The EMBO Journal vol.15 no.10 pp.2407–2416, 1996

The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism

Seamus J.Martin[1], Gustavo P.Amarante-Mendes, Lianfa Shi[2], Tsung-Hsien Chuang[3], Carlos A. Casiano[4], Geraldine A.O'Brien, Patrick Fitzgerald, Eng M.Tan[4], Gary M.Bokoch[3], Arnold H.Greenberg[2] and Douglas R.Green

La Jolla Institute for Allergy and Immunology, 11149 N. Torrey Pines Road, La Jolla, CA 92037, USA, [2]The Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba R3E 0V9, Canada, [3]Department of Immunology and Cell Biology and [4]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA

[1]Corresponding author

The major mechanism of cytotoxic lymphocyte killing involves the directed release of granules containing perforin and a number of proteases onto the target cell membrane. One of these proteases, granzyme B, has an unusual substrate site preference for Asp residues, a property that it shares with members of the emerging interleukin-1β-converting enzyme (ICE)/CED-3 family of proteases. Here we show that granzyme B is sufficient to reproduce rapidly all of the key features of apoptosis, including the degradation of several protein substrates, when introduced into Jurkat cell-free extracts. Granzyme B-induced apoptosis was neutralized by a tetrapeptide inhibitor of the ICE/CED-3 family protease, CPP32, whereas a similar inhibitor of ICE had no effect. Granzyme B was found to convert CPP32, but not ICE, to its active form by cleaving between the large and small subunits of the CPP32 proenzyme, resulting in removal of the prodomain via an autocatalytic step. The cowpox virus protein CrmA, a known inhibitor of ICE family proteases as well as granzyme B, inhibited granzyme B-mediated CPP32 processing and apoptosis. These data demonstrate that CPP32 activation is a key event during apoptosis initiated by granzyme B.

Keywords: apoptosis/cell-free/CPP32/granzyme B/proteolysis

Introduction

Recent studies have strongly implicated the interleukin-1β-converting enzyme (ICE)/CED-3 family of proteases as key participants in the apoptotic cell deaths that occur during development (Ellis and Horvitz, 1986; Yuan *et al.*, 1993), due to ligation of the Fas (CD95) or tumor necrosis factor (TNF) receptors (Enari *et al.*, 1995; Kuida *et al.*, 1995; Los *et al.*, 1995; Martin *et al.*, 1995a; Miura *et al.*, 1995; Tewari and Dixit, 1995; Tewari *et al.*, 1995a), trophic factor deprivation (Gagliardini *et al.*, 1994) and a number of other apoptosis-inducing stimuli (Lazebnik *et al.*, 1994; Boudreau *et al.*, 1995). Several ICE/CED-3 family members have now been identified, most of which induce apoptosis when ectopically expressed in various cell types (Miura *et al.*, 1993; Fernandes-Alnemri *et al.*, 1994; Kumar *et al.*, 1994; Wang *et al.*, 1994; Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995; for reviews see Kumar, 1995; Martin and Green, 1995). However, the significance of the latter observation is unclear since introduction of many proteases into the cell cytoplasm can initiate apoptosis (Williams and Henkart, 1994). Recent evidence indicates that ICE itself is unlikely to be a major participant in cell death during development, since targeted disruption of the *Ice* gene in mice did not alter dramatically the phenotype of these animals (Kuida *et al.*, 1995; Li *et al.*, 1995). Thus, current efforts are focused upon determining which, if any, of the ICE/CED-3 family of proteases are generally required for apoptosis.

A distinctive feature of the ICE/CED-3 family of proteases is that they cleave after Asp residues (Thornberry *et al.*, 1992; Nicholson *et al.*, 1995; Tewari *et al.*, 1995a; Xue and Horvitz, 1995) and typically are expressed as precursor proteins that require further processing by cleavage at Asp residues to form active heterodimers (Thornberry *et al.*, 1992; Faucheu *et al.*, 1995; Tewari *et al.*, 1995a). There is also some evidence that ICE/CED-3 family members can autoprocess, as well as process other family members (Thornberry *et al.*, 1992; Faucheu *et al.*, 1995; Tewari *et al.*, 1995a). Only one other protease with a substrate preference for Asp has been described—the cytotoxic lymphocyte (CL) protease, granzyme B (Shi *et al.*, 1992a,b). Granzyme B is a serine protease found in CL granules that, in combination with the pore-forming protein perforin, can initiate rapid internal destruction (apoptosis) in cells exposed to these proteins (Shi *et al.*, 1992a,b, 1994). Furthermore, targeted disruption of granzyme B in mice resulted in a profound defect in CL cell function, with cells from these animals incapable of inducing rapid target cell death (Heusel *et al.*, 1994; Shresta *et al.*, 1995). In contrast, granzyme A-deficient mice exhibit normal CL killing (Ebnet *et al.*, 1995). Whether granzyme B initiates apoptosis by activating ICE/CED-3 family proteases, or bypasses this requirement by acting directly upon a common set of substrates is not known. In this context, previous studies have shown that granzyme B is not capable of processing the 45 kDa proform of ICE to its active p20/p10 heterodimeric form (Darmon *et al.*, 1994). However, it has been found recently that granzyme B is capable of cleaving CPP32 *in vitro*, but whether this cleavage event is central to granzyme B-induced apoptosis remains unclear (Darmon *et al.*, 1995).

Recently, several cell-free systems have been described that faithfully reproduce essentially all of the changes seen in intact cells during apoptosis (Lazebnik *et al.*,

1993; Newmeyer *et al.*, 1994; Chow *et al.*, 1995; Martin *et al.*, 1995a). These extracts have proven very useful for analyzing apoptosis-associated proteolytic cleavage events, particularly since protease inhibitory peptides or proteins can be added directly to these extracts, a task which is made much more difficult in intact cells owing to the permeability barrier of the plasma membrane (Lazebnik *et al.*, 1994; Newmeyer *et al.*, 1994; Chow *et al.*, 1995; Martin *et al.*, 1995a; Nicholson *et al.*, 1995).

To test directly whether granzyme B, in the absence of perforin, is sufficent to initiate the whole process of apoptosis, we introduced purified granzyme B into cell-free extracts from healthy (non-apoptotic) Jurkat cells and evaluated these extracts at various times thereafter for nuclear changes and proteolytic cleavage events typical of apoptosis. Here, we report that granzyme B is capable of initiating the whole range of apoptotic changes in these extracts, including proteolysis of several key substrates and condensation and margination of chromatin in nuclei added to these extracts. We demonstrate that the ability of granzyme B to initiate these changes is achieved via processing and activation of the ICE/CED-3 family protease, CPP32, since inhibition of this protease also blocked the effects of granzyme B. Furthermore, granzyme B was found to be capable of directly processing CPP32 in a CrmA-inhibitable manner.

Results

The mechanism of Fas-independent CL killing has been the subject of much investigation. Both cytotoxic T lymphocytes and natural killer cells kill their targets by releasing cytoplasmic granules containing perforin (cytolysin) and a number of granule proteases, termed granzymes (fragmentins), into the intercellular space between the target and the CL (Henkart, 1985; Liu *et al.*, 1995; Smyth and Trapani, 1995). How these granzymes act to induce death of the target cell is unclear. Current evidence suggests that perforin acts as a conduit for entry of the granule proteases which then initiate rapid 'internal disintegration' of the target in a manner characteristic of apoptosis (Nakajima and Henkart, 1994). However, whether one or more granzymes are sufficient to initiate the whole process, in the absence of perforin, is not known.

Induction of apoptosis by cytotoxic granules or granzyme B in Jurkat cell-free extracts

As an approach to studying the mechanism of toxicity of CL granules, we explored whether purified granules, or granzyme B, could induce changes characteristic of apoptosis in a human cell-free system. Figure 1A demonstrates that CL granules were indeed capable of producing the changes in nuclear morphology (chromatin margination and condensation) that characterize apoptosis in whole cells. Interestingly, granzyme B alone was also capable of producing the same effects. In contrast, addition of purified perforin alone to these extracts did not produce any apoptotic changes, nor did it augment the ability of granzyme B to do so (Figure 1B).

Cell-free apoptosis, induced by either cytotoxic granules or granzyme B, was associated with cleavage of a number of proteins; poly(ADP-ribose) polymerase (PARP), fodrin (non-erythroid spectrin), the 70 kDa protein component of the U1 small nuclear ribonucleoprotein (U1snRNP) and D4 GDP dissociation inhibitor (GDI), previously shown to undergo proteolysis during apoptosis of intact cells (Kaufmann *et al.*, 1993; Casciola-Rosen *et al.*, 1994; Martin *et al.*, 1995a,b; S.Na, T-.H.Chuang, D.Danley, T.Turi, J.H.Hanke and G.M.Bokoch, submitted; Figure 2). In contrast, cleavage of β-actin or lamin A/C was not observed in apoptotic extracts during this time course, indicating that there is not a generalized degradation of all proteins during this process (Figure 2). Nuclei incubated in cell-free extracts in the absence of CL granules or granzyme B did not undergo apoptotic changes (Figure 1), nor was any significant proteolysis observed in these extracts (Figure 2).

The kinetics of cleavage of some of the substrates (PARP, fodrin) were rapid, being almost completed within 15 min of addition of either CL granules or granzyme B to the extracts (Figure 2). In contrast, cleavage of other substrates (U1snRNP, GDI) was markedly slower, suggesting that either these substrates were somehow protected during the initial phase (e.g. due to their location within the cell) or that granzyme B was activating other protease(s) in the cell-free extracts which then cleaved the latter substrates.

Selective inhibition of granzyme B-initiated apoptosis by inhibition of CPP32 but not ICE

Since intracellular substrates for granzyme B have been elusive, we considered it unlikely that granzyme B was directly cleaving all of the proteins we observed undergoing proteolysis in the cell-free extracts. Previously, it has been suggested that granzyme B may participate in CL killing by activating ICE (which requires cleavage after specific Asp residues for conversion from its inactive 45 kDa precursor to the active p20/p10 heterodimer) within the target cell (Vaux *et al.*, 1994). Since granzyme B- and CL granule-induced apoptosis was associated with proteolysis of PARP (Figure 2), a protein that was demonstrated recently to be cleaved during apoptosis by the ICE/CED-3 family protease CPP32 (Nicholson *et al.*, 1995; Tewari *et al.*, 1995a), this suggested that granzyme B may be activating CPP32 in the cell-free extracts. To address this question, we tested the ability of a recently described tetrapeptide inhibitor of CPP32 [Asp-Glu-Val-Asp (Ac-DEVD-CHO); Nicholson *et al.*, 1995] to inhibit granzyme B-induced apoptosis in the cell-free extracts. As a control, we used a similar peptide (Tyr-Val-Ala-Asp; Ac-YVAD-CHO) that is a selective inhibitor of ICE (Thornberry *et al.*, 1992) and has little effect on CPP32 activity (Nicholson *et al.*, 1995). Using these peptides, we found that inhibition of CPP32 activity dramatically blocked the ability of granzyme B to induce apoptosis in cell-free extracts (Figure 3). Significantly, cleavage of PARP, fodrin and U1snRNP in these extracts was blocked completely by addition of the CPP32 inhibitor, whereas the ICE inhibitory peptide had little effect at the same concentrations. Thus, these data suggested that granzyme B initiates apoptosis in the cell-free extracts by activating CPP32.

Processing of CPP32, but not ICE, by granzyme B

To explore whether granzyme B can convert CPP32 from its 36 kDa precursor form to the active p17/p12

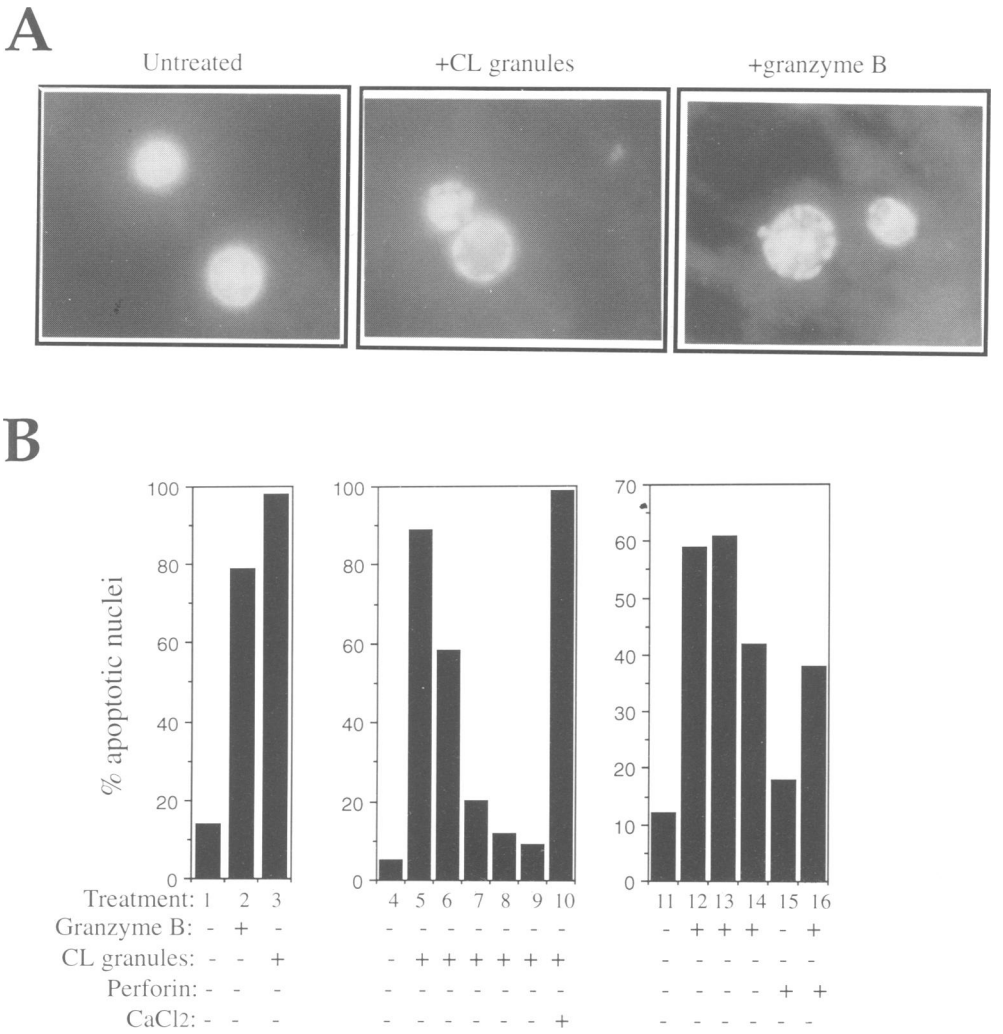

Fig. 1. CL granules and granzyme B induce apoptosis in cell-free extracts. (**A**) Rat liver nuclei were incubated at 37°C for 2 h in 25 µl of cytoplasmic extract (75 µg) from Jurkat cells (see Materials and methods) with or without addition of CL granules (240 ng), or purified granzyme B (75 ng), as indicated. (**B**) Apoptosis in Jurkat cell-free extracts after a 2 h incubation at 37°C under the indicated conditions. Cell-free reactions (25 µl) were assembled with 75 µg of cytoplasmic extract from Jurkat cells. Where indicated, purified proteins were added as follows; 75 ng of granzyme B (treatments 2 and 12), 50 ng of granzyme B (treatment 13), 25 ng of granzyme B (treatments 14 and 16); 240 ng of CL granules (treatments 3, 5 and 10), 120 ng of CL granules (treatment 6), 60 ng of CL granules (treatment 7), 30 ng of CL granules (treatment 8), 15 ng of CL granules (treatment 9); 95 ng of perforin (treatments 15 and 16). In order to inactivate perforin, cytotoxic granules were incubated in buffer containing 1 mM $CaCl_2$ for 30 min prior to addition to the extract, where indicated. Data shown are derived from counts of a minimum of 100 nuclei per treatment and are representative of five independent experiments.

heterodimer, we exposed [^{35}S]methionine-labeled CPP32, or hICE, prepared by *in vitro* transcription/translation, to purified granzyme B. Figure 4A demonstrates that granzyme B cleaved the 36 kDa CPP32 precursor to signature cleavage products of 17 and 12 kDa, characteristic of active CPP32 (Nicholson *et al.*, 1995; Tewari *et al.*, 1995a), whereas hICE remained completely intact under the same conditions. Interestingly, while CL granules also processed CPP32 to its p17/p12 form, they also proved capable of processing ICE to a major fragment of 10 kDa with a number of minor cleavage products also apparent (Figure 4A). This suggests that there is an ICE-cleaving activity within CL granules, distinct from granzyme B. However, in view of the fact that the active ICE heterodimer is composed of 20 and 10 kDa subunits, it is unlikely that CL granule-processed ICE is functional. Titration of granzyme B on pro-CPP32 revealed that processing of CPP32 occurred at concentrations of as little as 50 ng/ml of this protein (Figure 4B; and data not shown), whereas hICE remained intact even at 200-fold higher concentrations. These experiments also revealed that CPP32 was processed initially to 24 and 12 kDa cleavage products, with the 17 kDa fragment arising as a consequence of further cleavage of the 24 kDa fragment (Figure 4B). This cleavage pattern suggests that granzyme B initially cuts between the small and large subunits of the CPP32 proenzyme, with removal of the prodomain occurring subsequent to this.

As controls in these experiments, several other [^{35}S] methionine-labeled proteins (FADD, Bax, D4GDI), also prepared by *in vitro* transcription/translation, were assessed for cleavage by granzyme B and CL granules. Figure 4C illustrates that neither granzyme B nor CL granules cleaved any of these proteins, indicating that the cleavage of CPP32 by granzyme B is highly specific. The inability of granzyme B or CL granules to cleave D4GDI directly is

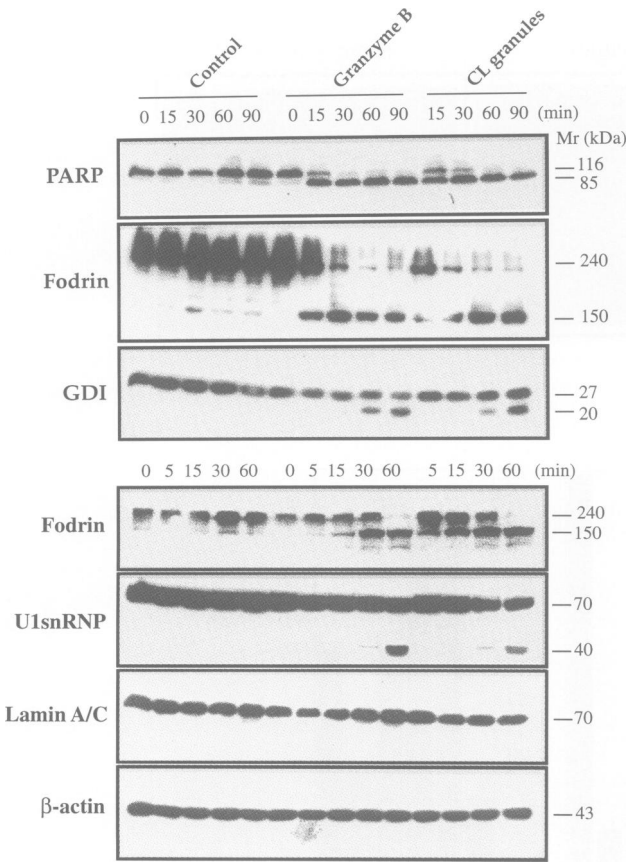

Fig. 2. Proteolysis of multiple protein substrates during granzyme B- or CL granule-induced cell-free apoptosis. A cell-free extract of Jurkat cells (75 μg of protein in 25 μl) was incubated at 37°C for the indicated times in the presence or absence of granzyme B (75 ng), or CL granules (240 ng), as indicated. Proteins (25 μg per lane) were then separated by SDS–PAGE followed by Western blotting and probing for the indicated proteins.

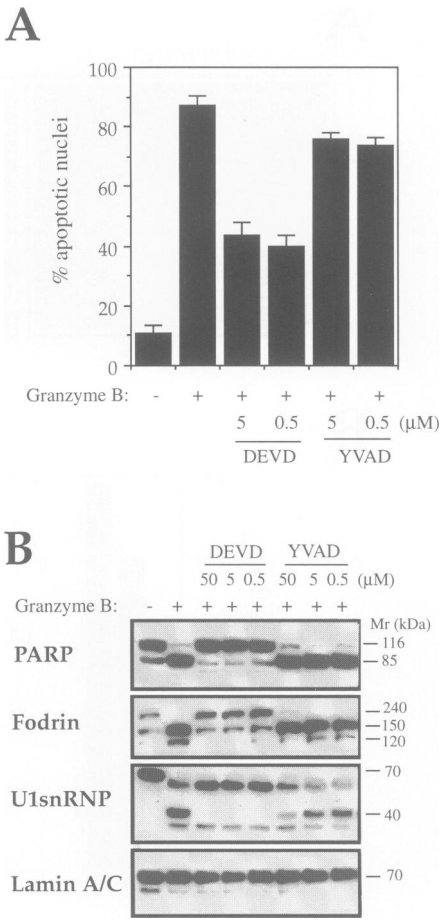

Fig. 3. Inhibition of granzyme B-initiated apoptosis by an inhibitor of CPP32, but not by an inhibitor of ICE. (**A**) Cell-free extracts of Jurkat cells (75 μg of protein in 25 μl) were incubated at 37°C for 2 h with or without granzyme B (75 ng), and the indicated concentrations of either Ac-DEVD-CHO or Ac-YVAD-CHO peptides, as shown. Data are the means of triplicates (±SEM), with a total of 300 nuclei per treatment counted. (**B**) Cell-free extracts of Jurkat cells (75 μg of protein in 25 μl) were incubated at 37°C for 3 h with or without granzyme B (75 ng), and the indicated concentrations of either Ac-DEVD-CHO or Ac-YVAD-CHO peptides, as in (A). Proteins were then separated by SDS–PAGE, followed by Western blotting and probing for the indicated proteins.

particularly significant since this protein became cleaved upon addition of granzyme B or CL granules to cell-free extracts (Figure 2). This provides further evidence that granzyme B-initiated cleavage of this protein was dependent upon its ability to activate CPP32 in cell-free extracts.

CPP32 inhibitor peptide arrests granzyme B-mediated processing of pro-CPP32 at a p24/p12 intermediate

We next examined the ability of the CPP32 inhibitor peptide (Ac-DEVD-CHO) that blocked apoptosis in the cell-free system, to inhibit processing of pro-CPP32 by granzyme B. As a control, we used the ICE inhibitor peptide (Ac-YVAD-CHO) at the same concentrations. These experiments were conducted to determine whether the CPP32 inhibitor peptide blocked apoptosis at a point distal to activation of CPP32 by granzyme B, or whether the peptide could also interfere directly with the activation of CPP32 by granzyme B. Figure 5 demonstrates that, in the presence of the CPP32 inhibitory peptide, pro-CPP32 was still processed by granzyme B to the 24 and 12 kDa cleavage products; however, the appearance of the 17 kDa fragment was completely blocked. In contrast, the ICE inhibitor peptide had no effect on the appearance of this fragment (Figure 5). The simplest explanation of these data, in conjunction with our earlier results (Figure 4B), is that granzyme B cleaves at a single site between the small and large subunits of the CPP32 proenzyme to generate cleavage products of 24 and 10 kDa, with partially active CPP32 then undergoing autoprocessing to remove the prodomain and yield the fully functional p17/p12 heterodimer. Thus, inhibition of CPP32 autoprocessing with the Ac-DEVD-CHO peptide blocked the appearance of the 17 kDa fragment but not the 24 or 12 kDa fragments.

Processing of CPP32 during granzyme B-induced apoptosis in cell-free extracts

Because granzyme B was capable of processing *in vitro* transcribed/translated CPP32 to its mature form and also because the CPP32 inhibitor, Ac-DEVD-CHO, blocked granzyme B-induced apoptosis in the cell-free system, this strongly suggested that granzyme B was activating CPP32 upon addition to the cell-free extracts (Figures 1–3). To confirm this directly, we conducted a time course to

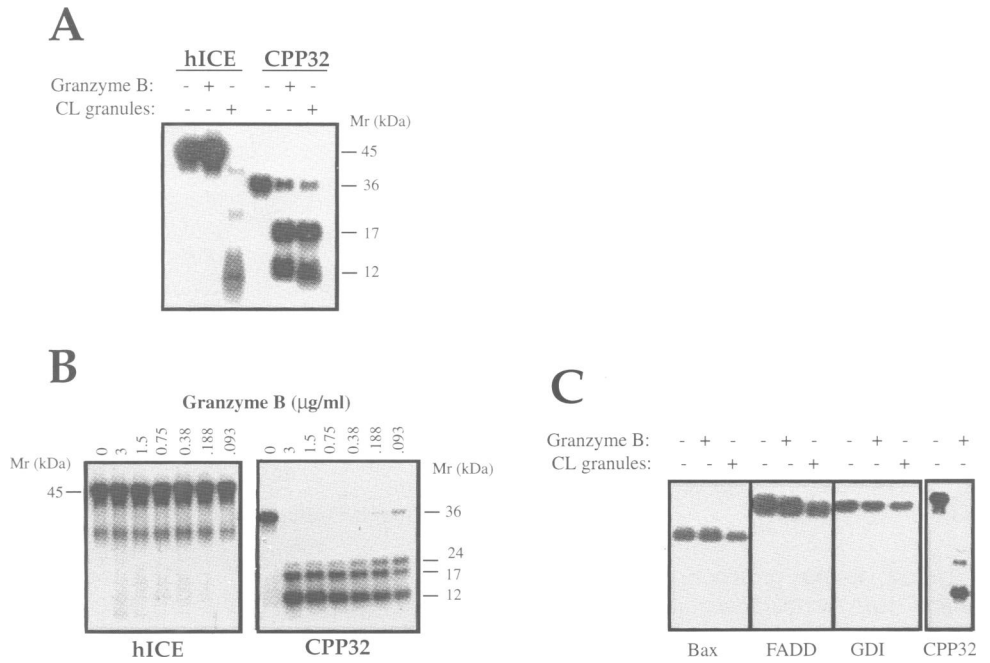

Fig. 4. Granzyme B processes CPP32 but not ICE. (**A**) Epitope-tagged [^{35}S]methionine-labeled hICE and CPP32 were synthesized by coupled *in vitro* transcription/translation and purified as described in Materials and methods. Purified proteins were then incubated at 37°C for 2 h in a 10 μl reaction volume with either buffer alone, purified granzyme B (30 ng) or CL granules (120 ng), as indicated. Reactions were then analyzed by SDS–PAGE, followed by detection of radiolabeled proteins by fluorography. (**B**) In a 10 μl reaction volume, 0.5 μl of *in vitro* transcribed/translated [^{35}S]methionine-labeled hICE, or CPP32, were incubated at 37°C for 2 h in the presence of the indicated amounts of granzyme B, followed by detection of cleavage products as in (A). (**C**) In a 10 μl reaction volume, 0.5 μl of *in vitro* transcribed/translated [^{35}S]methionine-labeled FADD, Bax, D4GDI or CPP32 were incubated at 37°C for 2 h with either buffer alone, purified granzyme B (30 ng) or CL granules (120 ng), as indicated. Reactions were then analyzed as in (A).

monitor conversion of CPP32 from its proenzyme form to its mature form during granzyme B-induced apoptosis in cell-free extracts. Figure 6 illustrates that CPP32 remained in the 36 kDa pro- form throughout the 60 min incubation period in control cell-free extracts. However, upon addition of granzyme B to the extracts, all of the CPP32 present was converted to the 24/12 kDa intermediate within 15 min (the 12 kDa subunit is not recognized by the anti-CPP32 antibody used), followed by the appearance of the 17 kDa chain as a result of further autoprocessing by CPP32, as was observed previously with *in vitro* transcribed/translated CPP32 (Figure 4B). Addition of Ac-DEVD-CHO peptide to the granzyme B-treated extracts arrested the maturation of CPP32 at the 24/12 kDa intermediate, as was also seen in the *in vitro* transcription/translation experiments (Figure 5), with the appearance of the 17 kDa chain being completely blocked (Figure 6). In contrast, Ac-YVAD-CHO peptide had little effect on CPP32 maturation, as before. Similar experiments were conducted to examine the status of ICE in cell-free extracts during granzyme B-induced apoptosis. These experiments revealed little detectable ICE in Jurkat cell-free extracts, which was not processed during granzyme B-induced apoptosis (data not shown).

CrmA prevents processing of CPP32 by granzyme B

CrmA is a cowpox-derived serpin (serine protease inhibitor) that has been reported to interact directly with and inhibit ICE and CPP32 (Ray *et al.*, 1992; Quan *et al.*, 1995; Tewari *et al.*, 1995a) and, more recently, has also been shown to form an SDS-stable complex with granzyme

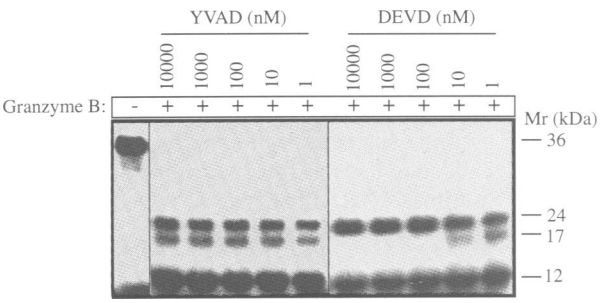

Fig. 5. Granzyme B-induced maturation of CPP32 is a two-step process involving CPP32 autoprocessing. In a 10 μl reaction volume, 0.5 μl of *in vitro* transcribed/translated [^{35}S]methionine-labeled CPP32 was incubated at 37°C for 2 h in the presence of either buffer alone, or granzyme B (30 ng), and Ac-DEVD-CHO or Ac-YVAD-CHO peptides, at the concentrations shown. Reactions were then analyzed by SDS-PAGE, followed by detection of radiolabeled proteins by fluorography.

B (Quan *et al.*, 1995). To explore whether CrmA could act as a pseudosubstrate for granzyme B and thereby block its ability to process CPP32, we tested whether [^{35}S]methionine-labeled *in vitro* transcribed/translated CrmA was cleaved by granzyme B. These experiments confirmed that CrmA formed an SDS-stable complex with granzyme B, and further revealed that CrmA became cleaved during this process (Figure 7A). Similar cleavage was also seen with purified CL granules, although the high molecular weight complex was not observed under these conditions (Figure 7A). To explore whether CrmA could prevent processing of pro-CPP32 by granzyme B, we incubated [^{35}S]methionine-labeled pro-CPP32 with

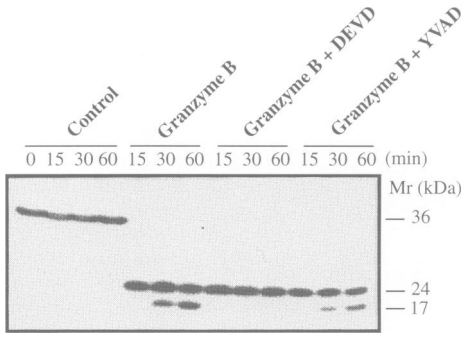

Fig. 6. Maturation of CPP32 during granzyme B-induced cell-free apoptosis. Cell-free extracts of Jurkat cells (150 μg of protein in 50 μl) were incubated at 37°C for the indicated times in the presence or absence of granzyme B (75 ng), and either 1 μM of Ac-DEVD-CHO or Ac-YVAD-CHO peptide, as indicated. Proteins (25 μg per lane) were then separated by SDS–PAGE followed by Western blotting and probing for CPP32.

granzyme B as before, in the presence or absence of purified recombinant CrmA, an inactive point mutant form of this protein (Tewari *et al.*, 1995a) or a glutathione-*S* transferase (GST)–CrmA fusion protein. Figure 6B demonstrates that native CrmA, as well as GST–CrmA, but not the CrmA point mutant, blocked the ability of granzyme B to process CPP32.

CrmA inhibits granzyme B- and CL granule-induced apoptosis in cell-free extracts

In view of its ability to prevent processing of CPP32 by granzyme B, it would be predicted that CrmA should block granzyme B- or CL granule-induced apoptosis in cell-free extracts, just as the CPP32 inhibitor peptide did (Figure 3). Figure 8 illustrates that this was found to be the case, with GST–CrmA, but not GST alone, efficiently inhibiting both the appearance of apoptotic changes in rat liver nuclei added to the cell-free extracts and the cleavage of PARP, fodrin and GDI that was seen in the presence of granzyme B or CL granules alone.

Discussion

Granzyme B is sufficient to trigger apoptosis via a CPP32-dependent mechanism

A major mechanism of cell-mediated cytotoxicity involves the release of perforin plus granzyme B from cytotoxic granules, which then act to induce apoptosis in target cells (for recent reviews, see Liu *et al.*, 1995; Smyth and Trapani, 1995). Perforin alone is capable of lysing cells but not of inducing apoptosis, and it has been suggested that this molecule facilitates the entry of granzyme B into cells (Liu *et al.*, 1995). Granzyme B, a serine protease with selectivity for Asp in the P_1 position, has been shown to be the major component of cytotoxic granules responsible for inducing apoptosis when combined with perforin (Shi *et al.*, 1992a,b).

Here we have shown that granzyme B is capable of inducing the changes in nuclear morphology characteristic of apoptosis (Figure 1), as well as a number of apoptosis-associated proteolytic events (Figure 2), when added to cell-free extracts of Jurkat cells. The ability of granzyme B to initiate these events was blocked by Ac-DEVD-CHO, a tetrapeptide inhibitor specific for the ICE/CED-3

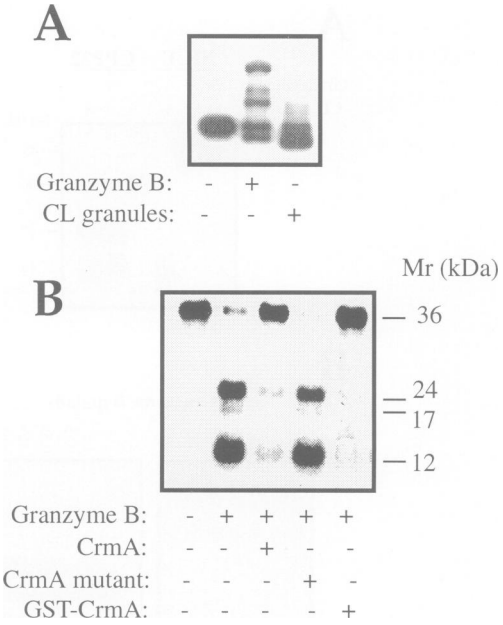

Fig. 7. CrmA directly interacts with granzyme B and inhibits its ability to process CPP32. (**A**) In a reaction volume of 10 μl, 0.5 μl of *in vitro* transcribed/translated [^{35}S]methionine-labeled CrmA was incubated at 37°C for 2 h with either buffer alone, purified granzyme B (30 ng) or CL granules (120 ng), as indicated. Reactions were then analyzed by SDS–PAGE followed by fluorography. (**B**) In a reaction volume of 10 μl, 0.5 μl of [^{35}S]methionine-labeled CPP32 was incubated at 37°C for 2 h with either buffer alone, or granzyme B (3.75 ng), in the presence or absence of purified recombinant CrmA (0.9 μM), mutant CrmA (0.9 μM) or GST–CrmA fusion protein (0.95 μM). Reactions were then analyzed by SDS–PAGE followed by fluorography.

family cysteine protease CPP32 (also known as YAMA or apopain). The possibility that granzyme B mediates its effects by activation of CPP32 is supported by our observations that granzyme B was capable of processing directly the 36 kDa CPP32 precursor to its characteristic p17/p12 form (Nicholson, *et al.*, 1995; Tewari, *et al.*, 1995a), via a p24/p12 intermediate, using *in vitro* transcribed/translated CPP32, as well as in the cell-free extracts. The processing of the p24 intermediate to the p17 chain was blocked by the CPP32 inhibitory peptide, but not by a similar ICE inhibitory peptide, suggesting that this is an autoproteolytic step. In contrast, processing of pro-CPP32 to the p24/p12 intermediate was not affected by either peptide, demonstrating that this step is mediated solely by granzyme B. Thus, there appears to be a single cleavage site for granzyme B within the CPP32 molecule, between the large and small subunits, with further autoprocessing removing the prodomain and yielding the mature form (Figure 9).

Recent studies have demonstrated a requirement for $p34^{cdc2}$ in apoptosis mediated by granzyme B and perforin (Shi *et al.*, 1994), although other forms of apoptosis do not exhibit this requirement (Martin *et al.*, 1995c). How Cdc2 participates in apoptosis mediated by granzyme B alone, as described in the present report, is not known. In future studies, it will be interesting, therefore, to explore whether Cdc2 or other cyclin-dependent kinases can affect the processing of CPP32 by granzyme B, or whether they interact with the cell death pathway downstream of CPP32 activation.

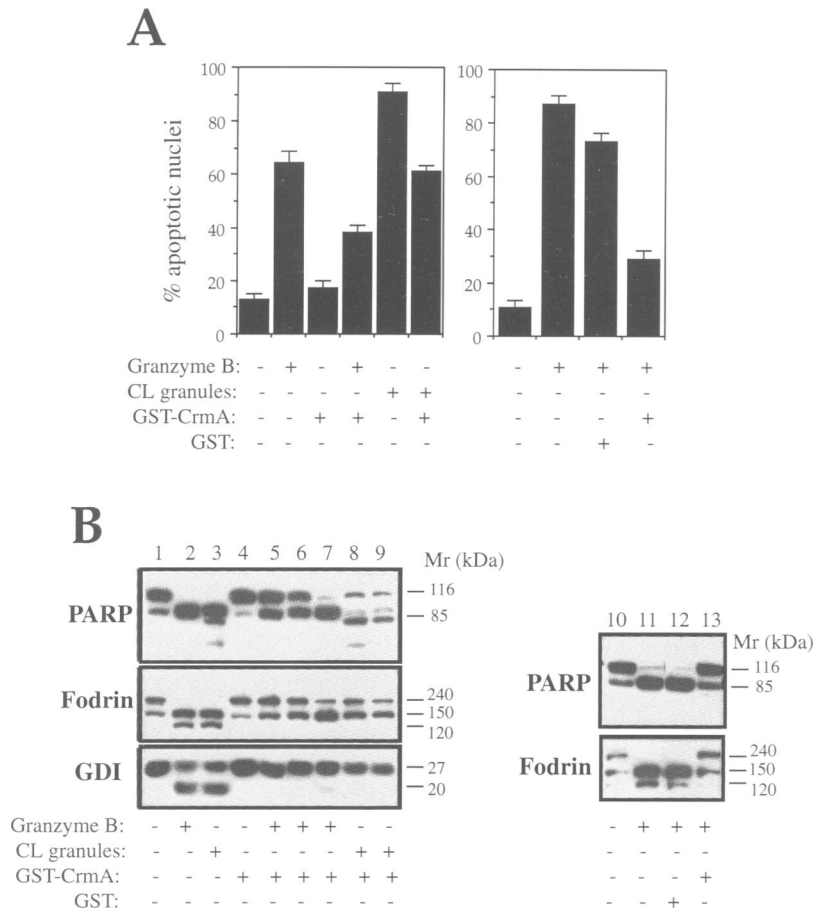

Fig. 8. CrmA inhibits granzyme B- and CL granule-induced apoptosis in cell-free extracts. (**A**) A cell-free extract of Jurkat cells (75 μg in 25 μl) was incubated at 37°C for 2 h with or without granzyme B (75 ng) or CL granules (240 ng), in the presence or absence of GST–CrmA fusion protein (0.6 μM in left panel, 0.8 μM in right panel) or GST alone (0.8 μM), as shown. Data shown are the means of triplicates (±SEM), with a total of 300 nuclei per treatment counted. (**B**) A cell-free extract of Jurkat cells (75 μg in 25 μl) was incubated at 37°C for 2 h with or without granzyme B (75 ng) or CL granules (240 ng), in the presence or absence of GST–CrmA fusion protein (0.6 μM lanes 4, 5 and 8; 0.4 μM lanes 6 and 9; 0.2 μM lane 7; 0.8 μM lane 13), or GST alone (0.8 μM). Proteins were then separated by SDS–PAGE, followed by Western blotting and probing for the indicated proteins.

CPP32: a key component of the executioner?

Recent findings strongly suggest that apoptosis is orchestrated via the activation of ICE/CED-3 family proteases. Upon activation, these proteases in turn are responsible for proteolytic events that dismantle the cell in a stereotypical way which we recognize as apoptotic cell death (reviewed in Martin and Green, 1995). We have therefore referred to the responsible proteases collectively as 'the executioner'. However, the precise manner in which proteolytic cleavage events produce the apoptotic cell phenotype remains largely speculative. Nevertheless, the evidence presented herein strongly implicates CPP32 as a key member of the executioner.

Since inhibition of CPP32 activity in the cell-free extracts, in the presence of active granzyme B, arrested cleavage of PARP, U1snRNP, fodrin and GDI, this suggests that granzyme B itself is not capable of cleaving these proteins and implies that either activated CPP32 directly cleaves these substrates, which has already been established for PARP (Nicholson *et al.*, 1995; Tewari *et al.*, 1995a), or that CPP32 activates other proteases in the extracts which do so (Figure 9).

Although several proteins are known to be cleaved during apoptosis (Kaufmann *et al.*, 1993; Browne *et al.*, 1994; Casciola-Rosen *et al.*, 1994; Jensen *et al.*, 1994; Lazebnik *et al.*, 1994; Kayalar *et al.*, 1995; Lazebnik *et al.*, 1995; Martin *et al.*, 1995a,b; Neamati *et al.*, 1995; Nicholson *et al.*, 1995; Tewari *et al.*, 1995a,b; Voelkel-Johnson *et al.*, 1995), the significance of these cleavage events to the cell death process is still unknown. Cleavage

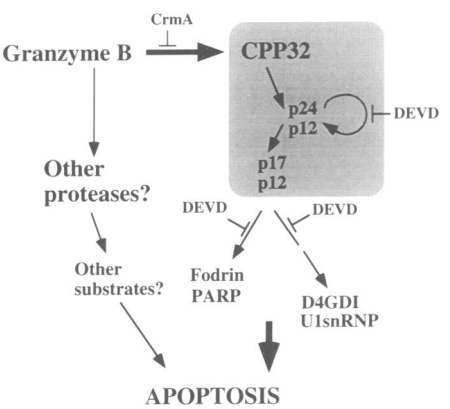

Fig. 9. Scheme of events downstream of introduction of granzyme B into the cytoplasm.

of PARP can result in inhibition of DNA repair and this might facilitate subsequent DNA damage (Kaufmann *et al.*, 1993), whereas cleavage of fodrin and α-actin, major components of the cortical cytoskeleton, may be responsible for the plasma membrane blebbing seen during apoptosis (Kayalar *et al.*, 1995; Martin *et al.*, 1995b). Clearly, further studies are required in order to define fully the impact of these cleavage events on the cell death process. Of course, it is entirely plausible that some of the cleavage events reported are merely bystander effects and that the important substrates for ICE/CED-3 family proteases, as participants in the death pathway, have yet to be found.

One other important set of substrates for ICE/CED-3 proteases are likely to be other family members such as Tx/ICH-2/ICErelII, Nedd-2/ICH-1, ICErelIII or Mch-2. Since there appears to be overlapping expression and substrate specificity of ICE/CED-3 family members, activation of one of these proteases may lead to explosive activation of multiple members of this family (Martin and Green, 1995).

As we have shown in the present report, granzyme B triggers the above proteolytic events by directly activating CPP32. Similarly, active ICE can also process pro-CPP32 to its active form *in vitro* (Tewari *et al.*, 1995a), although it is unclear whether this also occurs *in vivo*. However, unlike granzyme B, ICE (and other ICE/CED-3 family proteases) is not constitutively present in cells in its active form. Thus, signals culminating in apoptosis, apart from CL killing, must somehow activate these proteases by some other means. How any signal transduction pathway may culminate in the activation of an ICE family protease is currently unknown, but is clearly an important area of future investigation.

Neutralization of ICE/CED-3 family proteases as a general strategy of escape from cytotoxic lymphocyte killing

Our observations that CrmA can block activation of CPP32 by granzyme B, due to direct binding of CrmA to granzyme B (with CrmA becoming cleaved during this process), suggests a way in which cowpox virus may prolong its replication cycle by rendering its host cell resistant to attack by cytotoxic T lymphocytes. In support of this, overexpression of CrmA in a number of cell types renders these cells resistant to attack by cytotoxic T lymphocytes as well as due to engagement of Fas or exposure to TNF (Miura *et al.*, 1995; Tewari and Dixit, 1995; Tewari *et al.*, 1995c). However, recent observations have shown that the baculovirus-encoded p35 protein also exerts its anti-apoptotic effects by forming an inhibitory complex with ICE family proteases (Bump *et al.*, 1995). Since resistance to CLs is probably irrelevant for insect viruses (since insects do not have CLs), this suggests that the inhibitory activity of these viral proteins plays a more conserved role in the viral life-cycle and evolved for reasons other than evading CL attack. Furthermore, given that CrmA is a much more potent inhibitor of ICE than CPP32, it is likely that its ability to inhibit interleukin-1β production by the host cell (and the subsequent inflammatory response) is more important for prolongation of cowpox virus survival than its ability to neutralize ICE/CED-3 family proteases required for apoptosis, although the latter possibility cannot be ignored. In addition, the ability of viral proteins such as CrmA to interfere directly with the function of granzyme B is potentially very relevant in higher vertebrates, where cytotoxic granule-induced apoptosis is critical for the immunologic control of viral infection. Finally, a further reason for acquisition of anti-apoptotic proteins by viruses is that inhibition of apoptosis by the virus forces the cell to remain viable, despite the stress of the infection, until the viral burden finally overwhelms the cell.

Materials and methods

Materials

Monoclonal antibody to α-fodrin (non-erythroid spectrin) was purchased from Chemicon International; anti-β-actin monoclonal antibody was purchased from ICN. Autoantibodies to PARP and U1snRNP were derived from human subjects. Anti-CPP32 monoclonal was purchased from Transduction Laboratories (Kentucky). Polyclonal antibodies to lamin B_1 and lamin A/C were kindly provided by Dr Scott Kaufmann. Granzyme B was purified from CL granules as previously described (Shi *et al.*, 1992a,b). CL granules were purified from RNK cells and were solubilized as previously described (Shi *et al.*, 1992a) Ac-YVAD-aldehyde and Ac-DEVD-aldehyde peptides were purchased from BACHEM Bioscience Inc. CrmA and CrmA point mutant proteins were kindly provided by Dr Guy Salvesen. GST–CrmA fusion protein was kindly provided by Dr David Pickup.

Plasmids

The pCDNA3neo, pCDNA3YAMA/CPP32, pCDNA3hICE, pCDNA3-BAX, pCDNA3FADD, pCDNA3PARP and pET11dD4GDI plasmids were grown in *Escherichia coli* DH5α and were purified for *in vitro* transcription/translation on tip 100 Qiagen columns (Qiagen).

In vitro transcription/translation

Coupled transcription/translation of pro-CPP32β, pro-hICE, CrmA, Bax, FADD, D4GDI and PARP was carried out in rabbit reticulocyte lysates using the TNT kit (Promega). Briefly, 2 μg of each plasmid was incubated for 2 h at 30°C in a total volume of 50 μl containing the recommended proportions of each kit reagent and 4 μl of translation grade [^{35}S]methionine (1000 μCi/ml; Amersham). Reaction products were then aliquoted and stored at −70°C until required. For some experiments, *in vitro* transcribed/translated AU1 epitope-tagged CPP32 and hICE were purified by immunoprecipitation as follows. Reticulocyte lysates containing [^{35}S]methionine-labeled proteins (50 μl) were diluted to 500 μl with 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, followed by pre-clearing with normal mouse IgG and protein A/G-coupled beads (Santa Cruz). Ten μg of anti-AU1 antibody (Babco) was then added to each reaction, followed by incubation for 2 h at 4°C. Immune complexes were precipitated by incubation with protein A/G–agarose (20 μl per reaction) for 6 h at 4°C. Complexes were washed three times with 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS. Finally, bead-immobilized proteins were resuspended in the same buffer containing 10% sucrose and 5 μl portions were incubated either alone or with purified granzyme B or CL granules for 2 h, followed by addition of an equal volume of 2× reducing SDS–PAGE buffer, heating to 90°C for 5 min, and analysis by SDS–PAGE, followed by fluorography.

Analysis of cleavage of in vitro transcribed/translated substrates

Reactions between purified granzyme B or CL granules and [^{35}S] methionine-labeled *in vitro* transcribed/translated pro-ICE, pro-CPP32, CrmA, PARP, D4GDI, Bax, Bcl-2 and FADD, were assembled in a volume of 10 μl. Typically, this consisted of 0.5 μl of the [^{35}S]methionine-labeled substrate, 1 μl of either granzyme B or CL granules (at appropriate dilutions) and 8.5 μl of buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10% sucrose). Reactions were incubated for 2 h at 37°C, followed by addition of an equal volume of 2× reducing SDS–PAGE buffer, heating to 90°C for 5 min, and analysis on 12% SDS–PAGE gels. After electrophoresis, gels were fixed in isopropanol–acetic acid–water (25:10:65) for 30 min, followed by soaking in Amplify (Amersham) for 20–30 min, and exposure to Hyperfilm-MP overnight at −70°C.

Preparation of cell-free extracts

Cytoplasmic extracts were prepared essentially as previously described (Martin *et al.*, 1995a). Briefly, 2–5×10^8 Jurkat cells were washed twice with 50 ml of phosphate-buffered saline (PBS), pH 7.2, followed by a single wash with 5 ml of cell extract buffer [CEB; 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM cytochalasin B and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were pelleted, the supernatant aspirated, and were then transferred to a 2 ml glass Dounce homogenizer in the remaining droplet of buffer. Cells were then allowed to swell by addition of an equal volume of CEB to the volume occupied by the packed cell pellet (~100 μl per 10^8 cells) followed by incubation on ice for 20 min. Cells were gently lysed with 20 strokes of a B-type pestle. Lysis was monitored by staining a small aliquot of the lysate with trypan blue and examining under the light microscope. The cell lysate was then transferred to a 1 ml Eppendorf tube and centrifuged at 4°C for 20 min at 14 000 *g*. The clear supernatant was carefully removed without disturbing the nuclear pellet and then diluted to 7.5–15 mg/ml with extract dilution buffer (EDB; 10 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). Extracts were either used immediately or frozen at –80°C for later use.

Preparation of nuclei

Rat liver nuclei were isolated as previously described (Newmeyer and Wilson, 1991) and were resuspended in nuclei storage buffer (10 mM PIPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM PMSF and 50% glycerol) at 2×10^8 nuclei/ml. Nuclei were stored at –80°C in 20 μl aliquots until required.

Reconstitution of the cell-free extract

Cell-free reactions (25 μl) were comprised of 20 μl of cytoplasmic extract (7.5–15 mg/ml protein), 1 μl (2×10^5) of rat liver nuclei from frozen stocks and 4 μl of either EDB, or recombinant proteins diluted in this buffer. Extracts were then incubated at 37°C.

Quantitation of cell-free apoptosis

To enumerate apoptotic nuclei, a 3.5 μl aliquot of the cell-free reaction was removed and stained with 1.5 μl of a 10 μM solution of Hoechst 33342 in formalin, on a glass slide. Slides were then examined by fluorescence microscopy. Nuclei were scored as apoptotic if they exhibited margination and condensation of chromatin similar to that observed in apoptotic cells (Martin *et al.*, 1995a). In general, apoptotic changes proceeded with a high degree of synchrony in these extracts, as previously reported (Lazebnik *et al.*, 1993; Martin *et al.*, 1995a), and apoptotic nuclei were easily distinguishable from normal nuclei.

Electrophoresis and Western blotting

Proteins were separated under reducing conditions for 2 h at 70 V in SDS–polyacrylamide gels, as previously described (Martin *et al.*, 1995a,b). Separated proteins were then blotted at 100 mA for 2 h. Blots were blocked for 1 h in TBST (10 mM Tris–HCl, pH 8, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dried milk, then probed for 2 h with an appropriate dilution of the primary antibody diluted in the same buffer, washed for 1 h in several changes of TBST, followed by probing for a further 1 h with a peroxidase-coupled secondary antibody (Amersham, UK). Bound antibody was detected by enhanced chemiluminescence (Amersham).

Acknowledgements

We thank Dr Vishva Dixit for provision of cDNAs for CPP32/YAMA, hICE, CrmA and FADD; Dr David Pickup for provision of GST–CrmA fusion protein; Dr Scott Kaufmann for provision of anti-lamin antibodies and Dr Guy Salvesen for provision of recombinant CrmA and CrmA mutant proteins. S.J.M. is a Wellcome Trust International Travelling Fellow (041080). G.P.A-M. is a Brazilian Research Council (CNPq) Fellow. C.A.C. was the recipient of an Arthritis Foundation Fellowship. This research was also supported by grants from the National Institutes of Health (GM52735) and American Cancer Society (CB-82) to D.R.G. This is publication #136 from LIAI.

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Received on November 10, 1995; revised on January 15, 1996