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Yama/CPP32β, a Mammalian Homolog of CED-3, Is a CrmA-Inhibitable Protease That Cleaves the Death Substrate Poly(ADP-Ribose) Polymerase

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Summary

Although the mechanism of mammalian apoptosis has not been elucidated, a protease of the CED-3/ICE family is anticipated to be a component of the death machinery. Several lines of evidence predict that this protease cleaves the death substrate poly(ADP-ribose) polymerase (PARP) to a specific 85 kDa form observed during apoptosis, is inhibitable by the CrmA protein, and is distinct from ICE. We cloned a *ced-3/ICE*-related gene, designated *Yama*, that encodes a protein identical to CPP32β. Purified Yama was a zymogen that, when activated, cleaved PARP to generate the 85 kDa apoptotic fragment. Cleavage of PARP by Yama was inhibited by CrmA but not by an inactive point mutant of CrmA. Furthermore, CrmA blocked cleavage of PARP in cells undergoing apoptosis. We propose that Yama may represent an effector component of the mammalian cell death pathway and suggest that CrmA blocks apoptosis by inhibiting Yama.

Introduction

Apoptosis or programmed cell death (PCD) is of fundamental importance to biological processes ranging from embryogenesis to the development of the immune system (Ellis et al., 1991). Although the mechanism of mammalian PCD is not understood, three lines of evidence indicate that a protease is a component of the mammalian cell death pathway. First, investigations of PCD in the nematode Caenorhabditis elegans identified a gene, *ced-3*, that is essential for apoptosis (Ellis and Horvitz, 1986). Cloning and sequencing of *ced-3* revealed that it encodes a protein having homology to interleukin-1β (IL-1β)-converting enzyme (ICE) (Yuan et al., 1993), a mammalian Cys protease that processes pro-IL-1β to an active form by proteolytic cleavage at Asp residues (Thornberry et al., 1992; Cerretti et al., 1992). Initial reports suggested that ICE might be the functional mammalian homolog of *ced-3*, since overexpression of ICE induced apoptotic changes in Rat-1 cells (Miura et al., 1993). However, the recent analysis of mice with germline inactivation of ICE indicated that this is not the case, as these mice developed normally, with no obvious defects in apoptosis (Li et al., 1995). Given the compelling evidence that CED-3 is a death protease in C. elegans, however, it appears likely that there may be another mammalian protease that plays a key role in mammalian apoptosis.

A second independent line of evidence for protease involvement in mammalian PCD comes from experiments utilizing the cowpox virus CrmA protein, an inhibitor of ICE (Ray et al., 1992). Expression of CrmA was found to inhibit apoptosis in a wide range of systems, including PCD initiated by nerve growth factor withdrawal in primary chicken neuronal cultures (Gagliardini et al., 1994), by activation of either Fas or the tumor necrosis factor (TNF) receptor (Tewari and Dixit, 1995), by serum withdrawal in Rat-1 fibroblasts (Wang et al., 1994), and by detachment of mammary epithelial cells from the underlying extracellular matrix (Boudreau et al., 1995). This provides strong evidence for a CrmA-inhibitable ICE-like protease in the cell death pathway. The criterion that the death enzyme is CrmA inhibitable is important as it allows one to discount ICE family members such as ICH-1 that, when overexpressed, do induce apoptosis but appear not to be inhibitable by CrmA (Wang et al., 1994).

A third line of support for the activation of a protease during mammalian PCD comes from the study of a death substrate specifically cleaved during apoptosis. Kaufmann (1989) and colleagues (Kaufmann et al., 1993) found that the 116 kDa nuclear protein poly(ADP-ribose) polymerase (PARP) was specifically cleaved to produce an 85 kDa fragment in many forms of PCD, including that induced by chemotherapeutic drugs in cell lines and by dexamethasone in thymocytes. Further analysis by Lazebnik et al. (1994) using a cell-free system demonstrated that the cleavage occurred C-terminal to Asp and that the protease responsible resembled ICE in its susceptibility to chemical inhibitors, but was distinct from ICE, since purified ICE did not cleave PARP. Hence, an anticipated characteristic of the death protease is that it should cleave the death substrate PARP to generate the specific 85 kDa fragment observed during apoptosis.

Taken together, the data predict the existence of a mammalian protease that is a member of the CED-3/ICE family of Asp-specific Cys proteases, that cleaves the death substrate PARP to generate a specific 85 kDa apoptotic fragment, and that is susceptible to inhibition by CrmA. With this in mind, we adopted an approach of systematically cloning, expressing, and purifying mammalian members of the *ced-3/ICE* gene family and determining whether they possessed the above properties. We cloned a cDNA, designated *Yama* (after the Hindu god of death), which was

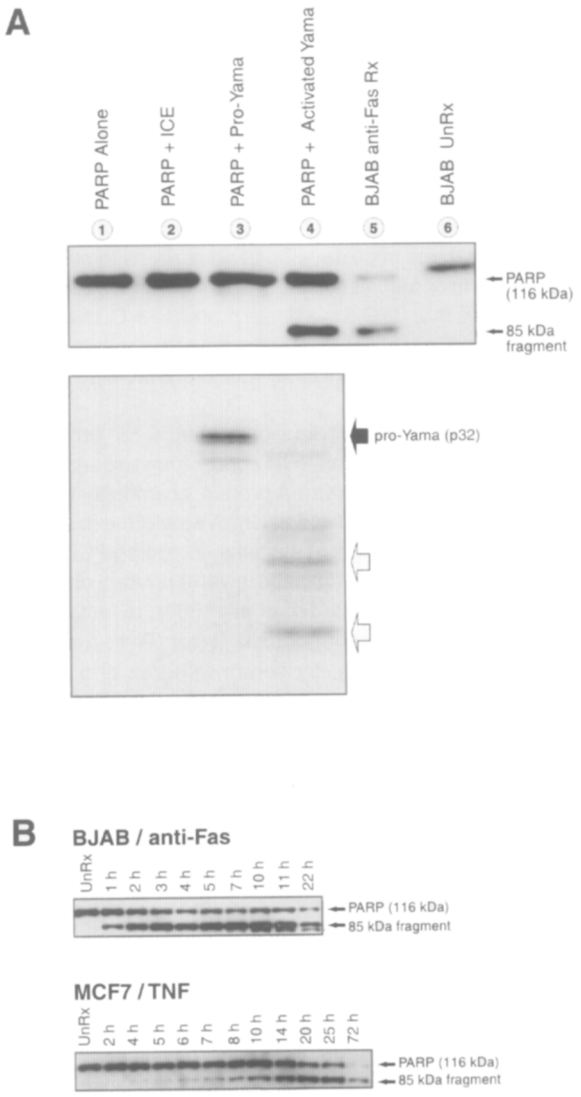

Figure 1. Cleavage of PARP by Activated Yama

(A) Yama is a protease zymogen that, upon activation, cleaves PARP in vitro to the 85 kDa apoptotic fragment.

(Top) His_6-tagged Yama was expressed and labeled using [^{35}S]Met in an in vitro transcription/translation reaction and purified by ion exchange and nickel chelate affinity chromatography as described in Experimental Procedures. In vitro reactions were assembled in which 0.586 μg of purified PARP was incubated for 2 hr at 37°C with either buffer only (lane 1), ICE (lane 2), purified pro-Yama (lane 3), or purified pro-Yama after activation with ICE (lane 4). Following incubation, one fifth of each reaction was analyzed by SDS–PAGE and immunoblotting using monoclonal antibody C-2-10 directed against PARP. Whole-cell lysates from BJAB cells undergoing anti-Fas-induced apoptosis (lane 5) or from untreated BJAB cells (lane 6) were run alongside the in vitro reaction samples.

(Bottom) Equal quantities of the in vitro reactions represented in lanes 1–4 in the top panel were resolved by SDS–PAGE, and the dried gel was subjected to phosphorimager analysis to assess the state of the radiolabeled Yama protein. The closed arrow indicates the migration of purified pro-Yama, which is designated as the full-length p32 form. The open arrows indicate the two major proteolytic fragments observed after activation of pro-Yama by ICE and are presumed to correspond to the putative p20 and p11 subunits predicted from cleavage at Asp residues in pro-Yama.

(B) Cleavage of PARP to an 85 kDa fragment is a characteristic feature of both Fas- and TNF-induced apoptosis.

(Top) BJAB cells were either left untreated (UnRx) or treated with agonist anti-Fas antibody (250 ng/ml) for the indicated time periods, and cell lysates were prepared and analyzed by immunoblotting using anti-PARP monoclonal antibody C-2-10 as described in Experimental Procedures.

(Bottom) MCF7 cells were either left untreated (UnRx) or treated with recombinant TNF (40 ng/ml) for the indicated time periods, and cell lysates were similarly analyzed.

almost identical to the cDNA encoding CPP32β identified independently by Fernandes-Alnemri et al. (1994). We demonstrate that purified Yama is a zymogen that, upon activation, assumes a proteolytically competent form that cleaves PARP to the signature 85 kDa apoptotic fragment. Furthermore, we show that the proteolytic activity of Yama is inhibited by purified CrmA but not by an equivalent quantity of an inactive point mutant of CrmA. Taken together, the results indicate that Yama possesses the expected properties detailed above of the mammalian death protease. Finally, in keeping with the in vitro results, we found that CrmA blocked the proteolytic cleavage of PARP in cells induced to undergo apoptosis. We propose that Yama represents a candidate for the effector protease of the mammalian cell death pathway and suggest that CrmA may block apoptosis by inhibiting the proteolytic activity of Yama.

Results

To identify genes related to *ced-3*, we took advantage of the conservation of the pentapeptide motif QACRG that encompasses the catalytic site Cys of ICE and is conserved among members of the CED-3/ICE protein family (Yuan et al., 1993). We adopted two approaches to identify related genes: one involved the use of degenerate polymerase chain reaction (PCR) primers encoding the QACRG motif, and the other was to search the GenBank database of human expressed sequence tags (ESTs). The results of the latter approach are described here. We identified an EST (T10341; deposited by M. Bento Soares) that, when translated, generated an open reading frame containing the QACRG motif. Using the cDNA corresponding to this EST as a probe, we screened a human umbilical vein endothelial cell library and cloned a cDNA encoding an open reading frame of 277 amino acids, designated Yama, that had homology to the CED-3/ICE family of proteins (data not shown). Soon thereafter, Fernandes-Alnemri et al. (1994) reported the cloning of a cDNA designated *CPP32β* that had an almost identical open reading frame.

We proceeded to determine whether Yama/CPP32β was indeed a protease and whether it fulfilled the requirements predicted for a death protease. The amino acid sequence suggested that it would be an Asp-specific Cys protease, as the residues thought to be important for Asp specificity in ICE are conserved in Yama (Walker et al., 1994; Wilson et al., 1994; Fernandes-Alnemri et al., 1994). Yama was expressed in vitro as a fusion to a His_6 purification tag at the N-terminus and isolated by ion exchange and nickel chelate affinity chromatography to determine

whether it possessed proteolytic activity capable of cleaving PARP. The full-length, p32 form of purified Yama had no proteolytic activity against PARP (Figure 1A, top, lane 3) and was thus designated pro-Yama. This result was not surprising, as ICE itself is a zymogen that requires proteolytic processing to a two-subunit form (p20 and p10 subunits) possessing proteolytic activity. The predicted amino acid sequence of Yama suggested that it, too, might be activated by proteolytic cleavage at Asp residues to generate a catalytically active enzyme (Fernandes-Alnemri et al., 1994). We found that purified ICE was able to cleave pro-Yama to yield two major products (Figure 1A, bottom, lane 4; putative p20 and p11 subunits are indicated by open arrows) and, more importantly, that Yama activated in this manner acquired proteolytic activity and cleaved PARP to the 85 kDa apoptotic form (Figure 1A, top, lane 4). Purified ICE did not cleave PARP (Figure 1A, top, lane 2), confirming earlier results (Lazebnik et al., 1994) and excluding the possibility that PARP cleavage was mediated by the added ICE. Given the interest of our laboratory in the mechanism of Fas- and TNF-induced apoptosis (Tewari and Dixit, 1995), we investigated whether PARP cleavage occurred in these cell death systems and whether the cleavage product was analogous to that observed in the in vitro experiments. Activation of either Fas (in BJAB lymphoma cells) or TNF receptors (in MCF7 breast carcinoma cells) induced PARP cleavage to the signature 85 kDa form (Figure 1B), and this product comigrated with the PARP cleavage fragment generated by purified Yama (Figure 1A, top, lanes 4 and 5). Thus, we concluded that Yama is a protease that cleaves PARP to the signature 85 kDa apoptotic fragment.

Since the mammalian cell death protease is expected to be susceptible to inhibition by CrmA, we asked whether Yama was CrmA inhibitable. To address this question definitively, we used purified proteins to reconstitute the PARP cleavage reaction in vitro such that purified recombinant CrmA protein could be added. To serve as a control, we designed a point mutant of CrmA, using as a guide results from mutational and structural analyses of members of the serpin family of protease inhibitors, a protein family of which CrmA is a member. Mutant CrmA carries a single amino acid substitution of Arg for Thr at amino acid 291 (Figure 2A). The corresponding residue in other inhibitory serpins is typically a small, uncharged residue and occupies a key position in the reactive site loop, which is a surface loop critically important for protease inhibitory function. Mutation of this residue to a charged residue in the serpins antitrypsin (Schulze et al., 1991; Hood et al., 1994), plasminogen activator inhibitor I (Lawrence et al., 1994) and C1 inhibitor (Davis et al., 1992) caused loss of inhibitory activity without significantly disrupting protein conformation. Hence, we expected that a similar mutation in CrmA would inactivate its protease inhibitory activity while allowing it to retain its native conformation. Both CrmA and mutant CrmA proteins were expressed as His_6-tagged fusion proteins in Escherichia coli and purified by nickel chelate affinity chromatography (data not shown). In vitro characterization of the mutant CrmA protein revealed that under conditions in which CrmA bound and inhibited

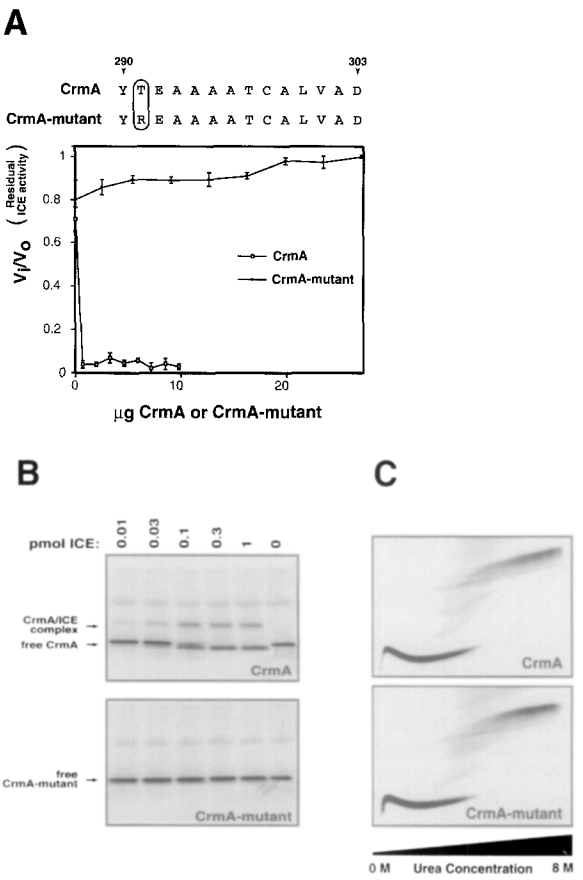

Figure 2. Biochemical Characterization of Wild-Type CrmA and Mutant CrmA Proteins

(A) A point mutation in the reactive site loop of CrmA inactivates its ability to inhibit ICE. (Top) The reactive site loop sequences of CrmA and mutant CrmA are compared. Amino acid 291 of wild-type CrmA, which corresponds to a critical reactive site loop residue in other serpins, was altered from Thr to Arg by site-directed mutagenesis. (Bottom) Both proteins were expressed in E. coli as His_6 fusions and purified as described in Experimental Procedures. Aliquots (44 ng) of ICE were titrated with the indicated amounts of purified CrmA protein (open squares) or mutant CrmA protein (closed circles). Residual ICE activity, expressed as the ratio of the inhibited rate (v_i) to the uninhibited rate (v_o), was determined with a chromogenic ICE substrate and plotted against the quantity of CrmA or mutant CrmA protein added. ICE activity was abolished by as little as 300 ng of CrmA, whereas no inhibition was detected with mutant CrmA, even using 30 μg, representing a 500-fold molar excess over the enzyme.

(B) Mutant CrmA does not form a complex with ICE. [^{35}S]Met-labeled CrmA or mutant CrmA proteins were produced by coupled transcription/translation of their respective genes. The indicated amounts of ICE were added directly to the diluted lysates and incubated as described in Experimental Procedures, following which samples were resolved by nondenaturing PAGE and the radioactive signals detected using a phosphorimager. Mutant CrmA failed to form a complex with ICE; indeed, ICE appeared to have no effect on this protein. In comparison, part of the wild-type CrmA formed a complex whereas the rest was cleaved in a manner that typifies the interaction of ICE with CrmA (Komiyama et al., 1994b).

(C) The tertiary structures of CrmA and mutant CrmA proteins are indistinguishable as assessed by TUG–PAGE. [^{35}S]Met-labeled CrmA and mutant CrmA proteins were generated by coupled transcription/translation and analyzed by TUG–PAGE as described in Experimental Procedures. The gels were dried and analyzed using a phosphorimager to detect the unfolding signature of each protein. No difference in the signatures was observed, indicating that the point mutation in mutant CrmA did not disrupt the tertiary structure of the protein.

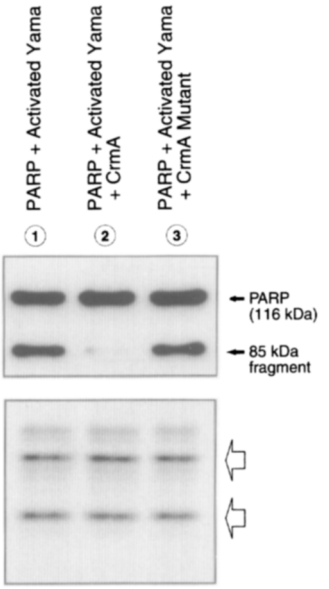

Figure 3. Cleavage of PARP by Activated Yama In Vitro Is Inhibitable by CrmA but Not by an Equivalent Amount of Mutant CrmA

(Top) [^{35}S]Met-labeled Yama was generated, purified, and activated by ICE as described in Experimental Procedures. Purified, activated Yama was then incubated for 2 hr at 37°C with 0.586 μg of purified PARP in the presence of either buffer (lane 1), 270 pmol of CrmA (lane 2), or 270 pmol of mutant CrmA (lane 3) protein as described in Experimental Procedures. Following the incubation with PARP, one fifth of each reaction was analyzed by immunoblotting using anti-PARP monoclonal antibody C-2-10.

(Bottom) Equivalent amounts of each of the above reactions were subjected to SDS–PAGE, and the dried gel was analyzed using a phosphorimager to assess the state of the labeled Yama protein. The open arrows indicate the position of the two major products that were observed in preparations of activated Yama and that are presumed to correspond to the putative p20 and p11 subunits predicted from the amino acid sequence of Yama.

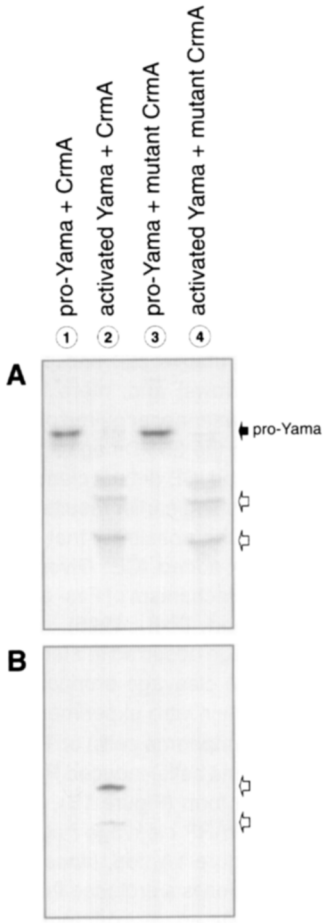

Figure 4. CrmA Directly Interacts with Activated Yama but Not with Pro-Yama

(A) Phosphorimager scan of reaction samples prior to immunoprecipitation analysis. Reactions (80 μl) were assembled in which either radiolabeled pro-Yama (lanes 1 and 3) or radiolabeled activated Yama (lanes 2 and 4) were mixed with either 358 pmol of CrmA (lanes 1 and 2) or 358 pmol of mutant CrmA (lanes 3 and 4) recombinant proteins. An aliquot (10 μl) of each reaction was resolved by SDS–PAGE, and pro-Yama or activated Yama was detected by phosphorimaging analysis. The closed arrow indicates the migration of pro-Yama (p32), whereas the open arrows indicate the putative p20 and p11 subunits of activated Yama.

(B) Immunoprecipitation of reaction samples with a polyclonal CrmA antiserum. An aliquot (35 μl) of each of the above reactions was subjected to immunoprecipitation using a rabbit polyclonal CrmA antiserum as described in Experimental Procedures. Immunoprecipitates were resolved by SDS–PAGE, and radiolabeled proteins were detected using a phosphorimager. The open arrows indicate the putative p20 and p11 subunits of activated Yama.

ICE, the mutant protein neither bound ICE (Figure 2B) nor inhibited its proteolytic activity (Figure 2A). The tertiary structure of mutant CrmA, however, was not significantly altered by the point mutation, as its conformational signature was indistinguishable from that of wild-type CrmA by transverse urea gradient–polyacrylamide gel electrophoresis (TUG–PAGE) (Figure 2C), a method used to probe the tertiary structures of serpins (Goldenberg, 1989; Mast et al., 1991; Komiyama et al., 1994a).

Using these purified recombinant proteins, we found that CrmA markedly inhibited the cleavage of PARP by Yama in vitro (Figure 3). When CrmA was replaced with an equivalent amount of mutant CrmA protein, no inhibition of PARP cleavage was observed (Figure 3), indicating that the effect of CrmA was a function specifically of its ability to act as a protease inhibitor.

To confirm that CrmA directly interacts with Yama, we examined the ability of either pro-Yama or activated Yama to form a complex with either native CrmA or mutant CrmA. Pro-Yama and activated Yama (both labeled with [^{35}S]Met) were each incubated with either native CrmA or an equivalent amount of mutant CrmA recombinant protein. Each reaction was subjected to immunoprecipitation analysis using a polyclonal CrmA antiserum, and the immunoprecipitates were resolved by SDS–PAGE and visualized by phosphorimaging. CrmA formed a complex with the activated two-subunit form of Yama but not with pro-Yama (Figure 4B). Mutant CrmA bound neither activated Yama nor pro-Yama (Figure 4B). We concluded that CrmA interacts directly with activated Yama to form an inhibitory complex.

Since CrmA inhibited the cleavage of PARP by Yama in vitro, we inferred that if Yama is indeed responsible

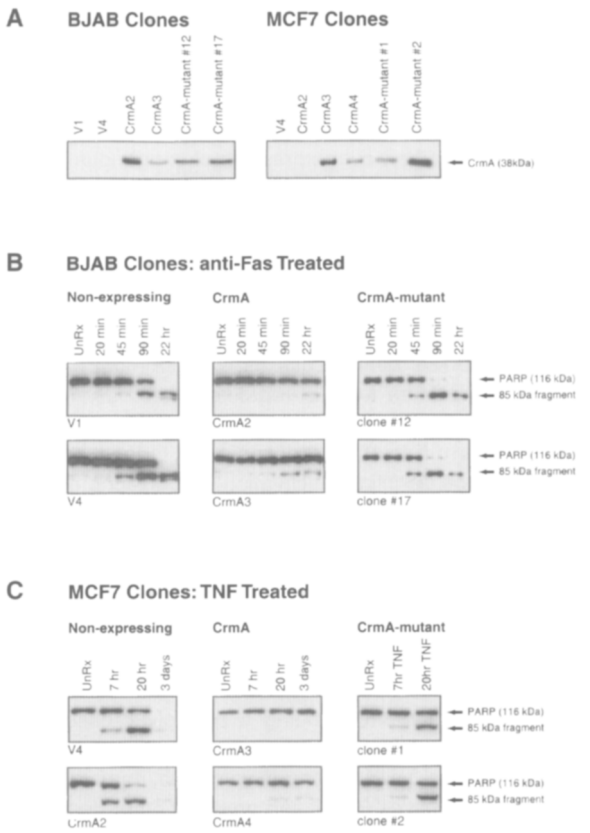

Figure 5. CrmA, but Not Mutant CrmA, Blocks PARP Cleavage In Vivo during Apoptosis

(A) Expression of CrmA or mutant CrmA in stably transfected clones. (Left) Clonal BJAB cell lines stably transfected with either vector control (V1 and V4), CrmA (CrmA2 and CrmA3), or mutant CrmA (CrmA mutant 12 and CrmA mutant 17) expression constructs were analyzed by Western blotting using a CrmA antiserum.

(Right) Clonal MCF7 cell lines stably transfected with either vector control (V4), CrmA (CrmA2, CrmA3, and CrmA4), or mutant CrmA (CrmA mutant 1 and CrmA mutant 2) expression constructs were similarly analyzed.

(B) PARP cleavage to the 85 kDa fragment during Fas-induced apoptosis is inhibited by CrmA but not by mutant CrmA. Clonal BJAB transfectants not expressing CrmA (V1 and V4), expressing CrmA (CrmA2 and CrmA3), or expressing mutant CrmA (CrmA mutant 12 and CrmA mutant 17) were treated with agonist anti-Fas antibody (250 ng/ml) for the indicated time periods (UnRx indicates untreated lane), and lysates were prepared and analyzed by Western blot using the anti-PARP monoclonal antibody C-2-10.

(C) PARP cleavage to the 85 kDa fragment during TNF-induced apoptosis is inhibited by CrmA but not by mutant CrmA. Clonal MCF7 transfectants not expressing CrmA (V4 and CrmA2), expressing CrmA (CrmA3 and CrmA4), or expressing mutant CrmA (CrmA mutant 1 and CrmA mutant 2) were treated with TNF (40 ng/ml) for the indicated time periods (UnRx indicates untreated lane), and lysates were prepared and analyzed by Western blot using the anti-PARP monoclonal antibody C-2-10.

for PARP cleavage during apoptosis, then CrmA should inhibit PARP cleavage in vivo. To investigate this, we utilized MCF7 breast carcinoma and BJAB lymphoma cell lines stably transfected with either vector, CrmA (Tewari and Dixit, 1995), or mutant CrmA expression constructs. Clonal cell lines that expressed the indicated proteins were selected, and protein expression was confirmed by immunoblotting using an anti-CrmA polyclonal antiserum (Figure 5A). In keeping with the in vitro findings, expression of CrmA inhibited proteolytic cleavage of PARP to the signature 85 kDa fragment normally generated during apoptosis induced by either Fas (BJAB cells) or TNF receptors (MCF7 cells), whereas in the vector and mutant CrmA lines, cleavage of PARP proceeded unabated (Figures 5B and 5C).

To investigate whether the in vivo blockade of PARP cleavage by CrmA and lack thereof by mutant CrmA correlated with the ability of these proteins to inhibit apoptosis, we examined the BJAB and MCF7 transfectants for their sensitivity to Fas- and TNF-induced apoptosis. Among MCF7 transfectants, CrmA afforded protection from TNF-induced apoptosis as expected, whereas mutant CrmA–expressing lines showed no protection and were as sensitive as a vector-transfected line (Figure 6, bottom). The protection conferred by CrmA and lack thereof by mutant CrmA was readily apparent on examination of nuclear morphology of propidium iodide–stained cells (Figure 6, top). When BJAB transfectants were examined for sensitivity to Fas-induced PCD, CrmA was protective, whereas mutant CrmA–expressing lines remained as sensitive as a vector control line (Figure 6, bottom). Thus, we concluded that the divergent abilities of CrmA and mutant CrmA to block PARP cleavage correlate with the abilities of these proteins to block cell death.

Discussion

The studies described herein were initiated to identify a mammalian CED-3-like protease that cleaved PARP to its signature 85 kDa apoptotic fragment and that was also inhibitable by CrmA. The data presented indicate that the protease Yama fulfills these criteria. The predicted protein sequence of Yama suggested it would be a Cys protease of the CED-3/ICE family and that it would have specificity for cleavage after Asp residues (Fernandes-Alnemri et al., 1994), two properties that are in common with the activity, designated prICE, that cleaves PARP in a cell-free system (Lazebnik et al., 1994). We now show by in vitro reconstitution employing purified components that Yama is indeed a protease that cleaves PARP (Figure 1). The specificity of cleavage to the signature 85 kDa fragment indicates that Yama might play a similar role in vivo, since the characterized members of the CED-3/ICE family have tightly restricted substrate specificities. ICE, for example, cleaves pro-IL-1β but not pro-IL-1α (Howard et al., 1991), even though the latter possesses several potential Asp sites for cleavage. Further, as shown previously (Lazebnik et al., 1994) and confirmed here, ICE did not cleave PARP (Figure 1). Since PARP cleavage is a biochemical event observed in virtually every form of PCD examined (Kaufmann, 1989; Kaufmann et al., 1993; M. T. and V. M. D., unpublished data), the protease responsible might be expected to play a central, universal role in mammalian apoptosis, a role perhaps akin to that of CED-3 in C. elegans. At this juncture, Yama is a candidate for such a protease. Additional studies will be required, however, before Yama can be definitively implicated as a death prote-

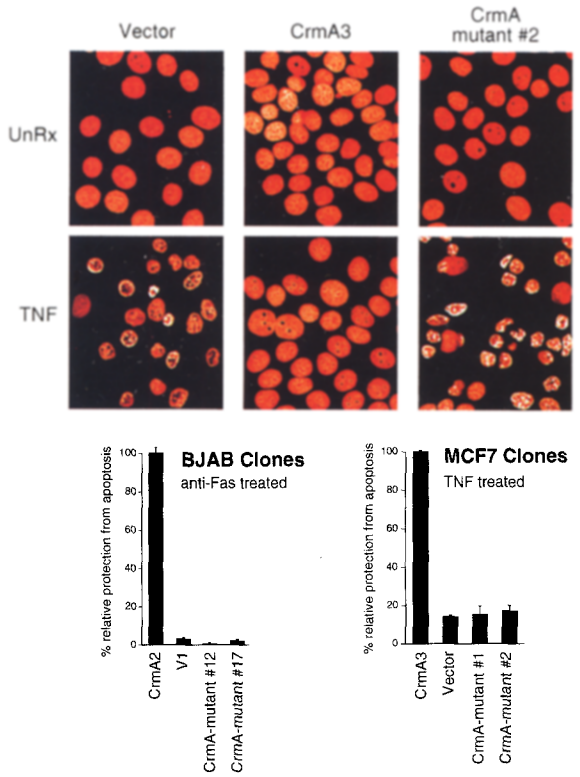

Figure 6. CrmA, but Not Mutant CrmA, Blocks Fas- and TNF-Induced Cell Death

(Top) The indicated MCF7 stably transfected clones were either kept untreated (UnRx) or treated with TNF (40 ng/ml) for 18 hr, following which cells were fixed and stained with propidium iodide and nuclear morphology examined by confocal microscopy. CrmA afforded significant protection from TNF-induced apoptosis, whereas both vector-transfected and mutant CrmA–expressing lines were sensitive to TNF-induced apoptosis.

(Bottom) (Left) The indicated BJAB stably transfected clones were quantitatively assessed for their sensitivity to Fas-induced PCD using an acridine orange–based apoptosis assay as described in Experimental Procedures. Mutant CrmA–expressing cell lines were uniformly sensitive, whereas CrmA expression afforded significant protection. (Right) The indicated MCF7 stably transfected clones were quantitatively assessed for their susceptibility to TNF-induced cell death as described in Experimental Procedures.

ase. These will include determining whether germline inactivation of Yama produces a phenotype analagous to that of the *ced-3* mutant in C. elegans.

The finding that CrmA inhibits the proteolytic activity of Yama (Figure 3) is significant, as it suggests that the well-documented ability of CrmA to inhibit apoptosis (Gagliardini et al., 1994; Tewari and Dixit, 1995; Wang et al., 1994; Boudreau et al., 1995) might be explained by its inhibition of Yama. Also, the finding that CrmA inhibits PARP cleavage in vivo is consistent with its inhibiting Yama. In this context, it is important to note that CrmA does not appear to be a general inhibitor of CED-3/ICE-family proteases given its inability to inhibit ICH-1 (Wang et al., 1994).

Importantly, our studies indicate that Yama is a zymogen that requires proteolytic activation (Figure 1). This has important ramifications, as it suggests a mode of regulation. Yama is likely synthesized as an inactive proenzyme that is activated by another protease during apoptosis. Hence, a role for a second protease, likely also Asp specific, is suggested. As our studies indicate that ICE itself can fulfill this role in vitro (Figure 1), this provides a possible explanation of how overexpression of exogenous ICE induces apoptosis in mammalian cells (Miura et al., 1993). However, since the *Ice* knockout mice have no overt defects in apoptosis (Li et al., 1995), it is likely that endogenous ICE is not a universal upstream proteolytic regulator of Yama in vivo. Nevertheless, since Yama is likely activated by cleavages at specific Asp residues, other members of the ICE family, such as ICH-1L/Nedd2, are potential candidates for this regulatory role. One cannot discount the possibility that different proteases may regulate Yama in different forms of apoptosis. Indeed, a second report on the germline inactivation of ICE found no overt defects in PCD except for one system: Fas-induced apoptosis of thymocytes (Kuida et al., 1995). ICE may not play a general role in even Fas-induced apoptosis, however, since the expected *lpr* phenotype (resulting from a defect in Fas-mediated peripheral T cell deletion) was not observed in the mice.

Taken together, the data presented are consistent with the cell death pathway being a proteolytic cascade in which apoptotic signals from diverse stimuli converge to activate proteolytically a common protease, perhaps Yama, that in turn cleaves PARP and probably other death substrates. Indeed, additional substrates cleaved during apoptosis have been identified recently, and one in particular, the 70 kDa protein component of the U1 small nuclear ribonucleoprotein, is cleaved by an activity that has characteristics of a Cys protease (Casciola-Rosen et al., 1994). It will be important to determine whether this cleavage can be mediated by Yama. The identification of Yama/CPP32β as a CrmA-inhibitable protease that cleaves PARP represents a focus point that may be useful for identifying other upstream and downstream components of the death pathway.

Experimental Procedures

Cloning of a cDNA Encoding Yama

The cDNA clone (b4HB3MA-COT8-HAP-Ft280 5′) corresponding to EST T10341 was provided by M. Bento Soares (Columbia University) and used to screen a random-primed cDNA library constructed from human umbilical vein endothelial cells treated with TNF and cycloheximide. Double-stranded DNA sequencing revealed an open reading frame, designated *Yama*, of 277 amino acids that was identical to that of CPP32β (Fernandes-Alnemri et al., 1994). Although the predicted protein sequence of Yama was identical to that of CPP32β, the nucleotide sequence of the two open reading frames differed at two positions, most likely representing sequence polymorphisms.

Expression and Purification of His_6-Tagged Yama

A 2.3 kb NcoI–BamHI fragment from the *Yama* cDNA described above was ligated into a vector (pTM1) that contained an N-terminal His_6 fusion to facilitate purification. This construct contained the His_6 tag followed by the complete coding region of *Yama* along with 1800 bp of 3′ untranslated DNA. Coupled transcription/translation was performed with the TNT kit (Promega) according to the recommendations of the manufacturer with modifications. In brief, 4 μg of plasmid DNA was incubated for 1 hr at 31°C in a total volume of 400 μl containing the kit reaction mixes and 160 μCi of translation-grade [^{35}S]Met. The trans-

lation reaction was diluted 1:20 with 20 mM HEPES buffer (pH 7.4), loaded onto an equilibrated 500 μl DEAE–Sepharose (Pharmacia) column, and then washed with 8 ml of HEPES buffer. The column was eluted with 5 ml of 20 mM HEPES, 0.5 M NaCl. This eluate was loaded onto a 300 μl nickel–NTA column (Qiagen) and then washed with 5 ml of reaction buffer (50 mM HEPES [pH 7.4], 0.1 M NaCl, 0.1% CHAPS, and 10% sucrose). The protein was eluted five times with 400 μl fractions of reaction buffer containing 50 mM imidazole.

Activation of Yama and In Vitro Reconstitution Experiments

Purified Yama (20 μl) was activated by incubating at 37°C for 4 hr with 1.5 pmol of ICE in reaction buffer supplemented with DTT (10 mM) in a total volume of 25 μl, after which 30 μl of reaction buffer was added and the reaction incubated at 37°C for an additional 15 min. Following activation, 30 μl of either control reaction buffer or reaction buffer containing 270 pmol of recombinant His_6-tagged CrmA or 270 pmol of recombinant His_6-tagged mutant CrmA was added and allowed to incubate at 37°C for 30 min. The recombinant proteins as well as the control buffer had been preincubated with DTT (2 mM) to preactivate CrmA. Following the 30 min incubation, 2 μl (0.586 μg) of purified PARP was added, and the DTT concentration was raised to 10 mM, following which the reaction was allowed to proceed for 2 hr at 37°C. The control reaction of PARP alone was carried out under identical conditions, except that no Yama, ICE, or CrmA proteins were added during the procedure. The ICE and PARP reaction was carried out identically as well, except that no Yama or CrmA proteins were added during the procedure.

Following the incubation with PARP, one fifth of each reaction sample was analyzed by immunoblotting using anti-PARP monoclonal antibody C-2-10 as described later. Additionally, an equivalent amount of each sample was resolved by SDS–PAGE and analyzed using a Molecular Devices phosphorimager to assess the state of radiolabeled Yama present in the reaction. Purified ICE was a gift of N. Thornberry (Merck). PARP was purified as described previously (Zahradka and Ebisuzaki, 1984).

CrmA Immunoprecipitation to Detect Complex Formation with Yama

Reactions were assembled by combining 20 μl of [^{35}S]Met-labeled pro-Yama or ICE-activated Yama with either 358 pmol of native CrmA or 358 pmol of mutant CrmA protein. Each reaction was diluted to a final volume of 80 μl in reaction buffer. Complex formation was allowed to occur at 37°C for 30 min. An aliquot (10 μl) of each reaction was resolved by SDS–PAGE and subjected to phosphorimaging to visualize the radiolabeled pro-Yama or activated Yama. A separate aliquot (35 μl) fo each reaction was diluted to 1 ml in PBS–TDS (O'Rourke et al., 1992) and immunoprecipitated using 25 μl of the rabbit polyclonal CrmA antiserum (described later in this section). Immunoprecipitation was carried out as described previously (O'Rourke et al., 1992), and precipitates were resolved by SDS–PAGE and subjected to phosphorimaging analysis to detect the presence of radiolabeled pro-Yama or activated Yama. Coomassie blue staining of the gel revealed that equivalent amounts of native CrmA and mutant CrmA were precipitated by the CrmA antiserum (data not shown).

Generation of Mutant CrmA Plasmids for Eukaryotic, Bacterial, and In Vitro Expression

A four-primer PCR-based method (Higuchi et al., 1988) was employed to convert codon 291 in the *crmA* gene from Thr to Arg. Initially, two independent PCRs were performed using the plasmid pcDNA3/crmA (Tewari and Dixit, 1995) as template. One reaction consisted of both an upstream primer (primer A) corresponding to nucleotides 682–711 of the *crmA* coding sequence (with nucleotide 1 representing the first nucleotide of the initiator Met codon) and of a downstream mutagenic primer (primer M2) complementary to nucleotides 853–896. Primer M2 contained a G to A transition that eliminated a PstI site and base changes that altered codon 291 to encode Arg instead of Thr and, additionally, introduced a diagnostic NruI site. The second PCR used an upstream sense mutagenic primer (primer M1) complementary to primer M2 and a downstream primer (primer B) complementary to the last 26 nucleotides of the *crmA* coding region with custom XbaI and XhoI sites. The PCR products were gel purified, combined, denatured by boiling, and annealed by slow cooling to room temperature. Following a 10 min extension reaction, PCR was carried out using the flanking primers A and B. The amplification product was digested with ClaI (cuts at nucleotide 692 in the *crmA* coding sequence) and XbaI and cloned into pcDNA3/crmA that had been similarly digested. The mutation was verified by DNA sequencing, as were all segments derived by PCR amplification. This recombinant plasmid was designated pcDNA3/crmA mutant.

The sequences of oligonucleotide primers were as follows: primer A, 5′-GCT ATG TTT ATC GAT GTG CAC ATT CCC AAG; primer M2, 5′-GCA CAA GTT GCT GCG GCT GCT TCG CGA TAC TCT TCA TTG ACA TC; primer B, 5′-GCT CTA GAC TCG AGT TAA TTA GTT GTT GGA GAG CAA TAT C; primer M1, 5′-GAT GTC AAT GAA GAG TAT CGC GAA GCA GCC GCA GCA ACT TGT GC.

For purposes of in vitro transcription and translation, the native *crmA* gene and its mutant version were digested with NcoI and XhoI and ligated into a plasmid based on pTM1 (Moss et al., 1990) that encoded an in-frame N-terminal Met–His_6 tag to facilitate purification. The coding sequence started with the initiator Met, followed by His_6, a Ser, and then the entire coding region of *crmA* or the mutant.

For expression in E. coli, the native and mutant *crmA* genes in pTM1 were digested with NcoI and XhoI and ligated into a derivative of the isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible plasmid pFLAG (IBI) that contained the same His_6 fusion tag. Additionally, the *crmA* gene from pcDNA3/crmA (Tewari and Dixit, 1995) was subcloned into the NcoI–XhoI-digested pGSTag bacterial expression vector (Dr. H. Dressler, Massachusetts General Hospital) (Ron and Dressler, 1992), generating a chimeric glutathione S-transferase (GST)–CrmA open reading frame.

Preparation of GST-CrmA Fusion Protein and Generation of Rabbit Polyclonal Antiserum

Antibodies were raised against recombinant CrmA fusion proteins. Initial immunization was with the His_6-tagged CrmA recombinant protein, and subsequent immunizations were with a GST–CrmA fusion protein produced as described previously (Hu et al., 1994). In brief, the BL21pLysS E. coli strain was transformed with pGSTag–CrmA plasmid, and production of fusion protein was induced in culture by the addition of IPTG to 50 μM. Following a 1.5 hr incubation at 25°C, the cells were recovered by centrifugation, resuspended in lysis buffer (20 mM Tris [pH 8.0], 0.5 M NaCl, 10% glycerol, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml pepstatin, and 0.1% Triton X-100), sonicated, clarified by centrifugation, and adsorbed to glutathione–agarose beads (Sigma). Soluble GST–CrmA fusion protein was eluted by incubating with 5 mM free glutathione (Sigma). Typical yield of fusion protein was 1 mg per liter of bacterial culture. Immunization of rabbits and screening of antisera were as previously described (O'Rourke et al., 1992).

Expression and Purification of Recombinant His_6-Tagged CrmA Proteins from E. coli

E. coli strain TG1 transformed with either the His_6–CrmA or His_6–mutant CrmA construct was induced with IPTG for 3 hr and harvested, and the cells were lysed by sonication and pelleted by centrifugation. The supernatant containing soluble CrmA was filtered through a 0.22 μm filter, loaded onto a 2 ml nickel–NTA column (Qiagen), and washed with 50 mM Tris (pH 8.0) containing 0.5 M NaCl. CrmA was eluted with 50 mM Tris, 50 mM imidazole (pH 8.0), containing 0.1 M NaCl. This material was diluted with 9 vol of 20 mM HEPES (pH 7.4) containing 2 mM DTT and applied to a 2 ml column of DEAE–Sepharose. This column was developed with a linear gradient of 0–1 M NaCl in 20 mM HEPES buffer (pH 7.4), and CrmA was eluted at approximately 0.4 M NaCl to give a yield of 4 mg of protein from 6 liters of culture. The material was greater than 95% pure as estimated by Coomassie blue staining and was stored at −70°C until use. In all experiments using this material, the CrmA was treated with 2 mM DTT for 5 min immediately before use. This resulted in CrmA with the highest inhibitory activity.

In Vitro Assay of ICE Inhibition by Recombinant CrmA or Mutant CrmA Protein

To assay for inhibition, 44 ng of purified ICE was activated with 10 mM DTT for 5 min at room temperature and then incubated at 37°C with various amounts of purified CrmA or mutant CrmA protein in a

total volume of 95 μl of reaction buffer: 20 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 0.5% NP-40, and 10 mM DTT. After 15 min, 5 μl of a 10 mM stock in DMSO of Boc-Ala-Ala-Pro-Asp-p-nitroanilide was added to determine the residual ICE activity by observing the release of p-nitroaniline at 410 nm using a Molecular Devices V_{max} plate reader operating in the kinetic mode. The data were expressed as the reaction velocity in the presence of inhibitor (v_i) divided by the velocity in its absence (v_o), which represents residual ICE activity. The data represent the mean and standard deviations of values derived from two independent experiments. Purified recombinant human ICE was supplied by N. Thornberry (Merck).

In Vitro Transcription/Translation of CrmA and Mutant CrmA

Coupled transcription/translation was performed using the TNT kit from Promega according to the recommendations of the manufacturer. In brief, 0.5 μg of plasmid DNA was incubated for 1 hr at 31°C in a total volume of 50 μl containing the kit reagents and 20 μCi of translation grade [^{35}S]Met. Once translated, the reaction mix was either used immediately or stored at −20°C until needed.

Gel Shift Assays to Detect Complex Formation between ICE and CrmA or Mutant CrmA

Serpin reactions with target proteases can be analyzed by gel shift analysis using purified proteinases and [^{35}S]Met-labeled serpins produced by in vitro translation (Komiyama et al., 1994a, 1994b). In vitro transcribed and translated CrmA or mutant CrmA was diluted with an equal volume of 50 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 10% sucrose, and 0.1% CHAPS. The diluted lysates (10 μl) were incubated with 10 μl of consecutive 3-fold dilutions of ICE in the same buffer containing 10 mM DTT for 30 min at 37°C. Samples were then resolved by native gel electrophoresis and visualized using a Molecular Devices phosphorimager.

TUG-PAGE

In vitro translated CrmA or mutant CrmA protein was subjected to electrophoresis in TUG-polyacrylamide gels (0–8 M) as previously described (Goldenberg, 1989; Mast et al., 1991). The gels were dried and analyzed using a Molecular Devices phosphorimager.

Stable Transfection of BJAB and MCF7 Cells

MCF7 or BJAB cells were electroporated with pcDNA3/crmA mutant plasmid and stable clonal cell lines generated as previously described (Tewari and Dixit, 1995).

Cell Culture

MCF7 cells, BJAB cells, and derived vector and crmA stable transfectants (Tewari and Dixit, 1995), along with the mutant CrmA-transfected stable lines generated in this study were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, penicillin/streptomycin, nonessential amino acids, and additionally supplemented with G418 sulfate (GIBCO BRL-Life Technologies) to 500 μg/ml for MCF7 transfectants and 3 mg/ml for BJAB transfectants.

Cell Death Assays

Apoptosis was quantitated using an assay described previously (Tewari and Dixit, 1995) that involves counting of apoptotic and nonapoptotic cells, with nuclear morphology visualized by fluorescence microscopy of cells stained with DNA-binding dyes (propidium iodide for MCF7 cells and acridine orange for BJAB cells). Laser-scanning confocal microscopy was carried out as previously described (Tewari and Dixit, 1995).

Treatment with Anti-Fas or TNF and Preparation of Cell Lysates for PARP Analysis

MCF7 cells or derived transfectants were plated in 100 mm dishes at a concentration of 2×10^6 cells per dish. On day 2, cells were treated with TNF at 40 ng/ml for the indicated time periods. Following a PBS rinse, cells were harvested by scraping into 15 ml PBS and protease inhibitors (1 mM PMSF, 0.5 mg/ml aprotinin, 0.5 mg/ml antipain, and 0.5 mg/ml pepstatin), recovered by centrifugation, and lysed in 2.5 ml of sample buffer (50 mM Tris-HCl [pH 6.8], 6 M urea, 6% 2-mercaptoethanol, 3% SDS, and 0.003% bromophenol blue). In cases in which nonadherent cells were present in the culture medium (e.g., at later timepoints), floating cells were also harvested by centrifugation and combined with the adherent cell pellet before lysis in sample buffer.

BJAB cells or derived transfectants were aliquoted at a concentration of 5×10^5 cells/ml into 6-well dishes, with 4 ml in each well. The following day, cells were treated with anti-Fas antibody (250 ng/ml) for the indicated time periods, harvested by centrifugation, washed once with PBS and protease inhibitors, and lysed in 2 ml of sample buffer.

Western Blotting

For detection of CrmA, whole-cell lysates (2×10^5 cells per lane) were resolved by SDS-PAGE, transferred to nitrocellulose, and processed as previously described (Shao et al., 1994). The GST-CrmA rabbit antiserum was used at a dilution of 1:1,000, and a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Amersham Life Sciences) was used at a 1:15,000 dilution. Visualization of signal was by ECL (Amersham).

Immunoblotting of lysates for PARP was carried out as previously described (Desnoyers et al., 1994). The anti-PARP mouse monoclonal antibody was used at a dilution of 1:10,000, and the secondary antibody, an anti-mouse immunoglobulin labeled with horseradish peroxidase, was used at a dilution of 1:1000. Visualization of signal was also by ECL.

TNF, Anti-Fas Antibody, and Anti-PARP Antibody

Recombinant TNF (specific activity, 6.27×10^7 U/mg) was a gift from Genentech (South San Francisco, CA). Anti-Fas monoclonal antibody (clone CH-11; IgM) was obtained from PanVera (Madison, WI). The anti-PARP monoclonal antibody was clone C-2-10, which, as described previously (Lamarre et al., 1988), recognizes an epitope near the N-terminal end of PARP, located between amino acids 216 and 375.

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