



# The adenomatous polyposis coli protein and retinoblastoma protein are cleaved early in apoptosis and are potential substrates for caspases

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## Abstract

Apoptosis in human monocytic THP.1 tumour cells, induced by diverse stimuli, was accompanied by proteolytic cleavage of the adenomatous polyposis coli gene product (APC) and by sequential cleavage of the retinoblastoma susceptibility gene product (Rb). Cleavage of poly(ADP-ribose) polymerase (PARP), APC and the initial cleavage of Rb at the carboxy terminal region all occurred at a similar time, early in the apoptotic process. Subsequently, Rb underwent a secondary cleavage to 43 kDa and 30 kDa protein fragments. Two caspase inhibitors, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD.FMK) and acetyl-Tyr-Val-Ala-Asp chloromethyl ketone (YVAD.CMK), had markedly different effects on the induction of apoptosis. Z-VAD.FMK inhibited the primary and secondary cleavage of Rb, cleavage of APC and PARP, and apoptosis assessed by flow cytometry. In marked contrast, YVAD.CMK inhibited cleavage of APC and the secondary cleavage of Rb to the 43 kDa and 30 kDa protein fragments but did not inhibit the primary carboxy terminal cleavage of Rb, PARP proteolysis or apoptosis assessed by flow cytometry. These results suggest that different caspases are responsible for the cleavage of different substrates at different stages during the apoptotic process and that a caspase may either cleave APC directly or may be involved in the pathway leading to APC proteolysis. This is the first report suggesting that a cytoplasmic tumour suppressor gene (APC) may be cleaved by a caspase during apoptosis.

**Keywords:** apoptosis; Z-VAD.FMK; YVAD.CMK; APC; Rb

**Abbreviations:** PARP, poly(ADP-ribose) polymerase; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone; YVAD.CMK, acetyl-Tyr-Val-Ala-Asp chloro-

methyl ketone; TLCK, tosyl-L-lysiny chloromethyl ketone; APC, adenomatous polyposis coli; Rb, retinoblastoma susceptibility gene product

## Introduction

Apoptosis is a physiological form of cell death characterised by cell shrinkage, nuclear condensation and fragmentation (Wyllie *et al*, 1984; Arends and Wyllie, 1991). Apoptosis is important in normal development and tissue homeostasis and perturbation of the apoptotic process may be important in many human diseases, including cancer (Wyllie *et al*, 1984; Arends and Wyllie, 1991). In the nematode, *Caenorhabditis elegans*, 131 cells die during development. The gene, *ced-3*, is required for these cells to die and it encodes a protein, CED-3, with considerable sequence homology to mammalian interleukin-1 $\beta$ -converting enzyme protease (ICE now termed caspase-1) (Yuan *et al*, 1993; Alnemri *et al*, 1996). Subsequently, a family of caspases has been identified, all containing an active QACXG (X=R, G or Q) pentapeptide motif (reviewed by Kumar, 1995; Martin and Green, 1995; Thornberry and Molineaux, 1995; Cohen, 1997). All caspases are synthesized as inactive proenzymes, which are processed proteolytically at aspartic acid (Asp) cleavage sites to generate a large and a small subunit, which form the active enzyme (Thornberry and Molineaux, 1995; Cohen, 1997). Activated caspases also cleave their substrates after Asp residues. Three sub-families of caspases have been identified, the CED-3 subfamily comprising caspase-3 (CPP32/Yama/apopain), caspase-7 (Mch3/ICE-LAP3/CMH-1), caspase-6 (Mch2) and caspase-9, the caspase-1 subfamily comprising caspase-1, caspase-4 (Tx/ICE rel-II/Ich2) and caspase-5 (ICE rel-III) and the NEDD-2 subfamily comprising caspase 2 (Ich-1) and its murine homologue, NEDD-2 (Chinnaiyan and Dixit, 1996; Takahashi and Earnshaw, 1996).

During the early stages of apoptosis, several nuclear proteins are degraded including histones, lamins, DNA topoisomerase I and II, poly(ADP-ribose) polymerase (PARP) and U1 small ribonucleoprotein. PARP cleavage has proved to be a useful early marker of apoptosis (Kaufmann, 1989; Kaufmann *et al*, 1993). In addition, two tumour suppressor gene products have been shown to be cleaved during apoptosis; the retinoblastoma susceptibility gene product Rb and the adenomatous polyposis coli gene product APC (Browne *et al*, 1994). The Rb tumour suppressor gene encodes a nuclear phosphoprotein that participates in the transition between G1 and S-phases of the cell cycle (reviewed in Ewen, 1994). Rb has been proposed to play a role in protecting cells from apoptosis (Haas-Kogan *et al*, 1995; Haupt *et al*, 1995), possibly through its interactions with

E2F as free E2F may induce apoptosis (Qin *et al*, 1994; Wu and Levine, 1994; Almasan *et al*, 1995).

APC protein is a cytoplasmic protein, which like Rb, is constitutively expressed in many tissue types, for example, lung, breast, prostate, pancreas, cervix, lymphoid and colon (Smith *et al*, 1993). The function of the APC protein is not known but it may play a role in regulating cell-cell adhesion through its binding to  $\beta$ -catenin (Rubinfeld *et al*, 1993; Su *et al*, 1993). APC competes for  $\beta$ -catenin binding with E-cadherin (Rubinfeld *et al*, 1993). The binding of  $\beta$ -catenin to E-cadherin activates cadherin mediated cell-cell adhesion (reviewed in Birchmeier *et al*, 1995). It is important to note however that E-cadherin is not expressed by all cell types expressing APC, for example lymphoid tissues. Interestingly APC may modulate the expression of  $\beta$ -catenin (Munemitsu *et al*, 1995) and has thus been implicated to have a role in signal transduction (reviewed by Kinzler and Vogelstein, 1996). In addition, APC binds to the tubulin cytoskeleton and has therefore been implicated to have a role in the control of cell shape (Munemitsu *et al*, 1994; Smith *et al*, 1994). APC also binds to DLG, the human homologue of the *Drosophila discs large* protein and has been suggested to have a role in neurone functioning (Matsumine *et al*, 1996). APC protein is cleaved in apoptotic cells (Browne *et al*, 1994). Although the functional significance of this cleavage is not fully understood, it suggests that APC may have a role in apoptosis (Browne *et al*, 1994). Consistent with this suggestion, full-length APC has recently been demonstrated to induce apoptosis in the colorectal tumour cell line HT29, which expresses two truncated APC proteins (Morin *et al*, 1996).

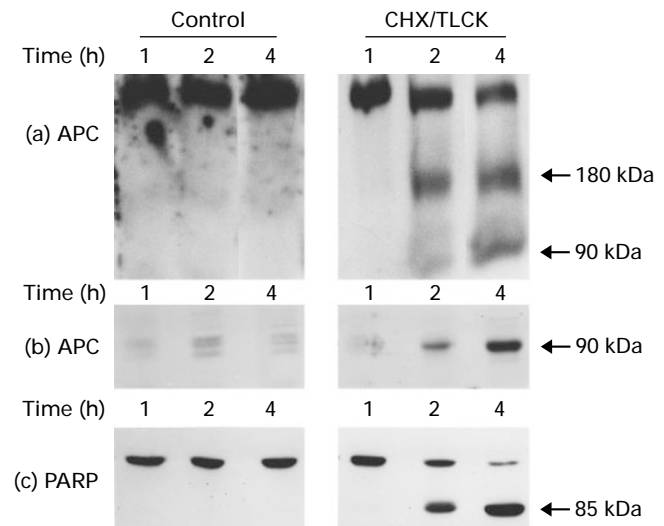
Apoptosis in the human monocytic cell line THP.1 can be induced with a wide range of stimuli (Zhu *et al*, 1995). In these cells, the trypsin-like serine protease inhibitor, N- $\alpha$ -tosyl-L-lysiny chloromethyl ketone (TLCK), enhances apoptosis induced by cycloheximide, an inhibitor of protein synthesis or thapsigargin, a microsomal  $\text{Ca}^{2+}$ -ATPase inhibitor. In contrast, the induction of apoptosis by the DNA topoisomerase II inhibitor, etoposide is inhibited by TLCK (Zhu *et al*, 1995). Apoptosis induced by DNA damaging agents in HL-60 and U937 cells was also inhibited by TLCK (Gong *et al*, 1993; An and Dou, 1996), whereas apoptosis in HL-60 cells induced by cycloheximide was potentiated by TLCK (Gong *et al*, 1993). Previously, we reported cleavage of APC and Rb proteins during apoptosis in three cell types, however the timing of these events in the apoptotic process has not been studied (Browne *et al*, 1994). Studying the timing of APC and Rb cleavage is important as it may give a clue to the importance of APC and Rb in apoptosis (Chen *et al*, 1997). In the present study apoptosis was induced in THP.1 cells using divergent stimuli and we have investigated the timing of APC and Rb cleavage relative to poly(ADP-ribose) polymerase (PARP) proteolysis, an early marker of apoptosis (Kaufmann *et al*, 1993). We have also utilised the two caspase inhibitors, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD.FMK) and Acetyl-Tyr-Val-Ala-Asp chloromethyl ketone (YVAD.CMK), to investigate whether one or more caspases are involved in the pathways leading to APC and Rb cleavage in apoptosis.

## Results

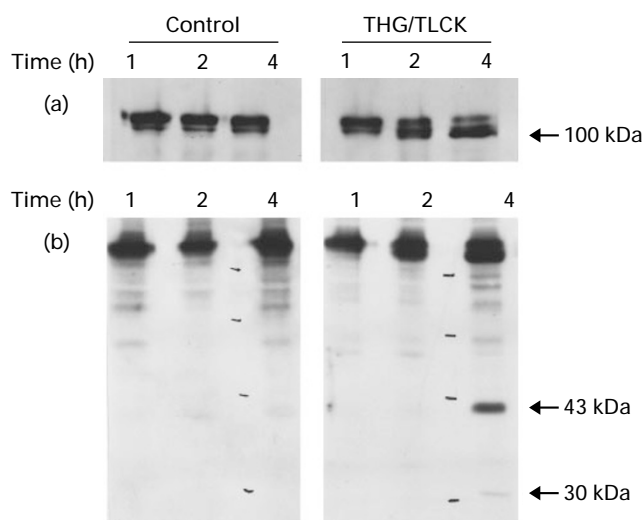
### APC and Rb proteolysis in THP.1 cell apoptosis induced by diverse stimuli

Apoptosis was induced in THP.1 cells by incubation for up to 4 h with cycloheximide/TLCK (25  $\mu\text{M}$ /100  $\mu\text{M}$ ) or thapsigargin/TLCK (100 nM/100  $\mu\text{M}$ ) or by incubation for up to 6 h with etoposide (25  $\mu\text{M}$ ) as previously described (Zhu *et al*, 1995). Protein samples were prepared from control and treated cells and the expression of APC and Rb proteins examined. APC protein expression was measured using the amino-terminal antibody FE9 (Smith *et al*, 1993) and Rb protein expression was detected using the Rb245 antibody. In apoptotic colonic epithelial cells, APC is cleaved to a 90 kDa protein fragment and Rb is cleaved to 43 kDa and 30 kDa protein fragments (Browne *et al*, 1994). Following exposure of THP.1 cells for 4 h to apoptotic stimuli, the 90 kDa cleavage product of APC protein (Figure 1) and the 43 kDa and 30 kDa cleavage products of the Rb protein (Figure 2) were also detected.

The timing of APC and Rb cleavage was determined relative to PARP cleavage and apoptosis. Cycloheximide/TLCK, thapsigargin/TLCK and etoposide all induced apoptosis in THP.1 cells (Table 1) and this occurred in a time dependent manner. Protein samples were prepared after 1, 2 and 4 h treatment with cycloheximide/TLCK and thapsigargin/TLCK, and after 2, 4 and 6 h treatment with etoposide as etoposide-induced apoptosis was delayed compared to the other stimuli. In untreated control cells almost all the PARP and APC were present as intact



**Figure 1** APC and PARP cleavage during cycloheximide/TLCK-induced apoptosis. THP.1 cells were treated for 1, 2 and 4 h with cycloheximide (25  $\mu\text{M}$ ) and TLCK (100  $\mu\text{M}$ ). Protein samples were prepared at these times and separated on either 3% agarose or 10% acrylamide gels to detect APC proteolysis (a and b respectively) or 7.5% acrylamide gel to detect PARP proteolysis (c). APC protein was detected using the amino terminal antibody FE9 and PARP protein was detected using the PARP antibody kindly provided by Dr. G Poirier. The 180 kDa and 90 kDa cleavage products of APC and the 85 kDa cleavage product of PARP are indicated. Although the 300 kDa intact APC protein was not transferred from the acrylamide gels, the 90 kDa cleaved fragment was more clearly observed



**Figure 2** Rb cleavage during thapsigargin/TLCK-induced apoptosis. THP.1 cells were incubated for up to 4 h either alone or in the presence of thapsigargin (THG) (100 nM) and TLCK (100  $\mu$ M). Protein samples were prepared at the indicated times and separated on a 6.5% and 10% acrylamide gel to detect (a) Rb primary carboxy terminal cleavage and (b) Rb secondary cleavage respectively. Rb protein was detected using the Rb245 antibody. The carboxy terminal cleaved product, 43 kDa and 30 kDa cleaved products of Rb are indicated

proteolysis of intact PARP (116 kDa) to its signature 85 kDa fragment was detected above control levels, whereas after 4 h treatment most but not all of the PARP was cleaved to this size of fragment (Figure 1c). Similarly, after 2 and 4 h treatment with cycloheximide/TLCK, loss of intact APC protein was accompanied by appearance of a 90 kDa APC cleavage product (Figure 1a and b). Interestingly in this cell line, in addition to the 90 kDa protein, a further band at approximately 180 kDa was detected in apoptotic cells (Figure 1a). This 180 kDa protein was not detected in three other apoptosis systems when APC was cleaved to a 90 kDa protein fragment (Browne *et al*, 1994) and may represent another cleavage product of the APC protein. These data suggested that the APC protein cleavage product accumulated at a similar time to the PARP cleavage product. The results shown for cycloheximide/TLCK were representative of the results found with either thapsigargin/TLCK or etoposide. Proteolysis of PARP and APC was detected above control levels after 2 h treatment with thapsigargin/TLCK and 4 h treatment with etoposide (data not shown). The timing of APC and PARP proteolysis was coincident with the detection of morphological apoptosis assessed by flow cytometry (H. Zhu - personal communication).

Rb protein cleavage has been previously shown to occur in apoptotic cells (Browne *et al*, 1994; An and Dou, 1996). Using the Rb245 antibody to detect Rb proteolysis, Rb protein was cleaved to 43 kDa and 30 kDa products in THP.1 cells exposed for 4 h to thapsigargin/TLCK (Figure 2b). Similarly, cleavage of Rb was observed after 4 h

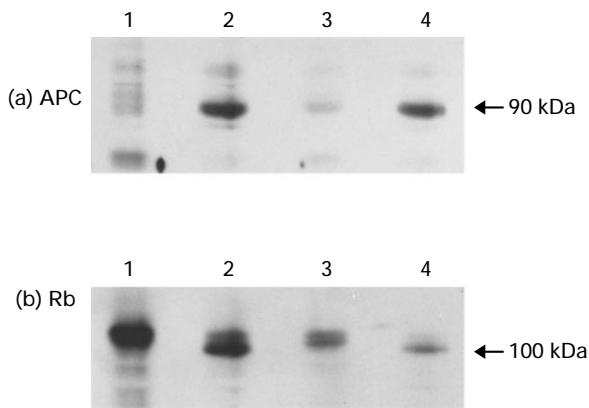
**Table 1** Inhibition of apoptosis by Z-VAD.FMK but not YVAD.CMK

Treatment	% Apoptosis		
	-	Z-VAD.FMK	YVAD.CMK
Control	1.90 $\pm$ 0.54 (4)	4.8 $\pm$ 1.30 (4)	1.9 (2)
Cycloheximide/ TLCK	48.2 $\pm$ 6.9 (4)	3.3 $\pm$ 2.5 (3)	46.3 (2)
Thapsigargin/ TLCK	33.3 $\pm$ 7.4 (4)	3.0 $\pm$ 2.0 (3)	22.1 (2)
Etoposide	39.7 (2)	2.1 (2)	36.5 (2)

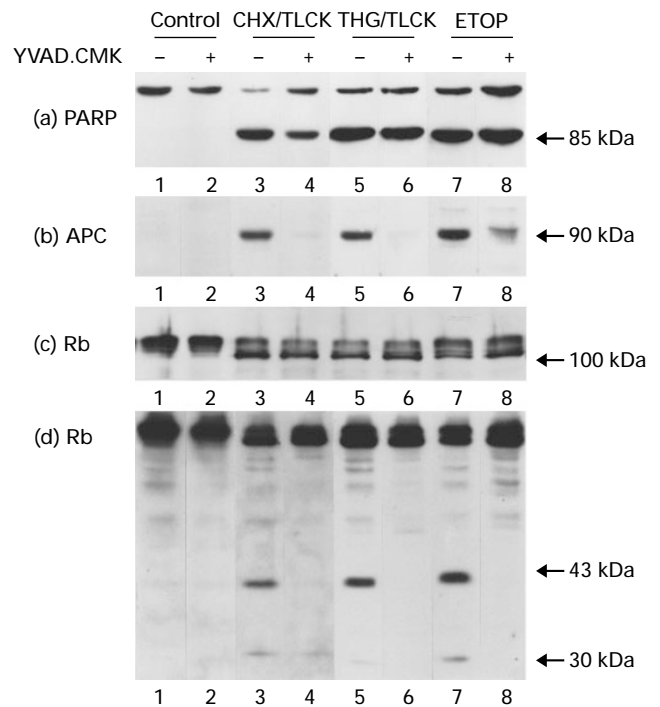
THP.1 cells were incubated for 1 h alone or in the presence of Z-VAD.FMK (50  $\mu$ M) or YVAD.CMK (100  $\mu$ M). They were then further incubated for 4 h in the presence of cycloheximide (25  $\mu$ M) together with TLCK (100  $\mu$ M) or thapsigargin (100 nM) together with TLCK (100  $\mu$ M). In the case of etoposide, cells were incubated for 6 h in order to induce a higher level of apoptosis. The percentage of apoptotic cells was assessed by flow cytometry as described in Materials and Methods. Results are expressed as the mean  $\pm$  S.E. and the number of experiments indicated in brackets

treatment with cycloheximide/TLCK or 6 h treatment with etoposide (Figures 4 and 5). Thus with all three apoptosis-inducing stimuli, cleavage of Rb to the 43 kDa and 30 kDa protein fragments occurred later than either PARP or APC proteolysis. Recently, Rb has also been shown to be cleaved early in apoptosis with loss of the carboxy terminal 42 amino acids (Janicke *et al*, 1996; Chen *et al*, 1997; Tan *et al*, 1997). Interestingly, a shift to a lower molecular weight form of Rb was detected after 2 h treatment with thapsigargin/TLCK (Figure 2a) or cycloheximide/TLCK and after 4 h with etoposide (data not shown). To determine whether the lower molecular weight form of Rb was due to either a change in phosphorylation state (An and Dou, 1996), or due to the carboxy terminal cleavage of Rb (Janicke *et al*, 1996; Chen *et al*, 1997; Tan *et al*, 1997), samples were separated on a 6.5% acrylamide gel, probed with the Rb antibody Rb245 and reprobbed with the carboxy terminal Rb antibody C-15. If the lower molecular weight form of Rb was due to an alteration in phosphorylation state, then the lower molecular weight protein should be detectable with both antibodies. However, if it was due to cleavage of the carboxy terminal region of Rb, then the protein would only be detected with the Rb245 antibody. When separated on a 6.5% acrylamide gel, an immunoreactive band was detected with a lower molecular weight than hypophosphorylated Rb (Figure 2a). As this was only detected with the Rb245 antibody, it suggested that the protein fragment was due to cleavage of the carboxy terminal region of Rb. This primary cleavage of Rb was observed after 2 h treatment with the apoptotic stimuli prior to the secondary cleavage of Rb to the 43 kDa and 30 kDa protein products (Figure 2 and data not shown).

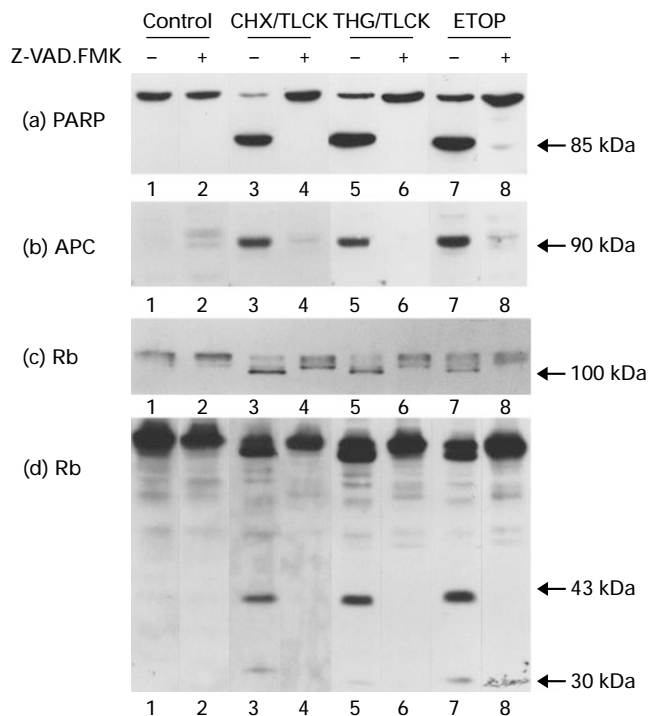
As treatment of THP.1 cells for 4 h with cycloheximide/TLCK did not result in 100% apoptosis (Table 1), a mixed population of cells with normal or apoptotic morphology was present. Some full-length APC (data not shown) and intact PARP protein was always detected at the 4 h time point as well as their cleavage products (Figure 1). In order to determine whether the remaining full-length protein was due to non-apoptotic cells in the mixed cell population, cells



**Figure 3** APC and Rb proteolysis in pure populations of normal and apoptotic cells. THP.1 cells were treated for 3 h either alone (lane 1) or with cycloheximide (25  $\mu$ M) and TLCK (100  $\mu$ M) (lane 2). The treated cells were then stained with Hoechst 33342 and sorted by flow cytometry into cells with low blue fluorescence which exhibited a normal morphology (lane 3), and cells with a high blue fluorescence, which displayed an apoptotic morphology (lane 4). These cells were separated on a 10% and 6.5% acrylamide gel to detect (a) APC proteolysis and (b) Rb primary cleavage respectively. APC protein expression was analyzed using the antibody FE9 and Rb proteolysis was detected using the antibody Rb245. The 90 kDa APC cleavage product and the carboxy terminal cleaved Rb fragment are indicated



**Figure 5** YVAD.CMK inhibits APC proteolysis and Rb secondary cleavage, but not PARP proteolysis or Rb primary cleavage. THP.1 cells were incubated in an identical manner to that described in the legend to Figure 4 except that cells in lanes 2, 4, 6 and 8 were preincubated for 1 h with YVAD.CMK (100  $\mu$ M) prior to exposure to the apoptotic stimuli



**Figure 4** Z-VAD.FMK inhibits PARP and APC proteolysis, and the primary and secondary cleavage of Rb. THP.1 cells were incubated for 6 h alone (lane 1). Cells were also incubated for 4 h with either cycloheximide (CHX)/TLCK (lane 3), thapsigargin (THG)/TLCK (lane 5), or for 6 h with etoposide (ETOP) (lane 7). Cells were preincubated for 1 h with Z-VAD.FMK (50  $\mu$ M) followed by an incubation either alone for 6 h (lane 2), with cycloheximide/TLCK for 4 h (lane 4), with thapsigargin/TLCK for 4 h (lane 6) or with etoposide for 6 h (lane 8). The proteolysis of (a) PARP and (b) APC, and the (c) primary and (d) secondary cleavage of Rb was investigated. The 90 kDa APC fragment, 85 kDa PARP fragment, carboxy terminal cleaved Rb fragment, and the 43 kDa and 30 kDa Rb fragments are indicated. Equal loading of samples was checked by Ponceau S staining of the PVDF membrane (data not shown)

were sorted using a flow cytometer into morphologically normal (low blue fluorescence) and morphologically apoptotic (high blue fluorescence) cell fractions as described in the methods. THP.1 cells were treated with cycloheximide/TLCK for 3 h, which resulted in approximately 40% apoptotic cells, and then sorted into normal and apoptotic cells. In morphologically normal cells, PARP was detected predominantly as the full-length 116 kDa protein, whereas in apoptotic cells it was present as the cleaved 85 kDa PARP fragment (MacFarlane *et al*, 1997). In the unsorted cells, APC protein was cleaved to a 90 kDa fragment and the carboxy terminus of Rb was also cleaved compared to control cells (compare Figure 3a and b, lanes 1 and 2). In the morphologically normal sorted cells, full-length APC was detected (data not shown) with only a small amount of the 90 kDa fragment (Figure 3a, lane 3) and Rb was predominantly in the hyperphosphorylated form with no detectable carboxy terminal cleaved Rb (Figure 3b lane 3). In contrast, in the apoptotic cells the 90 kDa protein fragment (Figure 3a lane 4) and the carboxy terminal cleaved form of Rb (Figure 3b, lane 4) were the predominant immunoreactive species detected. Thus the detection of both intact and cleaved APC and PARP in the 4 h samples after induction of apoptosis was consistent with the fact that not all the cells were apoptotic. Furthermore, the cleavage of APC to the 90 kDa fragment and the carboxy terminal cleavage of Rb occurred primarily in the apoptotic cells.

### Z-VAD.FMK inhibits proteolysis of PARP, APC and Rb

Having shown that APC and Rb proteins were cleaved in THP.1 cells during apoptosis, the possible role of caspases in this cleavage was investigated using the caspase inhibitor Z-VAD.FMK. Z-VAD.FMK inhibits apoptosis in THP.1 cells as measured by flow cytometry, PARP proteolysis, formation of large kilobase fragments of DNA and internucleosomal cleavage of DNA (Zhu *et al*, 1995). Pretreatment of THP.1 cells with Z-VAD.FMK (50  $\mu$ M) for 1 h completely inhibited apoptosis induced by diverse stimuli (Table 1). Treatment of THP.1 cells for 4 h with cycloheximide/TLCK, or thapsigargin/TLCK, or for 6 h with etoposide resulted in extensive cleavage of PARP to its characteristic 85 kDa fragment (Figure 4a lanes 3, 5 and 7) compared to control cells (Figure 4a lanes 1 and 2). This cleavage of PARP was almost totally inhibited by Z-VAD.FMK (Figure 4a lanes 4, 6 and 8). Similarly all three apoptotic stimuli resulted in extensive cleavage of APC protein to a 90 kDa fragment (Figure 4b lanes 3, 5 and 7), cleavage of the carboxy terminal region of Rb (Figure 4c lanes 3, 5 and 7) and Rb proteolysis to the 43 kDa and 30 kDa fragments (Figure 4d lanes 3, 5 and 7). All of these cleavages were almost totally inhibited by Z-VAD.FMK (Figure 4b–d lanes 4, 6 and 8) suggesting that Rb and APC proteins may be proteolytically cleaved by a caspase(s). Z-VAD.FMK blocked all the parameters of apoptosis measured in this study and further supported our previous suggestion that Z-VAD.FMK blocks apoptosis at an early stage in the apoptotic program (Zhu *et al*, 1995; MacFarlane *et al*, 1997).

### YVAD.CMK inhibits APC and Rb secondary cleavage but not PARP proteolysis or Rb primary cleavage

In order to gain further insight into the nature of the caspase(s) responsible for the cleavage of APC and Rb, we also used the caspase inhibitor YVAD.CMK. YVAD.CHO was designed initially to reversibly inhibit caspase-1 with a Ki in the nanomolar range (Thornberry *et al*, 1992). Using the same tetrapeptide motif, YVAD.CMK was synthesized as an irreversible inhibitor of caspase-1 but it also inhibits other caspases including the proteolytic activity described as p1ICE (Lazebnik *et al*, 1994). Prior to exposure to different apoptotic stimuli, THP.1 cells were pretreated for 1 h with YVAD.CMK (100  $\mu$ M), a concentration which inhibits Rb proteolysis in HL-60 cells and U937 cells (An and Dou, 1996). YVAD.CMK did not inhibit apoptosis as assessed by flow cytometry (Table 1) but it partially inhibited internucleosomal cleavage of DNA (data not shown) in agreement with previous results (Zhu *et al*, 1995). All three apoptotic stimuli again induced extensive cleavage of PARP to an 85 kDa fragment (Figure 5a lanes 3, 5 and 7). However, YVAD.CMK (100  $\mu$ M) caused a slight but clearly incomplete inhibition of the proteolysis of PARP induced by the three apoptotic stimuli (Figure 5a lanes 4, 6 and 8). In marked contrast, APC cleavage induced by all three apoptotic stimuli was almost totally inhibited by YVAD.CMK (Figure 5b compare lanes 3, 5 and 7 with 4, 6 and 8). Lower concentrations of YVAD.CMK (5–50  $\mu$ M) were also shown to inhibit cleavage of APC (data not shown). This data

demonstrated that the protease that cleaved APC was much more sensitive to inhibition by YVAD.CMK than the protease that cleaved PARP. Interestingly, the primary carboxy terminal cleavage of Rb induced by all three apoptotic stimuli (Figure 5c lanes 3, 5 and 7) was not inhibited by YVAD.CMK (Figure 5c lanes 4, 6 and 8) whereas secondary cleavage to 43 kDa and 30 kDa protein fragments was inhibited (Figure 5d compare lanes 3, 5 and 7 with 4, 6 and 8). These results suggested that different caspases may be responsible for the primary and secondary cleavage of Rb.

## Discussion

### APC proteolysis in apoptosis

The human monocytic THP.1 cell line expressed full-length APC protein, which was cleaved to a 90 kDa protein fragment during apoptosis induced by diverse stimuli (Figures 1, 4 and 5), consistent with the similar cleavage of APC in spontaneous apoptosis in colonic epithelial cell lines and in a SV40 transformed fibroblast cell line, and in calcium ionophore-induced apoptosis in a Burkitt lymphoma cell line (Browne *et al*, 1994). Although we have previously demonstrated cleavage of APC protein during apoptosis, when this proteolysis occurred relative to morphological and biochemical markers of apoptosis was unclear. Also the type of protease involved in APC cleavage was not known. In this study, cleavage of APC protein occurred at a similar time to proteolysis of PARP which has been proposed to be an early event in apoptosis (Kaufmann *et al*, 1993). In addition, we have shown that the two different caspase inhibitors, Z-VAD.FMK and YVAD.CMK, had markedly different effects on the cleavage of APC and PARP. Z-VAD.FMK, as well as inhibiting PARP proteolysis (Zhu *et al*, 1995; MacFarlane *et al*, 1997), also inhibited APC proteolysis (Figure 4). In contrast, YVAD.CMK (100  $\mu$ M) inhibited APC but not PARP cleavage (Figure 5). That both Z-VAD.FMK and YVAD.CMK inhibited APC cleavage suggests that a caspase may cleave APC or may be involved in the pathway leading to APC cleavage. In addition, the data suggests that APC protein may be cleaved by a different caspase(s) from that responsible for the cleavage of PARP. However, we cannot totally exclude the possibility that APC and PARP were cleaved by the same caspase but cleavage of PARP by this enzyme was much less susceptible to inhibition by YVAD.CMK.

Although the precise cleavage site(s) of APC are not known, potential caspase cleavage sites exist near the region of APC postulated to be cleaved during apoptosis. Proteolysis of PARP in intact cells is primarily carried out by caspase-3 (CPP32) and/or caspase-7 (Mch3) (Fernandes-Alnemri *et al*, 1995a; Nicholson *et al*, 1995; Tewari *et al*, 1995), although other caspases including recombinant caspase-1 (ICE), caspase-4 (TX) and caspase-6 (Mch2) when used at high concentrations can also cleave PARP (Fernandes-Alnemri *et al*, 1995b; Gu *et al*, 1995). In some model systems, the lamin protease is two orders of magnitude more sensitive to inhibition by YVAD.CMK than the PARP protease (Lazebnik *et al*, 1995). Caspase-6 (Mch2) but not caspase-3 or caspase-7 is capable of cleaving lamins (Orth *et al*, 1996; Takahashi *et al*, 1996). In

THP.1 cells undergoing apoptosis, cleavage of lamins is accompanied by the activation/processing of caspase-6 and YVAD.CMK inhibited the cleavage of the lamins but not the loss of intact caspase-6 suggesting that it inhibited the activity of caspase-6 but not its processing (MacFarlane *et al*, 1997). The susceptibility of cleavage of APC to inhibition by YVAD.CMK suggests that caspase-6 may be a possible candidate although the involvement of other caspases cannot be excluded. Further studies with purified caspases are necessary to clearly identify the enzyme(s) which cleaves APC.

The functional significance of APC cleavage in apoptosis is currently not known. Most of the known functional domains of APC are lost in proteolysis to the 90 kDa protein, including the catenin binding site, the region involved in modulation of  $\beta$ -catenin expression, and the EB1, DLG and cytoskeleton binding domains. Interestingly, APC has been demonstrated to bind tubulin and affect the construction of the tubulin cytoskeleton (Munemitsu *et al*, 1994; Smith *et al*, 1994). Cleavage of APC during apoptosis may therefore regulate some of the cytoplasmic changes that occur during apoptosis. Although the significance of APC proteolysis is unclear, we have previously suggested that APC may have a role in apoptosis/cell survival (Browne *et al*, 1994). APC protein is expressed in many normal cell types and its expression has been shown to increase in differentiated cells (Smith *et al*, 1993). In particular, in the colonic crypt APC protein expression has been shown to increase as the cells migrate towards the top of the crypt (Smith *et al*, 1993). It was suggested that the increased APC expression may result in apoptosis in the migrating cells as recent studies have shown that expression of wild-type APC induced apoptosis in a colorectal tumour cell line expressing two mutant APC proteins (Morin *et al*, 1996). Clearly it would be of interest to determine whether APC has a role in the induction of apoptosis in normal cells as well as in tumour cells. The expression of wild-type APC in colorectal carcinoma cells could result in apoptosis as a result of conflicting signals brought about by the introduction of a wild-type tumour suppressor gene.

Although APC has been shown to be cleaved during apoptosis, APC proteolysis is not necessary for apoptosis to occur. This is because YVAD.CMK was shown to inhibit APC proteolysis but did not inhibit morphological apoptosis. Z-VAD.FMK inhibits apoptosis at an early stage in the apoptosis programme (MacFarlane *et al*, 1997). This may possibly be after the cells are committed to die but prior to the morphological and biochemical measurements of apoptosis (McCarthy *et al*, 1997). YVAD.CMK inhibits only some features of the apoptotic phenotype, such as cleavage of lamins (Lazebnik *et al*, 1995; MacFarlane *et al*, 1997) and cleavage of APC and Rb possibly because its most susceptible targets are only activated at a later stage of the apoptotic programme. YVAD.CMK also inhibits internucleosomal cleavage of DNA (Zhu *et al*, 1995). Thus APC cleavage and DNA laddering may result from activation of a similar caspase(s). Further work is necessary in order to determine the significance of APC in apoptosis.

### Rb proteolysis in apoptosis

In this study, we have demonstrated that Rb protein is cleaved twice during apoptosis in THP.1 cells. Initially, it is cleaved early in the apoptotic process at a similar time to PARP and APC proteolysis. The size of the Rb cleavage product is consistent with the carboxy-terminal cleavage of Rb at the DEAD↓G site reported by Janicke *et al* (1996), Chen *et al* (1997) and Tan *et al* (1997). The secondary cleavage of Rb occurred later than PARP and APC proteolysis, coinciding with the detection of internucleosomal DNA cleavage. During the secondary cleavage, Rb protein was cleaved to 43 kDa and 30 kDa protein products, consistent with results found in three other apoptosis systems (Browne *et al*, 1994). This is the first report demonstrating that the primary and secondary cleavage of Rb occur with different timing in apoptosis. The functional significance of the primary and secondary cleavage of Rb protein in apoptosis is not known. Interestingly, it has been demonstrated that the carboxy-terminal cleaved form of Rb was no longer able to bind Mdm-2, however it was still able to bind E2F (Janicke *et al*, 1996; Chen *et al*, 1997). This may suggest that loss of Mdm-2 binding by Rb is an important event in apoptosis. Alternatively, retention of E2F binding by Rb may be important early in apoptosis. In support of this suggestion, Chen *et al* (1997) demonstrated that carboxy terminal cleavage of Rb enhanced E2F binding to Rb. The selective inactivation of Rb functions may have a role in ordering the timing of events in the apoptosis process.

The two caspase inhibitors had different effects on the primary and secondary cleavage of Rb. Z-VAD.FMK completely inhibited cleavage of Rb, however, YVAD.CMK inhibited the secondary cleavage but not the primary carboxy-terminal cleavage. This is the first report of a caspase inhibitor blocking secondary but not primary cleavage of Rb. Caspase-3 and/or caspase-7 have been proposed to cleave the carboxy terminal fragment of Rb based partly on the presence of the DEAD↓G sequence at the Rb cleavage site (Janicke *et al*, 1996; Chen *et al*, 1997; Tan *et al*, 1997), similar to the DEVD↓G sequence which is cleaved in PARP (Lazebnik *et al*, 1994), and compatible with the presence of a DXXD motif commonly found in most caspase-3 and caspase-7 substrates (Cohen, 1997). Our findings that YVAD.CMK did not inhibit PARP cleavage or Rb carboxy terminal cleavage (Figure 5) further supports the suggestion that the initial cleavage of Rb is mediated by caspase-3 and/or caspase-7. The identity of the caspase responsible for the secondary cleavage of Rb is not known. The different inhibitor profiles may suggest a different caspase was responsible for the primary and secondary cleavage. The susceptibility of this secondary cleavage to YVAD.CMK again suggested that caspase-6 may be involved. However, the different time course of the appearance of the 90 kDa fragment of APC and 43 kDa and 30 kDa fragments of Rb also suggested that different caspases may be involved in these cleavages. Thus our data provide indirect evidence for the involvement of at least three caspases in the cleavage of APC and Rb. Firstly, caspase-3 and/or caspase-7, which is responsible for the C-terminal cleavage of Rb as well as for PARP proteolysis, secondly an unidentified caspase which is

responsible for the early cleavage of APC to a 90 kDa fragment and thirdly a caspase responsible for the later cleavage of Rb to 43 kDa and 30 kDa fragments. Both the latter caspases are susceptible to inhibition by YVAD.CMK but are activated at different times in the apoptotic process. Again, we cannot exclude that these cleavages may be mediated by the same caspase which has different affinities to different substrates depending on factors, such as accessibility. Further studies using purified caspases will be necessary to clearly identify which proteases are responsible for the primary and secondary cleavage of Rb.

To summarise, we have demonstrated that APC protein was cleaved at a similar time to PARP proteolysis. Rb protein was sequentially cleaved during apoptosis with the initial cleavage occurring at a similar time to that of PARP cleavage, while the secondary cleavage occurred approximately 2 h later. Z-VAD.FMK inhibited all the morphological and biochemical features of apoptosis measured, including PARP, APC and Rb proteolysis whereas YVAD.CMK inhibited APC proteolysis and the secondary cleavage of Rb but not PARP proteolysis, the primary cleavage of Rb or morphological apoptosis as assessed by flow cytometry. The data presented suggests for the first time that a caspase may cleave APC protein or may be involved in the pathway leading to APC proteolysis. Our original observation that APC was cleaved in apoptosis and the recent observation that APC induces apoptosis (Morin *et al*, 1996) indicates that APC may have an important role in apoptosis. Further work will be necessary to determine the significance of APC in apoptosis.

## Materials and Methods

### Materials

All medium and serum used were purchased from Gibco (Paisley, UK). TLCK was obtained from Boehringer Mannheim UK (Lewes, UK), the caspase inhibitors Z-VAD.FMK and YVAD.CMK were obtained from Enzyme Systems Inc. (Dublin, CA, USA) and Bachem (Bubendorf, Switzerland) respectively. The purified mouse monoclonal antibody to APC, FE9 was purchased from Oncogene Science, Inc (Cambridge, MA, USA), purified mouse monoclonal to Rb, Rb245 was obtained from PharMingen (Cambridge, UK), rabbit polyclonal antibody to the carboxy terminus of Rb, C-15 was obtained from Santa Cruz Biotechnology, Inc (California) and the antibody to PARP (rabbit antiserum 318) was a kind gift from Dr. G Poirier (Laval University, Quebec, Canada).

### Cell culture

The human monocytic cell line THP.1 was obtained from Dr. K Whaley, University of Leicester and grown as described previously (Zhu *et al*, 1995). Apoptosis was induced in THP.1 cells by incubation for up to 4 h with cycloheximide/TLCK (25  $\mu$ M/100  $\mu$ M) or thapsigargin/TLCK (100 nM/100  $\mu$ M) or by incubation for up to 6 h with etoposide (25  $\mu$ M). In experiments with caspase inhibitors, cells were preincubated for 1 h with Z-VAD.FMK (50  $\mu$ M) or YVAD.CMK (100  $\mu$ M) in order to facilitate their entry into cells. Z-VAD.FMK was supplied with the Asp in the P1 position being esterified in order to increase cell permeability.

### Apoptosis assessed by flow cytometry

The method for the detection, separation and quantitation of morphologically normal and apoptotic THP.1 cells has been previously described (Zhu *et al*, 1995). Cells were stained with Hoechst 33342 (1.5  $\mu$ g/ml) and propidium iodide (0.05  $\mu$ g/ml) and analyzed using a Becton Dickinson FACS Vantage flow cytometer (Zhu *et al*, 1995). Non-viable cells, as indicated by propidium iodide inclusion, were gated out (mean  $3.2\% \pm 0.6$ ). Viable cells were displayed as a cytogram of blue fluorescence *versus* forward light scatter. The cells exhibiting low blue fluorescence appeared morphologically normal under fluorescence microscopy whereas the high blue cells displayed a typical apoptotic morphology (Zhu *et al*, 1995). The increased Hoechst staining reflects a change in membrane permeability (Ormerod *et al*, 1993). Cells were also sorted into two populations, one which exhibited low blue fluorescence and was morphologically normal and the other which exhibited high blue fluorescence and was morphologically apoptotic (MacFarlane *et al*, 1997).

### Protein sample preparation from whole cells

THP.1 cells ( $10^6$ ) were centrifuged at 13 000 r.p.m., for 3 min and washed in PBS. The cells were then resuspended in 50  $\mu$ l of gel sample buffer (63 mM Tris. HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.025% bromophenol blue), boiled for 5 min and stored at  $-20^\circ\text{C}$ .

### SDS-PAGE Western blotting

Proteins were resolved by SDS-PAGE, transferred onto PVDF membrane (Millipore, Watford, UK) and probed with monoclonal antibodies as described previously (Browne *et al*, 1994). 10% Acrylamide gels were used to look for the cleavage products of APC and Rb proteins, however 7.5% acrylamide gels were used to investigate PARP cleavage (Kaufmann *et al*, 1993). In addition, 6.5% acrylamide gels were used to distinguish between the hyperphosphorylated and carboxy terminal cleaved forms of Rb (Janicke *et al*, 1996).

### Agarose Western blotting

Full-length APC protein has a molecular weight of 300 kDa and is difficult to transfer using standard SDS-PAGE Western blotting techniques. Samples were therefore run on 3% agarose gels using the previously described method (Smith *et al*, 1993). Although this method detects both intact and cleaved APC products, the acrylamide blots were better for detecting the 90 kDa cleaved product and were therefore used in most of the experiments.

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