

## Protease Activity of *in Vitro* Transcribed and Translated *Caenorhabditis elegans* Cell Death Gene (*ced-3*) Product\*

(Received for publication, May 8, 1995, and in revised form, October 25, 1995)

Margaret Hugunin, Lucia J. Quintal, John A. Mankovich, and Tariq Ghayur‡

From the BASF Bioresearch Corporation, Worcester, Massachusetts, 01605-4314

The *Caenorhabditis elegans* cell death gene, *ced-3*, encodes one of the two proteins required for apoptosis in this organism. The primary sequence similarities between Ced-3 and the mammalian interleukin-1 $\beta$  converting enzyme (ICE) suggest that these two proteins may have functionally similar active sites and that Ced-3 may function as a cysteine protease. Here we report that *in vitro* transcribed and translated Ced-3 protein (p56) underwent rapid processing to smaller fragments. Replacement of the predicted active site cysteine of Ced-3 with serine (C364S) prevented the generation of smaller proteolytic fragments, suggesting that the processing might be an autocatalytic process. Peptide aldehydes with aspartic acid at the P1 position blocked Ced-3 autocatalysis. Furthermore, the protease inhibition profile of Ced-3 was similar to the profile reported for ICE. These functional data demonstrate that Ced-3 is an Asp-dependent cysteine protease with substrate specificity similar to that of ICE. Aurintricarboxylic acid, an inhibitor of apoptosis in mammalian cells, blocked Ced-3 autocatalytic activity, suggesting that an aurintricarboxylic acid-sensitive Ced-3/ICE-related protease might be involved in the apoptosis pathway(s) in mammalian cells.

Apoptosis, or programmed cell death, plays an important role in many multicellular organisms, in morphogenesis, maturation of B and T lymphocytes, development of the nervous system, and destruction of unwanted cells, as well as in the pathogenesis of some diseases. A better understanding of this process could lead to development of specific inhibitors to treat apoptosis-related diseases. The nematode *Caenorhabditis elegans* provides an excellent model in which to study the genetic pathway(s) involved in apoptosis. 11 genes have been identified and implicated in apoptosis in *C. elegans* (1). The functions of three of these genes are well characterized; the gene product Ced-9 prevents (2) and the Ced-3 and Ced-4 gene products promote apoptotic cell death (3, 4).

Some of the *C. elegans* apoptosis genes have homologs in mammalian cells. Correlation of the functional activities of these gene products with the activities of their mammalian homologs could lead to a better understanding of apoptosis in mammals. The mammalian gene *bcl-2* can functionally substitute for *ced-9* in *C. elegans* (5), suggesting that the BCL-2 proto-oncogene product is the mammalian homolog of Ced-9. Based on the amino acid similarities between Ced-3 and the

interleukin-1 $\beta$  converting enzyme (ICE)<sup>1</sup> it was suggested that ICE might be a mammalian homolog of Ced-3 (6). Although the *ced-4* gene has been cloned, no mammalian homolog of Ced-4 has been reported.

Ced-3 and ICE are two members of a growing family of proteins, which also includes the mouse product Nedd2 (neuronal precursor cell-expressed, developmentally down-regulated gene-2) (7), its human homolog Ich-1 (ICE and Ced-3 homolog-1) (8), Ich-2 (9) (also known as TX (10) and ICE<sub>rel</sub> II (11)), CPP32/YAMA (12–14)/prICE (15), ICE<sub>rel</sub> III (11), and Mch2 (mammalian Ced-3 homolog 2) (16). Functionally, overexpression of these Ced-3/ICE-related proteins in mammalian or insect cells induces apoptosis. Since ICE functions as a cysteine protease with an absolute requirement for aspartic acid at the P1 position (17, 18), it has been proposed that these Ced-3/ICE-related proteins function as Asp-dependent cysteine proteases. In fact, the catalytic cysteine and histidine as well as the residues defining the P1 carboxylate binding pocket of ICE, as determined by the three-dimensional x-ray crystal structure, are conserved in all these Ced-3/ICE-related proteins (19, 20).

ICE converts biologically inactive 31-kDa proIL-1 $\beta$  to its biologically active 17-kDa form. ICE is synthesized as a 45-kDa (p45) zymogen and is processed to generate the p20 and p10 subunits that comprise the active heterodimeric enzyme (17). Generation of the active enzyme requires four cleavages within p45 ICE (between residues 103 and 104, 119 and 120, 297 and 298, and 316 and 317). These cleavages occur between the Asp–X bonds, the primary substrate specificity of ICE itself, suggesting that conversion of p45 to its active heterodimeric form might be an autocatalytic event (17), that is, the result of the enzyme activity of ICE itself. The Asp residues at positions 103 and 297 in the ICE precursor are conserved in Ced-3 (residues 131 and 371 of the putative p56 Ced-3 zymogen) (6), suggesting that the active form of the Ced-3 protein might also be generated by autocatalysis.

We performed the studies presented here to determine protease activity of Ced-3 protein and its functional similarities to ICE. To this end, we developed an assay to detect autocatalysis of *in vitro* translated Ced-3 protein and used this assay together with various substrate and inhibitor studies to demonstrate that Ced-3 is an Asp-dependent cysteine protease.

### MATERIALS AND METHODS

***In Vitro* Transcription and Translation Reactions**—Full-length cDNA encoding Ced-3 was kindly provided by Dr. Horvitz (MIT, Cambridge, MA). For *in vitro* transcription and translation, this cDNA was subcloned into a T7 polymerase promoter containing plasmid (modified pSVb, Clontech, Palo Alto, CA). The cDNAs encoding human p45 ICE

\* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: BASF Bioresearch Corp., 100 Research Dr., Worcester, Massachusetts, 01605-4314. Tel.: 508-849-2563; Fax: 508-754-6742.

<sup>1</sup> The abbreviations used are: ICE, interleukin-1 $\beta$  converting enzyme; PARP, poly(ADP-ribose) polymerase; TNT, *in vitro* transcription and translation; WT, wild type; ATA, aurintricarboxylic acid; p56, full-length Ced-3 protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

precursor (amino acids 1–404), human and murine proIL-1 $\beta$ , and human poly(ADP-ribose) polymerase (PARP) were polymerase chain reaction-cloned. The polymerase chain reaction primers were designed based upon the published cDNA sequences of human ICE (21), human (22) and murine (23) proIL-1 $\beta$ , and human PARP (24).

The various  $^{35}\text{S}$ -labeled proteins were made by using a coupled transcription and translation (TNT) system (Promega, Madison, WI). Briefly, 1  $\mu\text{g}$  of DNA was added directly to TNT rabbit reticulocyte lysate, and reactions were carried out at 30  $^{\circ}\text{C}$ . For some experiments (see Fig. 3)  $^{35}\text{S}$ -labeled and nonlabeled wild-type (WT) Ced-3 proteins were synthesized in parallel reactions. The nonlabeled WT Ced-3 proteins were mixed with  $^{35}\text{S}$ -labeled C364S Ced-3 mutant or human and murine proIL-1 $\beta$  or human PARP at a ratio of 1:1. Control samples of  $^{35}\text{S}$ -labeled WT Ced-3 were mixed with fresh rabbit reticulocyte lysates at a 1:1 ratio to account for the dilution factor.

**Construction of C364S Ced-3 Mutant**—The C364S Ced-3 mutant template was generated by a two-step polymerase chain reaction-based overlapping site-directed mutagenesis (25). The internal overlapping primers used to generate the mutation of cysteine 364 to serine were 5' mutant oligo (5'-TGCAGGCTTCTCGAGGCGAACGTCGT-3') and 3' mutant oligo (5'-ACGACGTTCGCCTCGAGAAGCCTGCAC-3'). The mutagenized gene was then amplified by using oligonucleotides primers that placed a *Spe*I site at the 5'-end (5'-GGACTAGTATGATGCGTCAAGATAGAAGG-3') and a *Cla*I site at the 3'-end (5'-GGATCGATCCTCTATTAGACGGCAGAGTTTCGTGC-3'). The amplified product was digested with *Spe*I and *Cla*I and subcloned into a T7 polymerase promoter containing plasmid. The C364S Ced-3 mutant was verified by DNA sequence determination.

**Inhibition Studies**—The protease inhibitors iodoacetamide, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, and E-64 were purchased from Sigma. Two tetrapeptide aldehydes with Asp at P1 position (Ac-YVAD-CHO (17) and Ac-DEVD-CHO (12)) were purchased from Bachem Bioscience (Philadelphia, PA). The tetrapeptide aldehyde, Ac-YVAD-CHO, a potent ICE inhibitor, is based on one of the ICE cleavage site on the proIL-1 $\beta$  molecule (17); whereas, the tetrapeptide aldehyde Ac-DEVD-CHO, a potent inhibitor of CPP32, is based on the CPP32/YAMA (12–14)/prICE (15) cleavage site on the PARP molecule. Antiapoptosis compounds acetyl-L-carnitine, zinc sulfate ( $\text{ZnSO}_4$ ), and aurotricarboxylic acid (ATA) were obtained from Aldrich. Stock solutions of these compounds were made in the appropriate solvent, either distilled/deionized water, ethanol, or dimethyl sulfoxide (Sigma). Reactions ran overnight at room temperature and were analyzed on a 10–20% Tris-Tricine gradient polyacrylamide gel (Integrated Separation Systems, Natick, MA). Bands were visualized by autoradiography.

## RESULTS

**Protease Nature of Ced-3 Protein**—For functional characterization of Ced-3, we made full-length p56 Ced-3 protein using *in vitro* TNT reactions and collected aliquots at various time points after initiation of the reactions. As shown in Fig. 1, at time points up to 30 min, the p56 Ced-3 protein was the predominant form, but at 45, 60, and 90 min, several lower molecular weight bands (42, 40, 30, 26, 24, 18, and 10 kDa) were present. The intensity of the p56 band decreased between 30 and 90 min, while the intensity of the lower molecular weight bands increased during the same time period. These results demonstrate that the p56 Ced-3 protein degrades when made in rabbit reticulocyte lysates.

To determine whether the breakdown of the *in vitro* translated p56 Ced-3 protein, as shown in Fig. 1, was the result of autocatalysis of Ced-3 or an activity characteristic of the lysates, we performed the following experiments. Using the *in vitro* TNT reactions, we made Ced-3, human p45 ICE, and human proIL-1 $\beta$  proteins and incubated these proteins in the rabbit reticulocyte lysates overnight at room temperature in order to determine their stability in this assay system. The results in Fig. 2 show that unlike Ced-3, the p45 ICE and proIL-1 $\beta$  proteins were stable. The addition of recombinant ICE (derived from sf 9 cells infected with baculovirus encoding the ICE protein) resulted in the processing of the *in vitro* translated p45 ICE and proIL-1 $\beta$  proteins to their respective cleavage products (Fig. 2). The degradation of the p45 ICE and proIL-1 $\beta$  proteins, shown in Fig. 2, was therefore the result of

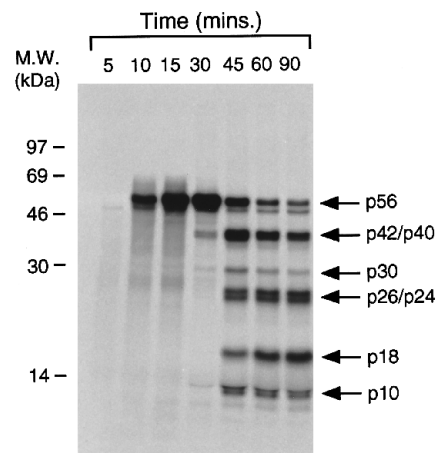


FIG. 1. Kinetics of p56 Ced-3 protein synthesis and degradation after the initiation of *in vitro* transcription and translation reaction. Samples were removed to denaturing buffer at the indicated time points and analyzed at the completion of the experiment on a 10–20% Tris-Tricine gradient polyacrylamide gel (Integrated Separation Systems). The molecular weight size markers are indicated on the left. The arrows on the right indicate the various lower molecular weight degradation products of the p56 Ced-3 protein.

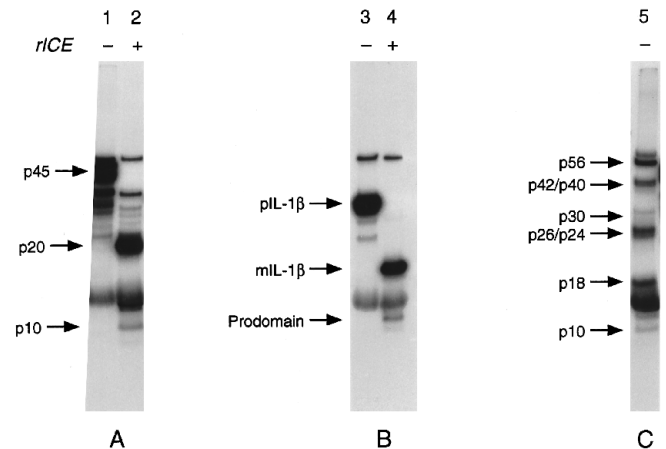


FIG. 2. Human p45 ICE and human proIL-1 $\beta$  proteins made in the *in vitro* transcription and translation reactions do not undergo degradation. A, human p45 ICE protein incubated overnight at room temperature in the absence (lane 1) or presence (lane 2) of rICE. B, human proIL-1 $\beta$  protein incubated overnight at room temperature in the absence (lane 3) or presence (lane 4) of rICE. C, Ced-3 protein incubated overnight at room temperature in the absence of rICE (lane 5). The arrows on the left indicate the various molecular species generated.

the activity provided by active ICE, not by a nonspecific activity found in the lysates. One can infer, therefore, that the observed degradation of Ced-3 also results from specific proteolysis rather than a nonspecific activity.

To demonstrate that the degradation of p56 Ced-3 protein was due to the proposed protease activity of Ced-3 (autocatalysis), we used a mutant called the C364S mutant, in which the putative active site cysteine, residue 364, was replaced by a serine. The results in Fig. 3A show that the TNT-synthesized C364S mutant was stable and did not generate the lower molecular weight bands, while the WT p56 Ced-3 protein did degrade, as evidenced by the presence of lower molecular weight bands. These data demonstrate that p56 Ced-3 protein degrades by autocatalysis and that cysteine 364 plays a crucial role in this proteolytic process.

To determine if WT p56 Ced-3 can cleave the C364S mutant and if the products of p56 Ced-3 autocatalysis possess proteolytic activity, WT p56 Ced-3 protein (30-min TNT reaction) or

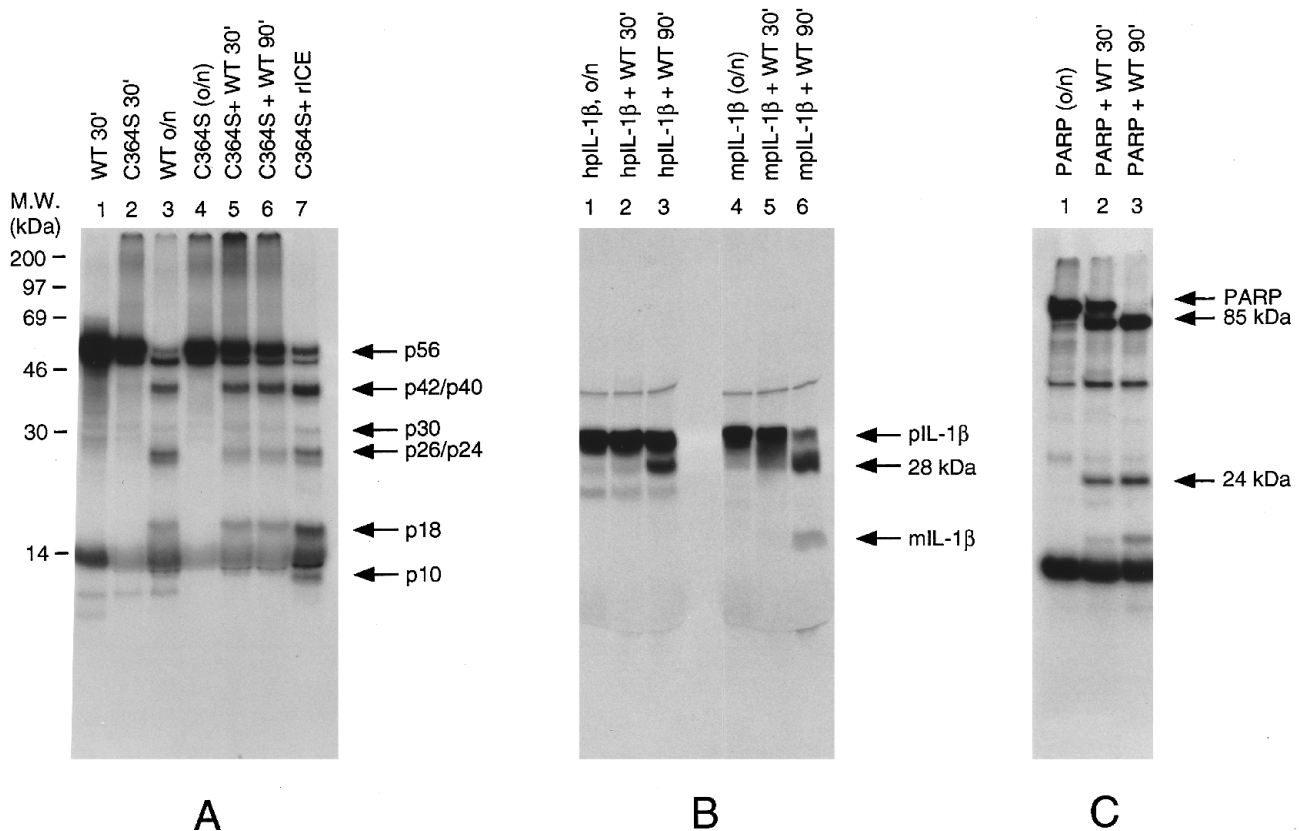


FIG. 3. **Enzymatic activities of *in vitro* transcribed and translated p56 CED-3 and the products of its autocatalysis.** A, WT Ced-3 (radiolabeled (lane 1) and nonradiolabeled) and C364S Ced-3 mutant (radiolabeled (lane 2)) proteins were synthesized in *in vitro* transcription and translation reactions. The radiolabeled WT Ced-3 (lane 3) and C364S mutant (lane 4) proteins were incubated overnight at room temperature. The radiolabeled C364S Ced-3 mutant protein was also incubated overnight with nonradiolabeled WT Ced-3 protein (30' reaction, lane 5), with nonradiolabeled WT Ced-3 (90' reaction, lane 6), or with rICE (lane 7). The molecular weight markers are depicted on the left. The arrows on the right depict the molecular sizes of the proteolytic fragments. B, radiolabeled human and murine proIL-1 $\beta$  proteins were synthesized in *in vitro* transcription and translation reactions (lanes 1 and 4). These proteins were incubated overnight with nonradiolabeled WT Ced-3 protein (30' reaction, lanes 2 and 5), or with nonradiolabeled WT Ced-3 protein (90' Ced-3 reaction, lanes 3 and 6)). Positions of 31-kDa proIL-1 $\beta$  (pIL-1 $\beta$ ), the 28-kDa intermediate (28 kDa), and the 17-kDa mature IL-1 $\beta$  (mIL-1 $\beta$ ) are indicated with arrows. C, *in vitro* transcribed and translated radiolabeled PARP protein (lane 1) was incubated with nonradiolabeled WT Ced-3 protein (30' reaction lane 2) or WT Ced-3 protein (90' reaction, lane 3). Positions of full-length PARP and the 85- and 24-kDa proteolytic fragments are indicated by arrows.

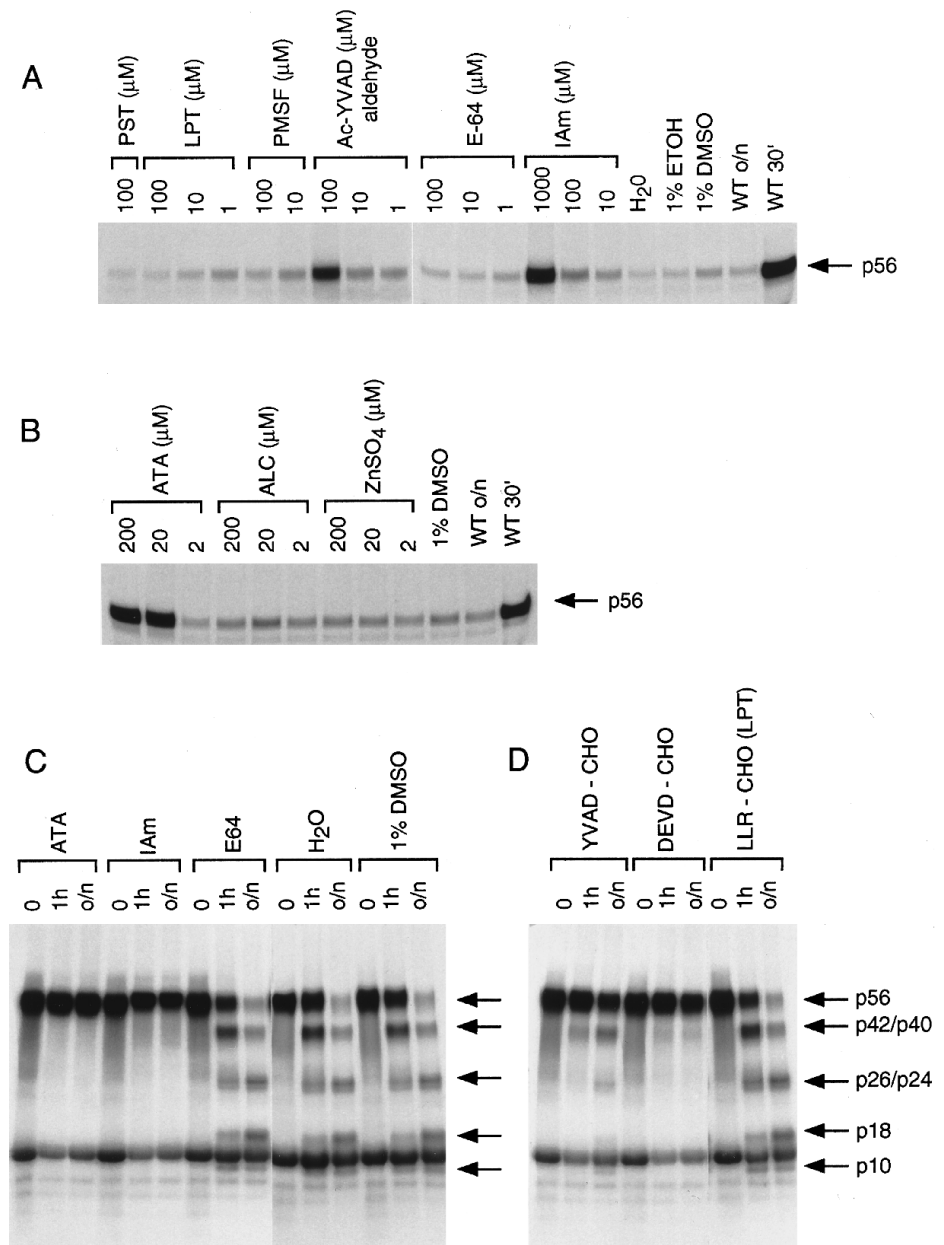
products of p56 Ced-3 autocatalysis (90-min TNT reaction) were mixed with the C364S mutant protein and incubated overnight at room temperature. Both the 30- and 90-min Ced-3 reaction products cleaved the C364S mutant protein, as demonstrated by the appearance of lower molecular weight bands shown in Fig. 3A. Cleavage of C364S mutant by WT p56 Ced-3 or by the autocatalysis products of p56 Ced-3 generated the same sized bands as did autocatalysis of WT Ced-3. Collectively, these data demonstrate that the products of p56 Ced-3 autocatalysis retain proteolytic activity. Recombinant ICE (produced by baculovirus-infected sf 9 cells) also cleaved the C364S mutant protein (Fig. 3A).

**Proteolysis of ProIL-1 $\beta$  by Ced-3**—ICE cleaves proIL-1 $\beta$  at two sites. Cleavage at the first ICE site, between residues Asp-27 and Gly-28, generates a 28-kDa IL-1 $\beta$  fragment; cleavage at the second site, between residues Asp-116 and Ala-117, generates a 17-kDa fragment (26, 27). To determine if Ced-3, like ICE, can process proIL-1 $\beta$ , we incubated p56 Ced-3 (30-min TNT reaction) or autoprocessed Ced-3 (90-min TNT reaction) with either human or murine proIL-1 $\beta$  (the proIL-1 $\beta$ s were also made using the TNT reaction). The results in Fig. 3B demonstrate that autoprocessed Ced-3 cleaved human and murine proIL-1 $\beta$  to varying degrees. Autoprocessed Ced-3 cleaved human proIL-1 $\beta$  to a 28-kDa form (probably acting at the first ICE cleavage site), but the 17-kDa IL-1 $\beta$  form (the product of the second ICE cleavage) was not detected. Autoprocessed

Ced-3 generated two proteolytic products, approximately 28 and 17 kDa from murine proIL-1 $\beta$ . In contrast, the p56 form of Ced-3 (30-min TNT reaction) did not process either human or murine proIL-1 $\beta$ . Incubation of p56 Ced-3 with proIL-1 $\beta$  prevents autocatalysis of Ced-3 and generation of the products of that reaction (data not shown).<sup>2</sup> Since both p56 Ced-3 and the products of its autocatalysis process the C364S Ced-3 mutant (Fig. 3A), the results presented in Fig. 3B suggest that autoprocessed Ced-3 may have a different substrate specificity and/or higher specific activity than the p56 Ced-3 form.

**Proteolysis of PARP by Ced-3**—Recent studies have shown a strong correlation between cleavage of PARP by ICE-related proteases and apoptosis in mammalian cells as well as in isolated nuclei (12–16). Also, recently, it has been suggested that Ced-3 can cleave human PARP (Ref. 28, and see “Addendum”). Therefore, we determined if both p56 Ced-3 and products of its autocatalysis would cleave *in vitro* TNT PARP. The results in Fig. 3C demonstrate that both the p56 as well as the autoprocessed forms of Ced-3 cleaved full-length PARP to generate 85- and 24-kDa fragments. However, the autoprocessed form of Ced-3 (90' TNT reaction) was more efficient at cleaving PARP than the p56 form (30' TNT reaction). The data in Fig. 3 also demonstrate that human PARP is a better substrate for

<sup>2</sup> M. Hugunin, L. Quintal, and T. Ghayur, unpublished observations.



**FIG. 4. Inhibition of p56 Ced-3 autocatalysis.** *A*, protease inhibition profile of p56 Ced-3 autocatalysis; *B*, effects of anti-apoptosis compounds on p56 Ced-3 autocatalysis. Wild-type p56 Ced-3 protein was made in a 30' *in vitro* transcription and translation reaction. Aliquots of p56 Ced-3 protein were incubated overnight at room temperature in the absence or presence of various test compounds. Only the p56 band is shown for each lane. *C*, effects of various compounds at blocking autocatalytic cleavages of p56 Ced-3. Except for iodoacetamide (1000 μM), all compounds were tested at a concentration of 100 μM. *D*, the ability of proIL-1β- and PARP-derived tetrapeptide aldehyde inhibitors to block autocatalytic cleavages of p56 Ced-3. Leupeptin was used as an aldehyde control. All peptides were tested at a concentration of 100 μM. The arrows on the right indicate the molecular weights of autocatalytic fragments of p56 Ced-3.

Ced-3 than human proIL-1β. A similar substrate specificity has also been demonstrated for the CPP32/YAMA/prICE.

**Protease Inhibition Profile of Ced-3 Autocatalysis**—To determine if ICE and Ced-3 have similar protease inhibition profiles, we compared our experimentally determined profile of p56 Ced-3 with published reports of the protease inhibitor profile of ICE (17, 18). The data in Fig. 4A show that the protease inhibitors leupeptin, pepstatin, phenylmethylsulfonyl fluoride, and E-64, which do not inhibit ICE activity, also do not inhibit Ced-3 autocatalysis. On the other hand, protease inhibitors that inhibit ICE activity, such as iodoacetamide and Ac-YVAD-aldehyde (17), did inhibit Ced-3 autocatalysis (also see Fig. 4C). That the inhibition profiles of ICE and Ced-3 are similar suggests that the catalytic specificities of the two proteins are similar and further supports the hypothesis that these proteins are functionally homologous.

We also determined if all the autocatalytic cleavages within the Ced-3 molecule are equally sensitive to various inhibitors. The results in Fig. 4C demonstrate that ATA and iodoacetamide blocked all autocatalytic cleavages of the p56 Ced-3 for up to 16 h of incubation at room temperature. In contrast and

as observed in Fig. 4, A and B, E-64 and pepstatin were completely ineffective at inhibiting Ced-3 autocatalysis.

Since *in vitro* transcribed and translated PARP protein was cleaved more efficiently by Ced-3 than proIL-1β (Fig. 3), we tested PARP-derived peptide (Ac-DEVD-CHO; a potent inhibitor of CPP32) and proIL-1β-derived peptide (Ac-YVAD-CHO; a potent inhibitor of ICE) for their abilities to block Ced-3 autocatalysis. The results in Fig. 4D demonstrate that the two peptide aldehydes inhibited Ced-3 autocatalysis to varying degrees. Ac-DEVD-CHO blocked all autocatalytic cleavages of p56 Ced-3 for up to 16 h of incubation, whereas, in the presence of Ac-YVAD-CHO, some p56 autocatalysis was observed at 16 h postincubation. The control peptide aldehyde Ac-LLR-CHO (leupeptin) was ineffective at blocking Ced-3 autocatalysis.

**Anti-apoptosis Compounds and Inhibition of Ced-3 Autocatalysis**—Since *ced-3* is required for apoptosis in *C. elegans*, we evaluated the effects of apoptosis inhibitors on Ced-3 autocatalysis *in vitro*. We tested three inhibitors of mammalian cell apoptosis, ATA (29), ZnSO<sub>4</sub> (30), and acetyl-L-carnitine (31), for their ability to inhibit Ced-3 autocatalysis. The results presented in Fig. 4B demonstrate that acetyl-L-carnitine and

ZnSO<sub>4</sub> do not inhibit Ced-3 autocatalysis but that ATA is a potent inhibitor of Ced-3 autocatalysis. ATA did not inhibit ICE activity at concentrations up to 200  $\mu$ M (data not shown).<sup>3</sup>

#### DISCUSSION

The data in this report demonstrate the protease nature of *in vitro* transcribed and translated Ced-3 protein. 1) *In vitro* translated p56 Ced-3 was rapidly degraded in our assay system, while other proteins were not, suggesting autocatalytic activity of Ced-3. 2) Replacement of the putative active site cysteine with serine (C364S mutant) completely abolished Ced-3 autocatalysis. 3) WT p56 Ced-3 and products of its autocatalysis cleaved the C364S mutant protein, generating proteolytic fragments of the same size as the WT p56 autocatalysis fragments. 4) Autoprocessed Ced-3 also cleaved certain of the ICE-sites in human and mouse proIL-1 $\beta$ , although it was far less effective than ICE with these substrates. 5) WT p56 Ced-3 and the products of its autocatalysis cleaved human PARP as well. Collectively, these observations clearly demonstrate that both the p56 and autoprocessed forms of Ced-3 are proteases.

We found an unusual inhibition profile of Ced-3 autocatalysis using various classes of protease inhibitors, similar to the profile previously demonstrated for ICE (17, 18). All active site protease inhibitors reported to inhibit ICE activity (18) also inhibited Ced-3 autocatalysis, while E-64, an inhibitor of many cysteine proteases, did not inhibit ICE activity (18) or Ced-3 autocatalysis. The three-dimensional structure of human ICE in complex with the ICE inhibitors Ac-YVAD-chloromethylketone (19) or Ac-YVAD-aldehyde (20) revealed that, in the crystalline state, ICE is a homodimer ((p20/p10)<sub>2</sub>) of p20/p10 heterodimers, with each p20/p10 subunit within the homodimer containing an active site. The catalytic cysteine and histidine residues as well as the residues defining the P1 carboxylate binding pocket of ICE are conserved in Ced-3 (19, 20), suggesting that mechanistically the active sites of ICE and Ced-3 are similar. The functional observations presented here support the above notion. However, only 2 out of 6 amino acid residues of ICE that interact with the P2-P4 residues of the tetrapeptide inhibitors are conserved in Ced-3 (6, 19, 20), suggesting that there might be differences in substrate binding/recognition. The data demonstrating a strong correlation between the efficiency of cleavage of PARP and proIL-1 $\beta$  by Ced-3 and the potency of PARP- and proIL-1 $\beta$ -derived peptides to inhibit Ced-3 autocatalysis might reflect such differences in substrate binding/recognition. Interestingly, 5 out of 6 amino acid residues that interact with P2-P4 residues of the substrate are conserved in Ced-3 and CPP32 and, like Ced-3, CPP32 cleaves PARP more efficiently than proIL-1 $\beta$ . Moreover, as with Ced-3, the PARP-derived peptide (Ac-DEVD-CHO) is a more potent inhibitor of CPP32 than the proIL-1 $\beta$ -derived peptide (Ac-YVAD-CHO) (12).

We found that p56 Ced-3 and autoprocessed Ced-3 showed differences in substrate specificity, as had previously been demonstrated for p45 ICE and p20/p10 ICE (18, 32, 33). The p56 form of Ced-3 cleaved the C364S mutant protein but did not cleave human or murine proIL-1 $\beta$ , while autoprocessed Ced-3 cleaved all three substrates. Similarly, the p45 ICE precursor cleaved the active site cysteine p45 ICE mutant (32) but did not cleave pro-IL-1 $\beta$  (33), while the active p20/p10 form of ICE derived from autocatalysis of the p45 ICE precursor cleaved both substrates (32, 33). These differences in the enzymatic activities of the precursor and the active forms of enzymes might reflect differences in substrate specificities and/or specific activities of the different forms of the enzymes.

Although Ced-3 autoprocessed to lower molecular weight

molecules retains proteolytic activity, we do not know which of the lower molecular weight fragment(s) constitutes the active Ced-3 component(s). It is possible that the processed form of Ced-3, like ICE, might function as a hetero/homodimer. The differences in the catalytic activities of p56 Ced-3 and autoprocessed Ced-3 could result from conformational changes in and around the active site that might accompany processing, as occurs in processing and activation of ICE. A 1000-fold excess of Ac-YVAD-chloromethylketone is required to inhibit the activity of p45 precursor ICE as compared with p20/p10 ICE (32). Furthermore, in an affinity labeling study using biotinylated tetrapeptide inhibitor, p45 precursor ICE was labeled with an EC<sub>50</sub> of 5  $\mu$ M, and p20/p10 ICE was labeled with an EC<sub>50</sub> of 1 nM (34). These observations demonstrate that the affinity of ICE for the inhibitor increased as ICE proceeded from the p45 precursor form to the p20/p10 active form, suggesting that active site conformational changes occurred during processing. Analogous conformation differences in the active sites of p56 Ced-3 and autoprocessed Ced-3 might explain the differences in substrate specificity or differences in specific activities between these forms of the enzyme.

Although members of the Ced-3/ICE family of proteases have been implicated in the process of apoptosis (6–8), our recent studies with ICE-deficient mice demonstrated that ICE *per se* is not a critical protease in several apoptosis pathways (35). We found no evidence of an apoptosis defect in ICE-deficient mice during embryogenesis, development of the immune system, *in vitro* spontaneous apoptosis of thymocytes, dexamethasone and  $\gamma$  irradiation-induced apoptosis of thymocytes, or lipopolysaccharide/ATP-induced apoptosis in peritoneal macrophages (35). However, ICE may play a role in the apoptosis pathway triggered via the Fas receptor (36). Apoptosis induced via the Fas receptor was blocked by the serpin, crmA (37), an inhibitor of ICE and possibly of ICE family members (6, 11). Collectively, these studies suggest that ICE *per se* is not critically involved in many apoptosis pathways. However, ICE may function redundantly with other ICE family members, certain of which may play a role in specific apoptosis pathways.

The involvement of Ced-3/ICE family of proteases in the process of apoptotic cell death, raises the possibility that some of the known inhibitors of apoptosis might interfere with the functioning of these proteases. Of the three agents we tested, only ATA inhibited Ced-3 autocatalysis, but ATA did not inhibit ICE activity, suggesting that one of the mechanisms by which ATA inhibits apoptosis might be its ability to inhibit a Ced-3/ICE family member involved in an apoptosis pathway, such as PrICE, Nedd2/Ich-1, Ich-2, CPP32, ICE<sub>rel</sub> III, Mch2, or some as yet unidentified member. Studies are underway to determine if ATA inhibits any of the other known ICE family member(s).

Since ICE is absolutely required for the generation of mature, biologically active IL-1 $\beta$  (35, 36), it may be possible to design selective inhibitors of ICE to block mature IL-1 $\beta$  production in order to treat inflammatory diseases without influencing the apoptotic pathway(s). Conversely, specific inhibitors of ICE family member(s) involved in apoptosis could be developed for the treatment of apoptosis-dependent diseases, such as neurodegenerative disorders and AIDS (38).

**Acknowledgments**—The authors wish to thank Drs. Hamish Allen, Robert Kamen, and Daniel Tracey for helpful comments and Dr. Lisa Christenson for editorial assistance.

**Addendum**—Since the submission of this manuscript, a manuscript by Xue and Horvitz has appeared showing protease activity of Ced-3 (Ref. 27).

<sup>3</sup> K. Brady, L. Quintal, and T. Ghayur, unpublished observations.

## REFERENCES

- Ellis, R. E., Yuan, J., and Horvitz, H. R. (1991) *Annu. Rev. Cell Biol.* **7**, 663–698
- Hengartner, M. O., Ellis, R. E., and Horvitz, H. R. (1992) *Nature* **356**, 494–499
- Yuan, J., and Horvitz, H. R. (1990) *Dev. Biol.* **138**, 33–41
- Yuan, J., and Horvitz, H. R. (1992) *Development* **116**, 309–320
- Vaux, D. L., Weissman, I. L., and Kim, S. K. (1992) *Science* **258**, 1955–1957
- Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993) *Cell* **75**, 641–652
- Kumar, S., Kinoshita, M., Noda, M., Copelan, N. G., and Jenkins, N. A. (1994) *Genes & Dev.* **8**, 1613–1626
- Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994) *Cell* **78**, 739–750
- Kamens, J., Paskind, M., Hugunin, M., Talanian, R. V., Allen, H., Banach, D., Bump, N., Hackett, M., Johnston, C. G., Li, P., Mankovich, J. A., Terranova, M., and Ghayur, T. (1995) *J. Biol. Chem.* **270**, 15250–15256
- C., Faucheu, Diu, A., Chan, A. W. E., Blanchet, A.-M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R. A., Lippke, J. A., Rocher, C., Su, S.-S. M., Livingston, D. J., Herchand, T., and Lalanne, J.-L. (1995) *EMBO J.* **14**, 1914–1922
- Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., Molineaux, S. M., Yamin, T.-T., Yu, V. L., and Nicholson, D. W. (1995) *J. Biol. Chem.* **270**, 15870–15876
- Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) *J. Biol. Chem.* **269**, 30761–30764
- Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) *Cell* **81**, 801–809
- Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, M. S., Smulson, M. E., Yamin, T.-T., Yu, V. L., and Miller, D. K. (1995) *Nature* **376**, 37–43
- Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) *Nature* **371**, 346–347
- Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995) *Cancer Res.* **55**, 2737–2742
- Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J. F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T. T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992) *Nature* **356**, 768–774
- Black, R. A., Kronheim, S. R., and Sleath, P. R. (1989) *FEBS Lett.* **2**, 386–390
- Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Houy, W., Mankovich, J. A., McGuinness, L., Orlewich, E., Paskind, M., Pratt, C. A., Reis, P., Summani, A.-K., Terranova, M., Welch, J. P., Xiong, L., Moller, A., Tracey, D. E., Kamen, R., and Wong, W. (1994) *Cell* **78**, 343–352
- Wilson, K. P., Black, J. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) *Nature* **370**, 270–275
- Black, R. A., Sleath, P. R., and Kronheim, S. R. (October 17, 1991) International Patent Application Wo 91/15577
- March, C. J., Mosley, B., Larson, A., Cerretti, D. P., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Grabstein, K., Conlon, P. J., Hopp, T. P., and Cosman, D. (1985) *Nature* **315**, 641–647
- Gray, P. W., Glaister, D., Chen, E., Goeddel, D. V., and Pennica, D. (1986) *J. Immunol.* **137**, 3644–3648
- Cherney, B. W., McBride, O. W., Chen, D., Alkhatib, H., Bhatia, K., Hensley, P., and Smulson, M. E. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 8370–8374
- Higuchi, R. (1990) in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) p. 177, Academic Press Inc., San Diego, CA
- Sleath, P. R., Hendrickson, R. C., Kronheim, S. R., March, C. J., and Black, R. A. (1990) *J. Biol. Chem.* **265**, 14526–14528
- Howard, A. D., Kostura, M. J., Thornberry, N. A., Ding, G. J. F., Limjuco, G., Weidner, J., Salley, J. P., Hogquist, K. A., Chaplin, D. D., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1991) *J. Immunol.* **147**, 2964–2969
- Xue, D., and Horvitz, R. (1995) *Nature* **377**, 248–251
- Mogil, R. J., Bissonnette, R. J., Bromley, P., Yamaguchi, I., and Green, D. R. (1994) *J. Immunol.* **152**, 1674–1683
- Cohen, J. J., and Duke, R. C. (1984) *J. Immunol.* **132**, 38–42
- Galli, G., and Fratelli, M. (1993) *Exp. Cell Res.* **204**, 54–60
- Rolando, A. M., Palyha, O. C., Ding, G. J.-F., Howard, A. D., and Molineaux, S. M. (1993) *J. Cell. Biochem.* **18**, 147
- Ayala, J. M., Yamin, T.-T., Eggar, L. A., Chin, J., Kostura, M. J., and Miller, D. K. (1994) *J. Immunol.* **153**, 2592–2599
- Yamin, T. T., Ayala, J., and Miller, D. K. (1994) *FASEB J.* **8**, 644
- Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., Towne, E., Tracey, D., Wardwell, T., Wei, F.-Y., Wong, W., Kamen, R., and Seshadri, T. (1995) *Cell* **80**, 401–411
- Kuida, K., Lippke, J. A., Ku, G., Harding, M. W., Livingston, D. J., Su, M. S.-S., and Flavell, R. A. (1995) *Science* **267**, 2000–2003
- Tewari, M., and Dixit, V. A. (1995) *J. Biol. Chem.* **270**, 3255–3260
- Vaux, D. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 786–789