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Caspase-4 and caspase-5, members of the ICE/CED-3 family of cysteine proteases, are CrmA-inhibitable proteases

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Abstract

Proteases of the caspase family are implicated in mammalian apoptosis and constitute a protease cascade. We characterized caspase-4 (TX/ICH-2/ICE$_{rel}$II) and caspase-5 (ICE$_{rel}$III/TY), which are most closely related to caspase-1 (ICE) among the caspase family. Although overexpression of caspase-4 and caspase-5 induced apoptosis, confirming previous observations, this apoptosis was not inhibited by a caspase-1-specific tetrapeptide inhibitor (Ac-YVAD-CHO), suggesting that caspase-4 and caspase-5 have different substrate specificities from caspase-1 and also that caspase-4- and caspase-5-induced apoptosis is not mediated by caspase-1. CrmA, a cowpox virus-derived caspase-1 inhibitor that prevents apoptosis induced by various stimuli, was cleaved by caspase-4 and caspase-5, and inhibited their proteolytic activity as assessed by cleavage of pro-caspase-3 (pro-CPP32/Yama/apopain). Thus, caspase-4 and caspase-5 are CrmA-inhibitable proteases like caspase-1 and might be involved in apoptosis.

Keywords: Apoptosis; cysteine protease; caspase-4; caspase-5; CrmA; baculovirus p35

Abbreviations: ICE, interleukin-1β-converting enzyme; TNF, tumour necrosis factor; PAGE, polyacrylamide gel electrophoresis

Introduction

Apoptosis, or programmed cell death, is a fundamental process crucial to morphogenesis and the maintenance of homeostasis in both vertebrates and invertebrates. Genetic studies in the nematode *Caenorhabditis elegans* have led to the isolation of genes, including *ced-3*, *ced-4* and *ced-9*, that are involved in programmed cell death (Yuan and Horvitz, 1990; Hengartner *et al*, 1992). *ced-3* and *ced-4* are required for such cell death to occur. *ced-3* encodes a protein that is similar to interleukin-1β (IL-1β)-converting enzyme (ICE), a cysteine protease that cleaves the inactive 31 kDa precursor of IL-1β to generate the active cytokine (Thornberry *et al*, 1992; Yuan *et al*, 1993). Ten proteins homologous to ICE (caspase-1) have been identified in mammals and are classified into three subfamilies, caspase-1-, caspase-3 (CPP32/Yama/apopain)-, and caspase-2 (NEDD2/ICH-1)-like proteases, based on their structures (Alnemri *et al*, 1996). The caspase-1-like subfamily includes caspase-1 (Thornberry *et al*, 1992), caspase-4 (TX/ICH-2/ICE$_{rel}$II) (Faucheu *et al*, 1995; Kamens *et al*, 1995; Munday *et al*, 1995), and caspase-5 (ICE$_{rel}$III/TY) (Munday *et al*, 1995; Faucheu *et al*, 1996). The caspases have been suggested to constitute a protease cascade (Martin and Green, 1995; Enari *et al*, 1996; Shimizu *et al*, 1996). Among the caspase family, caspase-1(-like) and caspase-3(-like) proteases have been implicated in physiological cell death, based upon the inhibition of cell death by tetrapeptide inhibitors of caspase-1 and caspase-3 (Los *et al*, 1995; Milligan *et al*, 1995; Nicholson *et al*, 1995; Enari *et al*, 1996; Chen *et al*, 1996; Jacobson *et al*, 1996; Schlegel *et al*, 1996; Hasegawa *et al*, 1996; Dubrez *et al*, 1996). Studies of a shorter isoform of *caspase-2* have also revealed its involvement in cell death (Wang *et al*, 1994). However, roles of other members of this family in cell death remain to be elucidated. Since apoptosis induced by a variety of stimuli is inhibited by baculovirus p35 (Clem *et al*, 1991; Rabizadeh *et al*, 1993; Hay *et al*, 1994; Sugimoto *et al*, 1994; Beidler *et al*, 1995), which inhibits the activities of caspases (Xue and Horvitz, 1995; Bump *et al*, 1995), and by a cowpox virus-derived caspase-1 inhibitor, CrmA (Pickup *et al*, 1986; Ray *et al*, 1992; Gagliardini *et al*, 1994; Tewari and Dixit, 1995; Los *et al*, 1995; Enari *et al*, 1995; Tewari *et al*, 1995b; Smith *et al*, 1996; Li *et al*, 1996), it would be interesting to identify CrmA-inhibitable members of the caspase family.

In the present study, we characterized caspase-4 and caspase-5, which are most closely related to caspase-1 among the caspase family, and showed that these two proteases have similar substrate preferences and are both CrmA-inhibitable.

Results

Caspase-4- and caspase-5-induced apoptosis is not inhibited by Ac-YVAD-CHO

Among the caspase family, caspase-4 and caspase-5 are most closely related to caspase-1 (53% identity for caspase-4 and 51% identity for caspase-5). To study the apoptosis-inducing activity of caspase-4 and caspase-5, we employed the same transient expression system as that previously used for *caspase-1* (Miura *et al*, 1993). We fused human *caspase-4*

and *caspase-5* cDNAs in frame with the *E. coli lacZ* gene and placed the fused gene under the control of the chicken β-actin promoter in pcas-4-lacZ and pcas-5-lacZ, respectively. We also used constructs of the fusion gene of the *caspase-1* and *caspase-2*$_L$ cDNAs with the *lacZ* gene, pcas-1-lacZ and pβactH37Z (Wang *et al*, 1994), respectively. The fusion genes were transfected into Rat-1 cells and their expression was examined with the X-Gal reaction. Most blue Rat-1 cells (X-Gal-positive) transfected with the *lacZ* gene alone were flat and well attached to the plates (data not shown). In contrast, most blue cells transfected with pcas-4-lacZ and pcas-5-lacZ contained round or small membrane-bound fragments representing apoptotic bodies, similar to those in cells transfected with pcas-1-lacZ and pβactH37Z. These results indicate that overexpression of caspase-4 and caspase-5 proteins induces Rat-1 cells to undergo apoptosis, confirming previous observations (Faucheu *et al*, 1995; Kamens *et al*, 1995; Munday *et al*, 1995). The tetrapeptide caspase-1 inhibitor Ac-YVAD-CHO (Thornberry *et al*, 1992) effectively blocked caspase-1-induced apoptosis in a concentration-dependent manner, whereas caspase-4-, caspase-5- and caspase-2$_L$-induced apoptosis was not significantly inhibited (Figure 1). These findings suggest that caspase-4 and caspase-5 have different substrate specificities from caspase-1 and also that caspase-4-, caspase-5-, and caspase-2$_L$-induced apoptosis are not mediated by caspase-1.

Caspase-4 and caspase-5 show similar substrate preferences in the *in vitro* cleavage assay

We compared the substrate preferences of caspase-4 and caspase-5 with cowpox virus CrmA, baculovirus p35, and pro-caspase-3 as potential substrates using recombinant proteases and *in vitro* translated ^{35}S-labeled substrates. The results are summarized in Table 1. Caspase-4 and caspase-5

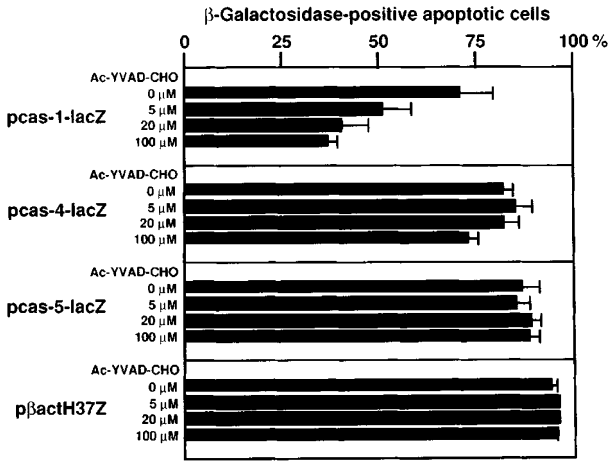

Figure 1 Effect of Ac-YVAD-CHO on caspase-1-, caspase-4-, caspase-5-, and caspase-2$_L$-induced apoptosis. One day after plating Rat-1 cells at a density of 2×10^5 in each well of 6-well dishes, 1.0 μg of the indicated DNA constructs was transfected. pβactH37Z is the caspase-2$_L$-expressing construct (Wang *et al*, 1994). The cells were incubated for 3 hr in serum-free medium containing DNA and lipofectamine, washed with serum-free medium, cultured in medium containing 10% FBS and the indicated concentrations of inhibitor (Ac-YVAD-CHO) for an additional 20 h and stained with X-Gal. The percentages (mean $\pm$ S.D.) of morphologically apoptotic cells among all β-galactosidase-positive blue cells are shown. More than 500 blue cells were counted in three independent experiments.

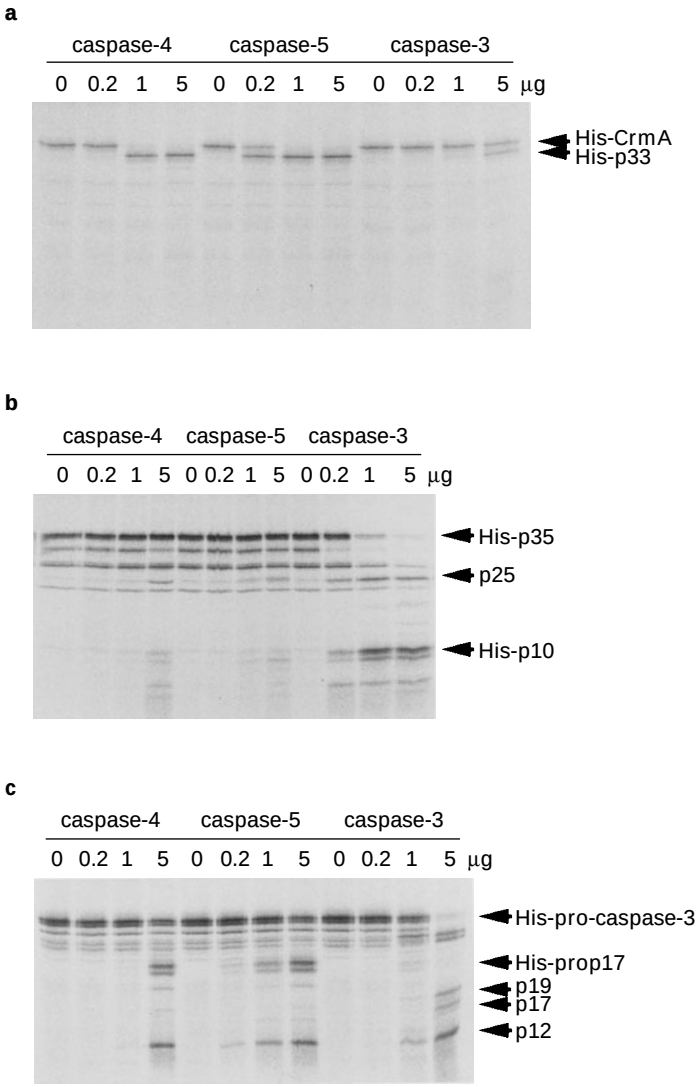

Figure 2 Substrate preferences of recombinant caspase-4 and caspase-5. (**A**) Cleavage of CrmA by recombinant caspase-4, caspase-5, and caspase-3. ^{35}S-labeled CrmA protein was incubated with the indicated amounts of purified caspase-4, caspase-5, or caspase-3 for 3.5 h and reaction products were analyzed by 15% SDS–PAGE. The His-p33 band corresponds to the His-tagged amino-terminal cleavage product resulting from cleavage at Asp-304. The 5 kDa carboxy-terminal cleavage product was not seen because it contains no methionine residues. (**B**) Cleavage of baculovirus p35 by caspase-4, caspase-5, and caspase-3. ^{35}S-labeled p35 protein was incubated with purified caspase-4, caspase-5, or caspase-3 and the reaction products were analyzed as described in (**A**). The p25 band corresponds to the carboxy-terminal cleavage product resulting from cleavage at Asp-87. The His-p10 bands correspond to the His-tagged amino-terminal cleavage product. (**C**) Cleavage of pro-caspase-3 by caspase-4, caspase-5, and caspase-3. ^{35}S-labeled mutant pro-caspase-3 protein was incubated with purified caspase-4, caspase-5, or caspase-3 and the reaction products were analyzed as described in (**A**). The p17 and p12 bands correspond to two subunits of active caspase-3. The His-prop17 bands may be the His-tagged prodomain with the p17 subunit resulting from cleavage at Asp-175. The p19 band may be the amino-terminal cleavage product resulting from cleavage at Asp-9 and Asp-175.

proteases showed a preference for CrmA rather than p35 as a substrate, whereas caspase-3 effectively cleaved p35 and only poorly cleaved CrmA (Figure 2a and b). As shown in Figure 2c, purified recombinant caspase-3 cleaved pro-caspase-3 bearing active site mutations at both p17 and p12 (subunits of the active enzyme) to generate p12 and p17, confirming previous observations (Wang *et al*, 1996; Xue *et al*, 1996). Purified recombinant caspase-4 and caspase-5 also cleaved pro-caspase-3 to generate p12 and pro-p17 (Figure 2c). The *in vitro* cleavage of CrmA, baculovirus p35, and mutant pro-caspase-3 by caspase-4, caspase-5, and caspase-3 was dependent on the concentration of the respective protease. Lysates of bacteria expressing the respective proteases cleaved the substrates, but not lysates of bacteria with a control vector (data not shown), indicating that cleavage was not due to contaminating bacterial proteases. These data show that caspase-4 and caspase-5 have a similar substrate preference for CrmA and p35.

CrmA inhibits cleavage of pro-caspase-3 by caspase-4 and caspase-5

Detection of the cleavage of CrmA by caspase-4 and caspase-5 led us to examine whether CrmA directly inhibits the proteolytic activities of caspase-4 and caspase-5 using the *in vitro* cleavage assay. As shown in Figure 3, CrmA effectively inhibited the cleavage of pro-caspase-3 by recombinant caspase-4 and caspase-5, indicating that caspase-4 and caspase-5 are CrmA-inhibitable proteases.

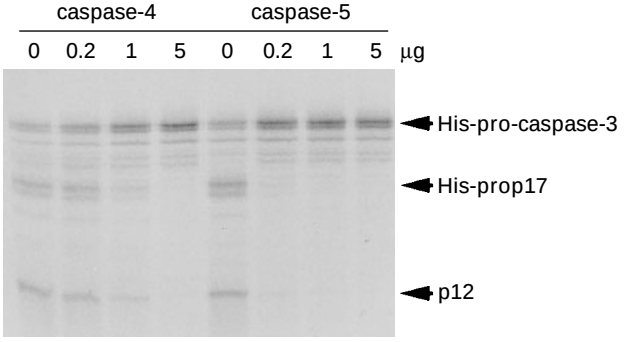

Figure 3 CrmA-mediated inhibition of the cleavage of pro-caspase-3 by caspase-4 and caspase-5. ^{35}S-labeled mutant pro-caspase-3 protein was incubated with 5 μg of purified caspase-4 and caspase-5 and the indicated amounts of purified CrmA for 3.5 h after which the reaction products were analyzed by 15% SDS–PAGE.

Table 1 Substrate preferences of caspase-3, caspase-4, and caspase-5

Protease	Substrate
caspase-4	CrmA>pro-caspase-3>p35
caspase-5	CrmA>pro-caspase-3>p35
caspase-3	p35>pro-caspase-3>CrmA

Substrate preference was assessed by the extent of cleavage of three substrates with the respective protease at fixed concentrations. Data from Figure 2 are summarized here.

Caspase-4- and caspase-5-induced apoptosis is inhibited by CrmA

To further confirm the inhibitory effect of CrmA on caspase-4 and caspase-5 *in vivo*, Rat-1 cells were cotransfected with *lacZ* fusion plasmids (pcas-1-lacZ, pcas-4-lacZ, pcas-5-lacZ, and pβactH37Z) together with a *crmA* expression construct (pCAG-crmA). As shown in Figure 4, apoptosis induced by overexpression of caspase-4 and caspase-5 was inhibited by CrmA as effectively as caspase-1-induced apoptosis. In contrast, caspase-2$_L$-induced apoptosis was not affected by CrmA, confirming the previous observation (Wang *et al*, 1994).

Discussion

The implication of caspase-1 in physiological cell death is based upon the prevention of apoptosis by inhibitors of the protease, including a tetrapeptide caspase-1 inhibitor Ac-YVAD-CHO (Los *et al*, 1995; Milligan *et al*, 1995; Enari *et al*, 1996; Chen *et al*, 1996), that was prepared based on the cleavage site in proIL-1β (Thornberry *et al*, 1992). However, it has not previously been determined whether Ac-YVAD-CHO only inhibits caspase-1. We showed that apoptosis induced by caspase-4 and caspase-5, which are most closely related to caspase-1, was not inhibited by Ac-YVAD-CHO, a finding consistent with the previous observation that caspase-4 and caspase-5 could not cleave proIL-1β (Faucheu *et al*, 1995, 1996; Munday *et al*, 1995). Although the *Ki* of caspase-1 inhibition by Ac-YVAD-CHO is <1 nM, caspase-1-induced apoptosis is only partially inhibited by 100 μM inhibitor. This is probably due to poor cell permeability of the tetrapeptide inhibitor. Thus, the available data suggest that Ac-YVAD-CHO is a quite specific inhibitor of caspase-1 itself.

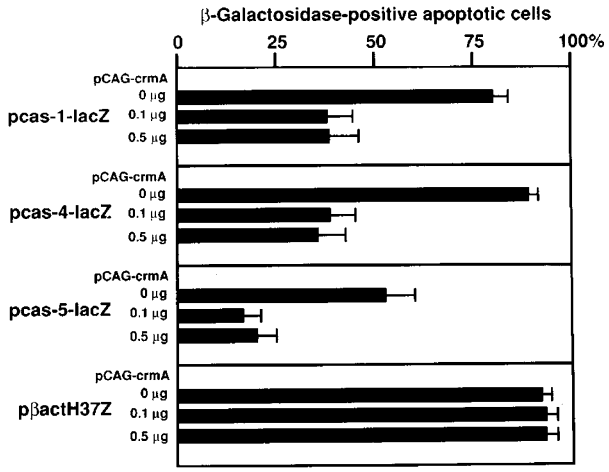

Figure 4 Effects of CrmA on caspase-1-, caspase-4-, caspase-5- and caspase-2$_L$-induced apoptosis. One day after plating Rat-1 cells at a density of 2×10^5 in each well of 6-well dishes, 0.1 μg of the indicated plasmids and various amount of pCAG-crmA or pUC-CAGGS were transfected in a total of 1.0 μg of DNA. The cells were incubated for 3 h in serum-free medium containing DNA and lipofectamine. Then a threefold volume of medium containing 10% FBS was added without removing the transfection mixture, and incubation was done for 22 h followed by staining with X-Gal. The data (mean $\pm$ S.D.) were analyzed as described in the legend to Figure 1.

Xue *et al*. (1996) suggested that caspases could be categorized by their ability to cleave CrmA and p35 after studying CED-3, caspase-3, caspase-1, and caspase-2; CED-3 and caspase-3 effectively cleave pro-caspase-3 and baculovirus p35 but not CrmA, caspase-1 effectively cleaves CrmA, and caspase-2 weakly cleaves only baculovirus p35. The preference of caspase-4 and caspase-5 for CrmA rather than baculovirus p35 as a substrate (Figure 2 and Table 1) suggests that these proteases are functionally related to caspase-1, consistent with the findings on structural analysis.

The cowpox virus gene product CrmA is a potent inhibitor of caspase-1 (Ray *et al*, 1992) and has also been shown to inhibit the induction of apoptosis by various stimuli. CrmA efficiently inhibits granzyme B, but only inhibits caspase-3 and caspase-7 (Mch3/ICE-LAP3/CMH-1) at high concentrations (Quan *et al*, 1995; Fernandes-Alnemri *et al*, 1995). Here, we have shown that CrmA efficiently inhibits caspase-4 and caspase-5. It was recently reported that a Fas-associated caspase family protease, caspase-8 (MACH/FLICE/Mch5) (Boldin *et al*, 1996; Muzio *et al*, 1996; Fernandes-Alnemri *et al*, 1996), is a CrmA-inhibitable protease (Srinivasula *et al*, 1996). This suggests that caspase-8 is the critical target of CrmA when inhibiting Fas- and TNF-induced apoptosis, because caspase-8 appears to be a protease that is furthest upstream in the caspase cascade. It is not known whether caspase-8 is generally involved in apoptosis. However, since caspase-4 and caspase-5 are also CrmA-inhibitable protease, they may be involved in CrmA-inhibitable physiological apoptosis triggered by a variety of stimuli.

The caspase cascade is implicated in the apoptotic cell death pathway (Enari *et al*, 1996; Shimizu *et al*, 1996). In Fas-mediated apoptosis, caspase-1(-like) proteases seem to act downstream of caspase-8 and/or Mch-4 (caspase-10) (Srinivasula *et al*, 1996) and upstream of caspase-3(-like) protease (Enari *et al*, 1996). We have recently shown that a caspase-4(-like) protease is involved in Fas-mediated apoptosis, probably by transducing a death signal directly to caspase-3(-like) proteases (Kamada *et al*, 1997). Since caspase-4 and caspase-5 cleave pro-caspase-3 to release p12 but not p17, both of which are components of active caspase-3, the generation of p17 might be mediated by other members of caspase family. Alternatively, the release of p12 might lead to auto-activation of caspase-3. *In vitro* cleavage of pro-caspase-3 by caspase-5 suggests that caspase-5 as well as caspase-1 and caspase-4 might be involved in physiological apoptotic cell death by activating caspase-3(-like) proteases. Thus, multiple protease cascade pathways might exist to activate caspase-3(-like) proteases, one of the executioner protease

Materials and Methods

Cell culture

Rat-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfection was performed using Lipofectamine (Life Technologies) according to the manufacturer's instructions. For analysis of β-galactosidase expression, cells were rinsed once with phosphate-buffered saline (PBS), fixed with 1% glutaraldehyde for 5 min, rinsed twice with PBS, and stained in X-Gal buffer (0.5 mg/ml 5-bromo-4-chloro-3-indoxyl β-galactoside, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$-3H20, 1 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100 in 0.1 M sodium phosphate buffer [pH 7.5]) (Miura *et al*, 1993) at 37°C for 3 h.

Construction of plasmids

To construct expression plasmids, PCR was carried out using the cDNAs of *caspase-1, caspase-4, and caspase-5* as templates. The following PCR primers were used: for human *caspase-1*, C1-5 (5′-CGGTCGACAGCCATGGCCGACAAGGTCCTG-3′) and C1-3 (5′-CGGGTACCCAATGTCCTGGGAAGAGGTAGAA-3′); and for human caspase-4, C4-5 (5′-CGGTCGACCCCTATGGCAGAAGGCAACCAC-3′) and C4-3 (5′-CGGGTACCCAATTGCCAGGAAAGAGGTAGAA-3′); and for human *caspase-5*, C5-5 (5′-CGGGTACCAGCTATGTT-CAAAGGTA-3′) and C5-3 (5′-CGGGTACCCAATTGCCAGGAAA-GAG-3′). The C1-5 and C4-5 primers had a *Sal*I at the 5′ end, while the C1-3, C4-3, C5-5, and C5-3 primers had a *Kpn*I site at their 5′ end. The amplified human *caspase-1* and *caspase-4* fragments and the *caspase-5* fragment were respectively cloned into *Sal*I-*Kpn*I sites and the *Kpn*I site of pactβGal, which possesses the chicken β-actin promoter (Maekawa *et al*, 1991) to generate pcas-1-lacZ, pcas-4-lacZ and pcas-5-lacZ, respectively. In these constructs, the sequences coding for the proteases were joined in-frame to the amino terminal of β-galactosidase. The same fragments were also cloned into *Sal*I-*Kpn*I sites and the *Kpn*I site of pBluescript SK(−) (Stratagene) and sequenced.

The *crmA* gene was isolated as a 1.4 kb *Eco*RI fragment by digestion of plasmid p996 (Pickup *et al*, 1986) and was inserted into the *Eco*RI site of the pUC-CAGGS vector (Niwa *et al*, 1991), generating pCAG-crmA.

Purification of recombinant His_6-tagged proteases

DNA sequences encoding the 105–377th amino acid residue of caspase-4, 122–418th amino acid residue of caspase-5 and 29–277th amino acid residue of caspase-3 were amplified by PCR and subcloned into the *E. coli* expression vector pRSET A (Invitrogen), in which the protease sequences were placed under the control of the T7 promoter and joined in-frame to sequences encoding an N-terminal fusion peptide, which includes an ATG translation initiation codon and the sequence for six successive histidine residues that function as a metal-binding domain in the translated proteins. The resultant plasmids (pHiscas4, pHiscas5 and pHiscas3) were transformed into *E. coli* strain JM109. Induction of recombinant His_6-tagged proteases was achieved according to the manufacturer's instructions. Cells were harvested and recombinant proteases were purified essentially as described elsewhere (Lippke *et al*, 1996). Fractions showing proteolytic activity in an *in vitro* cleavage assay (described below) were pooled and dialyzed against buffer A (50 mM HEPES, 0.1 M NaCl, 10% (v/v) glycerol, pH 7.5) to remove excess imidazole and were stored at −80°C. The purity of the proteins thus obtained was greater than 80% for His-caspase-4 or His-caspase-5, and approximately 20% for His-caspase-3, as estimated by Coomassie blue staining.

Purification of His_6-tagged CrmA protein

crmA DNA containing the entire coding region was amplified by PCR using the p996 plasmid (Pickup *et al*, 1986) as a template and two primers, HisCrm5 (5′-CGCTCGAGGAATGGATATCTTCAGG) and

HisCrm3 (5′-CGGAATTCTTAATTAGTTGTTGG), encompassing the initiation and termination codons of CrmA, respectively. HisCrm5 had an *Xho*I site at the 5′ end and HisCrm3 had an *Eco*RI site. The amplified fragment was subcloned into the *Xho*I-*Eco*RI sites of pRSET C to generate pHiscrmA. Expression of His_6-tagged CrmA was achieved according to the manufacturer's instructions. Cells were lyzed by sonication in a buffer containing 20 mM sodium phosphate (pH 7.8) and 0.5 M NaCl, after which the lysates were cleared by centrifugation. Then the supernatants containing soluble His-CrmA were loaded onto a 1 ml nickel-NTA column (Qiagen) and washed extensively with 20 mM sodium phosphate (pH 6.0) and 0.5 M NaCl. His-CrmA protein was eluted with 100–200 mM imidazole in 20 mM sodium phosphate (pH 7.8) and 0.5 M NaCl, and was found to elute at approximately 200 mM imidazole. It was dialyzed against buffer A to remove excess imidazole and stored at -80°C. The protein thus obtained showed greater than 90% purity by Coomassie blue staining.

In vitro cleavage assay

To construct an expression plasmid for His_6-tagged baculovirus p35, the 1 kb *NdeI p35* fragment containing entire coding region was blunt-ended and inserted into the *Pvu*II site of pRSET A, generating pHisp35. To substitute Cys 163 for Ser and Arg 207 for Glu in *caspase-3*, a PCR-based method was used. The final PCR product was cloned into the *Eco*RI site of pBluescript SK (−) and sequenced. The same fragment encoding caspase-3 containing active site mutations at both p17 and p12 subunits was subcloned into pRSET A to generate pHiscas3^{m}. ^{35}S-methionine-labeled His_6-tagged CrmA, p35 and mutant pro-caspase-3 were prepared for *in vitro* cleavage assays using expression constructs (pHiscrmA, pHisp35, and pHiscas3^{m}) and a TNT T7 coupled *in vitro* transcription and translation system (Promega) according to the manufacturer's instructions. ^{35}S-methionine-labeled proteins were purified essentially as described elsewhere (Tewari *et al*, 1995a) and stored at -80°C until use. Recombinant caspase-4, caspase-5, and caspase-3 with or without His-CrmA were preincubated in 20 μl of buffer A supplemented with 20 μM dithiothreitol at room temperature for 15 min. Reactions were initiated by addition of 20 μl of ^{35}S-methionine-labeled proteins and incubation was done at 37°C for 3.5 h. The reactions were stopped by the addition of SDS–PAGE sample buffer and cleaved products were analyzed by 15% SDS–PAGE.

Acknowledgements

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