# An interleukin-1β-converting enzyme-like protease is a common mediator of apoptosis in thymocytes

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Received 13 October 1995

**Abstract Apoptosis was induced in thymocytes using diverse stimuli in order to identify events within a common apoptotic pathway. Benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone (Z-VAD.FMK), an interleukin-1β-converting enzyme (ICE)-like protease inhibitor, inhibited apoptosis assessed by flow cytometry, proteolysis of poly (ADP)-ribose polymerase (PARP), an early biochemical marker of apoptosis, and cleavage of DNA to both large kilobase pair fragments (30–50 and 200–300 kbp) and to nucleosomal fragments. Z-VAD.FMK also blocked all the classical ultrastructural features of apoptosis including chromatin condensation to one pole of the nucleus, nucleolar disintegration and cytoplasmic vacuolation. These results suggest the involvement of an ICE-like protease as a common mediator of apoptosis in thymocytes.**

*Key words:* Poly (ADP)-ribose polymerase; Z-VAD.FMK; Thymocyte; Apoptosis; ICE inhibitor

## 1. Introduction

There is a growing realisation that apoptosis acts as a counterbalance to cell proliferation and is of vital importance for normal development and tissue homeostasis [1]. Apoptosis is a common response in many different cell types elicited by diverse stimuli including chemicals, radiation and viruses [1–3]. To explain this, a model of apoptosis has been proposed in which disparate stimuli activate private signalling pathways, which converge on a single pathway characterised by a series of common morphological and biochemical changes [4]. Apoptosis has been divided into distinct stages using both genetic and biochemical criteria. Different genes are associated with the decision to die, the execution of a common death programme and the subsequent engulfment and degradation of the dying cells [5]. A series of common biochemical and morphological changes is associated with apoptosis [1,4,6] and these are well characterised in thymocytes [7,8]. Briefly, the cell shrinks and the chromatin condenses with an attendant cleavage of DNA [1,7]. Initially DNA is cleaved to 200–300 and 30–50 kilobasepair size fragments, which are then further degraded to produce oligonucleosomes and the typical DNA ladder pattern [9–12]. The cytoplasmic changes of apoptosis are characterised by dilatation of the endoplasmic reticulum [1].

Protein degradation has also been implicated in apoptosis in both invertebrate and mammalian experimental systems. In the nematode, *Caenorhabditis elegans*, the gene *ced-3* is essential for apoptosis and encodes a protein similar to the mammalian protease, interleukin-1β-converting enzyme (ICE) [13]. Further work has identified a family of *ced-3* related mammalian genes whose overexpression results in apoptosis [13–20] and one, *ich-1*$_L$, is expressed in thymocytes [17]. Thymocyte apoptosis is accompanied by calcium-dependent proteolysis and a role for calpain, a calcium-dependent neutral protease, has been suggested [21]. Protease inhibitors can prevent internucleosomal DNA cleavage in thymocytes [22], apoptosis in HL-60 cells [22,23] and T-cell receptor mediated cell death [24]. In addition introduction of exogenous proteases (trypsin, chymotrypsin and proteinase K) into cells induces chromatin condensation and DNA fragmentation characteristic of apoptosis [25]. We have recently shown in thymocytes that TLCK prevents apoptosis induced by diverse stimuli and acts at an early stage of the apoptotic process, prior to the formation of large kilobase pair fragments of DNA, suggesting that a TLCK-sensitive trypsin-like protease is required early in the apoptotic process [26]. Specific proteins, including histones, lamins, topoisomerases I and II, poly (ADP ribose)-polymerase (PARP) and U1 small nuclear ribonucleoprotein, are degraded during apoptosis [18,19,23,27,28]. Proteolysis of PARP has been proposed as an early marker of apoptosis [27,29]. Subsequent work has shown that cleavage of PARP is similar in nuclei undergoing chromatin condensation typical of apoptosis and in cells undergoing apoptosis [29]. Thus there is strong evidence that PARP is at least one of the cellular targets for an ICE-like protease (prICE) and its breakdown may be required for the chromatin condensation of apoptosis [18,29].

To ensure the events studied lie on a common pathway and are not specific to a particular agent, we have used several different apoptotic stimuli with diverse modes of action and assessed the effects of ICE-like protease inhibitors on apoptosis. The stimuli included dexamethasone, a glucocorticoid [7], etoposide, a DNA topoisomerase II inhibitor [9] and thapsigargin, an inhibitor of endoplasmic reticular $Ca^{2+}$-ATPase [30]. We have also used several criteria to assess apoptosis in order to ascertain at what stage(s) the various inhibitors are blocking the apoptotic process. Benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone (Z-VAD.FMK) possesses an aspartic acid residue in the P1 position and therefore inhibits ICE-like proteases [31,32]. We now demonstrate that in thymocytes Z-VAD.FMK inhibits the induction of apoptosis by diverse stimuli supporting the involvement of an ICE-like protease as a common mediator of thymocyte apoptosis.

## 2. Materials and methods

### 2.1. Preparation and incubation of thymocyte suspensions

Suspensions of thymocytes from immature male F344 rats (4–5 weeks old, 65–85 g) were prepared as described previously [33]. Thymocytes ($2 \times 10^7$ cells·ml$^{-1}$) were incubated in RPMI 1640 medium supplemented with 10% foetal bovine serum in a humidified incubator

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at 37°C under 5% $CO_2$ and 95% air. To induce apoptosis, thymocytes were incubated for up to 4 h with either dexamethasone (0.1 μM), etoposide (10 μM) or thapsigargin (50 nM). Thymocytes were preincubated with either Z-VAD.FMK or TLCK for 1 h prior to exposure to apoptotic stimuli.

*2.2. Apoptosis assessed by flow cytometry*

Cells ($1 \times 10^6$) were stained with Hoechst 33342 and propidium iodide [34] and analysis carried out using a Becton Dickinson Vantage flow cytometer with Lysis II software (Becton Dickinson). Viable apoptotic thymocytes were separated from normal cells based upon both smaller size and higher blue fluorescence with Hoechst 33342 [34]. The increased Hoechst staining reflects a change in membrane permeability and is quantified by flow cytometry [35]. Protease inhibitors were used at nontoxic concentrations as assessed by exclusion of propidium iodide.

*2.3. Field inversion and conventional gel electrophoresis*

Agarose plugs (100 μl) containing $1 \times 10^6$ cells were prepared and then subjected to FIGE as previously described [10]. Whole cells ($2 \times 10^6$) were loaded per lane and conventional electrophoresis carried out to detect DNA laddering [36].

*2.4. Detection of PARP proteolysis*

Cells ($5 \times 10^6$) were prepared for SDS-PAGE as previously described with some modifications [37]. Briefly, $5 \times 10^6$ cells were lysed in sample buffer (62.5 mM Tris, 4 M urea, 1 mM EDTA, 1 mM phenylmethanesulfonylfluoride, 2% SDS, 5% β-mercaptoethanol and 0.005% bromophenol blue pH 6.8), sonicated and boiled for 3 min. Proteins were resolved on a 7% SDS-polyacrylamide gel, transferred onto nitrocellulose and Western blotting carried out using rabbit antiserum (318) to PARP (diluted 1:8000 in Tris buffered saline 0.1% Tween 20, pH 7.4). The antibody to PARP was a gift from Dr. G. Poirier Quebec, Canada. Detection was achieved by using a secondary antibody (goat anti-rabbit IgGs) conjugated to horseradish peroxidase (diluted 1:2000 in Tris buffered saline, 0.1% Tween 20, pH 7.4) and an ECL detection kit (Amersham Life Science, UK).

*2.5. Electron microscopy*

Cells ($2 \times 10^6$) were fixed and prepared as previously described [38] and examined in a Zeiss 902A electron microscope.

*2.6. Materials*

All media and serum were from Gibco (Paisely UK). Pronase and TLCK were from Boehringer Mannheim UK (Lewes, UK). Z-VAD.FMK was from Enzyme Systems Inc (Dublin, CA, USA). *N*-Acetyl tyrosinyl-valinyl-alaninyl-aspartyl chloromethyl ketone was from Bachem (Bubendorf, Switzerland). YVAD.CHO and benzyloxycarbonyl-valinyl-alaninyl-δ-ethylaspartyl- (2,6-dichlorobenzoyl-oxy) methyl ketone were provided by Professor L. Rubin (Eisai Laboratories, London, UK). All other chemicals were from Sigma Chemical Company (Poole, UK).

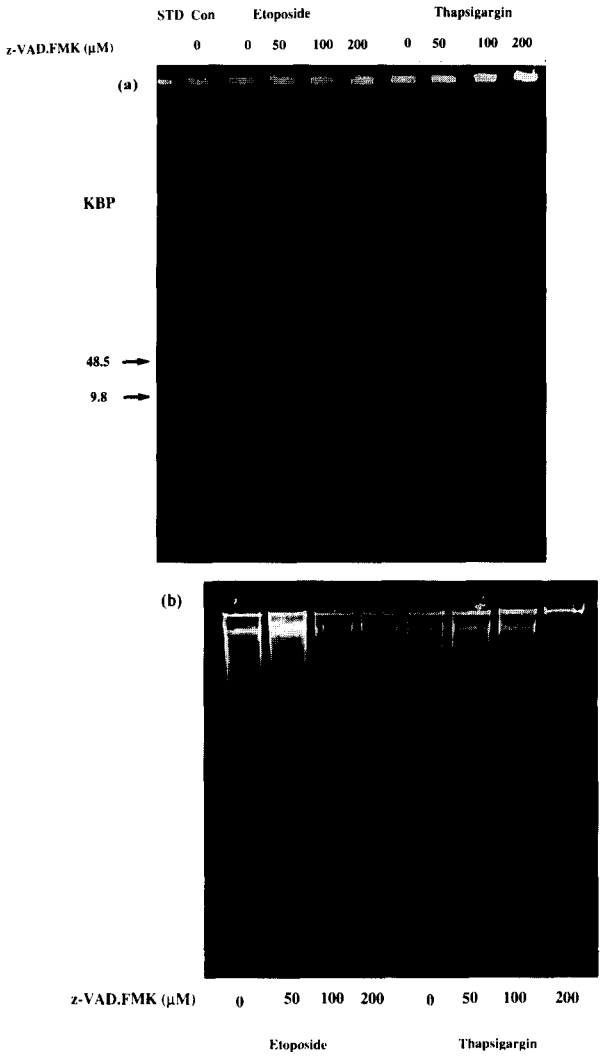


Fig. 1. Z-VAD.FMK prevents DNA fragmentation. Thymocytes were incubated for 1 h either alone or with Z-VAD.FMK (50–200 μM). Apoptosis was induced by further incubation for 4 h with either etoposide (10 μM) or thapsigargin (50 nM). (a) The formation of large kilobase pair sized fragments of DNA was assessed by FIGE. b) Internucleosomal cleavage of DNA was detected by conventional agarose gel electrophoresis.

## 3. Results

*3.1. Z-VAD.FMK inhibits apoptosis induced by diverse stimuli*

ICE-like proteases play an important role in apoptosis [13–20]. Therefore the effects of an ICE-like protease inhibitor, Z-VAD.FMK, on thymocyte apoptosis were investigated. Thymocytes were incubated for 1 h with Z-VAD.FMK (50, 100 or 200 μM) and subsequently incubated for a further 4 h with dexamethasone, etoposide or thapsigargin. Apoptosis induced by dexamethasone, etoposide and thapsigargin was markedly inhibited by Z-VAD.FMK (200 μM) (Table 1). In order to ascertain at what stage of the apoptotic process Z-VAD.FMK was exerting its inhibitory action, DNA fragmentation was assessed by both field inversion gel electrophoresis (FIGE) and conventional agarose gel electrophoresis. Etoposide, thapsigargin and dexamethasone induced both the formation of large kilobase pair sized fragments, primarily of 10–50 kbp, and also internucleosomal cleavage compared with control cells (Fig. 1 and data not shown). Z-VAD.FMK caused a concentration dependent inhibition of both the formation of large kilobase pair sized fragments and DNA laddering (Fig. 1).

Other inhibitors of ICE-like proteases were examined for their ability to prevent apoptosis as assessed by flow cytometry. Dexamethasone-induced apoptosis was not inhibited by either *N*-(*N*-acetyl-tyrosinyl-valinyl-alaninyl)-3-amino-4-oxobutanoic acid (YVAD.CHO) (100 μM) or benzyloxycarbonyl-valinyl-alaninyl-δ-ethylaspartyl-(2,6-dichlorobenzoyl-oxy) methyl ketone (50 μM) (data not shown). At higher concentrations both these inhibitors were insoluble in the culture medium. *N*-Acetyl-tyrosinyl-valinyl-alaninyl aspartyl chloromethyl ketone (200 μM) did not inhibit either dexamethasone- or etoposide-induced apoptosis as assessed by either flow cytometry or conventional agarose gel electrophoresis (data not shown).

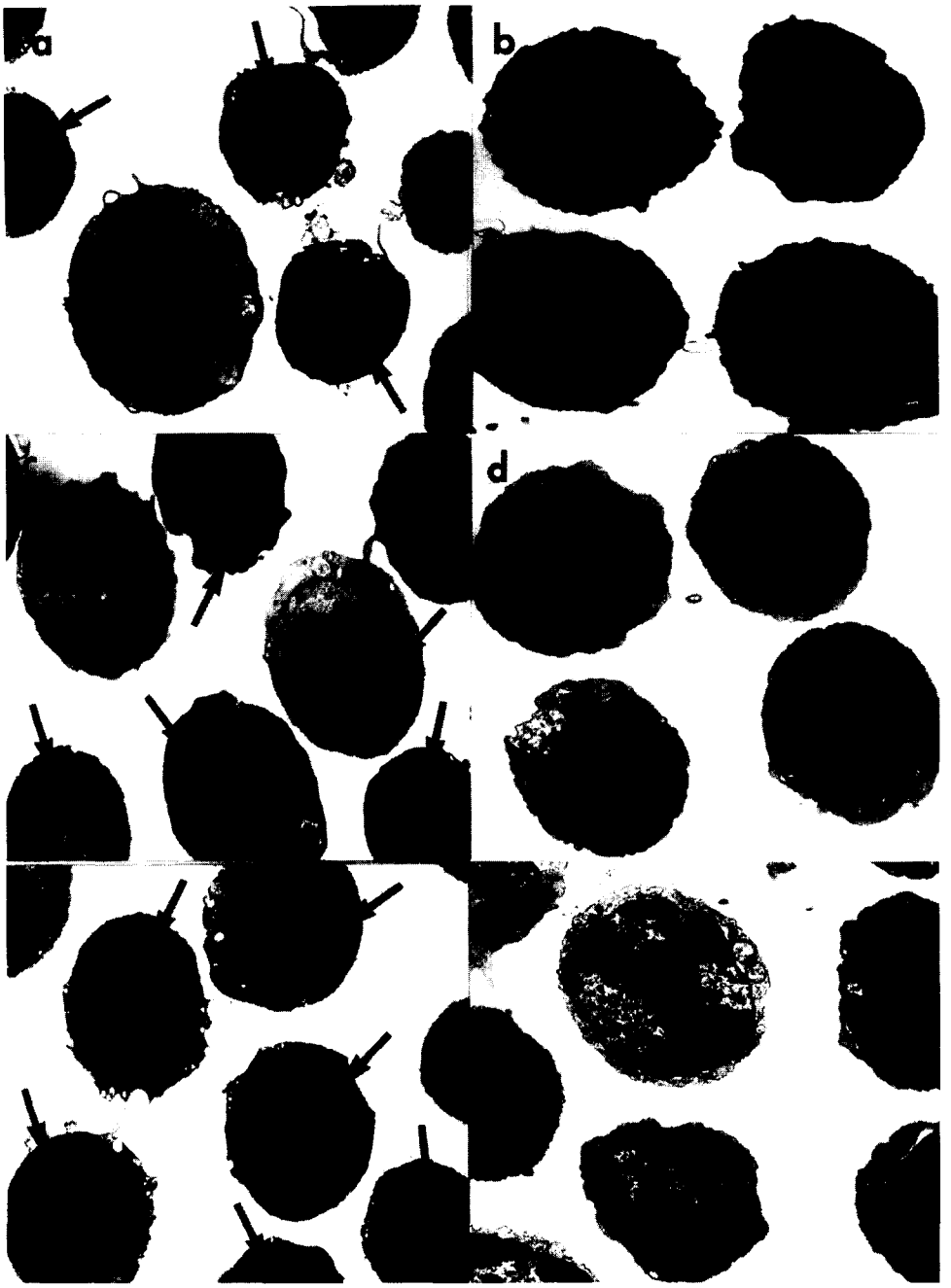


Fig. 2. Z-VAD.FMK prevents the morphological changes of apoptosis. Thymocytes were incubated for 1 h either alone (a, c and e) or with Z-VAD.FMK (200 μM) (b, d and f). They were then further incubated for 4 h with dexamethasone (0.1 μM) (a and b), etoposide (10 μM) (c and d) or thapsigargin (50 nM) (e and f). Z-VAD.FMK inhibited the characteristic chromatin condensation of apoptosis induced by dexamethasone, etoposide and thapsigargin. Magnification = × 5,600.

### 3.2. *Morphological changes of apoptosis were inhibited by Z-VAD.FMK*

Dexamethasone, etoposide and thapsigargin induced classical ultrastructural features of apoptosis (Fig. 2) including cell shrinkage, chromatin condensation to one pole of the nucleus, nucleolar disintegration and cytoplasmic vacuolation. Z-VAD.FMK (200 μM). inhibited all these ultrastructural features of apoptosis induced by dexamethasone, etoposide and thapsigargin (Fig. 2). The cytoplasm of cells protected with Z-VAD.FMK exhibited normal ultrastructure. The nuclei of these cells were within the range of morphologies found in preparations of untreated thymocytes but the proportion showing slight condensation of perinuclear heterochromatin was increased. In addition to causing ultrastructural changes typical of apoptosis, thapsigargin also produced in 5–10% of thymocytes mitochondrial changes, which are not normally associated with apoptosis. These mitochondrial changes were not inhibited by Z-VAD.FMK (data not shown). Thapsigargin, inhibits the microsomal calcium pump resulting in an increase in intracellular calcium [30], which may lead to mitochondrial

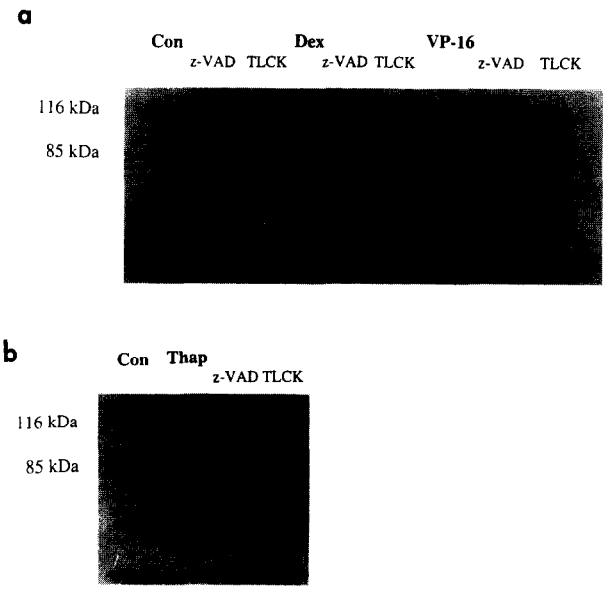


Fig. 3. Z-VAD.FMK and TLCK inhibit PARP proteolysis. Thymocytes were incubated for 1 h alone, or with either Z-VAD.FMK (200 μM) or TLCK (50 μM). Proteolysis of intact PARP (116 kDa) to an 85 kDa fragment was induced by further incubation for 4 h with either (a) dexamethasone or etoposide (VP16) or (b) thapsigargin. The proteolysis of PARP caused by the apoptotic stimuli was completely or partially prevented by Z-VAD.FMK or TLCK, respectively.

damage by initiating biochemical changes independent of apoptosis.

### 3.3. Z-VAD.FMK inhibits PARP proteolysis

As PARP proteolysis has been reported to be an early biochemical marker of apoptosis [27,29], we wished to investigate whether this was also influenced by Z-VAD.FMK. We have previously reported that TLCK (50 μM) prevents apoptosis induced by a number of agents [26]. In order to extend these observations and compare them with the present studies, thymocytes were incubated for 1 h in the presence of Z-VAD.FMK (200 μM) or TLCK (50 μM) and subsequently exposed for 4 h to dexamethasone (0.1 μM), etoposide (10 μM) or thapsigargin (50 nM). The extent of PARP degradation in these cells was then assessed by Western blotting using a polyclonal antibody (318). In control cells and in cells treated with Z-VAD.FMK or TLCK alone, the majority of PARP was intact (116 kDa) with little or no degradation to an 85 kDa product (Fig. 3). Dexamethasone, etoposide and thapsigargin caused a decrease in intact PARP accompanied by an increase in the 85 kDa product, showing an increased proteolysis (Fig. 3). This was more marked with etoposide and thapsigargin than with dexamethasone. Z-VAD.FMK (200 μM) almost totally prevented the proteolysis of PARP induced by dexamethasone, etoposide and thapsigargin (Fig.3). TLCK was less effective at inhibiting the proteolysis of intact PARP (116 kDa) and the extent of inhibition varied with the nature of the apoptotic stimulus (Fig. 3). TLCK was most effective at inhibiting dexamethasone-induced proteolysis of PARP (Fig. 3).

## 4. Discussion

### 4.1. An ICE-like protease is required at an early stage of thymocyte apoptosis

Dexamethasone, etoposide and thapsigargin induced classical ultrastructural features of thymocyte apoptosis involving both nuclear and cytoplasmic changes, which were prevented by Z-VAD.FMK (Fig. 2). Z-VAD.FMK inhibited dexamethasone-, etoposide- and thapsigargin-induced apoptosis (Table 1) and prevented both cleavage of DNA to large kilobasepair size fragments and the formation of DNA ladders (Fig. 1). Thus the inhibition of the biochemical changes by Z-VAD.FMK correlated closely with the inhibition of morphological signs of apoptosis. These data support the involvement of an ICE-like protease at an early stage of a common apoptotic pathway in thymocytes prior to any detectable biochemical or ultrastructural alterations characteristic of apoptosis.

Recently Z-VAD.FMK has also been shown to inhibit Fas-induced apoptosis in Jurkat cells and apoptosis induced by diverse stimuli in the human monocytic tumour cell line, THP-1 [39,40]. The high concentrations of Z-VAD.FMK required for inhibition of apoptosis in thymocytes may have been due to poor cellular permeability compared to Jurkat or THP-1 cells or to nonspecific effects. Fluoromethyl ketones are, however, much less reactive than the corresponding chloromethyl ketones [41] and so their lack of toxicity at high concentrations (Table 1) suggested they were not reacting nonspecifically. Alternatively high concentrations of Z-VAD.FMK may have been required to inhibit a homologue(s) of ICE different from those found in Jurkat and THP-1 cells [39,40]. Specific inhibitors of ICE have an aspartate in the $P_1$ position together with four amino acid residues to the left of the cleavage site [31]. The lack of inhibition of apoptosis by more specific ICE inhibitors, such as YVAD.CHO and *N*-acetyl-tyrosinyl-valinyl-alanyl-aspartyl chloromethylketone, suggested that an ICE-like protease other than ICE per se may be involved in thymocyte apoptosis. This suggestion is in agreement with the recent finding that induction of apoptosis in thymocytes and macrophages from ICE-deficient mice appeared normal [42] although some differences were observed in Fas-induced apoptosis [43]. Inhibition of other ICE-like proteases, whilst requiring an aspartate in the $P_1$ position, will require different residues in the $P_2$–$P_4$ positions [18]. In contrast Z-VAD.FMK, with an aspartate in the $P_1$ position but no amino acid in the $P_4$ position would be expected

Table 1
Z-VAD.FMK inhibits apoptosis induced by diverse stimuli

| Treatment | Z-VAD.FMK | % Apoptotic cells |
|---|---|---|
| Control | – | 9.8 ± 1.6 |
| Control | + | 8.9 ± 3.4 |
| Dexamethasone | – | 29.9 ± 2.6 |
| Dexamethasone | + | 10.9 ± 3.8 |
| Etoposide | – | 41.5 ± 11.6 |
| Etoposide | + | 17.1 ± 3.8 |
| Thapsigargin | – | 40.0 ± 8.6 |
| Thapsigargin | + | 18.9 ± 2.6 |

Thymocytes were incubated for 1 h either alone or with Z-VAD.FMK (200 μM) and then further incubated for 4 h with either dexamethasone (0.1 μM), etoposide (10 μM) or thapsigargin (50 nM). The percentage of apoptotic cells was then assessed by flow cytometry [34]. The data represent the mean ( ± S.E.M.) of at least 3 experiments.

to have a lower affinity for ICE but should inhibit other ICE-like proteases [32].

### 4.2. PARP proteolysis

Degradation of PARP has been proposed as an early marker of apoptosis [27]. In thymocytes dexamethasone, and etoposide induced proteolysis of intact PARP (116 kDa) with the concomitant generation of an 85 kDa fragment (Fig. 3), consistent with previous reports [23,27,29]. Thapsigargin also induced proteolysis of PARP and the appearance of an 85 kDa fragment (Fig. 3). Z-VAD.FMK was very effective at inhibiting PARP proteolysis induced by all three stimuli (Fig. 3) further supporting our hypothesis that Z-VAD.FMK was inhibiting apoptosis at an early stage of a common pathway. As one of the mammalian homologues of ICE, variously known as pICE, CPP32 or Yama, has been implicated in cleaving PARP and being important for the initiation of apoptotic cell death [8,19,29], it was possible that in thymocytes, Z-VAD.FMK was inhibiting this protease either directly or indirectly.

Our previous studies had implicated both a trypsin-like and a chymotrypsin-like serine protease at an early and late stage of thymocyte apoptosis [26]. In the present study, we also implicate an ICE-like protease suggesting the involvement of multiple proteases in apoptosis in thymocytes in agreement with a similar proposal of multiple proteases being involved in Fas-induced apoptosis in Jurkat cells [39]. TLCK also inhibited PARP proteolysis induced by all three stimuli (Fig. 3) supporting our earlier suggestion that TLCK was inhibiting thymocyte apoptosis at an early stage [26]. However in our recent study with THP-1 cells, Z-VAD.FMK inhibited apoptosis whereas TLCK potentiated apoptosis by most stimuli [40]. These results suggested that the TLCK target was not part of a common effector mechanism but that it had an important function either positively or negatively regulating apoptosis dependent on the cell type. In contrast the ability of Z-VAD.FMK to inhibit apoptosis induced by diverse stimuli in several different systems [39, 40, 44 and the present study] support the hypothesis that it is inhibiting a common mediator of apoptosis. This common mediator of apoptosis in both the nematode and mammals appears to be a ced-3/ICE-like cysteine protease [13–20]. The specific homologue(s) of ICE involved in the induction of apoptosis in thymocytes remains to be elucidated.

*Acknowledgements*: We thank Dr. G. G. Poirier for PARP antibody, Dr. L. Rubin for ICE inhibitors and Mrs R. Morgan for help with the manuscript. The able technical assistance of Mr. D. Brown, Mrs J. McWilliam and Mr. R. Snowden is gratefully acknowledged.

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