# A pre-existing protease is a common effector of thymocyte apoptosis mediated by diverse stimuli

Howard O. Fearnhead[a], A. Jennifer Rivett[b], David Dinsdale[a], Gerald M. Cohen[a,*]

[a]*MRC Toxicology Unit and* [b]*Department of Biochemistry, University of Leicester, P.O. Box 138, Lancaster Road, Leicester, LE1 9HN, UK*

Received 1 December 1994

**Abstract Data from a number of model systems support a role for proteolysis in apoptotic cell death. Using immature rat thymocytes, we demonstrate that the inhibitors *N*-tosyl-L-lysyl chloromethylketone (TLCK) and *N*-tosyl-L-phenylalanyl chloromethylketone (TPCK) have very different effects on apoptosis. TLCK inhibits apoptosis induced by diverse stimuli at an early stage prior to both DNA fragmentation and cytoplasmic changes. We show that the TLCK-sensitive target is pre-existing and not synthesized in response to apoptotic stimuli. The contrasting effects of TLCK and TPCK support the hypothesis that the TLCK target is a trypsin-like protease which is a common effector of thymocyte apoptosis.**

*Key words:* Thymocyte apoptosis; Protease inhibitor; Trypsin-like protease

## 1. Introduction

Apoptosis is a major mechanism of cell death of vital importance for normal development and tissue homeostasis [1]. Thymocyte apoptosis is one of the best characterised experimental models of apoptosis [2,3]. During apoptosis there are dramatic morphological and biochemical changes in the cytoplasm and nucleus of a dying cell. The chromatin condenses with an attendant cleavage of DNA [1–3], initially to 200–300 and 30–50 kilobasepair size fragments [4–7], which are then further degraded to produce oligonucleosomes and the typical DNA ladder pattern [3,6,7]. Cytoplasmic changes of apoptosis are characterized by dilatation of the endoplasmic reticulum [1], cell shrinkage [1,8,9] and alterations in membrane permeability [10].

Recently, protein degradation has also been implicated in apoptosis in both invertebrates and mammals. In the nematode, *Caenorhabditis elegans*, the gene *ced-3* is essential for apoptosis and encodes a protein similar to the mammalian protease, interleukin-1$\beta$-converting enzyme (ICE) [11]. In rat fibroblasts, over expression of *ced-3*, ICE or the related *nedd-2* results in apoptosis [12,13]. In thymocytes, inhibitors of proteolysis prevent internucleosomal DNA cleavage [14] and a role for serine and cysteine proteases has been proposed [15,16]. Proteolysis is also seen during apoptosis in HL-60 cells [17], T-cell receptor mediated cell death [18], natural killer cell killing [19] and TNF mediated cytotoxicity [20].

The aim of the present study was to determine the role of proteolysis in the sequence of events leading to apoptosis in thymocytes. Apoptosis was induced by agents acting by different mechanisms, i.e. dexamethasone, a glucocorticoid [3], etoposide, a DNA topoisomerase II inhibitor [4,21], staurosporine, a protein kinase inhibitor [22 ] and thapsigargin, an inhibitor of endoplasmic reticular $Ca^{2+}$-ATPase [23]. In order to ascertain at what stage of the apoptotic process the inhibitors were acting, several techniques were used to assess apoptosis or properties associated with apoptotic cells. Nuclear changes of apoptosis were assessed by field inversion and conventional gel electrophoresis to detect formation of large kilobase pair fragments and internucleosomal cleavage of DNA respectively and DNA fragmentation was quantified by the diphenylamine method [3]. Cytoplasmic changes were assessed both by Coulter analysis to detect cell shrinkage and also by a flow cytometric method, which separates and quantifies normal and apoptotic cells based on changes in cell size and membrane permeability [10,24]. We have used two protease inhibitors, *N*-tosyl-L-lysyl-chloromethylketone (TLCK) and *N*-tosyl-L-phenylalanyl-chloromethylketone (TPCK), which prevent internucleosomal cleavage of DNA in both thymocytes and a tumour cell line [14,15,17]. Both TLCK and TPCK inhibit some cysteine and serine proteases [25]. However, TLCK irreversibly inhibits trypsin-like proteases, which require a basic amino acid in the P1 position (Schecter and Berger nomenclature), while TPCK irreversibly inhibits chymotrypsin-like proteases, which require an aromatic amino acid in the P1 position [25].

Our results demonstrate that more than one protease is required for apoptotic cell death. A TPCK-sensitive protease is required for internucleosomal cleavage. A pre-existing TLCK-sensitive protease is a common effector of early apoptotic changes induced by diverse stimuli.

## 2. Materials and methods

### 2.1. Materials

All media and serum were from Gibco (Paisley, UK). Pronase, TLCK and TPCK were from Boehringer Mannheim (Mannheim, Germany). All other chemicals were from Sigma Chemical Company (Poole, UK).

### 2.2. 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 [26]. To induce apoptosis, thymocytes ($2 \times 10^7$ cells·ml$^{-1}$) were incubated at 37°C for 4 h in RPMI 1640 medium supplemented with 10% foetal bovine serum with either dexamethasone (0.1 $\mu$M), etoposide (10 $\mu$M), staurosporine (1 $\mu$M) or thapsigargin (0.05 $\mu$M). Thymocytes were either preincubated with TLCK for 1 h prior to exposure to the apoptotic stimuli or were cotreated with TLCK or TPCK and dexamethasone or etoposide for 4 h. In experiments to investigate whether the TLCK-sensitive target was pre-existing or was synthesised in response to apoptotic stimuli, TLCK (100 $\mu$M) was incubated without cells for up to 1 h in culture medium before dilution with a cell suspension to

*Corresponding author. Fax: (44) (533) 525 616.

*Abbreviations:* ICE, interleukin-1$\beta$-converting enzyme; TLCK, *N*-tosyl-L-lysyl chloromethylketone; TPCK, *N*-tosyl-L-phenylalanyl chloromethylketone; FIGE, field inversion gel electrophoresis.


*SSDI* 0014-5793(94)01367-5

give a final TLCK concentration of 50 μM and a final cell density of $2 \times 10^7$ cells·ml$^{-1}$. These cultures were then further incubated for 4 h with either dexamethasone or etoposide.

*2.3. Apoptosis assessed by flow cytometry*

Thymocytes ($1 \times 10^6$) were stained with Hoechst 33342 and propidium iodide and viable apoptotic and normal cells separated by flow cytometry [10]. The apoptotic cells exhibit a higher blue fluorescence due to greater Hoechst 33342 staining and when sorted by flow cytometry display apoptotic morphology and internucleosomal cleavage [10]. All protease inhibitors were used at nontoxic concentrations as assessed by exclusion of propidium iodide.

*2.4. DNA analysis and cell size*

Cells ($5 \times 10^6$) were lysed and high molecular weight DNA separated from degraded chromatin as described [3]. The amounts of DNA in the supernatant (degraded) and the pellet (high molecular weight) were quantified using the diphenylamine reaction [27]. Conventional and field inversion gel electrophoresis (FIGE) were carried out as previously described [5,28]. Cell sizing and electron microscopy were carried out as described [7,29].

## 3. Results

*3.1. TLCK and TPCK inhibit internucleosomal cleavage of DNA and DNA fragmentation*

Control cells showed a small amount of internucleosomal cleavage of DNA (Fig. 1, lane 1) consistent with a low level of spontaneous apoptosis. Incubation of thymocytes for 4 h with dexamethasone (0.1 μM) or etoposide (10 μM) caused an increase in internucleosomal cleavage of DNA (Fig. 1, lanes 2 and 5, respectively). Both TPCK (25 μM) and TLCK (50 μM) caused a marked inhibition of DNA laddering induced by both dexamethasone (Fig. 1, lanes 3 and 4, respectively) and etoposide (Fig. 1, lanes 6 and 7, respectively). TLCK also inhibited internucleosomal cleavage induced by staurosporine (1 μM) and thapsigargin (0.05 μM) (data not shown).

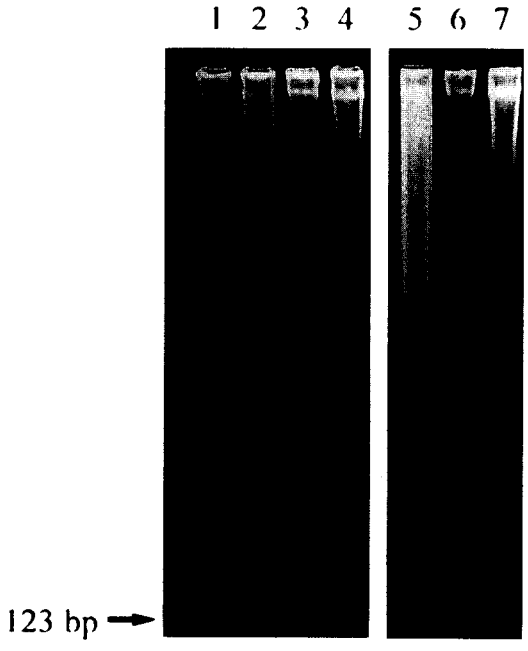


Fig. 1. Conventional agarose gel electrophoresis of DNA from thymocytes treated with TLCK and TPCK. Cells ($2 \times 10^6$/well) were loaded and electrophoresis carried out as described [28]. Thymocytes were incubated for 4 h either alone (lane 1), with dexamethasone (0.1 μM) alone (lane 2), or in the presence of TPCK (25 μM) (lane 3), or TLCK (50 μM) (lane 4). Thymocytes were also incubated with etoposide (10 μM) alone (lane 5), or in the presence of TPCK (25 μM) (lane 6) or TLCK (50 μM) (lane 7). The distance migrated by 123 basepair marker is indicated.

Table 1
Inhibition of dexamethasone and etoposide induced DNA fragmentation by TLCK and TPCK

| Treatment | % DNA fragmentation |
|---|---|
| Control | 6.8 ± 1.6 |
| Dexamethasone | 28.0 ± 2.4 |
| Dexamethasone + TLCK | 8.6 ± 1.8 |
| Dexamethasone + TPCK | 11.0 ± 1.5 |
| Etoposide | 39.8 ± 3.6 |
| Etoposide + TLCK | 16.3 ± 2.5 |
| Etoposide + TPCK | 9.3 ± 1.4 |
| TLCK | 2.0 ± 0.8 |
| TLCK | 9.4 ± 1.2 |

Cells were incubated for 4 h with either dexamethasone (0.1 μM) or etoposide (10 μM) to induce apoptosis and the ability of TLCK (50 μM) and TPCK (25 μM) to inhibit DNA fragmentation. The data represent the mean (± S.E.M.) of at least 3 experiments.

DNA fragmentation is quantifiable by measurement of the percentage of diphenylamine-reactive material present in the supernatant fraction obtained after centrifugation of lysed cells [3]. In agreement with previous studies [3,21], both dexamethasone and etoposide caused an increase in DNA fragmentation (Table 1). These increases were abrogated by both TLCK and TPCK (Table 1).

*3.2. TLCK inhibits apoptosis detected by flow cytometry*

In agreement with previous results [10,21,29], both dexamethasone and etoposide caused an increase in apoptotic cells (Table 2). Similarly staurosporine and thapsigargin also caused an increase in high blue fluorescent cells. The increase from all four stimuli was markedly inhibited by TLCK (Table 2).

*3.3. TLCK inhibits the formation of large kilobasepair fragments of DNA*

Following incubation of rat thymocytes with dexamethasone or etoposide, a marked increase in the formation of large fragments of DNA, particularly of 10–50 kbp, was detected by FIGE (Fig. 2A, lanes 4 and 6, respectively) compared to control cells (Fig. 2A, lane 3). This DNA cleavage was inhibited by

Table 2
TLCK inhibits a common effector of thymoctye apoptosis induced by diverse stimuli

| Treatment | % Apoptotic cells |
|---|---|
| Control | 10.4 ± 0.3 |
| Dexamethasone | 25.6 ± 1.2 |
| Dexamethasone + TLCK | 7.7 ± 1.1 |
| Etoposide | 35.7 ± 4.3 |
| Etoposide + TLCK | 13.7 ± 1.9 |
| Thapsigargin | 39.5 ± 2.0 |
| Thapsigargin + TLCK | 16.2 ± 2.8 |
| Staurosporine | 23.7 ± 0.7 |
| Staurosporine + TLCK | 15.3 ± 0.7 |

Cells were incubated for 1 h with TLCK (50 μM) and then further incubated for 4 h with either dexamethasone (0.1 μM), etoposide (10 μM), thapsigargin (0.05 μM) or staurosporine (1 μM). The percentage of apoptotic cells was then assessed by flow cytometry [10]. The data represent the mean (± S.E.M.) of at least 3 experiments.

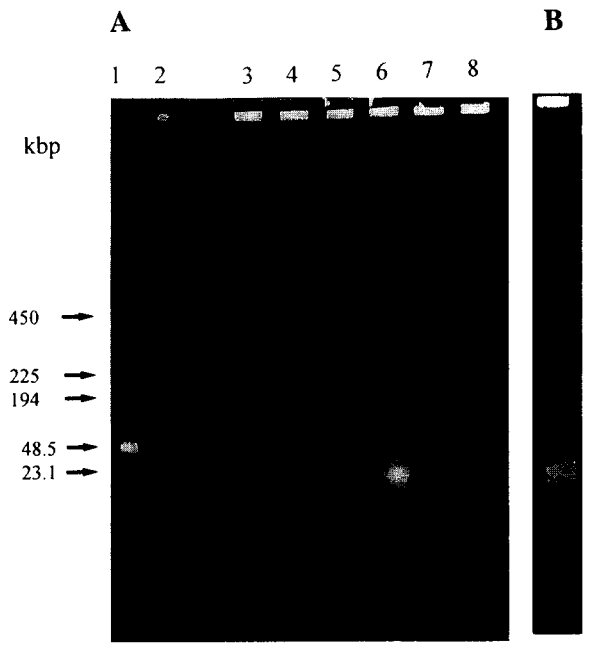


Fig. 2. FIGE of DNA from thymocytes treated with TLCK or TPCK. A. Thymocytes were incubated for 4 h either alone (lane 3), with dexamethasone (0.1 μM) alone (lane 4) or in the presence of TLCK (50 μM) (lane 5), with etoposide (10 μM) alone (lane 6) or in the presence of TLCK (50 μM) (lane 7) or with TLCK (50 μM) alone (lane 8). B. Thymocyes were also incubated for 4 h with TPCK (25 μM). Size markers, 0.1–100 kbp (lane 1) and 245–2200 kbp (lane 2) are shown. Agarose plugs containing $1 \times 10^6$ cells were prepared and electrophoresis carried out as described [5].

TLCK (Fig. 2A, lanes 5 and 7, respectively). TLCK alone caused a slight decrease in the large fragments (10–50 kbp) observed in the control cells (Fig. 2A compare lanes 3 and 8). Thapsigargin and staurosporine also caused an increase in the formation of large fragments which was inhibited by TLCK (data not shown).

### 3.4. *TLCK inhibits the decrease in cell volume induced by dexamethasone and etoposide*

Thymocytes undergoing apoptosis show a marked decrease in cell volume [8,9]. Following dexamethasone treatment, a proportion of the cells shrink, a change which was prevented by TLCK (Fig. 3A). Etoposide also caused a loss of cell volume that was inhibited by TLCK (data not shown).

### 3.5. *A TLCK sensitive target was present in thymocytes prior to the induction of apoptosis*

Preincubation of media with TLCK (100 μM) alone demonstrated that TLCK became progressively less effective at inhibiting apoptosis confirming the known instability of TLCK above neutral pH (Fig. 4). The length of incubation of TLCK with medium alone clearly affected its ability to subsequently inhibit DNA fragmentation (Fig. 4). Thus after 1 h preincubation, insufficient TLCK remained to exert any inhibitory effect on either dexamethasone- or etoposide-induced apoptosis (Fig. 4). Only when TLCK was preincubated for 15 min or less was there sufficient remaining to inhibit dexamethasone- or etoposide-induced DNA fragmentation (Fig. 4) or the appearance of apoptotic cells detected by flow cytometry (data not shown).

In contrast when cells rather than medium were pretreated with TLCK for 1 h and subsequently exposed to apoptotic stimuli, marked inhibition of apoptosis was observed (Table 2). These results suggested that TLCK bound to and inactivated its target, a putative protease, which was already present in thymocytes and which was not synthesised in response to apoptotic stimuli.

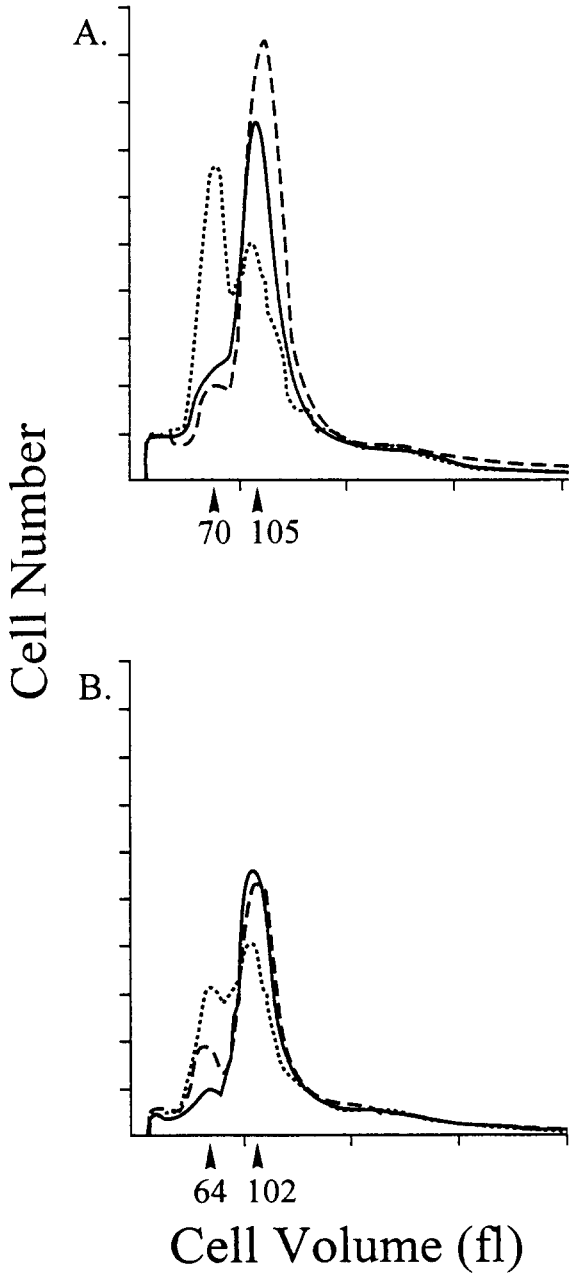


Fig. 3. The effect of TLCK and TPCK on dexamethasone induced cell shrinkage. Thymocytes were incubated for 4 h either alone (——) or in the presence of dexamethasone (0.1 μM) (···) and cell volume assessed as described in experimental procedures. The volumes of normal and apoptotic cells are shown in fl. (A) Thymocytes were also incubated with dexamethasone in the presence of TLCK (50 μM) (- - -). The size distribution of cell treated with TLCK alone was identical to that of cells treated with dexamethasone plus TLCK and is not shown for clarity. (B) The size distribution of cells treated with TPCK (25 μM) alone (- - -) was identical to that of cells treated with dexamethasone plus TPCK.

### 3.6. TPCK induces changes characteristic of early apoptosis

The effects of TPCK on large fragment formation were very different from those of TLCK. TPCK (25 μM) alone caused an increase in large fragments of 10–50 kbp and ~200 kbp (Fig. 2B) compared to control cells (Fig. 2A, lane 3), similar to that seen when thymocytes were treated with dexamethasone in the presence of zinc [5]. These thymocytes displayed a distinct ultrastructure, characterised by condensed chromatin abutting the nuclear membrane [29]. Ultrastructural examination of cells following 4h incubation with TPCK (25 μM) revealed that most thymocytes (80%) showed a very similar morphology (data not shown). Several of the cells treated with TPCK showed disintegration of the nucleolus together with the formation of cytoplasmic vacuoles. Consistent with the cytoplasmic morphology, TPCK (25 μM) alone caused a decrease in cell volume in a proportion of the cells (Fig. 3B). Incubation of thymocytes for 4 h with TPCK (25 μM) resulted in the induction of apoptosis (22.2% ± 2.2, mean ± S.E., $n$=3), as assessed by flow cytometry, compared to control cells (9.3% ± 0.3). These studies demonstrated that TPCK induced both biochemical and morphological changes associated with early nuclear changes of apoptosis without producing internucleosomal cleavage.

## 4. Discussion

### 4.1. A pre-existing TLCK-sensitive target is a common effector of thymocyte apoptosis

Apoptosis was induced in thymocytes by four stimuli with different mechanisms of action (Table 2). TLCK inhibited all the characteristic changes of apoptosis induced by these stimuli, i.e. both large fragment formation (Fig. 2A) and internucleosomal cleavage (Fig. 1) of DNA, cell shrinkage (Fig. 3A) and plasma membrane changes (Table 2). Thus a TLCK-sensitive target is required at an early stage of thymocyte apoptosis prior to both the nuclear and cytoplasmic changes of apoptosis. The data obtained following preincubation of media or cells for 1 h with TLCK (Fig. 4 and Table 2) suggested that the TLCK-sensitive target was pre-existing and not synthesised in response to apoptotic stimuli.

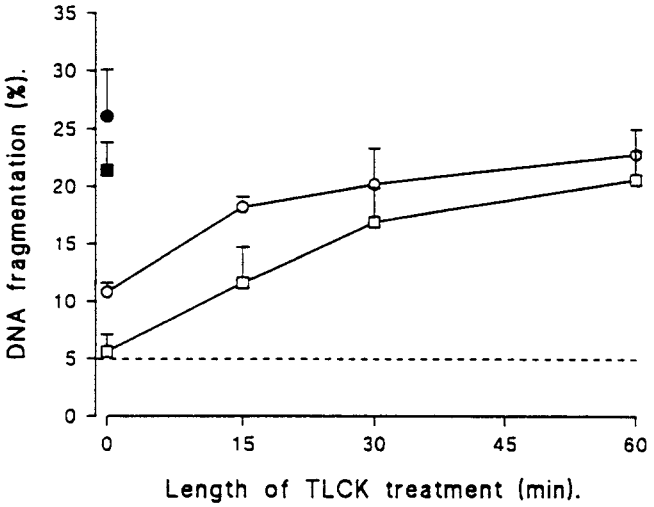


Fig. 4. The TLCK sensitive protease is already present and not synthesized in response to apoptotic stimuli. TLCK (100 μM) was incubated in culture medium for the indicated times before being added to cells to a final concentration of 50 μM with either dexamethasone (0.1 μM) (□) or etoposide (10 μM) (○) and further incubated for 4 h. Some cells were exposed to only dexamethasone (0.1 μM) (■) or etoposide (10 μM) (●) for 4 h. After this time, DNA fragmentation was measured as described (3,27). The amount of DNA fragmentation seen in untreated thymocytes is shown by a dotted line. Results are the mean (± S.E.M.) of 3 experiments.

As transcription and translation are often required for thymocyte apoptosis [30], the possibility that TLCK was affecting these was examined by comparing its effects to those of cycloheximide. Apoptosis induced by dexamethasone, etoposide and thapsigargin were inhibited by both TLCK and cycloheximide, whereas that induced by staurosporine was inhibited only by TLCK (data not shown). These data support a model in which the apoptotic machinery is pre-existing and staurosporine induces apoptosis by acting downstream of de novo protein synthesis.

### 4.2. TPCK inhibits DNA laddering but itself induces early apoptotic changes

TPCK exerted markedly different effects compared to TLCK. TPCK inhibited internucleosomal cleavage induced by dexamethasone and etoposide (Fig. 1) but alone caused the formation of large DNA fragments (Fig. 2B). Weaver et al. [15] showed that the induction of internucleososmal cleavage of DNA by dexamethasone or teniposide but not the formation of large fragments in rat thymocytes was prevented by TPCK. On ultrastructural examination of TPCK-treated cells, we obseved both formation of cytoplasmic vacuoles and condensation of chromatin into sharply defined clumps abutting the nuclear membrane (data not shown). Both the pattern of DNA fragmentation and the morphology are typical of early nuclear changes of apoptosis [5,21,29,31,32]. Consistent with the alterations in cytoplasmic morphology, TPCK alone induced shrinkage in a proportion of cells (Fig. 3B). TPCK also increased the percentage of high blue fluorescent cells reflecting an altered membrane permeability [24]. These data demonstrate that the effects of TLCK and TPCK are different and that TPCK alone induces many of the early changes of apoptosis and only inhibits the terminal stages of DNA fragmentation, i.e. internucleosomal cleavage and full chromatin condensation.

### 4.3. A trypsin-like protease is required for early apoptotic changes in thymocytes

In this study no direct evidence of proteolysis is presented. However, TLCK and TPCK at the concentrations we have used, inhibit intracellular proteolysis [33,34]. Under identical conditions, TLCK stabilised cdc-2 kinase activity, consistent with an inhibition of cyclin degradation [35]. While intracellular proteases are the most likely targets of these inhibitors, we cannot totally exclude the possibility of other cellular targets. As TLCK and TPCK both inhibit cysteine proteases [25], it is possible that they exert their effects by inhibition of a cysteine protease similar to ICE. The known substrate specificity of ICE [36] together with a recent report that a TLCK- and TPCK-insensitive ICE-like protease is required for the nuclear changes of apoptosis [37] make it unlikely that the effects described in the present study are due to a direct inhibition of an ICE-like protease. This was supported by our preliminary studies, which showed that three inhibitors of ICE were ineffective at inhibiting either dexamethasone or etoposide-induced apoptosis (data not shown). The cysteine protease calpain has also been impli-

cated in thymocyte apoptosis [16]. We cannot exclude the possibility that calpain is a target for TLCK or TPCK. However the substrate specificity of calpain [38] and the observation that a calpain inhibitor, *N*-acetyl-Leu-Leu-norleucinal, alone causes the formation of large DNA fragments (data not shown) suggest that calpain is not the target. TLCK inhibits trypsin-like proteases but does not inhibit chymotrypsin-like proteases. Conversely, TPCK inhibits chymotrypsin-like proteases but not trypsin-like proteases [39]. Thus the contrast between the effects of TLCK and TPCK described here support a role for at least two distinct proteases in thymocyte apoptosis. A TPCK-sensitive chymotrypsin-like protease is required for the terminal changes of apoptosis, i.e. both the internucleosomal cleavage of DNA and the complete condensation of the chromatin and a TLCK-sensitive trypsin-like protease is required early in the apoptotic process. This TLCK-sensitive protease mediates both cytoplasmic and nuclear changes of thymocyte apoptosis induced by diverse stimuli. Therefore this protease, which is pre-existing and is not synthesised in response to apoptotic stimuli, is a common effector of thymocyte apoptosis.

*Acknowledgements:* We thank Mr. R. Snowden for help with the flow cytometry and Mrs. J. Symons for preparation of the manuscript.

## References

[1] Arends, M.J. and Wyllie, A.H. (1991) Int. Rev. Exp. Path. 32, 223–254.
[2] Compton, M.M. and Cidlowski, J.A. (1992) Trends Endocrinol. Metab. 3, 17–23.
[3] Wyllie, A.H. (1980) Nature 284, 555–556.
[4] Walker, P.R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J.F. and Sikorska, M. (1991) Cancer Res. 51, 1078–1085.
[5] Brown, D.G., Sun, X.-M. and Cohen, G.M. (1993) J. Biol. Chem. 268, 3037–3039.
[6] Oberhammer, F., Wilson, J.W., Dive, C., Morris I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R. and Sikorska, M. (1993) EMBO. J. 12, 3679–3684.
[7] Cohen, G.M., Sun, X.-M., Fearnhead, H. MacFarlane, M., Brown, D.G., Snowden, R.T. and Dinsdale, D. (1994) J. Immunol. 153, 507–516.
[8] Thomas, N. and Bell, P.A. (1981) Mol. Cell. Endocrinol. 22, 71–84.
[9] Wyllie, A.H. and Morris, R.G. (1982) Am. J. Pathol. 109, 78–87.
[10] Sun, X-M., Snowden, R.T., Skilleter, D.N., Dinsdale, D., Ormerod, M.G. and Cohen, G.M. (1992) Anal. Biochem. 204, 351–356.
[11] Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993) Cell 75, 641–652.
[12] Miura, M., Zhu, H., Rotello, R., Hartweig, E.A. and Yuan, J. (1993) Cell 75, 653–660.
[13] Kumar, S., Kinoshita, M., Noda, M., Copeland, N.G. and Jenkins, N.A. (1994) Genes Dev. 8, 1613–1626.
[14] Bruno, S., Bino, G.D., Lassota, P., Giaretti, W. and Darzynkiewicz, Z. (1992) Leukemia 6, 1113–1120.
[15] Weaver, V.M., Lach, B., Walker, P.R. and Sikorska, M. (1993) Biochem. Cell Biol. 71, 488–500.
[16] Squier, M.K.T., Miller, A.C.K., Malkinson, A.M. and Cohen J.J. (1994) J. Cell Physiol. 159, 229–237.
[17] Kaufmann, S.H. (1989) Cancer Res. 49, 5870–5878.
[18] Sarin, A., Adams, D.H. and Henkart, P.A. (1993) J. Exp. Med. 178, 1693–1700.
[19] Shi, L. Kraut, R.P., Aebersold, R. and Greenberg, A.H. (1992) J. Exp. Med. 175, 553–566.
[20] Suffys, P., Beyaert, R., Van Roy, F. and Fiers, W. (1988) Eur. J. Biochem. 178, 257–265.
[21] Sun, X.-M., Snowden, R.T., Dinsdale, D., Ormerod, M.G. and Cohen, G.M. (1994) Biochem. Pharmacol. 47, 187–195.
[22] Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C. and Raff, M.C. (1993) Nature 361, 365–368.
[23] Jiang, S., Chow, S.C., Nicotera, P. and Orrenius, S. (1994) Exp. Cell Res. 212, 84–92.
[24] Ormerod, M.G., Sun, X.-M., Snowden, R.T., Davies, R., Fearnhead, H. and Cohen, G.M. (1993) Cytometry 14, 595–602.
[25] Powers, J.C. and Harper, J.W. (1986) in: Proteinase Inhibitors (Barrett and Salvesen, Eds) Elsevier, Amsterdam, pp. 55–151.
[26] Raffray, M. and Cohen, G.M. (1991) Arch. Toxicol. 65, 135–139.
[27] Burton, K. (1956) Biochem. J. 62, 315–323.
[28] Sorenson, C.M., Barry, M.A. and Eastman, A. (1990) J. Natl. Cancer Inst. 82, 749.
[29] Cohen, G.M., Sun, X-M, Snowden, R.T., Dinsdale, D. and Skilleter, D.N. (1992) Biochem. J. 286, 331–334.
[30] Wyllie, A.H., Morris, R.G., Smith, A.L. and Dunlop, D. (1984) J. Path. 142, 67–77.
[31] Kerr, J.F.R., Searle, J., Harmon, B.V. and Bishop, C.J. (1987) in: Perspectives on Mammalian Cell Death. (Potten, C.S., Ed.) Oxford University Press, pp. 93–128.
[32] Cohen, G.M., Sun, X.-M., Snowden, R.T., Ormerod, M.G. and Dinsdale, D. (1993) J. Immunol. 151, 566–574.
[33] Mellits, K.H., Hay, R.T. and Goodbourn, S. (1993) Nucleic Acids Res. 21, 5059–5066.
[34] Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y. and Baeuerle, P.A. (1993) Nature 365, 182–185.
[35] Norbury, C., MacFarlane, M., Fearnhead, H.O. and Cohen G.M. (1994) Biochem. Biophys. Res. Commun. 202, 1400–1406.
[36] Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J., Elliston, K.O., Ayala, J.M., Casano, F.J., Chin, J., Ding, G.J.-F., Egger, L.A., Gaffney, E.P., Limjuco, G., Palyha, O.C., Raju, S.M., Rolando, A.M., Salley, J.P. Yamin, T-T., Lee, T.D., Shively, J.E., MacCross, M., Mumford, R.A., Schmidt, J.A., and Tocci, M.J. (1992) Nature 356, 768–774.
[37] Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C. (1994) Nature 371, 346–347.
[38] Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N. and Murachi, T. (1984) J. Biol. Chem. 259, 12489–12494.
[39] Crawford, C. (1990) in: Intracellular Calcium Dependent Proteolysis (R.L. Mellgren and T. Murachi, Eds.) CRC Press, pp. 75–89.