2×10^6 cells/ml) were incubated for 1 h either alone or in the presence of the indicated concentrations of Z-VAD.FMK, Ac-YVAD-CHO or Ac-DEVD-CHO. Apoptosis was then induced by incubation of the cells for 4 h with cycloheximide (CHX; 25 μM) together with TLCK (100 μM). Apoptosis was quantified by flow cytometry as described in the Materials and methods section. Results are the means ± S.E.M. of at least three determinations except * where the values are the means of two determinations. Similar results were obtained when apoptosis was induced by etoposide.

	Apoptosis (%)
Control	1.8 ± 1.0
Z-VAD.FMK (10 μM)	2.1 ± 1.5
Ac-YVAD-CHO (20 μM)	4.7*
Ac-DEVD-CHO (20 μM)	4.2*
CHX + TLCK	52.0 ± 1.4
CHX + TLCK + Z-VAD.FMK	1.5 ± 0.4
CHX + TLCK + Ac-YVAD-CHO	32.5*
CHX + TLCK + Ac-DEVD-CHO	38.9*

have any noticeable effect on apoptosis (results not shown). Ac-YVAD-CHO (10 μM), a specific cell-permeable ICE inhibitor [5], had relatively little effect on apoptosis in intact THP.1 cells (Table 1), nor did it affect the formation of the 24 kDa PARP product [Figure 1 (top), lanes 7–10]. In marked contrast to its complete inhibition of apoptosis and PARP proteolysis in intact THP.1 cells [26] (Table 1), Z-VAD.FMK was a poor inhibitor of PARP protease activity in cell lysates [Figure 1 (top), lanes 11–14]. Our results suggested that Z-VAD.FMK was not inhibiting CPP32 activity but was blocking apoptosis either by inhibiting the processing of CPP32 or at some earlier stage. To further corroborate these findings, cell lysates, which have been shown to possess apoptotic nuclei promoting activity, were prepared from Fas-treated Jurkat cells [27]. Z-VAD.FMK (10 μM) is a potent inhibitor of Fas-induced apoptosis, assessed by its ability to inhibit both morphological and biochemical changes associated with apoptosis [27]. As illustrated in Figure 1, bottom (lane 2), lysates derived from Fas-treated Jurkat cells also contained PARP protease activity, and this activity was potently inhibited by Ac-DEVD-CHO [Figure 1 (bottom), lanes 3–6], but not by Ac-YVAD-CHO [Figure 1 (bottom), lanes 7–10] or Z-VAD.FMK [Figure 1 (bottom), lanes 11–14]. Thus the inhibition of apoptosis by Z-VAD-FMK in Jurkat and THP.1 cells was not due to the inhibition of PARP protease as such, but rather due to inhibition of the activation of CPP32 or at some earlier stage.

Z-VAD.FMK inhibits activation of PARP protease activity in control cell lysates

Previous studies [8] have shown that the incubation of control THP.1 cell lysates at 37 °C in the presence of DTT for 1 h activates the processing of CPP32 and resulted in detectable PARP protease activity. In good agreement with Nicholson et al. [8], the 24 kDa PARP product was formed when [^{35}S]PARP was incubated with control THP.1 cell lysates which were pre-incubated for 1 h at 37 °C (Figure 2, lane 1). Using this approach we examined the effect of Z-VAD.FMK on the activation of this PARP protease activity in control THP.1 cell lysates. Activation of this PARP protease activity, which presumably results from processing of CPP32, and possibly other ICE homologues, was prevented by preincubation of control cell lysates with Z-VAD.FMK (Figure 2, lanes 6–8). Similar concentrations of

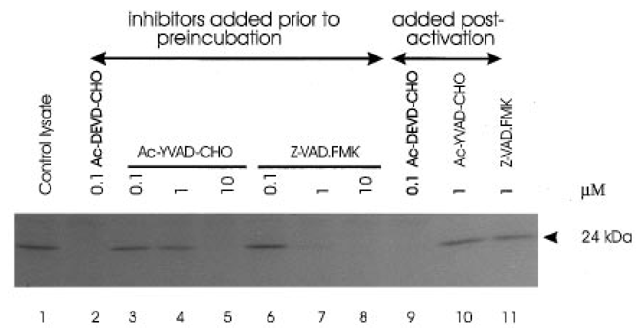

Figure 2 Z-VAD.FMK inhibits activation of PARP protease activity in control cell lysates

Lysates (30 μg of protein) from control THP.1 cells were preincubated with DTT for 1 h at 37 °C either alone (lanes 1, 9–11) or with Ac-DEVD-CHO (100 nM) (lane 2), Ac-YVAD-CHO (100 nM–10 μM) (lanes 3–5) or Z-VAD.FMK (100 nM–10 μM) (lanes 6–8). In some reactions (lanes 9–11), inhibitors were added after this initial 1 h preincubation. All lysates were then further incubated at 37 °C with [^{35}S]PARP for 30 min and PARP cleavage was determined as described in Figure 1. The 24 kDa cleavage product from the autoradiograph is shown. Preincubation of control THP.1 cell lysates resulted in activation of CPP32-dependent PARP protease activity (lane 1). Z-VAD.FMK (1 μM) inhibited this activation when preincubated with lysates (lane 7) but not when added following a 1 h incubation (lane 11).

Z-VAD.FMK did not inhibit PARP protease activity once activated (Figure 2, lane 11), further demonstrating that the Z-VAD.FMK target was upstream of CPP32 and was involved directly or indirectly in the processing of CPP32. Ac-YVAD-CHO (10 μM) (Figure 2, lane 5) also inhibited this activation but required higher concentrations than Z-VAD.FMK, suggesting that a Z-VAD.FMK-inhibitable ICE-like protease other than ICE may be involved in the activation of CPP32 (Figure 2, lanes 3–5). Although our data with Ac-YVAD-CHO, both in intact cells (Table 1) and in cell lysates (Figures 1 and 2), do not support a major role for ICE in the induction of apoptosis in THP.1 cells, we cannot totally exclude an involvement of this enzyme. We cannot discern whether the inhibition of PARP proteolysis by Ac-DEVD-CHO (Figure 2, lane 2) was due to inhibition of the processing of CPP32 or by direct inhibition of CPP32 PARP protease activity (Figure 2, lane 9).

Z-VAD.FMK blocks the processing of CPP32

In order to obtain more supporting evidence for the hypothesis that Z-VAD.FMK was inhibiting processing of CPP32, we used polyclonal antibodies directed against the p17 subunit. The CPP32 proenzyme comprises a short N-terminal pro-domain followed by p17 and p12 subunits, with Asp residues present at both the pro-domain/p17 and the p17/p12 junctions [8,12]. In THP.1 cells exposed to an apoptotic stimulus, CPP32 was cleaved at both these junctions to yield the p17 subunit [Figure 3 (top), lane 2]. This was accompanied by a slight decrease in intact CPP32 compared with control cells [compare lanes 1 and 2 in Figure 3 (top)]. Similar results were obtained following exposure of THP.1 cells to other apoptotic stimuli such as etoposide (results not shown). These results were consistent with the activation of CPP32 during apoptosis. Z-VAD.FMK clearly inhibited the processing of CPP32 to the p17 subunit [Figure 3 (top), lane 3] under identical conditions where it inhibited apoptosis (Table 1). In Jurkat cells induced to undergo apoptosis by Fas antibody, a marked decrease in intact CPP32 was observed after 60 min [Figure 3 (bottom), lane 2], together with the corresponding appearance of a 17 kDa product [Figure 3 (bot-

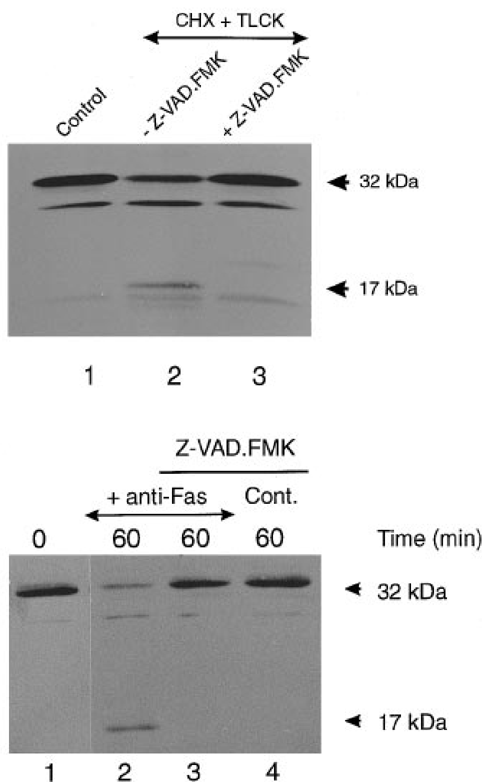

Figure 3 Z-VAD.FMK inhibits the processing of CPP32 in intact THP.1 and Jurkat cells

Top: THP.1 cells were incubated for 4 h either alone (lane 1) or with cycloheximide and TLCK in the presence (lane 3) or absence (lane 2) of Z-VAD.FMK (10 μM) as described in the Materials and methods section. On induction of apoptosis, intact CPP32 in control THP.1 cells (lane 1) was cleaved to a 17 kDa product (lane 2), which was inhibited by Z-VAD.FMK (lane 3). Bottom: Jurkat cells were treated with Fas antibody for 60 min either alone (lane 2) or in the presence of Z-VAD.FMK (10 μM) (lane 3). Jurkat cells were also incubated either alone or in the presence of Z-VAD.FMK (lanes 1 and 4). CPP32 was detected by Western blotting, as described in the Materials and methods section.

tom), lane 2], which is indicative of CPP32 processing occurring in Jurkat cells exposed to anti-Fas antibody. Both the loss of pro CPP32 and the appearance of the 17 kDa product were completely inhibited by Z-VAD.FMK (10 μM) [Figure 3 (bottom), lane 3] under conditions where it completely inhibited apoptosis [27]. Z-VAD.FMK alone did not have any detectable effect on CPP32 [Figure 3 (bottom), lane 4].

Taken collectively, our present results demonstrate that Z-VAD.FMK inhibits apoptosis not by blocking CPP32 activity as such, but by inhibiting the activation of CPP32, presumably by blocking the process leading to the activation of CPP32 or by interfering directly with the processing of CPP32. Recently it has also been shown that the cytoxic T-lymphocyte-specific serine protease granzyme B cleaves and activates CPP32, leading to PARP cleavage and apoptosis [30]. Thus, in different systems, induction of apoptosis is accompanied by processing and activation of CPP32. This is the first demonstration that inhibition of apoptosis may be accomplished by inhibiting the processing of proforms of ICE-like proteases, such as CPP32, that are activated in apoptosis. Whether Z-VAD.FMK can inhibit the activation of other ICE homologues is not known. Z-VAD.FMK is a prototype of a molecule which blocks processing of CPP32, thus explaining its ability to inhibit apoptosis in diverse systems [26,27,31,32]. Our results highlight the possibility of developing novel therapeutic agents based on this action, which would prevent the activation of proforms of members of the ICE family.

Received 1 February 1996; accepted 10 February 1996

We thank R. Snowden for help with the flow cytometry. E. A. S. is the recipient of an MRC studentship.

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