Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells

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The activity of ICE-like proteases or caspases is essential for apoptosis. Multiple caspases participate in apoptosis in mammalian cells but how many caspases are involved and what is their relative contribution to cell death is poorly understood. To identify caspases activated in apoptotic cells, we developed an approach to simultaneously detect multiple active caspases. Using tumor cells as a model, we have found that CPP32 (caspase 3) and Mch2 (caspase 6) are the major active caspases in apoptotic cells, and are activated in response to distinct apoptosis-inducing stimuli and in all cell lines analyzed. Both CPP32 and Mch2 are present in apoptotic cells as multiple active species. In a given cell line these species remained the same irrespective of the apoptotic stimulus used. However, the species of CPP32 and Mch2 detected varied between cell lines, indicating differences in caspase processing. The strategy described here is widely applicable to identify active caspases involved in apoptosis.

Keywords: affinity labels/apoptosis/cancer therapy/caspases (ICE-like proteases)/proteases

Introduction

Apoptosis is a fundamental biological process critical for the development of organisms and for maintaining tissue homeostasis. Consequently, deregulation of apoptosis contributes to diseases such as cancer and neurodegenerative disorders (Thompson, 1995). The role of apoptosis in disease makes the apoptotic machinery a promising target for therapeutic intervention. However, such therapy appears to be a distant goal since the knowledge of apoptosis is still fragmentary.

The emerging view of apoptosis is that diverse regulatory pathways activate a conserved execution machinery which carries out cell disassembly. Although this execution machinery is poorly understood, it appears that an essential component is caspases, a family of cysteine proteases (Martin and Green, 1995b; Chinnaiyan and Dixit, 1996). Caspases are expressed as precursors that must be proteolytically processed to become active enzymes (Thornberry, 1994). This processing yields three polypeptides, a pro-domain, a small subunit and a large subunit which contains the catalytic cysteine. The two subunits associate to form the active protease and both contribute to the catalytic activity. Caspases cleave their substrates after an aspartate

residue, a very unusual substrate specificity for eukaryotic proteases, although not every aspartate provides a cleavage site. In fact, only about a dozen proteins cleaved by caspases *in vivo* have been identified.

The key observation linking caspases to apoptosis was the finding that CED-3, a protein essential for apoptosis in *Caenorhabditis elegans*, is a caspase (Xue and Horvitz, 1995; Yuan *et al.*, 1993). This finding prompted the search for the mammalian equivalent of CED-3. The observation that poly(ADP-ribose) polymerase (PARP) is cleaved during apoptosis (Kaufmann *et al.*, 1993) by a caspase activity (Lazebnik *et al.*, 1994) led to the identification of this activity as CPP32 (Nicholson *et al.*, 1995; Tewari *et al.*, 1995), a caspase cloned previously (Fernandes-Alnemri *et al.*, 1994). Since CPP32 could cleave PARP and because inhibitors of CPP32 blocked apoptosis, it was concluded that CPP32 is the mammalian CED-3 equivalent.

However, while only one caspase is known in *C.elegans*, 10 caspases have been identified in humans (Alnemri *et al.*, 1996). Of these only ICE, which processes cytokines, has a well established function (Thornberry, 1994; Gu *et al.*, 1997). The diversity of human caspases raised the question of whether CPP32 is the only apoptotic caspase or if other caspases are also required. The answer to this question is critical both for understanding apoptosis and for developing therapeutic strategies that target this process.

Several lines of evidence strongly suggest that in mammalian cells multiple caspases are involved. First, deficiency in CPP32 prevented apoptosis in some cell types while it had no effect in others, indicating that CPP32 is redundant in many cell types (Kuida *et al.*, 1996). This redundancy was consistent with the finding that caspases other than CPP32 can cleave PARP and are sensitive to CPP32 inhibitors (Gu *et al.*, 1995). Furthermore, studies in a cell-free system provided evidence that nuclear changes of apoptosis require more than one caspase (Lazebnik *et al.*, 1995; Takahashi *et al.*, 1996). In addition, several caspases were found to be processed in apoptotic cells (Chinnaiyan and Dixit, 1996), suggesting that their activation is linked to apoptosis. Finally, FLICE/MACH (caspase 8) was identified as a part of the receptor complexes that mediate apoptosis, implying that some caspases act as an interface between signaling pathways while others are involved in cell execution (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Thus, while there is little doubt that multiple caspases are implicated in apoptosis, the number and the roles in cell death remain poorly understood.

In this study, we used peptide inhibitors to identify directly the pool of caspases activated in apoptotic cells. Peptide inhibitors that mimic caspase substrates can block apoptosis. However the identity of the caspases inhibited remained elusive since peptide inhibitors have broad

specificity and can inactivate multiple caspases. To overcome this problem, we have used irreversible peptide inhibitors tagged with biotin as affinity labels (Thornberry *et al.*, 1994; Nicholson *et al.*, 1995) and developed an approach to simultaneously detect and identify multiple labeled caspases by their mobility in two-dimensional polyacrylamide gels. This approach provides a snapshot that identifies the caspases activated in apoptotic cells, estimates their relative abundance and indicates their post-translational modifications.

The approach that we developed relies solely on the biochemical activity of caspases and does not depend on caspase primary sequence, cell type or the signal inducing apoptosis. Using this approach we found that in all cell lines tested two major active caspases were CPP32 and Mch2. Intriguingly, both caspases were present as multiple species which varied with the cell line, perhaps indicating differences in post-translational modification. We also found that CPP32 and Mch2 were consistently activated in response to distinct apoptosis-inducing stimuli, suggesting that distinct stimuli ultimately activate the same set of caspases.

Results

Affinity labeling of caspases activated in apoptotic cells

The key point of our approach was to make no assumptions about the substrate specificity of the apoptotic caspases. Although all caspases cleave after an aspartate, their specificity varies. For example, CPP32 cleaves preferentially after a DxxD↓x site while Mch2 cleaves at a VxxD↓x site and ICE at YxxD↓x. As peptide inhibitors of caspases mimic a cleavage site, any inhibitor would display some selectivity between caspases. However, our goal was to use an inhibitor to label all caspases activated in apoptotic cells. Therefore, to decrease the selectivity of the inhibitor we use the following strategy. First, we used the observation that selectivity of caspase inhibitors decreases with increasing concentration (Nicholson, 1996). Second, we used an irreversible inhibitor and extended labeling times which improves labeling of caspases with low affinity for the peptide moiety of the inhibitor. The key requirement for such an approach is low non-specific binding at a high concentration of the label. Thus, we tested several available affinity labels: biotin-YVAD-acyloxymethyl ketone (biotin-YVAD-amk); biotin-VAD-chloromethyl ketone (biotin-VAD-cmk); and biotin-DEVD-chloromethyl ketone (biotin-DEVD-cmk).

Each label was tested for its ability to reveal active caspases present in apoptotic Jurkat cells and for non-specific labeling. Jurkat cells were treated with etoposide, an anti-cancer drug, to induce apoptosis or left untreated. Cells were harvested and snap-frozen 6 h after treatment when ~60% of the treated cells had characteristic apoptotic morphology. To label active caspases, cells were lysed by freezing and thawing in a buffer that contained one of the three affinity labels at concentrations from 0.1 to 10 μ M. The lysates were incubated for 15 min at 37°C, clarified by centrifugation, subjected to SDS-PAGE and the separated proteins were transferred to a membrane. The blot was probed with avidin followed by biotin-horseradish peroxidase and the labeled proteins were visualized by

enhanced chemiluminescence (ECL). We will refer to such blots as affinity blots.

Several polypeptides were labeled with 10 μ M biotin-YVAD-amk in etoposide-treated cells (Figure 1A). Several observations suggested that these proteins are caspases activated during apoptosis. First, they had apparent molecular weights between 17 and 21 kDa, a range consistent with the sizes of large caspase subunits. Second, the labeled proteins were detected only in cells treated with etoposide, an apoptosis-inducing agent. Finally, these proteins were only revealed in the presence of the affinity label. Labeling with biotin-DEVD-cmk and biotin-VAD-cmk revealed a set of polypeptides similar to those detected with biotin-YVAD-amk. However, at concentrations >1 μ M, both biotin-DEVD-cmk and biotin-VAD-cmk also labeled a wide range of proteins in untreated cells (data not shown). This apparent non-specific binding may be explained by the chemical reactivity of chloromethyl ketones, which react with a wide range of proteins (Grammer and Blenis, 1996). In contrast, acyloxymethyl ketones are quiescent in solution and are reactive only when bound to a cysteine protease (Krantz, 1994) although we found that increasing the concentration of biotin-YVAD-amk to 100 μ M resulted in increased background labeling (data not shown). Thus, for further studies, we chose to use 10 μ M biotin-YVAD-amk as the affinity label.

Since large subunits of caspases have similar molecular weights, we reasoned that one-dimensional (1D) gel electrophoresis may not resolve all labeled caspases. Therefore, we used two-dimensional (2D) gel electrophoresis. A broad pH range (3–10) was used for isoelectrofocusing to accommodate the labeled subunits of all known caspases with the possible exception of TY (Faucheu *et al.*, 1996) which has a calculated isoelectric point (pI) of 9.5. Proteins resolved by 2D electrophoresis were transferred to a membrane which was processed as an affinity blot. This 2D affinity blot (Figure 1B) indeed revealed more putative caspases than the 1D affinity blot (Figure 1A).

To confirm that the labeled proteins were related to apoptosis rather than to etoposide treatment, we used 293 cells which are resistant to apoptosis induced by etoposide (Figure 1C). We treated both Jurkat and 293 cells with etoposide, labeled active caspases with biotin-YVAD-amk and obtained affinity blots (Figure 1C). While putative caspases were labeled in Jurkat cells which underwent apoptosis after the treatment, no proteins were labeled in 293 cells which did not undergo apoptosis. Thus, the labeled proteins were linked to apoptosis rather than to etoposide treatment.

To determine whether the proteins labeled with biotin-YVAD-amk were required for apoptosis, we set up a cell-free system. In such systems, isolated nuclei added to extracts from apoptotic cells undergo morphological and biochemical changes characteristic of apoptosis (Lazebnik *et al.*, 1993). We and others have demonstrated that these changes depend on caspase activity and can be inhibited by peptide inhibitors of caspases, including YVAD-cmk (Lazebnik *et al.*, 1994; Martin *et al.*, 1995). Here we prepared extracts from apoptotic Jurkat cells that were lysed either in the absence (mock labeling) or presence of 10 μ M biotin-YVAD-amk, a concentration used for the affinity blots. Nuclei added to the mock labeled extract

Fig. 1. Affinity labeling reveals putative active caspases in apoptotic cells. (A) Jurkat cells were either treated with etoposide or left untreated. Cells were then lysed either in the presence of biotin-YVAD-amk or in dilution buffer and labeled proteins were visualized by 1D affinity blot. The percent of apoptotic cells is indicated below the blot. Arrows indicate labeled proteins. (B) 2D affinity blot of Jurkat cells treated with etoposide and labeled with biotin-YVAD-amk as in (A). (C) Putative caspases are not labeled in 293 cells, a cell line resistant to etoposide-induced apoptosis. The percent of apoptotic cells is indicated below the blot. (D) Biotin-YVAD-amk abolishes the activity required for apoptosis in the cell-free system. Jurkat cells were treated with etoposide and then lysed either with or without biotin-YVAD-amk (10 μ M). HeLa nuclei (1×10^5) were incubated with an aliquot of each lysate (10 μ l). After incubation for 60 min at 37°C nuclei were fixed, stained with DAPI and examined by fluorescence microscopy. Shown are nuclei representative for each treatment.

underwent apoptotic changes whereas nuclei added to the labeled extract did not (Figure 1D). Thus, at least one of the proteins labeled and, therefore, inactivated by biotin-YVAD-amk was required for the nuclear apoptotic changes in the cell-free system.

Purification of caspases

To identify the labeled putative caspases, we decided to purify them and to obtain their protein sequence. Sixty-six liters of Jurkat cells were treated with etoposide and cells were harvested when 60–70% of the cells acquired characteristic apoptotic morphology. Active caspases were labeled by lysing the cells in the presence of biotin-YVAD-amk. In preliminary experiments we determined that the labeled proteins were soluble by comparing 2D affinity blots of the pellet and supernatant obtained from the labeled cell lysate (data not shown). Therefore, lysates were clarified by centrifugation and the lysate was incubated with avidin agarose to bind labeled proteins. No biotinylated proteins remained unbound (Figure 2A), suggesting that all detectable, labeled caspases were bound

to the resin. Bound proteins were eluted by boiling in 1% SDS (Figure 2A), resolved by 2D electrophoresis and visualized by Coomassie-staining (Figure 2B, 3). An aliquot of the purified proteins was used to obtain a corresponding 2D affinity blot (Figure 2B, 4). We also obtained a Coomassie-stained 2D gel of total cell lysate (Figure 2B, 1) and the corresponding affinity blot (Figure 2B, 2). The sets of affinity-labeled proteins in cell lysate (Figure 2B, 2) and in purified sample (Figure 2B, 4) were similar, indicating that all detectable, labeled proteins were recovered by purification, although the efficiency of purification varied slightly between the proteins.

Multiple species of CPP32 and Mch2 are activated in apoptotic Jurkat cells

By comparing the 2D Coomassie-stained gel to the corresponding 2D affinity blot (Figure 3A), we identified eight spots that contained labeled proteins. These spots were excised from the Coomassie-stained gel, the proteins were digested, and the resulting peptides were sequenced. We obtained partial peptide sequences for six out of eight

Fig. 2. Purification of labeled caspases from Jurkat cell lysate. Cells were treated with etoposide and lysed in the presence of biotin-YVAD-amk as described in Material and methods. **(A)** Affinity chromatography of lysates on avidin-agarose. Shown is an affinity blot of input lysate (input), lysate incubated without the resin (mock-depletion), lysate after incubation with avidin-agarose (unbound) and the eluate from avidin-agarose (eluate). **(B)** 2D gel electrophoresis. Coomassie-stained 2D gel (1) and the matching affinity blot (3) of input lysate. Coomassie-stained 2D gel (2) and the matching affinity blot (4) of proteins eluted from avidin-agarose. Dashed boxes indicate location of affinity labeled proteins selected for sequencing.

proteins. Peptides derived from proteins contained in spots 1, 2, 5 and 6 matched the sequence of the caspase CPP32 while peptides derived from spots 3 and 4 matched the sequence of the caspase Mch2 (Figure 3B). We were unable to obtain sequences from proteins in spots 7 and 8 because of insufficient amount of recovered peptides.

The identified species of CPP32 and Mch2 differed in both molecular weight and pI. The apparent molecular weights of CPP32 species from spots 1 and 2 differed from those in spots 5 and 6 (Figure 3A), a difference that could be attributed to proteolytic processing. In fact, a peptide sequence obtained from spot 5 matched the sequence of CPP32 from Ser29 to Lys39 (Figure 3B). Since the residue preceding Ser29 in CPP32 is an aspartate and the protease used for protein digestion cleaves only after a lysine, Ser29 should be the N-terminal residue of the CPP32 species recovered from spot 5. Such a species was described previously and is referred to as p17 CPP32 subunit (Nicholson *et al.*, 1995). Peptides derived from two other CPP32 species (spots 1 and 2) spanned the

Asp28/Ser29 cleavage site, indicating that these species were processed differently from the p17 subunit. However, some species of CPP32 (spots 1 and 2) and Mch2 (spots 3 and 4) had similar apparent molecular weight but differed in their pIs. These results indicated that both CPP32 and Mch2 are present in apoptotic cells as multiple active species which have different post-translational modification.

To determine whether either of the two unidentified proteins (Figure 3A, spots 7 and 8) was CPP32, we probed a 2D affinity blot of apoptotic Jurkat cells with a monoclonal anti-CPP32 antibody (Transduction Laboratories). This antibody recognized the protein in spot 7, indicating that it is CPP32 (Figure 3C). The antibody also recognized another CPP32 species (Figure 3A and C, spot 2) previously identified by sequencing, but not the p17 subunit of CPP32 (Figure 3A and C, spot 5), indicating that the epitope for this antibody is located upstream of Asp28. Another CPP32 species (Figure 3A and C, spot 1) was also not detected with this antibody, perhaps due to low abundance of the CPP32 species. The lack of reliable antibodies to other caspases prevented us from using immunoblots to identify the protein in spot 8. Thus, by using peptide sequencing and immunoblotting we identified that the major active caspases in apoptotic Jurkat cells are multiple species of CPP32 and Mch2.

CPP32 and Mch2 are activated early in apoptosis

Having determined the position of CPP32 and Mch2 species on a 2D affinity blot, we used this caspase map to answer the following questions: when during apoptosis are CPP32 and Mch2 activated? Is the same set of caspases activated in response to distinct cytotoxic agents? Are the caspases that are activated during apoptosis conserved between cell lines?

To determine when CPP32 and Mch2 are activated during apoptosis, we treated Jurkat cells with etoposide and harvested the cells at different times during the treatment (Figure 4). For each sample, we determined the proportion of cells that had apoptotic nuclear morphology (Figure 4A), the extent of PARP cleavage, a marker of caspase activity (Figure 4B), and the presence of active caspases, as revealed by labeling with biotin-YVAD-amk (Figure 4C and D). Consistent with previous reports (Kaufmann *et al.*, 1993) we observed a delay between addition of etoposide and an increase in the proportion of apoptotic cells (Figure 4A). This increase was preceded by the appearance of the characteristic 85 kDa fragment of PARP (Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994) (Figure 4B). As revealed by the 1D affinity blot (Figure 4C), caspases were activated concurrently with the cleavage of PARP (Figure 4C). The increase in the intensity of labeling correlated with both the amount of cleaved PARP and the proportion of apoptotic cells. As revealed by 2D affinity blots (Figure 4D), most abundant labeled proteins were CPP32 and Mch2. Other weakly labeled proteins with apparent molecular weights similar to that of large caspase subunits were also detected (indicated by arrows in Figure 4D) transiently during etoposide treatment. Whether these labeled proteins are transiently activated caspases (Enari *et al.*, 1996) or are processing intermediates remains to be established. Labeled CPP32 was detected earlier than Mch2 (Figure 4D) although this could

Fig. 3. Identification of affinity labeled caspases purified from apoptotic Jurkat cells. (A) Coomassie-stained 2D gel and the matching 2D affinity blot of purified proteins labeled with biotin-YVAD-amk. Shown are regions indicated in Figure 2B by dashed boxes. Numbers 1–8 indicate the spots that were excised for peptide sequencing. (B) Partial peptide sequences obtained from proteins extracted from spots 1 to 6. Sequences are indicated by the line under the matched protein sequence. The number below the line corresponds to the spot in (A). The protease used for digestion of excised proteins cleaves after lysine residues which are highlighted in bold. (C) A 2D affinity blot (top) of lysate from apoptotic Jurkat cells was re-probed with an anti-CPP32 monoclonal antibody (bottom). Arrows indicate species of CPP32 detected by the antibody. (D) 2D map of affinity labeled caspases in Jurkat cells as identified by both sequencing (spots 1–6) and immunoblotting (spots 2 and 7). Positions of identified caspases are shown by circles. Unidentified putative caspases are indicated by a square.

be attributed to greater abundance of CPP32 rather than the sequence of activation. Thus, these experiments demonstrated that both CPP32 and Mch2 were activated early during apoptosis.

Apoptosis induced by distinct agents involves activation of CPP32 and Mch2

Whether apoptosis induced by distinct stimuli is mediated by the same set of caspases is a question fundamental to understanding apoptosis and to the use of caspases as targets for therapeutics. To address this question, we treated Jurkat cells with staurosporine, a kinase inhibitor (Jacobson *et al.*, 1993), anti-Fas antibody that activates the Fas pathway (Trauth *et al.*, 1989; Itoh *et al.*, 1991) and etoposide. When 60–70% of cells were apoptotic, active caspases were labeled with biotin-YVAD-amk to obtain 1D and 2D affinity blots. Both 1D and 2D affinity blots revealed no difference in activated caspases among cells killed by the three agents (Figure 5). To rule out errors caused by gel-to-gel differences in caspase mobility we used the following approach. The 2D affinity blot of etoposide-treated cells (Figure 5B, 1) was used as an

arbitrary standard. In addition, for each tested agent, we obtained two 2D affinity blots: one of the sample prepared from the cells treated with the agent (Figure 5B, 2 for anti-Fas and Figure 5B, 3 for staurosporine), and another of the mixture of this sample with the sample prepared from etoposide treated cells (Figure 5B, 4 for anti-Fas treated cells and Figure 5B, 5 for staurosporine treated cells). If the same caspases were activated by both the tested agent and etoposide, then the patterns of caspase labeling in all three 2D affinity blots should be identical whereas caspases specific for one agent will be detected on only two of the three blots. We found not only that CPP32 and Mch2 were activated by all three treatments but that the ratio between CPP32 and Mch2 species was similar regardless of the agent used. Hence, we concluded that at least three distinct signaling pathways activate the same set of CPP32 and Mch2 species in Jurkat cells.

Sets of active CPP32 and Mch2 species vary between cell lines

Whether the same set of caspases mediates apoptosis in various cell types is also a critical question both to

Fig. 4. Activation of CPP32 and Mch2 in Jurkat cells correlates with cleavage of PARP and the onset of apoptosis. Apoptosis in Jurkat cells was induced by etoposide and aliquots of cells were collected at different times during the treatment. The cells were scored for apoptotic nuclear morphology (A), and the cell lysates were analyzed by immunoblotting for cleavage of PARP (B), and by 1D (C) and 2D (D) affinity blotting for caspase activation. Arrows in (C) indicate active caspases. Arrows in (D) indicate putative caspases that appeared transiently during etoposide treatment. (D) Bottom: 2D map of active caspases as identified in Figure 3. Positions of identified caspases are shown by circles. Unidentified putative caspases detected transiently are indicated by squares.

understanding apoptosis and to evaluating the possibility of using caspases as target for therapeutics. In particular, since activation of caspases is a critical step in cell death induced by anti-cancer drugs, it is important to determine whether the set of caspases induced by a drug varies among tumor cells. To address this question, we induced apoptosis with etoposide in the following cells: BJAB and Namalwa (Burkitt's lymphomas), CEM and Molt-4 (adult lymphocytic leukemias, ALL), HL-60 (promyeloid leukemia) and M-07 (granulocytic leukemia). To compare sets of labeled caspases activated in these cell lines, we used the same approach that we used to compare caspases activated by distinct agents. We compared 2D affinity blots of the tested cells, of Jurkat cells (standard) and of a mixture of these two samples (Figure 6).

This analysis provided several observations. First, active CPP32 and Mch2 were detected in all cell lines (Figure 6 and data not shown). Second, CPP32 and Mch2 were the

most abundant labeled proteins. Third, the sets of active CPP32 and Mch2 species varied between cell lines (compare Figure 6B parts 1, 2 and 3). While the sets of CPP32 and Mch2 in BJAB, Namalwa, Molt-4 and HL-60 were similar to that of Jurkat (Figure 6, 1, and data not shown), the sets detected in M-07 and CEM were different (Figure 6, parts 2 and 3). In addition, we detected unidentified labeled proteins that could be caspases since they had molecular weights similar to that of large caspase subunits. These putative caspases were prominent in both M-07 and CEM cells (Figure 6B, parts 2 and 3) and were minor in other cell lines (data not shown). Although these putative caspases remain unidentified, we noticed that the location of the putative caspase from M-07 cells on 2D affinity blots appears to coincide with that of a minor CPP32 species detected in the pool of caspases purified from Jurkat cells (Figure 3A and B, spot 6). Thus, we found that active species of CPP32 and Mch2 vary with the cell line.

Fig. 5. CPP32 and Mch2 are activated in Jurkat cells treated with distinct apoptotic stimuli. Jurkat cells were treated with etoposide, an anti-Fas antibody or staurosporine to induce apoptosis, or left untreated. Caspases were labeled with biotin-YVAD-amk and visualized by 1D (A) and 2D (B, 1–3) affinity blots. (B, 4) is a 2D affinity blot of a mixture of samples used to obtain blots (B, 1) and (B, 2). (B, 5) is a 2D affinity blot of a mixture of samples used to obtain blots (B, 1) and (B, 3). (B, 6) is a map of active caspases detected by 2D affinity blots in etoposide-treated Jurkat cells.

Discussion

Although there is little doubt that caspases are a critical element of the apoptotic machinery, which of the human caspases are involved is not clear. To address this question we developed an approach that directly identifies caspases activated in apoptotic cells. This approach generates snapshots that simultaneously identify multiple labeled caspases, estimate their relative abundance and indicate their post-translational modifications. Using tumor cell lines as a model, we found that the major active caspases in apoptotic cells are CPP32 and Mch2. We also demonstrated that these caspases are present as multiple active species. In a given cell line these species remain the same irrespective of the apoptotic stimulus used, suggesting that distinct signaling pathways ultimately activate the same set of caspases. However, these species vary among cell lines, reflecting differences in their post-translational modifications.

Our approach relies only on the biochemical activity of caspases and does not depend on cell type, species or on the primary caspase sequence. Therefore, this study lays the groundwork for identification of caspases activated in apoptotic cells irrespective of the species or type of apoptotic stimulus. Our approach, however, does not provide information about what caspases do to bring about cell death, but rather directly identifies the pool of caspases activated in apoptotic cells, providing candidates to focus functional studies on.

Previously, the reversible peptide inhibitor DEVD-aldehyde was used to purify CPP32 from a cell lysate (Nicholson *et al.*, 1995). The affinity labeling approach described here is distinct from that of Nicholson *et al.* in two major aspects. First, our approach was designed to provide every caspase activated in apoptotic cells and not

only CPP32 a chance to be labeled. Second, the use of irreversible inhibitors in this study allows identification of the labeled caspases by affinity blotting.

The approach described here is simple and widely applicable. The large scale purification of labeled caspases, a costly step that we used to identify labeled caspases, can be avoided, by using reliable antibodies when they become available. Alternatively, caspases can be identified by their co-localization with known caspases on a 2D affinity blot. The latter approach requires as little as 1–10 µg of total cell protein or $\sim 3 \times 10^5$ apoptotic cells, an amount affordable in many experimental systems. Furthermore, using multi-layer avidin-biotin complexes may improve the sensitivity further. However, identification of labeled proteins that neither react with available antibodies nor co-migrate with known labeled caspases will require purification and sequencing. Considering the progress in protein sequencing (Muzio *et al.*, 1996) the amount of protein required to obtain peptide sequence can be significantly less than that used in this study. Other affinity labels can be used as they become available, in particular those that have low selectivity between caspases. It is important to keep in mind, however, that the 2D map of affinity labeled caspases may change with a label.

Since one molecule of a caspase binds only one molecule of an affinity label, affinity labeling can be used to quantify amounts of active caspases in the extract, given that the caspases are saturated with the label. Although in this study we did not attempt to quantify labeled caspases in cell extracts, we established by using recombinant ICE and CPP32 that the amount of caspase revealed by affinity labeling is proportional to the caspase concentration in a sample, suggesting that the described approach may be used to evaluate relative abundance of active caspases.

Fig. 6. Sets of active CPP32 and Mch2 species vary between cell lines. Jurkat, BJAB, M-07 and CEM cells were treated with etoposide and harvested when a majority of cell were apoptotic which was 60% for Jurkat and CEM, and 70% for BJAB and M-07. Active caspases were labeled with biotin-YVAD-amk and visualized by 2D affinity blots. **(A)** The 2D affinity blot of Jurkat cells. **(B)** 2D affinity blots of BJAB (1), M-07 (2) and CEM (3). **(C)** 2D affinity blots of a mixture of samples prepared from Jurkat cells and one of the following: BJAB (1), M-07 (2) or CEM (3). **(D)** 2D map of active caspases present in BJAB (1), M-07 (2) and CEM (3). Positions of caspases identified in etoposide-treated Jurkat cells are shown by circles. Unidentified putative caspases are indicated by squares.

That CPP32 and Mch2 are the only active caspases detected raises the question of why other caspases implicated in apoptosis, such as Mch3 (caspase 7) (Fernandes-Alnemri *et al.*, 1995b) and FLICE/MACH (Muzio *et al.*, 1996), were not revealed. One explanation is that some apoptotic caspases bind the affinity label at a rate much slower than CPP32 and Mch2. However, the high concentration of the label used, the irreversible nature of the binding and the extended time of labeling all favor labeling of caspases, even those with low affinity for the label. In fact, CPP32 and Mch2 bind poorly to inhibitors containing the YVAD motif (Fernandes-Alnemri *et al.*, 1995a; Nicholson *et al.*, 1995) but, nevertheless, were labeled. Therefore, if active unlabeled caspases were present, then their specificity was not like that of CPP32, Mch2 and ICE, a possibility that we cannot exclude at this point. However, considering that specificity of many caspases to peptide substrates overlaps with either CPP32 or ICE, the number of caspases that avoided labeling is likely to be small.

Another possibility is that the cell extract analyzed contained only a subset of total active caspases found in the cell. However, this is also unlikely since 2D affinity blots of soluble and insoluble fraction of total cell lysates revealed no new labeled caspase (data not shown). It is possible that some caspases are localized in some vesicles that are inaccessible to the label. However, since we were using cells displaying morphological and biochemical features of apoptosis that are believed to be caused by caspases, it is unclear how caspases enclosed in such vesicles could contribute to apoptosis.

A more probable explanation is that only active CPP32 and Mch2 were detected because other active caspases involved in apoptosis were present at much lower levels. This explanation is consistent with the cascade model of caspase activation (Martin and Green, 1995a; Fraser and Evan, 1996). In this model, signaling pathways of apoptosis lead to activation of caspase(s) that are at the beginning of this cascade. The activated caspases, in turn, process the caspases that are at the next step of the cascade, and this sequential activation continues until the caspases at the end of this cascade become activated. A hallmark of characterized protease cascades is a dramatic difference between the amount of protease activity required to trigger a protease cascade and the activity generated by the cascade (Jesty *et al.*, 1993; Jesty and Nemerson, 1995).

The notion that active apoptotic caspases can differ dramatically in abundance is consistent with the results of Enari *et al.* (1996). Enari *et al.* found that two unidentified caspase activities, one of which cleaved YVAD and another DEVD substrates, are activated sequentially during apoptosis mediated by Fas. Importantly, the YVAD caspase activity, which was activated first, constituted only 3% of the DEVD caspase activity. Assuming comparable catalytic rates, this translates into a 30-fold difference in concentration of active caspases. Such a difference would explain why some caspases evade detection in our assay. Hence, the relative abundance of active caspases present in apoptotic cells may reflect the hierarchy of the caspase cascade. According to this model, our finding that CPP32 and Mch2 are the major active caspases suggests that these proteases are the end product

of the caspase cascade and that other caspases involved in apoptosis may contribute to activation of CPP32 and Mch2.

The end product of a protease cascade, such as thrombin in blood clotting, often carries out the main function of the whole protease system, suggesting that the most abundant active caspases in apoptotic cells are directly involved in cell destruction. This argument is consistent with the predicted function of Mch2, which is thought to cleave lamins during apoptosis (Orth *et al.*, 1996; Takahashi *et al.*, 1996) thereby causing the destruction of the nuclear lamina and the collapse of the nucleus. Although putative CPP32 substrates are known, how their cleavage contributes to cell death is not clear. Nevertheless a recent study with CPP32-deficient mice indicated that CPP32 is required for developmental apoptosis in neurons although it is dispensable for apoptosis in other tested cell types (Kuida *et al.*, 1996).

Our study demonstrated that active CPP32 and Mch2 are present in apoptotic cells as multiple species. Some of the CPP32 species are cleaved at distinct sites and differ in their molecular weight, suggesting alternative or multistep proteolytic processing of these caspases. However, other species differed only in their isoelectric points, suggesting post-translational modifications other than proteolysis. These species may have different substrate specificity, intracellular localization or other properties relevant to their function in apoptosis.

Intriguingly, the sets of active CPP32 and Mch2 species varied between cell lines. This variability may reflect the differences in how CPP32 and Mch2 were activated. One possibility is that the caspases which process CPP32 or Mch2 vary between cell types and, therefore, cleave CPP32 or Mch2 precursors at different sites. Another explanation is that the processing caspase is the same but the access of this protease to cleavage sites is regulated differently. For example, regulation may be achieved by non-proteolytic, post-translational modifications that either change the conformation of the precursor or modify a residue next to a cleavage site rendering the site inaccessible to the processing caspase. Such post-translational modifications could also provide a link between signaling pathways and the caspase cascade involved in apoptosis.

The observation that induction of apoptosis by the same anti-cancer drug resulted in different pattern of caspase activation is intriguing and prompts the speculation that alterations in caspase processing may be related to cell transformation. Further characterization of caspase activation in untransformed and transformed cells would provide an insight as to whether this hypothesis is true.

In summary, this study describes a novel strategy that directly identifies the pool of caspases activated in apoptotic cells. Using tumor cell lines as a model we found that CPP32 and Mch2 are the major active caspases present in apoptotic cells. We suggest that other caspases if involved in apoptosis play a regulatory role by contributing to activation of CPP32 or Mch2. Our finding that active CPP32 and Mch2 are present as various multiple species suggests that pathways that activate these enzymes vary with cell type. The methodology described here is widely applicable to experimental systems of apoptosis and should facilitate the understanding of this process.

Materials and methods

Cell culture

All cells except 293 were maintained in RPMI tissue culture medium (Gibco-BRL) supplemented with 10% fetal bovine serum. 293 cells were maintained in DMEM tissue culture medium supplemented with 10% fetal bovine serum. M-07 cells were provided by Nick Carpino (Cold Spring Harbor Laboratory).

Induction of apoptosis

Apoptosis in cells ($5\text{--}10 \times 10^5$ cells/ml) was induced with one of the following agents: 50 μM etoposide (Sigma), 1 μM staurosporine (Sigma) or 100 ng/ml CH-11 anti-Fas antibody (Kamiya). The proportion of apoptotic cells was measured as described previously (Lazebnik *et al.*, 1993). Briefly, cells were fixed in 4% paraformaldehyde (Ted Pella), permeabilized with 0.1% Triton-100 (Sigma) and stained with 1 $\mu\text{g/ml}$ DAPI. Nuclear morphology was examined using fluorescence microscopy and cells with characteristic chromatin condensation were scored as apoptotic.

SDS-PAGE and 2D gel electrophoresis

For 1D SDS-PAGE, cells lysates were diluted to 2 mg/ml with SDS sample buffer, boiled for 5 min and stored at -20°C . 10 μg of protein from each sample was separated on a 15% SDS-PAGE gel. For 2D gels, cell lysates were diluted to 1 mg/ml in dSDS buffer (0.3% SDS, 1% β -mercaptoethanol, 0.05 M Tris, pH 8), heated to 100°C for 5 min and cooled on ice. One-tenth volume of DNase/RNase solution (1 mg/ml DNase, 0.5 mg/ml RNase, 0.5 M Tris, 0.5 M MgCl_2 , pH 7.0) was added to the samples which were then snap-frozen, lyophilized and resuspended in urea sample buffer [9.5 M urea, 2% NP40, 5% β -mercaptoethanol, 2% ampholites 1.34% pH 3.5–10 (Pharmacia), 0.44% pH 7–9 (Pharmacia) and 0.22% pH 5–6 (Serva)]. The samples (either 10 or 50 μg) were separated in a 3% acrylamide-IEF gel for 19 000 Vh. The IEF gel was then loaded onto a 20 cm $\times$ 24 cm 15% polyacrylamide gel that was run at 60 W for 3.5 h.

Labeling of caspases and affinity blotting

All affinity labels were obtained from Biosyn (Ireland) and stored at -70°C as a 10 mM stock solution in DMSO. The stock solution was diluted before use into MDB buffer (50 mM NaCl, 2 mM MgCl_2 , 5 mM EGTA, 10 mM HEPES, 1 mM DTT, pH 7) to prepare a 20 μM solution. Cells were resuspended at 1×10^7 cells/ml in KPM buffer (50 mM KCl, 50 mM PIPES, 10 mM EGTA, 1.92 mM MgCl_2 , pH 7, 1 mM DTT, 0.1 mM PMSF, 10 $\mu\text{g/ml}$ of cytochalasin B and 2 $\mu\text{g/ml}$ of the following: chymostatin, pepstatin, leupeptin, antipain), pelleted and the pellet was snap-frozen in liquid nitrogen. An equal volume of 20 μM biotin-YVAD-amk was added to the cell pellet and cells were lysed by three cycles of freezing-thawing. The lysates were incubated at 37°C for 15 min, clarified at 4°C for 20 min at 16 000 g, and mixed with either SDS-PAGE or 2D electrophoresis buffer.

The labeled proteins were separated by 1D SDS-PAGE or 2D electrophoresis and transferred to PVDF Immobilon P^{5Q} membrane (Millipore) for 1 h at 100 V (1D gels) or 4 h at 45 V (2D gels) using the standard transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). Membranes were then soaked in methanol and dried at room temperature overnight or at 80°C for 15 min. Dried membranes were incubated in avidin-Neutralite (Molecular Probes) at 1 $\mu\text{g/ml}$ in PTB (20 mM Tris, 150 mM NaCl, 0.02% Tween-20) supplemented with 1% BSA (PBT-BSA). Membranes were washed and then incubated in biotinylated horseradish peroxidase (Molecular Probes) at 25 ng/ml in PBT-BSA. The labeled proteins were visualized by ECL (Amersham). Using recombinant ICE as a standard, we estimated that this assay detects as little as 0.1 ng of an active caspase, the amount present in 1 μg of a lysate prepared from apoptotic Jurkat cells.

Purification of labeled caspases

Five batches (total of 66 l) of Jurkat cells grown to $6\text{--}7 \times 10^5$ cells/ml were treated with etoposide (50 μM) to induce apoptosis. Cells were harvested 6–7 h after treatment when greater than 60–70% of the cell population had apoptotic nuclear morphology. Cells were collected by centrifugation, lysed in the presence of 10 μM biotin-YVAD-amk to label caspases and the lysates were clarified by centrifugation at 100 000 g for 1 h at 4°C . The protein concentration of the pooled supernatant was 8.9 mg/ml with total of 68 ml. To remove unbound probe, the lysate was dialyzed against four changes of MDB buffer (50 mM NaCl, 2 mM MgCl_2 , 5 mM EGTA, 10 mM HEPES, 1 mM DTT, pH 7) supplemented

with 2 µg/ml of each chymostatin, leupeptin, antipain and pepstatin, and 0.1 mM PMSF. 68 ml of dialyzed extract was incubated with 6 ml of Immopure Immobilized Avidin (Pierce) for 1 h. The resin was washed with 1% SDS and bound proteins were eluted by boiling in the same buffer. The eluted proteins were precipitated by adding 0.25 volume of TCA solution (100% trichloroacetic acid, 0.4% sodium deoxycholate) to the eluate, washed once with 1 mM HCl in acetone and once with pure acetone. The precipitate was resuspended in 150 µl of the urea sample buffer. 0.2 µl of the sample were saved and the rest was resolved by 2D electrophoresis. The gel was stained for 1 h with 0.05% Coomassie Blue (Sigma) in 5% acetic acid containing 10% methanol and then destained in 5% acetic acid containing 10% methanol. To obtain the matching affinity blot, the saved sample (0.2 µl) was resolved by 2D electrophoresis and the gel was used to obtain the affinity blot. The labeled proteins were identified on the Coomassie-stained gel by comparing the gel with the matching affinity blot. The identified spots were excised and used to obtain the protein sequence.

Sequencing of purified caspases

The excised gel fragments were washed twice with 50% methanol and treated with Achromobacter protease I (Waco Chemical) in (50 mM Tris-HCl, 0.05% Tween 20, pH 9.0) for 20 h at 30°C to digest the proteins. Peptide fragments were extracted in a solution of 50% acetonitrile containing 0.065% trifluoroacetic acid and separated by HPLC (Hewlett-Packard, model 1090) using a Vydac C-18 column (1.0×250 mm/100 µM, 300 Å). Separated peptides were sequenced using an automated protein sequencer (Applied Biosystems, model 494).

Immunoblotting

Immunoblotting with monoclonal antibody to PARP (gift of Dr Guy Poirier) was carried out as described previously (Kaufmann *et al.*, 1993). Probing of an affinity blot with monoclonal anti-CPP32 antibody (Transduction Lab) was carried out after the blot was soaked in methanol to inactivate the horseradish peroxidase bound to avidin.

Cell-free system of apoptosis

The cell-free assays were carried out as described (Lazebnik *et al.*, 1993). Briefly, purified HeLa nuclei were added to a Jurkat cell extract (10 µl) supplemented with ATP regeneration system (2 mM ATP, 10 mM creatine phosphate and 50 mg/ml creatine kinase) and incubated for 60 min at 37°C. To observe nuclear apoptotic changes nuclei were fixed with 4% paraformaldehyde, stained with DAPI (1 µg/ml) and examined by fluorescence microscopy. Images were acquired using a Photometrics PXL CCD camera (Photometrics Ltd) controlled by Oncor Image software (Oncor Inc.) and figures prepared using Adobe Photoshop software.

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References

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J.Y. (1996) Human ICE/CED-3 protease nomenclature. *Cell*, **87**, 171.

Boldin, M., Goncharov, T., Goltsev, Y. and Wallach, D. (1996) Involvement of MACH, a novel MORT1/FADD-Interacting Protease, in Fas/Apo-1 and TNF receptor-induced cell death. *Cell*, **85**, 803–815.

Chinnaiyan, A.M. and Dixit, V.M. (1996) The cell-death machine. *Curr. Biol.*, **6**, 555–562.

Enari, M., Talanian, R.V., Wong, W.W. and Nagata, S. (1996) Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature*, **380**, 723–726.

Faucheu, C., Blanchet, A.M., Collarddutilleul, V., Lalanne, J.L. and Diuhercend, A. (1996) Identification of a cysteine protease closely related to interleukin-1 beta-converting enzyme. *Eur. J. Biochem.*, **236**, 207–213.

Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1994) CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J. Biol. Chem.*, **269**, 30761–30764.

Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1995a) Mch2, a new member of the apoptotic ced-3/ICE cysteine protease family. *Cancer Res.*, **55**, 2737–2742.

Fernandes-Alnemri, T. *et al.* (1995b) Mch3, a novel human apoptotic cysteine protease highly related to cyp32. *Cancer Res.*, **55**, 6045–6052.

Fraser, A. and Evan, G. (1996) A license to kill. *Cell*, **85**, 781–784.

Grammer, T.C. and Blenis, J. (1996) The serine protease inhibitors, tosylphenylalanine chloromethyl ketone and tosyllysine chloromethyl ketone, potentially inhibit pp70(s6k) activation. *J. Biol. Chem.*, **271**, 23650–23652.

Gu, Y., Sarnacki, C., Aldape, R.A., Livingston, D.J. and Su, M.S. (1995) Cleavage of poly(ADP-ribose) polymerase by interleukin-1 beta converting enzyme and its homologs TX and Nedd-2. *J. Biol. Chem.*, **270**, 18715–18718.

Gu, Y. *et al.* (1997) Activation of interferon-γ inducing factor mediated by interleukin-1β converting enzyme. *Science*, **275**, 206–208.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. and Nagata, S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66**, 233–243.

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C. and Raff, M.C. (1993) Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature*, **361**, 365–368.

Jesty, J. and Nemerson, Y. (1995) In Beutker, E., Lichtman, M.A., Collier, B.S. and Kipps, T.J. (eds), *The Pathway of Blood Coagulation*. McGraw-Hill, New York, pp. 1227–1238.

Jesty, J., Beltrami, E. and Willems, G. (1993) Mathematical analysis of a proteolytic positive-feedback loop: dependence of lag time and enzyme yields on the initial conditions and kinetic parameters. *Biochemistry*, **32**, 6266–6274.

Kaufmann, S.H., Desnoyers, S., Ottaviano, Y., Davidson, N.E. and Poirier, G.G. (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.*, **53**, 3976–3985.

Krantz, A. (1994) Peptidyl (acyloxy)methanes as quiescent affinity labels for cysteine proteinases. *Methods Enzymol.*, **244**, 656–671.

Kuida, K., Zheng, T.S., Na, S.Q., Kuan, C.Y., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature*, **384**, 368–372.

Lazebnik, Y.A., Cole, S., Cooke, C.A., Nelson, W.G. and Earnshaw, W.C. (1993) Nuclear events of apoptosis *in vitro* in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis. *J. Cell Biol.*, **123**, 7–22.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.E. (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, **371**, 346–347.

Lazebnik, Y.A., Takahashi, A., Moir, R.D., Goldman, R.D., Poirier, G.G., Kaufmann, S.H. and Earnshaw, W.C. (1995) Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. *Proc. Natl Acad. Sci. USA*, **92**, 9042–9046.

Martin, S. and Green, D. (1995) Protease activation during apoptosis: death by a thousand cuts? *Cell*, **82**, 349–352.

Martin, S.J., Newmeyer, D.D., Mathias, S., Farschon, D.M., Wang, H.G., Reed, J.C., Kolesnick, R.N. and Green, D.R. (1995) Cell-free reconstitution of Fas-, UV radiation- and ceramide-induced apoptosis. *EMBO J.*, **14**, 5191–5200.

Muzio, M. *et al.* (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell*, **85**, 817–827.

Nicholson, D.W. (1996) ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. *Nature Biotechnol.*, **14**, 297–301.

Nicholson, D.W. *et al.* (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, **376**, 37–43.

Orth, K., Chinnaiyan, A.M., Garg, M., Froelich, C.J. and Dixit, V.M. (1996) The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J. Biol. Chem.*, **271**, 16443–16446.

Takahashi, A. *et al.* (1996) Cleavage of lamin A by Mch2 alpha but not CPP32: Multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc. Natl Acad. Sci. USA*, **93**, 8395–8400.

- Tewari,M., Quan,L.T., O'Rourke,K., Desnoyers,S., Zeng,Z., Beidler, D.R., Poirier,G.G., Salvesen,G.S. and Dixit,V.M. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell*, **81**, 801–809.
- Thompson,C. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456–1462.
- Thornberry,N.A. (1994) Interleukin-1 beta converting enzyme. *Methods Enzymol.*, **244**, 615–631.
- Thornberry,N.A., Peterson,E.P., Zhao,J.J., Howard,A.D., Griffin,P.R. and Chapman,K.T. (1994) Inactivation of interleukin-1 beta converting enzyme by peptide (acyloxy)methyl ketones. *Biochemistry*, **33**, 3934–3940.
- Trauth,B.C., Klas,C., Peters,A.M.J., Matzku,S., Moller,P., Falk,W., Debatin,K.-M. and Krammer,P.H. (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science*, **245**, 301–305.
- Xue,D. and Horvitz,H.R. (1995) Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature*, **377**, 248–251.
- Yuan,J., Shaham,S., Ledoux,S., Ellis,H.M. and Horvitz,H.R. (1993) The *C.elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β -converting enzyme. *Cell*, 641–652.

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