THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 52, Issue of December 25, pp. 35109–35117, 1998

*Printed in U.S.A.*

# Identification of Multiple *Caenorhabditis elegans* Caspases and Their Potential Roles in Proteolytic Cascades*

(Received for publication, July 15, 1998, and in revised form, October 15, 1998)

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**Proteases of the caspase family play a central role in the execution of programmed cell death in all metazoans examined. The *Caenorhabditis elegans* caspase CED-3 is essential for programmed cell death in this organism. Three additional *C. elegans* caspase-related genes, *csp-1* (caspase homolog-1), which encodes the *csp-1A*, *csp-1B*, and *csp-1C* RNA species; *csp-2*, which encodes the *csp-2A* and *csp-2B* RNA species; and *csp-3* are identified. CSP-1A, CSP-1B, CSP-2A, and CSP-2B proteins are similar in sequence to caspase proproteins. CSP-1C is similar only to large caspase subunits, and CSP-3 is similar only to small caspase subunits. CSP-1B can be activated to become a cysteine protease by processing at internal aspartate residues. Activated CSP-1B can cleave the CSP-1B, CED-3, and CSP-2B proproteins, and activated CED-3 can cleave the CED-3 and CSP-2B proproteins. Inhibitor and synthetic substrate studies further suggest that activated CSP-1B and activated CED-3 have different substrate specificities. These results suggest that *C. elegans* encodes several caspases that might act in proteolytic cascades to regulate processes such as programmed cell death.**

Programmed cell death is a highly regulated process involving components conserved from nematodes to humans (1). The most downstream elements of this process described so far are proteases termed caspases (2). Caspases play a central role in programmed cell death and have also been implicated in the control of interleukin-1$\beta$ production during inflammation in humans (3, 4) and oocyte maturation in *Drosophila melanogaster* (5).

Active caspases are formed by cleavage of a proprotein precursor at internal aspartate residues (2). These residues divide the proprotein into N-terminal, middle, and C-terminal domains. After proprotein processing, the middle and C-terminal domains associate to form the active enzyme (2–4, 6), a cysteine protease with a well conserved QAC(R/Q/G)G amino acid motif containing the active site cysteine. Active caspases cleave substrates following aspartate residues (2). However, not all aspartates can direct cleavage (3), suggesting that additional amino acid residues within a substrate are important in determining cleavage specificity.

N-terminal domains of some caspase proproteins bind to proteins thought to regulate caspase activity. For example, the N-terminal domain of the caspase-8 proprotein binds the FADD protein, which, in turn, binds the Fas receptor. Activation of the Fas receptor can lead to activation of caspase-8 (7, 8). Similarly, the N-terminal domain of the *Caenorhabditis elegans* caspase CED-3 proprotein binds the CED-4 protein, resulting in CED-3 activation (9).

Caspases can also be processed and activated by other caspases. Human caspase-3 has been shown to process caspase-1 and caspase-2 *in vitro* (6). Similar processing and activation have also been documented *in vivo*. For example, caspase-8 activation by the Fas receptor can lead to processing and activation of caspase-3 (10). Caspase-9 activation by Apaf-1 (a protein similar to CED-4) and cytochrome C can lead to activation of caspase-3 (11), and caspase-1 can be activated by caspase-11 (12). Thus, proteolytic cascades are important in regulating caspase activity in mammals.

Although the protease activities of caspases are essential for their functions, the mammalian FLIP/Casper protein is an exception. This protein is similar to caspases but lacks the catalytic cysteine residue required for protease activity (13, 14). The observation that FLIP/Casper can either promote or prevent apoptosis (13, 14) suggests that protease activity may not be the sole biochemical activity of caspases.

In the nematode *C. elegans* only a single caspase, CED-3, has been described. Mutations in *ced-3* prevent programmed cell deaths from occurring (15), and overexpression of CED-3 can induce death in cells that normally live (16). The key molecular regulators of cell death in *C. elegans*, *ced-3*, *ced-4*, and *ced-9*, all have mammalian counterparts (caspases (17), Apaf-1 (18), and *bcl-2* family members (19), respectively), suggesting that mechanisms that regulate the activities of these proteins may be conserved as well. Although CED-3 can be activated *in vitro* by CED-4, it remains unclear whether other activation mechanisms exist *in vivo*. Here the identification of three novel *C. elegans* caspase-related genes, *csp-1*, *csp-2*, and *csp-3*, encoding six different proteins, CSP-1A, CSP-1B, CSP-1C, CSP-2A, CSP-2B, and CSP-3, is described. Activated CSP-1B is shown to cleave the CSP-1B, CED-3, and CSP-2B proproteins, whereas activated CED-3 can cleave the CED-3 and CSP-2B proproteins. These results suggest that proteolytic cascades might be used in *C. elegans* to regulate caspase activation.

## EXPERIMENTAL PROCEDURES

*Northern, YAC[1] Filter, and cDNA Library Hybridizations*—Northern hybridization procedures followed those in Ref. 20. *C. elegans* total RNA was purified as described previously (17) from mixed stage wild-type strain N2 animals. Poly(A)$^+$ RNA was isolated using the FastTrack kit (Invitrogen). YAC filter probing conditions were identical to those rec-

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF088285 (csp-1A), AF088286 (csp-1B), AF088287 (csp-1C), AF088288 (csp-2A), AF088289 (csp-2B), AF088290 (csp-3).*

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[1] The abbreviations used are: YAC, yeast artificial chromosome; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; RACE, rapid amplification of cDNA ends; bp, base pair(s); kb, kilobase pair(s); AFC, 7-amino-4-trifluoromethyl-coumarin.

ommended for Southern blotting (20). The cDNA library used was constructed in a pBluescript-derived vector[2] (Stratagene) and was transformed into *Escherichia coli* DH5$\alpha$ bacteria. $1 \times 10^6$ bacterial colonies were screened by hybridization (20) using probes specific for *csp-1* or *csp-2/csp-3*. The *csp-1* probe was amplified from YAC Y48E1 using primers SH161 (5′-ACGGACGCCAAGAAATCG-3′) and SH203 (5′-TAATACGAACGCGCTGGAG-3′). The *csp-2/csp-3* probe was amplified from YAC Y47H9 using primers SH97 (5′-CGTATTCCAAAGTTCTTCCAG-3′) and SH98 (5′-TCACAATTGATAGAAAATCCG-3′). Probes were labeled by random primer extension (20). Plasmids from hybridizing bacterial colonies were isolated by alkali lysis minipreps (20). *csp-1A* cDNA sequence was determined using primers SH241 (5′-ATGAGATGAGATCTGAGG-3′), SH232 (5′-ACTGAAGATTTTAATACG-3′), SH194 (5′-CTATGGTGCAGGAATTCG-3′), SH200 (5′-TATACAAGAGCCATCTCC-3′), SH87 (5′-GCAAATTACTGTGTACTG-3′), SH162 (5′-ACACGGCAACCCATTTGG-3′), SH203, and the universal T3 primer. *csp-1*B and *csp-1*C cDNA sequences were determined using primers SH87, SH162, SH203, and the universal T3 primer. *csp-2*A cDNA sequence was determined using primers SH131 (5′ ATGAAGATTGGCTGGTTATTG 3′), SH124 (5′ GAGCGATACAACGTGTGTCAC 3′), SH132 (5′ AACGTGAGATGTTTCGTG 3′), SH133 (5′ AGGAGATTTTCACGAAAC 3′), SH134 (5′ TCTCCAGACTTGTGACGG 3′), SH105 (5′ CATCAGTGACGTCATCAATGC 3′), SH136 (5′ ATCCTTCTTGAATCTCGG 3′), SH142 (5′TGGCGGGTGTTGCAAAGG 3′), SH144 (5′ GCCGGAACATCGTCAGAATCGG 3′), SH164 (5′ CGAATGAATCAACGGATG 3′), SH104 (5′ CATAAAGAAGTCCCTGATCTC 3′), SH158 (5′ CTGTTGGTCTCCATTAGG 3′), SH159 (5′ CACTTTATTGACTAGTTAAAC 3′), and the universal T3 primer. *csp-2B* cDNA sequence was determined using primers SH138 (5′-CGGCACTTTGAAGATCG-3′), SH104, SH158, SH159, and the universal T3 primer. *csp-3* cDNA sequence was determined using primers SH97, SH92 (5′-CTTGCGACGACGAGGTTTGG-3′), SH93 (5′-ACACATGGTATATTCAGG-3′), SH98, and the universal T3 primer. All sequencing runs were performed using an ABI sequencer (Applied Biosystems).

*PCR Techniques*—PCR used AmpliTaq DNA polymerase and followed the instructions of the manufacturer (Perkin-Elmer). 100 ng of genomic DNA or YAC DNA were added to each PCR reaction. To confirm the chromosomal location of *csp-2,* primer pairs SH133/SH134 and SH142/SH163 (5′-TCATTTGACTGTTCCAGC-3′) were used to amplify DNA by PCR from YACs Y48F8 and Y73B6. Products of the expected sizes of 1 and 0.55 kb, respectively, were observed. RT-PCR was performed using the GeneAmp RT-PCR kit (Perkin-Elmer). Primers used were SH241 and SH239 (5′-CGTCGTTGGAAAACTGC-3′). Rapid amplification of cDNA ends (RACE) was performed using the 5′-RACE kit (Life Technologies, Inc.) using primer SH124 as the 3′-most primer and primer SH125 (5′-CTCAATCACATCATTAACACC-3′) as the adjacent 5′-primer.

*C. elegans and Yeast Genomic DNA Preparations—C. elegans* genomic DNA was prepared as described previously (21). YAC DNA for PCR was crudely isolated in a genomic DNA preparation of yeast harboring the YAC (22).

*csp-2 Genomic Structure Determination—C. elegans* genomic DNA was amplified by PCR using the following primer pairs derived from the *csp-2A* cDNA sequence: SH131 and SH132, SH133 and SH134, SH135 (5′-CTGGAGACCCTATGAGAG-3′) and SH136, SH142 and SH144, SH164 and SH138, SH153 (5′-GTGAGGACGCATCAGATG-3′) and SH104, SH139 (5′-GATCTTCAAAGTGCCGGG-3′) and SH158, and SH160 (5′-TCAACATCTCCTGGCTTC-3′) and SH159. End sequences of amplified products were determined using an ABI sequencer.

*Expression of Proteins in E. coli*—All expression constructs used the pET3a vector (Novagen) for expression in *E. coli* strain BL21(DE3). pET-3a-CED-3-FLAG, pET-3a-CED-3(C358S), pET-3a-CSP-1B-FLAG, and pET-3a-CSP-1B(C138S)-FLAG plasmids were named pSH179–3, pSH66–1, pSH192–2, and pSH194–2, respectively. CED-3-FLAG was expressed as follows. An overnight culture of pSH179–3-containing cells was diluted 1:10 in LB broth (20) and incubated at 37 °C until an $A_{600}$ of 0.5 was reached. Isopropyl $\beta$-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was incubated at 25 °C for 2 h. CSP-1B-FLAG and CSP-1B(C138S)-FLAG were expressed in a similar manner to CED-3-FLAG except that isopropyl $\beta$-D-thiogalactopyranoside induction was performed for 3 h. Coomassie Blue staining was done as described previously (20).

*Protease Purification*—Activated CSP-1B-FLAG protease was partially purified as follows: 50 ml of bacteria expressing CSP-1B-FLAG were centrifuged at 5000 $\times$ *g* for 5 min. The pellet was resuspended in 1 ml of 50 mM Tris, pH 7.5, 5 mM EDTA and sonicated for 5 s to achieve cell lysis. The lysed cells were centrifuged at 12,000 $\times$ *g* for 10 min at 4 °C, and the supernatant was passed over an anti-FLAG antibody column (Eastman Kodak Co.). Bound proteins were eluted using 200 $\mu$g/ml FLAG peptide and concentrated in a Centricon-10 concentrator (Amicon). Activated CED-3-FLAG was partially purified as described for activated CSP-1B-FLAG.

*Peptide Sequencing*—Partially purified CSP-1B-FLAG was run on a 15% SDS-polyacrylamide gel and blotted onto Immobilon-P polyvinylidine difluoride membrane (Millipore Corp.). The membrane was stained with Coomassie Blue (20), and the 14- and 16-kDa bands were excised and subjected to four rounds of N-terminal protein sequencing (23).

*Protease Assays*—2 $\mu$l of partially purified CSP-1B-FLAG or CED-3-FLAG protease were added to 1 $\mu$l of *in vitro* translated $^{35}$S-labeled substrate and incubated at 25 °C for 1–3 h. 1 $\mu$l of either inhibitor or buffer control was preincubated with protease for 10 min prior to the addition of substrate. The reaction was stopped by the addition of 20 $\mu$l of protein gel loading buffer (20). The entire reaction was run on a 15% SDS-polyacrylamide gel, which was dried and exposed to x-ray film (Eastman Kodak Co.). Plasmids encoding the CED-3, CSP-1B, CSP-2B, and CSP-2B(D8A) *in vitro* translated substrates were pSH149–1, pSH200–1, pSH199–1, and pSH237–1, respectively. Coupled *in vitro* transcription-translation reactions to produce $^{35}$S-labeled substrates were performed using the TNT kit (Promega). Fluorescent substrate assays were performed as described by the manufacturer (Enzyme Systems Products). AFC amounts were calculated using a standard curve.

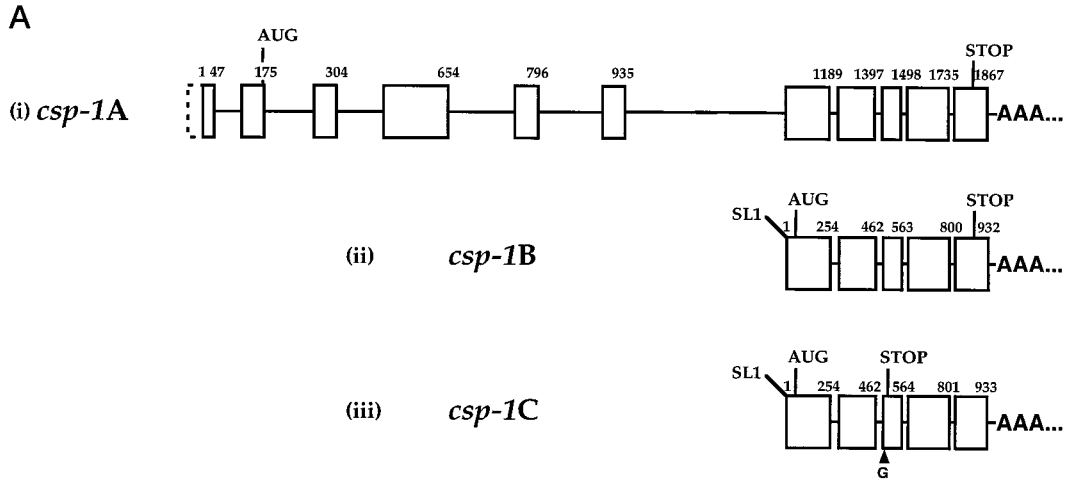
*Western Blots*—Bacterial extracts were separated on 15% SDS-polyacrylamide gels and blotted onto polyvinylidine difluoride membranes. Membranes were blocked in 2% milk in 1× TBS (100 mM Tris-HCl, pH 7.5, 0.5 M NaCl) for 1 h, incubated with anti-FLAG antibody (10 $\mu$g/ml; Eastman Kodak Co.) in 1% milk in 1× TBS for 1 h, washed three times in 1% milk in 1× TBS, and incubated with Goat anti-mouse horseradish peroxidase-coupled antibody (Bio-Rad) for 30 min. Three final washes were done in 1% milk in 1× TBS. Horseradish peroxidase activity was detected using an ECL chemiluminescence kit (Amersham Pharmacia Biotech).

## RESULTS

*csp-1 Encodes at Least Three Distinct Transcripts*—To identify new *C. elegans* caspases, translated genomic sequences compiled by the *C. elegans* genome sequencing project (24) were searched for similarities to the CED-3 caspase using the BLAST sequence comparison program (25). Two sequences with similarities to CED-3 were identified. One of these sequences was located on YAC Y48E1, which contains DNA from the right arm of *C. elegans* chromosome II. To determine if this Y48E1 region was actively transcribed, a *C. elegans* cDNA library[2] was probed with $^{32}$P-labeled DNA amplified from Y48E1 by the PCR using primers SH161 and SH203. cDNAs of 1.8, 1.0, and 1.0 kb were isolated, and their sequences were determined (see "Experimental Procedures"; Fig. 1). The gene from which these cDNAs were derived was named *csp-1* (caspase homolog-1), and the respective transcripts were named *csp-1A*, *csp-1B*, and *csp-1C*.

Comparison of the *csp-1A* cDNA sequence with the sequence of Y48E1 suggested that this cDNA was incomplete at its 5′-end and did not contain the full-length *csp-1A* open reading frame. To determine the location of the 5′-end of the *csp-1A* open reading frame, a 0.5-kb DNA fragment was amplified by RT-PCR using poly(A)$^+$ *C. elegans* RNA, a primer located within the *csp-1A* cDNA, and a primer located upstream of the *csp-1A* cDNA 5′-end. Sequence determination of the 0.5-kb fragment revealed an open reading frame starting at position 173 of the *csp-1A* sequence (Fig. 1*A*, *i*) and preceded by an in frame stop codon 57 bp upstream. Transcripts *csp-1B* and *csp-1C* contained 8- and 6-bp sequences, respectively, identical to the *C. elegans* SL1 trans-spliced leader sequence (26) 41 bp upstream of their putative start codon, thus defining their 5′-ends. All three *csp-1* transcripts contained poly(A) sequences at their 3′-ends. These results suggested that the full-length open reading frames of the *csp-1A*, *csp-1B*, and *csp-1C* mRNAs

[2] S. Shaham, unpublished data.




B

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1    MVLKTIEDNCKSQFDDDLVEDFNNFQTTSSMSSSTTISTEDFNTIEIEST
51   FEICRSGSYTEEPILGENDEFLIDFEMERFLKFLKDKTKQVEKRKEPFSQ
101  KEIYAVFQRRIKSELCIETVKKKFQPLLPNAIQTCEFDEETMIRMIYGAG
151  IRIDSVDFWNRFTSKATISLDCYSRLISYSSDSLTLSGTHRSGFTYHWIS
201  TPPVTYHRTENKDPNIQEPSPVEFLDVQSSLGSSMKPPILDKPTKLDDPA
251  ETRHDCSYSLEEYDSQSRMPRTDAKKSNHKHKYCYEMNSNPRGTVLILSN
301  ENFKNMERRVGTKQDEVNLTKLFQKLQYTVICKRNLEAESMLEAIKEFAE
351  MAHTDSIILFLLSHGDGAGSVFGIDDMPVNVMEVSTYLAYHQNLLLKPKW
401  VAVSACRGGKLNMGVPVDGLPALEDKCAPISKFWNLMMSRIMPGTFTSLN
451  ADVIISFSTTDGFTSYRDEEAGTWYIKSMCKVFNKHSKTMHLLDILTETG
501  RNVVTKYENVQGNVVLKQAPEILSRLTKQWHFSRSM
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FIG. 1. ***csp-1* encodes at least three RNAs.** *A*, *i*, *csp-1A*; ii, *csp-1B*; iii, *csp-1C* pre-mRNA structures as deduced from cDNA sequences and RT-PCR (see "Results"). *Boxes*, exons; *horizontal lines*, introns. The *dashed box* indicates that the exact 5′-end of the *csp-1A* mRNA is not known. Locations of the start codons (AUG), stop codons, and poly(A) tails are indicated. SL1 trans-spliced leader sequences are indicated by a *diagonal line*. *Numbers* at the ends of exons indicate the mRNA nucleotide positions, not including the SL1 sequences or the poly(A) tails. G nucleotide insertion in *csp-1C* is indicated. *B*, CSP-1A protein sequence. *Underlined* amino acids indicate the CSP-1B protein sequence. The CSP-1C sequence diverges from the CSP-1B sequence following amino acid position 410 and continues for 7 amino acids (IEHGCSR). *Boldface* residues represent either aspartate residues that direct processing in *E. coli* (see Fig. 4) or residues corresponding to the conserved pentapeptide surrounding the putative active site cysteine. *Numbers* indicate CSP-1A amino acid positions.

had been defined.

The genomic structure of *csp-1* (Fig. 1*A*) was deduced by comparing the *csp-1* RNA sequences with the sequence of Y48E1. The 3′ 932 bp (not including poly(A) sequences) of *csp-1A*, *csp-1B*, and *csp-1C* mRNAs were identical, with the exception of a G nucleotide insertion following position 463 of the *csp-1C* sequence (Fig. 1*A*, *iii*; see below).

To assess the relative abundance of the *csp-1* transcripts, a Northern blot of mixed stage wild-type *C. elegans* poly(A)$^+$ RNA was probed with the same probe used for the initial library screen. Two bands, one of 2.3 kb and one of 1.1 kb, were detected that corresponded in size to *csp-1A* and to *csp-1B* and *csp-1C*, respectively. The 2.3-kb band was 5–10-fold less abundant than the 1.1-kb band, suggesting that *csp-1A* may be expressed at lower levels than *csp-1B* and *csp-1C* (data not shown).

As shown in Figs. 1*B* and 2, the putative proteins encoded by *csp-1A* and *csp-1B* showed extensive similarities to caspase proproteins. Amino acids 285–536 of CSP-1A and amino acids 17–269 of CSP-1B share 38% identity with amino acids 235–503 of CED-3. CSP-1A contains a putative N-terminal domain (amino acids 1–273; Fig. 1*B*) with no similarity to known sequences in current data bases. CSP-1B contains a short N-terminal domain of 5 amino acids (Fig. 1*B*).

CSP-1C and CSP-1B are identical in their N-terminal 142 amino acids. Alternative splice-acceptor site selection in intron 2 of the *csp-1C* pre-mRNA results in the insertion of an extra G nucleotide at position 463 with respect to the *csp-1B* sequence (see Fig. 1*A*). This insertion results in an altered reading frame causing CSP-1C to terminate 7 amino acids downstream of amino acid 142 (see legend to Fig. 1*B*). Thus, CSP-1C appears to lack a C-terminal caspase domain, suggesting that it is unlikely to be an active enzyme.

*Genomic Organization of csp-2 and csp-3*—In addition to the *csp-1* sequence described above, a sequence with similarity to *ced-3* was also identified on the overlapping YACs, Y47H9 and Y53C10, which contain DNA from chromosome I. To determine if this region was actively transcribed, a *C. elegans* cDNA library was probed as described above with $^{32}$P-labeled DNA amplified by PCR from YAC Y47H9. Three cDNAs with lengths of 2.8, 1.1, and 0.7 kb were isolated. Sequence determination of these cDNAs revealed that the 0.7-kb cDNA was derived from YAC Y47H9. Nucleotides 1657–2743 of the 2.8-kb cDNA (Fig. 3*A*, *i*) were identical to nucleotides 1–1087 of the 1.1-kb cDNA

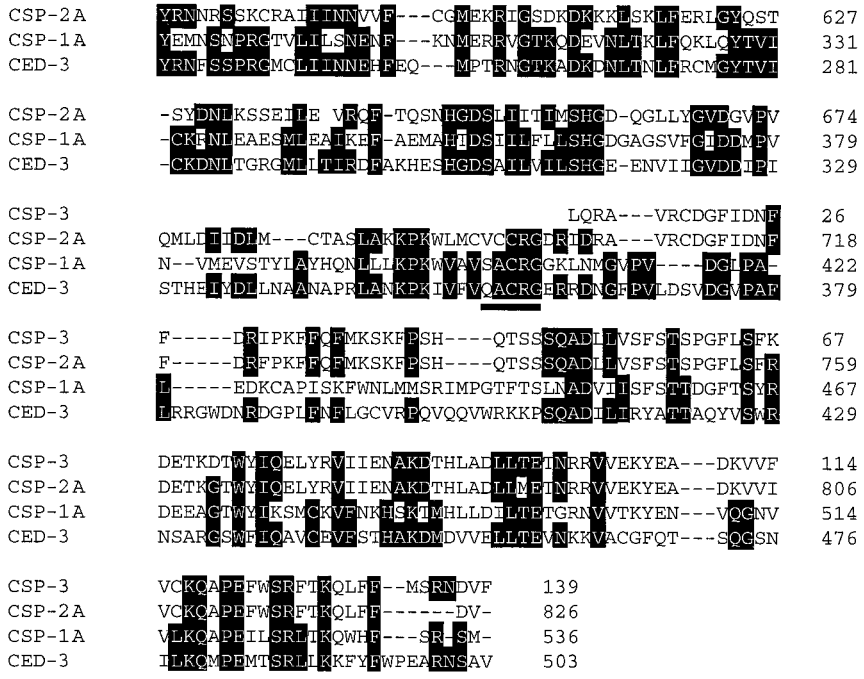


FIG. 2. **CSP-1A, CSP-2A, and CSP-3 are most similar to CED-3.** Alignment of CSP-1A, CSP-2A, CSP-3, and CED-3 caspase proprotein sequences. Residues identical to CED-3 are *highlighted*. *Numbers* indicate amino acid positions. A *bar* indicates the conserved pentapeptide flanking the active site cysteine.

(Fig. 3*A*, *ii*), and the 3′ 603 nucleotides of both were similar to yet distinct from nucleotides 94–695 of the 0.7-kb cDNA (95% identity; Fig. 3*A*, *iii*). These results suggested that the 2.8- and 1.1-kb cDNAs were both derived from transcripts encoded by the same gene and that the 0.7-kb cDNA was derived from the transcript of a different gene. The gene encoding transcripts corresponding to the 2.8- and 1.1-kb cDNAs was named *csp-2* (caspase homolog-2), and the respective transcripts were named *csp-2A* and *csp-2B*. The gene encoding the transcript corresponding to the 0.7-kb cDNA was named *csp-3* (caspase homolog-3).

To determine the 5′-end of the *csp-2*A open reading frame, *csp-2*A cDNA 5′-ends were amplified from mixed stage wild-type RNA using RACE (see "Experimental Procedures"). Sequence determination of the resulting product suggested that the 5′-end of *csp-2A* was identical to that defined by the 2.8-kb cDNA (Fig. 3*A*, *i*). However, the possibility that a longer *csp-2* transcript exists that was not detected in the cDNA library screen cannot be excluded. The 5′-end of the 1.1-kb *csp-2B* cDNA contained 8 bp of sequence identical to the *C. elegans* SL1 trans-spliced leader sequence (26), thus defining the 5′-end of the *csp-2B* transcript. The *csp-3* cDNA contained an in frame stop codon 24 bp upstream of the first ATG start codon. Both *csp-2* cDNAs as well as the *csp-3* cDNA contained poly(A) sequences at their 3′-ends. These results suggested that the full-length open reading frames encoded by the *csp-2A*, *csp-2B*, and *csp-3* mRNAs had been defined. A Northern blot of mixed stage wild-type *C. elegans* poly(A)$^+$ RNA probed with the *csp-2*/*csp-3*-specific $^{32}$P-labeled DNA described above failed to reveal hybridizing bands, suggesting that *csp-2* and *csp-3* are either expressed at low levels or only in a small number of cells.

To determine the genomic structure of *csp-2*, eight different primer pairs spanning the length of the *csp-2A* sequence were used to amplify wild-type *C. elegans* genomic DNA (see "Experimental Procedures"). The exon and exon/intron sequences of amplified bands were determined by comparison with the *csp-2A* and *csp-2B* sequences. The genomic structure of *csp-3* (Fig. 3*A*, *iii*) was determined by comparing the sequence of the *csp-3* cDNA with that of YAC Y47H9.

To determine the chromosomal location of *csp-2*, the same $^{32}$P-labeled DNA described above was used to probe a nitrocellulose filter spotted with an ordered array of *C. elegans* YACs (24). Hybridization was detected with the overlapping YACs Y47H9 and Y53C10 on chromosome I, which contain *csp-3*. In addition, hybridization was detected with the overlapping YACs Y48F8 and Y73B6 located on the left arm of chromosome IV (data not shown). These results suggested that *csp-2* was located in the overlap region of YACs Y48F8 and Y73B6. To test this notion, DNA from these YACs was amplified by PCR using *csp-2A*-specific primers (see "Experimental Procedures"). Genomic DNA bands of the expected sizes were detected. These results confirmed that *csp-2* was located in the overlap region of YACs Y48F8 and Y73B6 on chromosome IV.

*The N-terminal Domain of CSP-2A Is Weakly Similar to E. coli Colicin E1—csp-2A* and *csp-2B* encode proteins with similarities to caspase proproteins. The region of similarity is most similar to the corresponding region of the CED-3 proprotein. Specifically, there is 33% identity between amino acids 18–263 of the CSP-2B protein and amino acids 235–503 of CED-3. The sequence, VCCRG, containing the putative active site cysteine of CSP-2A and CSP-2B (Figs. 2 and 3*B*) is highly diverged from the consensus caspase sequence of QAC(R/Q/G)G (2).

The putative N-terminal domain of CSP-2B is only 7 amino acids long, suggesting that it is unlikely to interact with other proteins (Fig. 3*B*). However, the putative N-terminal domain of CSP-2A is 571 amino acids long (Fig. 3*B*) and is weakly similar in sequence to *E. coli* colicin E1. Specifically, there is 27% identity between amino acids 104–201 of CSP-2A and amino acids 205–302 of colicin E1 (Fig. 3*C*). Colicin E1 is encoded by the ColE1 plasmid of *E. coli* and can kill bacterial cells by creating pores in the plasma membrane (27–30). The similarity between the N-terminal domain of CSP-2A and colicin E1 is restricted to the receptor-binding domain of this colicin (see "Discussion").

The *csp-3* transcript encodes a protein highly similar to the C-terminal domain of CSP-2A and CSP-2B (Fig. 3*D*). Amino acids 15–139 of CSP-3 are 92% identical to amino acids 705–826 of CSP-2A (Fig. 2). Since the CSP-3 open reading frame does not contain a middle caspase domain, it is unlikely that it encodes an active caspase.

*CSP-1B Can Be Processed in E. coli*—To determine if *csp-1* could encode an active protease, the CSP-1B protein was stud-

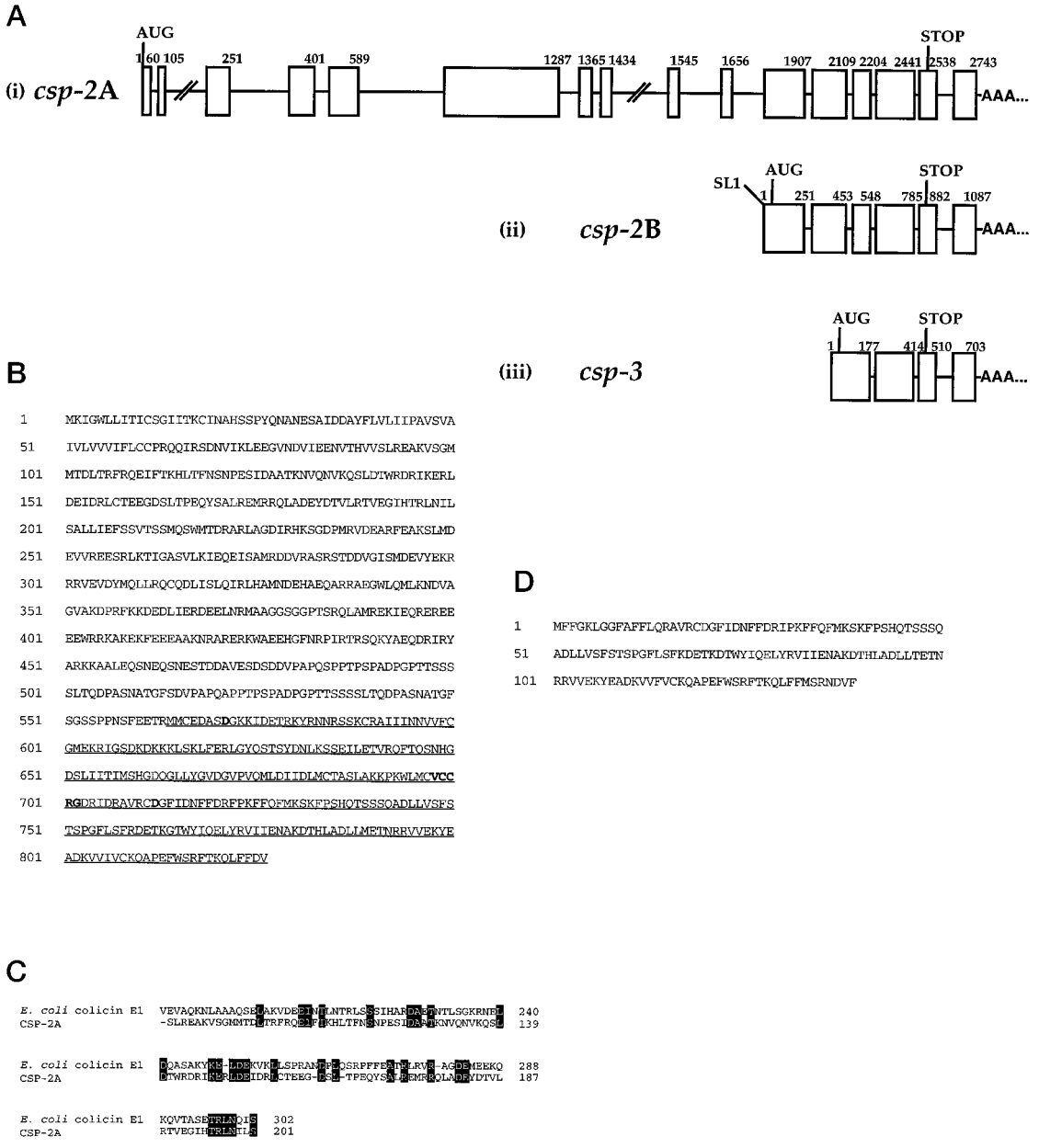


FIG. 3. ***csp-2* encodes at least two RNAs.** *A*, *i*, *csp-2*A; *ii*, *csp-2*B; *iii*, *csp-3* pre-mRNA structures as deduced from cDNA sequences and RACE (see "Results"). *Boxes*, exons; *horizontal lines*, introns. *Double slashes* indicate intron regions of greater than 800 bp whose sequences were not determined. Locations of the start codons, stop codons, and poly(A) tails are indicated. SL1 trans-spliced leader sequences are indicated by a *diagonal line*. *Numbers* at the ends of exons indicate the mRNA nucleotide positions, not including the SL1 sequences or the poly(A) tails. *B*, CSP-2A protein sequence. *Underlined* residues indicate the CSP-2B sequence. *Boldface* residues indicate potential sites of processing by activated CSP-1B or CED-3 caspases (delimiting the putative N-terminal, middle, and C-terminal caspase domains; see "Results") or residues corresponding to the conserved pentapeptide surrounding the putative active site cysteine. *Numbers* indicate CSP-2A amino acid positions. *C*, amino acid sequence alignment of the region of similarity between *E. coli* colicin E1 and CSP-2A. Identical residues are *highlighted*. *Numbers* indicate amino acid positions of colicin E1 and CSP-2A. *D*, CSP-3 protein sequence. *Numbers* indicate CSP-3 amino acid positions.

ied, since the activated CSP-1A and activated CSP-1B caspases were likely to be identical. The CSP-1B protein was tagged at its C terminus with a FLAG peptide (amino acid sequence DYKDDDDK) and was expressed in *E. coli* (see "Experimental Procedures"). As shown in Fig. 4*A*, a Western blot of bacterial extracts from cells expressing CSP-1B-FLAG and probed with an anti-FLAG antibody revealed the presence of both the full-length 31-kDa wild-type CSP-1B-FLAG proprotein as well as a smaller 14-kDa protein. The 14-kDa protein was not detected when a CSP-1B-FLAG protein containing a C138S mutation in the putative catalytic cysteine residue was expressed in *E. coli* under identical conditions. These results suggested that the CSP-1B-FLAG proprotein could be specifically processed when expressed in bacteria. Similar processing has been shown for several other caspases (6).

To confirm that the CSP-1B-FLAG proprotein was processed in *E. coli*, extracts expressing the proprotein were passed over an anti-FLAG antibody column, and the bound proteins were eluted with FLAG peptide (see "Experimental Procedures"). The resulting eluate was run on a 15% SDS-polyacrylamide gel and stained with Coomassie Blue. As shown in Fig. 4*B*, three major bands were detected. The 31-kDa band corresponded in size to the CSP-1B-FLAG proprotein. To identify the 14- and 16-kDa proteins, their N-terminal sequences were determined (see "Experimental Procedures"). The N terminus of the 14-kDa protein contained the sequence GLPA, corresponding to

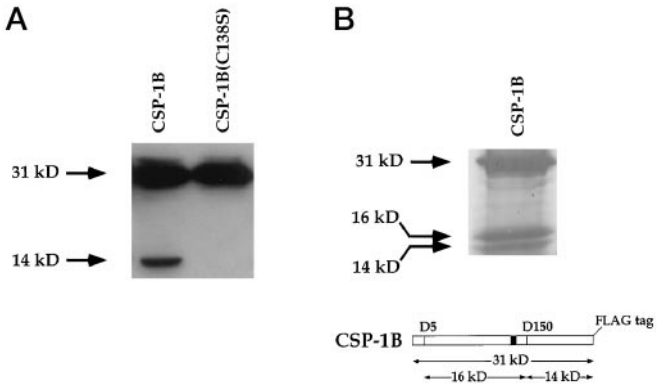


FIG. 4. **CSP-1B is processed in *E. coli*.** *A*, Western analysis of *E. coli* extracts expressing either CSP-1B-FLAG or CSP-1B(C138S)-FLAG using a monoclonal anti-FLAG antibody (see "Results"). Band sizes are indicated. *B*, Coomassie Blue-stained gel of partially purified CSP-1B-FLAG. Sizes of major bands corresponding to full-length and processed CSP-1B-FLAG are indicated. The *schematic diagram* indicates the CSP-1B-FLAG proprotein, aspartate residues where processing was shown to take place, and predicted sizes of processed fragments. *Black box* indicates the pentapeptide surrounding the active site cysteine.

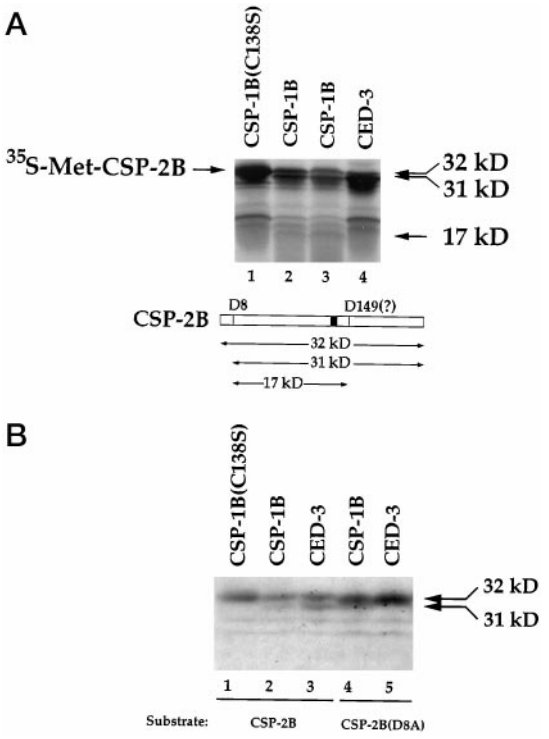


FIG. 5. **CSP-2B is cleaved by both CSP-1B and CED-3.** *A*, 40 $\mu$g of partially purified CSP-1B(C138S)-FLAG (*lane 1*), activated CSP-1B-FLAG (two different purified extracts; *lanes 2* and *3*), or activated CED-3 (*lane 4*) were incubated with 1 $\mu$l of *in vitro* translated $^{35}$S-labeled CSP-2B. Reactions were separated on a 15% SDS-polyacrylamide gel. Band sizes indicating full-length and processed CSP-2B are indicated. The *schematic diagram* indicates the CSP-2B proprotein, aspartate residues where processing might take place, and predicted sizes of processed fragments. Cleavage at aspartate 8 is shown in *B*. The *black box* indicates the pentapeptide surrounding the active site cysteine. *B*, 40 $\mu$g of partially purified CSP-1B(C138S)-FLAG (*lane 1*), activated CSP-1B-FLAG (*lanes 2* and *4*), or activated CED-3 (*lanes 3* and *5*) were incubated with 1 $\mu$l of *in vitro* translated $^{35}$S-labeled CSP-2B or CSP-2B(D8A) as indicated. Reactions were separated on a 15% SDS-polyacrylamide gel. The 17-kDa fragment is not visible. Band sizes indicating full-length and processed CSP-2B are shown.

amino acids 151–154 of CSP-1B (amino acids 419–422 of the CSP-1A sequence; Fig. 1*B*). The N terminus of the 16-kDa protein contained the sequence AKKS, corresponding to amino acids 6–9 of CSP-1B (amino acids 274–277 of the CSP-1A sequence; Fig. 1*B*). These results suggested that the CSP-1B proprotein was processed following aspartate 5 and aspartate 150. Furthermore, since the FLAG tag was located at the C-terminal end of the CSP-1B-FLAG proprotein, these results suggested that the 16-kDa CSP-1B protein could tightly associate with the 14-kDa FLAG-containing protein. Such processing at aspartate residues and tight association of the resulting subunits is characteristic of all known active caspases (2, 6).

*CSP-2B Proprotein Is a Substrate of both Activated CSP-1B and Activated CED-3*—The finding that *C. elegans* encodes at least two potentially active caspases in addition to CED-3 suggested that all of these proteases might cleave and activate each other as can occur in mammalian systems. To test this hypothesis, cleavage of the CSP-2B proprotein by activated CSP-1B or CED-3 was assessed. As shown in Fig. 5*A*, two different preparations of activated CSP-1B-FLAG could cleave the *in vitro* translated 32-kDa CSP-2B protein to generate two main cleavage products of 31 and 17 kDa (*lanes 2* and *3*). The 31-kDa band corresponded to the predicted C-terminal protein remaining after cleavage following aspartate 8 (aspartate 571 of the CSP-2A sequence; Fig. 3*B*). The N-terminal peptide released by such cleavage would have been too small to detect in the assay. The 17-kDa band corresponded to the predicted N-terminal protein remaining after cleavage following aspartate 149 (aspartate 712 of the CSP-2A sequence; Fig. 3*B*). The C-terminal portion of this protein may have not been detected, since it could potentially contain only two $^{35}$S-labeled methionine residues (compared with 5 methionines in the 17-kDa fragment), and cleavage at this second site was very inefficient. As shown in Fig. 5*A*, activated CED-3 tagged at the C terminus of its 15-kDa subunit with the FLAG peptide (see "Experimental Procedures") could also cleave CSP-2B (*lane 4*). However, only one cleavage product, perhaps corresponding to cleavage at aspartate 8, could be detected.

To determine if activated CSP-1B-FLAG and CED-3-FLAG cleave CSP-2B following aspartate 8, the abilities of these enzymes to cleave a CSP-2B protein containing an aspartate-to-alanine mutation at position 8 was examined. As shown in Fig. 5*B*, no 31-kDa cleavage product was detected, suggesting that CSP-1B and CED-3 both cleave CSP-2B following aspartate 8.

CSP-2B cleavage by activated CSP-1B or CED-3 suggested that CSP-2B or CSP-2A processing could produce an active caspase. To test this idea, a CSP-2B-FLAG or CSP-2A-FLAG protein was expressed in *E. coli* as described above. Although full-length proteins were easily detected in *E. coli* extracts, specific processing was not detected under a variety of experimental conditions (data not shown). Furthermore, extracts expressing the CSP-2B-FLAG or CSP-2A-FLAG proteins did not cleave a variety of *in vitro* translated substrates (data not shown). These results suggested that processed CSP-2B and CSP-2A might not be active caspases. Consistent with this notion, these proteins contain divergent active site sequences that might not allow protease activity. Alternatively, the assay conditions used might not have been optimal to detect processing and activation.

*Activated CSP-1B Can Cleave the CED-3 Proprotein*—To determine whether activated CSP-1B could cleave other proteins, the ability of activated CSP-1B-FLAG to process the CED-3 proprotein was examined. As shown in Fig. 6*A*, wild-type activated CSP-1B-FLAG could process a 56-kDa *in vitro* translated CED-3 proprotein (*lane 4*). The sizes of the CED-3 proprotein cleavage products suggest that CSP-1B might cleave CED-3 at aspartates 144 and 374. These results suggest

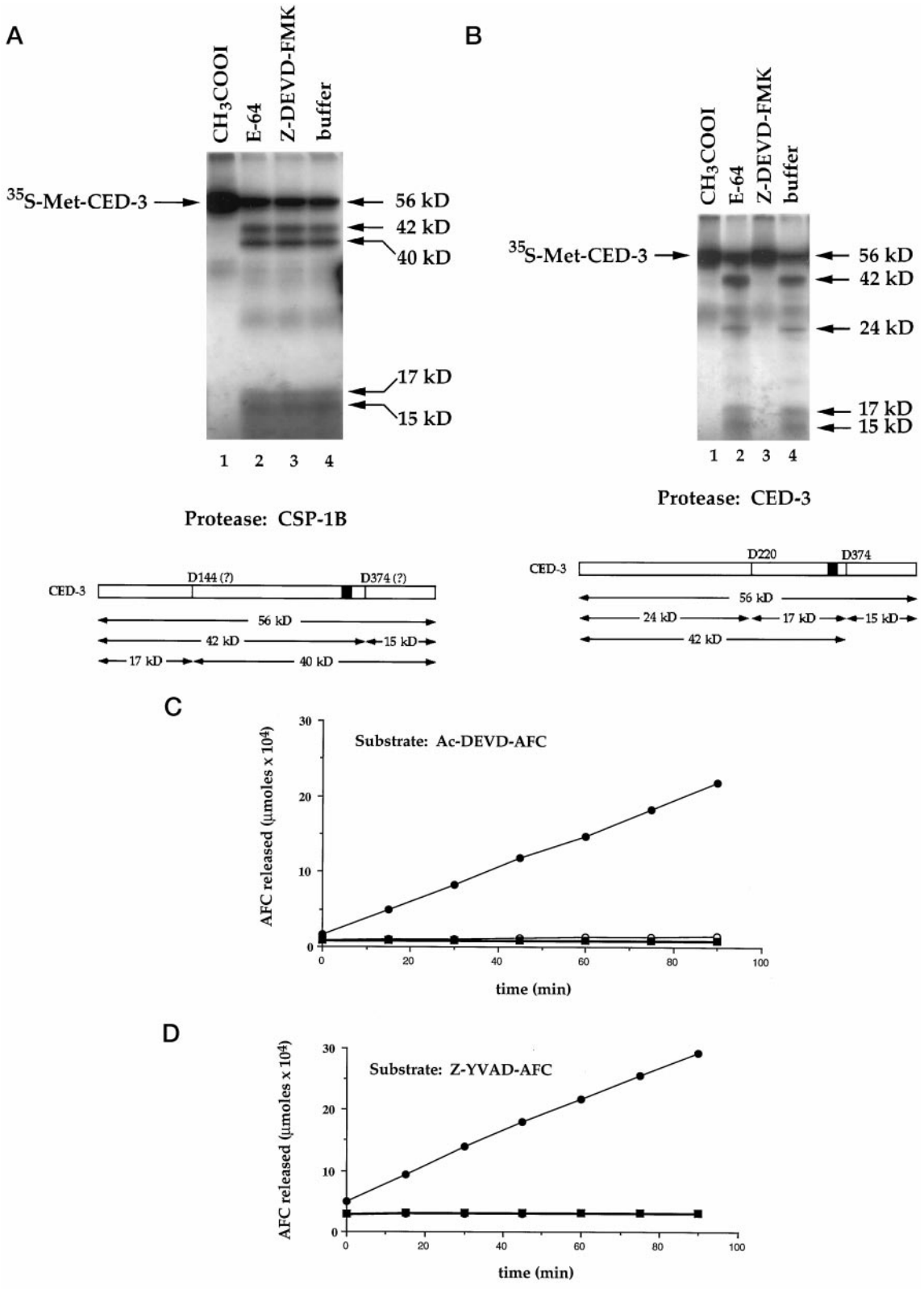


FIG. 6. **Activated CED-3 and CSP-1B have different substrate specificities.** *A*, 40 $\mu$g of partially purified activated CSP-1B-FLAG were incubated with *in vitro* translated $^{35}$S-labeled full-length CED-3 in the presence of inhibitors or buffer control (see "Experimental Procedures"). Final inhibitor concentrations were as follows: iodoacetic acid (*lane 1*), 5 mM; E-64 (*lane 2*), 0.2 mM; benzyloxycarbonyl-DEVD-fluoromethyl ketone (*lane 3*), 10 $\mu$M. Band sizes indicating full-length and cleavage fragments of CED-3 are shown. The *schematic diagram* indicates the CED-3 proprotein, aspartate residues where processing might take place, and predicted sizes of processed fragments. The *black box* indicates the pentapeptide surrounding the active site cysteine. *B*, same as *A* except that activated CED-3-FLAG was used as protease. Schematic diagram indicates the CED-3 proprotein, aspartate residues where processing was shown to take place (6), and predicted sizes of processed fragments. *C*, graph depicting cleavage of acetyl-DEVD-AFC by bacterial extracts containing CED-3-FLAG (*filled circles*), CSP-1B-FLAG (*open circles*), CED-3(C358S)-FLAG (×), or CSP-1B(C138S)-FLAG (■). Released AFC was measured using a spectrofluorimeter. *D*, graph depicting cleavage of benzyloxycarbonyl-YVAD-AFC by bacterial extracts containing CSP-1B-FLAG (*filled circles*), CED-3-FLAG (○), CED-3(C358S)-FLAG (×), or CSP-1B(C138S)-FLAG (■).

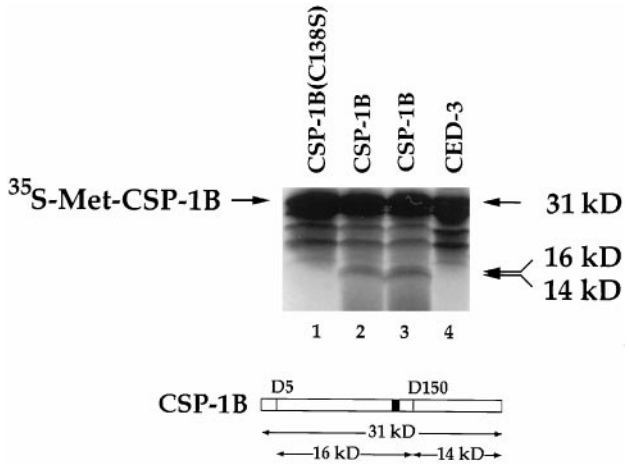


FIG. 7. **Activated CSP-1B but not activated CED-3 can cleave the CSP-1B proprotein.** This *figure* is the same as Fig. 5*A* except that 1 $\mu$l of *in vitro* translated $^{35}$S-labeled CSP-1B was used as substrate. The *schematic diagram* indicates the CSP-1B proprotein, aspartate residues where processing is likely to take place (based on Fig. 4), and predicted sizes of processed fragments.

that activated CSP-1B is a caspase capable of processing the CED-3 proprotein, perhaps leading to CED-3 activation or inhibition.

*Activated CSP-1B and CED-3 Have Different Substrate Specificities*—As shown in Fig. 6, *A* and *B*, both activated CSP-1B-FLAG and activated CED-3-FLAG could cleave the CED-3 proprotein. However, whereas both proteases probably cleaved efficiently at aspartate 374 of the CED-3 proprotein, cleavage at other sites was enzyme-dependent. This result suggests that activated CSP-1B and CED-3 have different substrate specificities. To confirm this notion, the effect of protease inhibitors on activated CSP-1B and CED-3 activities was examined. The cysteine protease inhibitor iodoacetic acid (Sigma) blocked processing of the CED-3 proprotein by either activated CSP-1B-FLAG (Fig. 6*A*, *lane 1*) or CED-3-FLAG (Fig. 6*B*, *lane 1*). The cysteine protease inhibitor E-64 (Sigma) was unable to block activated CSP-1B-FLAG (Fig. 6*A*, *lane 2*) or CED-3-FLAG (Fig. 6*B*, *lane 2*) protease activity. Interestingly, the caspase peptide inhibitor benzyloxycarbonyl-DEVD-fluoromethyl ketone (Enzyme Systems Products) inhibited processing of the CED-3 proprotein by activated CED-3-FLAG (Fig. 6*B*, *lane 3*) but not by activated CSP-1B-FLAG (Fig. 6*A*, *lane 3*), suggesting that activated CSP-1B has a different substrate specificity from activated CED-3.

To further demonstrate the differing substrate specificities of activated CSP-1B and CED-3, their abilities to cleave synthetic caspase substrates were tested. As shown in Fig. 6*C*, activated CED-3, but not activated CSP-1B, could efficiently cleave the acetyl-DEVD-AFC substrate (Enzyme Systems Products). Conversely, the same preparations of activated CSP-1B, but not of activated CED-3, could cleave the benzyloxycarbonyl-YVAD-AFC substrate (Fig. 6*D*). These observations further indicate that activated CSP-1B and CED-3 have different substrate specificities.

*Activated CSP-1B but Not Activated CED-3 Can Cleave the CSP-1B Proprotein*—Since both activated CED-3-FLAG and activated CSP-1B-FLAG can cleave the CED-3 proprotein, the ability of these proteases to cleave the CSP-1B proprotein was assessed. As shown in Fig. 7, two different preparations of activated CSP-1B-FLAG could process *in vitro* translated CSP-1B proprotein to generate two specific cleavage products of 14 and 16 kDa (*lanes 2* and *3*). These products corresponded in size to the CSP-1B middle and C-terminal caspase subunits (see Fig. 4). Interestingly, activated CED-3-FLAG could not process the *in vitro* translated CSP-1B proprotein (*lane 4*) although the same preparation of CED-3-FLAG protease could efficiently process the CED-3 proprotein (data not shown). These results suggest that CSP-1B could act upstream of CED-3 in a proteolytic cascade.

## DISCUSSION

*C. elegans Encodes Multiple Caspases*—Many metazoans contain several caspases with different substrate specificities (6, 10). At least 11 caspases have been described in humans (12, 31), and at least three have been described in *Drosophila melanogaster* (32–34). In the nematode *C. elegans*, one caspase, CED-3, has been demonstrated to be important for the execution of the cell death program (15–17). Here it is shown that *C. elegans* expresses at least three different caspase-related genes, *csp-1*, *csp-2*, and *csp-3*, in addition to *ced-3*. Furthermore, the *csp-1* and *csp-2* genes express multiple transcripts encoding distinct caspase-related proteins. *C. elegans* thus possesses a repertoire of at least seven different caspase-related transcripts potentially encoding the CED-3, CSP-1A, CSP-1B, CSP-1C, CSP-2A, CSP-2B, and CSP-3 proteins.

CSP-1C and CSP-3 are similar only to the large and small catalytic domains of caspases, respectively, suggesting that they are unlikely to be active enzymes. However, such proteins might act in a dominant-negative fashion to inhibit the activities of activated CSP-1B and CSP-2B proteins, respectively. Alternatively, it has been previously shown that large and small subunits can be shared between different caspases (35). Thus, CSP-1C and CSP-3 might interact with subunits of activated CED-3, CSP-1B, or CSP-2B.

CSP-1A, CSP-1B, CSP-2A, and CSP-2B have divergent active site sequences. The activated CSP-1B caspase, for example, contains the sequence SACRG surrounding the active site cysteine (cysteine 138 of CSP-1B; Fig. 1*B*). This sequence differs from the consensus QAC(R/Q/G)G sequence present in most active caspases (2). Interestingly, the results presented here suggest that such sequence variation is still compatible with enzymatic activity. The CSP-2A and CSP-2B proteins contain the sequence VCCRG surrounding the putative active site cysteine (cysteine 137 of CSP-2B). Unlike CSP-1B, however, caspase activity could not be demonstrated for CSP-2B, suggesting that the sequence divergence in the CSP-2B active site may not be compatible with enzymatic activity.

*C. elegans Caspases Have either Short or Long N-terminal Domains*—CSP-1B and CSP-2B are caspase proproteins with short N-terminal domains. In mammals, such proteases (for example, caspase-3) are thought to be the most downstream effectors of programmed cell death (10) and are thought to be primarily regulated by cleavage by other caspases (2, 10). CSP-1A and CSP-2A are caspase proproteins with long N-terminal domains. In mammals and in *C. elegans*, such long N-terminal domains are thought to be important for binding to proteins that regulate caspase activation (7–9). Thus, *C. elegans* encodes the same classes of caspases seen in mammals, suggesting that interactions among *C. elegans* caspases might be similar to those among mammalian caspases.

Although the sequence of the N-terminal domain of CSP-1A is uninformative as to its potential function, the N-terminal domain of CSP-2A shows weak similarity to *E. coli* colicin E1. Colicins are plasmid-encoded bacterial proteins that are involved in killing of bacteria. Colicin E1 can insert into the plasma membranes of bacteria via specific receptors on the recipient bacterial membrane. Pore formation then kills the bacterial cell (27–30). The domain structure of colicin E1 has been roughly characterized and consists of an N-terminal re-

gion required for membrane translocation, a middle domain required for receptor binding, and a C-terminal domain required for killing (30). The CSP-2A N-terminal domain was only similar to the receptor-binding domain of colicin E1. However, it is possible that a longer *csp-2* transcript exists that was not detected in the cDNA library screen and might encode a protein similar to additional colicin E1 domains.

The similarity of the N-terminal domain of CSP-2A to the receptor-binding domain of colicin E1 suggests that the CSP-2A domain might interact with other proteins in a manner similar to N termini of other caspases. If *csp-2* plays a role in programmed cell death, several roles for the N-terminal domain of CSP-2A could be imagined. One possibility is that following processing of the CSP-2A proprotein by CED-3, CSP-1B, or another protein, the released N-terminal domain could bind to a protein on the surface of the CSP-2A-expressing cell, thus marking the cell for degradation by neighboring cells. Alternatively, it is possible that the N-terminal domain of CSP-2A does contain pore-forming sequences, in which case this domain could form pores in CSP-2A-expressing cells, hastening their demise. The possible lack of protease activity by CSP-2B and CSP-2A suggests that they could be similar to the mammalian FLIP/Casper caspase that lacks a catalytic cysteine residue yet can still promote and prevent cell death (13, 14).

*C. elegans Caspases Might Act in a Proteolytic Cascade*—Although *ced-3* has a clear role in programmed cell death, the roles of *csp-1*, *csp-2*, or *csp-3* in this or other processes are unknown. *csp-1*, *csp-2*, and *csp-3* might play a role in programmed cell death that is partially redundant with that of *ced-3* or be involved in tissue-specific activation of *ced-3*. It is also possible that all four *C. elegans* caspase-related genes are involved in processes distinct from programmed cell death.

Several experiments described here suggest that the products of the *ced-3*, *csp-1*, and *csp-2* genes may interact. Activated CSP-1B could process the CSP-1B, CED-3, and CSP-2B proproteins *in vitro*, suggesting that cells containing activated CSP-1B might also contain cleaved CED-3 and CSP-2B. Thus, CSP-1B activation may either activate or inhibit CED-3 and CSP-2B.

Activated CED-3 did not cleave the CSP-1B proprotein, yet still processed the CED-3 and CSP-2B proproteins. These results suggest that active CED-3 and CSP-1B have different substrate specificities, a notion bolstered by experiments using the caspase inhibitor benzyloxycarbonyl-DEVD-fluoromethyl ketone, which could inhibit CED-3 protease activity but not CSP-1B protease activity. Furthermore, CSP-1B and CED-3 differentially cleaved synthetic caspase substrates. Thus, as in mammals, *C. elegans* encodes caspases with differing substrate specificities.

The different target specificities of activated CSP-1B and CED-3 suggest that there might be a directionality in the interactions of CED-3, CSP-1B, and CSP-2B *in vivo*. Specifically, CSP-1B might activate or inhibit CED-3, which might, in combination with CSP-1B, activate or inhibit CSP-2B. All or parts of this cascade may be used *in vivo* to regulate caspase activation.

The finding that *C. elegans* encodes a number of different caspases suggests that this organism could be useful for understanding how caspases are regulated and how different caspases are targeted to different tasks, a problem currently poorly understood. Directed mutations in the *csp-1*, *csp-2*, and *csp-3* genes should help to reveal the cellular roles played by each of these genes.

*Acknowledgments*—Many thanks are due to Ira Herskowitz for providing space and support for the experiments described here and for stimulating discussions concerning the manuscript. Thanks are also due to M. Grether, L. Huang, M. Maxon, and M. Shuman for helpful discussions; to K. Benjamin, M. Grether, M. Maxon, J. Shih, M. Shuman, and A. Sil for helpful comments on the manuscript; to A. Coulson and S. Chissoe for YAC clones; to P. Peluso in the Walter laboratory for help with spectrofluorimetry; and to S. Zhou in the Tjian laboratory for help with protein sequence determinations.

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