An Alternatively Spliced C. elegans ced-4 RNA Encodes a Novel Cell Death Inhibitor

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Summary

The C. elegans gene ced-4 is essential for programmed cell death. We report that ced-4 encodes two transcripts and that whereas the major transcript can cause programmed cell death, the minor transcript can act oppositely and prevent programmed cell death, thus defining a novel class of cell death inhibitors. That ced-4 has both cell-killing and cell-protective functions is consistent with previous genetic studies. Our results suggest that the dual protective and killer functions of the C. elegans bcl-2-like gene ced-9 are mediated by inhibition of the killer and protective ced-4 functions, respectively. We propose that a balance between opposing ced-4 functions influences the decision of a cell to live or to die by programmed cell death and that both ced-9 and ced-4 protective functions are required to prevent programmed cell death.

Introduction

Programmed cell death is a major aspect of metazoan development (e.g., Glücksmann, 1950; Duvall and Wyllie, 1986; Ellis et al., 1991; Oppenheim, 1991). During the development of the nematode Caenorhabditis elegans, 131 of the 1090 cells generated undergo programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). Mutations affecting the process of programmed cell death in C. elegans have been isolated and have defined 14 genes (reviewed by Horvitz et al., 1994). Loss-of-function (lf) mutations in the genes ced-3 and ced-4, a gain-of-function mutation in the gene ced-9, or overexpression of the wild-type ced-9 gene can prevent programmed cell death (Ellis and Horvitz, 1986; Hengartner et al., 1992; Hengartner and Horvitz, 1994b). By contrast, ced-9(+) mutations or overexpression of the wild-type ced-3 or ced-4 genes can cause cells that normally survive to undergo programmed cell death (Hengartner et al., 1992; Shaham and Horvitz, 1996). These results indicate that ced-3 and ced-4 can cause and ced-9 can prevent programmed cell death. Loss-of-function mutations in either ced-3 or ced-4 can prevent both ectopic and normal cell deaths that occur in ced-9(+) animals, suggesting that the function of ced-9 is to prevent ced-3, ced-4, or both from acting (Hengartner et al., 1992). In addition, the killing of the C. elegans ALM neurons by overexpression of ced-4 is greatly reduced in ced-3 mutants, whereas killing of these neurons by overexpression of ced-3 is unaffected by mutations in ced-4, suggesting that ced-3 might act downstream of ced-4 (Shaham and Horvitz, 1996). ced-3, ced-4, and ced-9 all act cell autonomously (Yuan and Horvitz, 1990; Shaham and Horvitz, 1996).

The CED-9 protein is similar in sequence to the mammalian cell death inhibitor protein BCL-2, which like CED-9 functions to prevent programmed cell death (Vaux et al., 1988; Nunez et al., 1990; Garcia et al., 1992; Sentman et al., 1992). The CED-3 protein is similar in sequence to members of the interleukin-1 converting enzyme (ICE) family of cysteine proteases, which can induce apoptotic cell deaths (Miura et al., 1993; Yuan et al., 1993; Fernandes-Alnemri et al., 1994, 1995; Kumar et al., 1994; Wang et al., 1994; Faucheu et al., 1995; Munday et al., 1995). A number of reports indicate that cell death induced by mammalian CED-3-like proteins can be inhibited by overexpression of BCL-2 (Miura et al., 1993; Kumar et al., 1994; Wang et al., 1994), suggesting that BCL-2 might prevent the actions of CED-3-like proteases to inhibit programmed cell death just as CED-9 protein prevents the action of CED-3 in worms. No mammalian protein structurally similar to CED-4 has been described. Nevertheless, our recent finding that ced-4 might mediate protection by ced-9 against ced-3-induced death (Shaham and Horvitz, 1996) suggests that in mammals a CED-4-like protein might mediate protection by BCL-2-like proteins against death induced by CED-3-like proteases. If so, studies of how ced-4 acts could provide insights concerning universal mechanisms that regulate programmed cell death.

In this report we show that the ced-4 gene encodes two alternatively spliced mRNAs, called ced-4L and ced-4S, that encode opposing functions. Whereas ced-4S, the previously identified ced-4 transcript (Yuan and Horvitz, 1992), can induce programmed cell death, ced-4L can protect cells from programmed cell death, thus defining a novel class of cell death inhibitors. Both transcripts are likely to be conserved between C. elegans and the related nematodes C. briggsae and C. vulgaris, since the ced-4L alternative exon is conserved among all three species. We report that ced-4 encodes two opposing genetic cell death functions and suggest that these two functions correspond to the two distinct ced-4 transcripts, indicating that alternative splicing is normally used in vivo to regulate programmed cell death. Finally, our results suggest that the activities of both ced-4L and ced-4S are inhibited by the activity of the ced-9 gene. This finding can account for the ability of ced-9 both to promote and to prevent programmed cell death (Hengartner and Horvitz, 1994a) and suggests that other CED-9-like proteins might also be able both to promote and to prevent cell death by inhibiting CED-4L-like and CED-4S-like proteins, respectively. We propose that ced-9 and ced-4L are both required to prevent programmed cell death and that the balance between ced-4L and ced-4S determines whether a cell will live or die by programmed cell death.

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Results

A Conserved ced-4 Sequence Is Used as an Alternative Exon

To define functionally important regions of ced-4, we cloned and determined the sequences of ced-4 homologs from the nematodes C. briggsae and C. vulgaris. By comparing complete C. briggsae and partial C. vulgaris genomic sequences with the C. elegans genomic sequence, we found that exons were in general more conserved among these genes than were introns, with the exception of intron 3 (Figure 1A). The 72 bp at the 3’ end of intron 3 (as defined by previously described C. elegans ced-4 cDNAs; Yuan and Horvitz, 1992) were highly conserved (Figure 1B), suggesting that this region is important for ced-4 function. In all three species, this 72 bp region was immediately preceded by a consensus splice-acceptor sequence (TTNAG; T, A, and G are present in >90% of sites, while N represents a more variable position; Emmons, 1988) (Figure 1B), suggesting that this region might be used as an exon.

To determine whether C. elegans ced-4 encodes an alternative transcript that uses this presumptive splice-acceptor site, we hybridized a radioactive probe corresponding to the 72 bp conserved sequence to a Northern blot of mixed-stage C. elegans poly(A)’ RNA prepared from wild-type animals. As shown in Figure 1C, a 2.2 kb transcript was seen. This transcript is 10- to 30-fold less abundant than the previously described ced-4 transcript (data not shown) and is similar to it in size (Yuan and Horvitz, 1992). To determine whether this ced-4 transcript was produced by splicing at an acceptor site at position 114 of intron 3 (see Figure 1B), we prepared cDNAs from the same RNA preparation used for the Northern blot. These cDNAs were used with primers flanking intron 3 to amplify the region encompassing this intron by the polymerase chain reaction (PCR). We observed two products (Figure 1D). Sequence analysis of these products confirmed that the longer one (called ced-4L, for ced-4 long) corresponded to a ced-4 RNA spliced at position 114 of intron 3 and the shorter one (called ced-4S, for ced-4 short) corresponded to the previously described ced-4 mRNA (data not shown). These results indicate that ced-4 generates two alternatively spliced transcripts (Figures 1B and 1E). The ced-4L transcript contains an in-frame insertion of 72 nt relative to the previously described ced-4S transcript and could encode a protein with a 24 amino acid insertion relative to CED-4S (see Figure 1B).

Overexpression of ced-4L Can Prevent Programmed Cell Death

We previously demonstrated that the ALM neurons, which normally express the mec-7 gene (Savage et al., 1989), undergo programmed cell death in animals carrying a transgene consisting of the ced-4S cDNA fused to the mec-7 promoter (P_mec-7; ced-4S) (Shaham and Horvitz, 1996). This result suggested that ced-4S normally promotes programmed cell death. To determine whether ced-4L also promotes programmed cell death, we established transgenic lines containing a ced-4L cDNA fused to two different heat-shock promoters (A. Fire and P. Candido, personal communication). Again, no ectopic cell deaths were observed; instead, extra cells accumulated in the anterior pharynx of transgenic embryos subjected to a heat shock (Table 1), as in animals containing death-preventing mutations. This result suggested that programmed cell death was inhibited in these animals.

To test further the hypothesis that overexpression of ced-4L inhibited programmed cell death, we introduced a transgene containing a ced-4L cDNA fused to the constitutive promoter of the dpy-30 gene (D. Hsu and B. Meyer, personal communication) into animals containing a ced-9(II) mutation. ced-9(II) homozygous animals derived from ced-9(II)/+ heterozygous mothers generate progeny that die from massive ectopic cell death and can be rescued by mutations that prevent programmed cell death (Hengartner et al., 1992). ced-9(II) mutants carrying the P_dpy-30;ced-4L transgene were rescued from lethality (Table 2), supporting the notion that ced-4L can prevent programmed cell death.

ced-4 Encodes Protective as Well as Killer Genetic Functions

The observation that ced-4 encodes a death-preventing as well as a death-promoting transcript suggests that ced-4 might function in vivo not only to cause but also to protect against programmed cell death. Evidence consistent with such a protective in vivo function of ced-4 was obtained some years ago by Ellis and Horvitz (1986), although there was no basis for interpreting their data at that time. Specifically, the egl-1(n487) mutation causes the HSN neurons to undergo programmed cell death, and the percentage of dying HSNs is lower in egl-1(n487)/+ heterozygous animals than in ced-4(n1162)/+; egl-1(n487)/+ doubly heterozygous animals. Since the n1162 mutation eliminates ced-4 function (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992; S. S. and H. R. H., unpublished data), this observation indicates that a reduction of ced-4 activity can lead to an increase in programmed cell death, i.e., that ced-4 can protect against programmed cell death.

The ced-4 Allele n2273 Perturbs Both Killer and Protective ced-4 Functions

To confirm further that ced-4 encodes a death-preventing function and to examine whether this function and the death-promoting function of ced-4 might be encoded by the ced-4L and ced-4S transcripts, respectively, we characterized the effects of the ced-4(n2273) mutation, which changes a conserved G to an A at position 114 of intron 3 (Yuan and Horvitz, 1992; see Figure 1B) and thus might be useful for distinguishing the functions of the two ced-4 transcripts. We examined ced-4 transcripts in ced-4(n2273) mutants by probing a Northern blot of poly(A)’ RNA from ced-4(n2273) animals with either a probe consisting of the 72 bp conserved region of intron 3 (detecting only the ced-4L transcript) or a full-length ced-4S cDNA probe (detecting both the ced-4S and ced-4L transcripts). As shown in Figure 1C, expression of ced-4L in ced-4(n2273) animals was enhanced compared
C. elegans ced-4 Encodes Opposing Death Functions

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Figure 1. ced-4 Encodes Two Alternatively Spliced Transcripts

(A) Diagram showing the conservation of ced-4 genomic nucleotide sequences among C. elegans, C. briggsae, and C. vulgaris. Boxes represent exons, horizontal lines represent introns, broken lines indicate alignments, numbers above and below the C. elegans diagram indicate the percent nucleotide identity between C. elegans and C. vulgaris and C. elegans and C. briggsae, respectively. Only a partial C. vulgaris clone was isolated.

(B) Alignment of the 77 bp sequence at the 3' end of intron 3 from C. elegans, C. briggsae, and C. vulgaris. Numbers at the ends of the C. elegans sequence indicate positions within intron 3. Residues conserved among all species are boxed. The hypothetical C. elegans protein sequence encoded by this region is indicated below the alignment. Consensus splice-acceptor sites (Emmons, 1988) (TTN NAG) are indicated by horizontal bars. Arrows indicate positions of splicing in ced-4L and ced-4S transcripts. Nucleotide and corresponding amino acid changes in ced-4(n2273) animals are indicated.

(C) Left: Northern blot of wild-type and ced-4(n2273) poly(A) RNA probed with a 72 bp fragment corresponding to the ced-4L-specific sequence (2.2 kb) or with a probe for the gene that encodes the C. elegans L5 ribosomal protein (M. Koelle, personal communication) (0.9 kb) as a loading control. Right: equivalent blot of wild-type and ced-4(n2273) poly(A) RNA probed with a full-length ced-4S probe (2.2 kb) or with a probe for the gene that encodes the C. elegans L5 ribosomal protein as a loading control (0.9 kb).

(D) Agarose gel stained with ethidium bromide showing products generated by PCR using primers flanking intron 3 from wild-type and ced-4(n2273) RNA (see Experimental Procedures for details). Numbers show 123 bp ladder marker sizes.

(E) Drawing showing splicing patterns that generate the ced-4S and ced-4L transcripts. Open boxes represent exons, and V-shaped lines represent introns. AUG start and UAA stop codons are indicated. Numbers indicate nucleotide positions within an unspliced ced-4 mRNA beginning at the AUG and ending at the poly(A) addition site (Yuan and Horvitz, 1992).
with expression in wild-type animals, suggesting that in ced-4(n2273) animals the ced-4L-specific splice-acceptor site might be used more often than in wild-type animals.

To determine the splicing pattern of ced-4 transcripts in ced-4(n2273) animals, we amplified by PCR the ced-4S and ced-4L transcripts using primers flanking the ced-4L-specific exon. Separation of the amplified products on an agarose gel yielded two bands (Figure 1D).

Sequence determination revealed that the larger product corresponded to the wild-type ced-4L transcript, except for a G-to-A change at the ced-4(2273) mutation site (see Figure 1B), presumably causing an arginine-to-lysine substitution in the CED-4L protein. The smaller band contained two distinct products. One product corresponded to the wild-type ced-4S transcript except for a 3 bp deletion immediately downstream of the ced-4S-specific splice-acceptor site. This transcript would presumably result in the deletion of Ser-211 of CED-4S. The second product corresponded to a ced-4S transcript spliced at position 185 instead of 186 of the sequence shown in Figure 1B, resulting in an insertion of an A nucleotide relative to the wild-type sequence. Translation of this frameshifted product would presumably terminate prematurely at a TGA stop codon 85 bp downstream of the insertion site.

That ced-4S transcripts are abnormal in ced-4(n2273) mutants is consistent with a weak defect in cell killing observed in these animals both by us and by M. Hengartner and H. R. H. (unpublished data). Specifically, the anterior pharynx of ced-4(n2273) animals contained on average 2.9 extra cells that failed to undergo programmed cell death (see Table 4). Since ced-4(n2273) animals produced a mutated ced-4L product, we surmised that they also might have a defect in a death-preventing function of ced-4. To test this hypothesis, we examined the effect of introducing the weak loss-of-function ced-9 allele n1653 (Hengartner et al., 1992) into ced-4(n2273) animals. Although both ced-9(n1653) animals and ced-4(n2273) animals produce mostly viable progeny, doubly mutant ced-4(n2273) ced-9(n1653) animals derived from ced-4(n2273) ced-9(n1653)/+ parents produced mostly dead progeny (Table 3; M. Hengartner, personal communication), and this effect was blocked by preventing programmed cell death with a mutation in ced-3. This result suggests that ced-4(n2273) enhanced programmed cell death caused by the ced-9(n1653) allele. This enhancement of cell death is opposite to the reduction of cell death in ced-4(n2273) single mutant animals and suggests that ced-4(n2273) is defective in a death-preventing as well as a death-promoting function.

To test this hypothesis further, we showed that ced-4(n2273)/ced-4(+/+) animals did not contain extra surviving cells in the anterior pharynx and that ced-4(n2273)/ced-4(null) animals had more surviving cells than ced-4(n2273)/ced-4(+/+) mutants (data not shown), which supports the hypothesis that ced-4(n2273) results in a reduction of ced-4S activity. Overexpression of a ced-4L cDNA harboring the n2273 mutation reduced (but did not eliminate) extra cell survival compared with overexpression of ced-4L(+) (Table 1; data not shown). Furthermore, in a ced-9(n1653) background, ced-4(n2273)/ced-4(+) animals were alive, whereas both ced-4(n2273)/

Table 1. Overexpression of ced-4L Can Prevent Programmed Cell Death

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Heat Shock</th>
<th>Number of Extra Cells (n)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp-ced-4L-1</td>
<td>+</td>
<td>7.0 ± 0.9 (21)</td>
<td>0–13</td>
</tr>
<tr>
<td>hsp-ced-4L-1</td>
<td>-</td>
<td>0.07 ± 0.07 (15)</td>
<td>0–1</td>
</tr>
<tr>
<td>hsp-ced-4L-2</td>
<td>+</td>
<td>10 ± 0.6 (15)</td>
<td>5–14</td>
</tr>
<tr>
<td>hsp-ced-4L-2</td>
<td>-</td>
<td>0.2 ± 0.1 (15)</td>
<td>0–1</td>
</tr>
<tr>
<td>hsp-ced-4L-3</td>
<td>+</td>
<td>7.7 ± 0.7 (15)</td>
<td>4–12</td>
</tr>
<tr>
<td>hsp-ced-4L-3</td>
<td>-</td>
<td>0.07 ± 0.07 (15)</td>
<td>0–1</td>
</tr>
</tbody>
</table>

Heat-shock constructs were made by cloning a ced-4 cDNA into the heat-shock promoter vectors ppD49.79 and ppD49.83 (A. Fire, personal communication) and injected into wild-type animals. Transgenic adults were allowed to lay eggs for 3–5 days. Animals progressing past the L3 stage were scored as viable. n, number of eggs laid. Experiments were done at 20°C.

Table 2. Overexpression of ced-4L Can Rescue ced-9(ef) Animals from Lethality

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Number of Progeny per 20 Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>ced-9(n1950n2161)</td>
<td>ced-9(n1950n2077)</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>P$_{H}$-ced-4L</td>
<td>192 ± 24</td>
</tr>
<tr>
<td>P$_{H}$-ced-4L(frameshift)</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

Constructs were injected into unc-69(e587) ced-9(n1950n2161)/qC1, unc-69(e587) ced-9(n1950n2077)/qC1, and ced-9(n2812)/qC1 animals, and progeny of Ced-9 animals were examined. We allowed 20 animals of each genotype and containing a given transgene to produce progeny. The number of live progeny produced is shown. For the transgenic animals, this number represents the average number ± standard deviation of progeny produced by three independent lines. ND, not determined.

Table 3. ced-4(n2273) Can Enhance Killing by Programmed Cell Death

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent Viable Progeny (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ced-4(n2273)</td>
<td>98 (707)</td>
</tr>
<tr>
<td>ced-4(n2273)</td>
<td>99.6 (926)</td>
</tr>
<tr>
<td>ced-4(n2273) ced-9(n1653)</td>
<td>0.3 (532)</td>
</tr>
<tr>
<td>ced-4(n2273) ced-9(n1653); ced-3(n2427)</td>
<td>98 (651)</td>
</tr>
<tr>
<td>ced-4(n2273) ced-9(n1653); ced-3(n2438)</td>
<td>97 (583)</td>
</tr>
</tbody>
</table>

We allowed 10–20 animals of each genotype to lay eggs for 3–5 days. Animals progressing past the L3 stage were scored as viable. n, number of eggs laid. Experiments were done at 20°C.
BCL-2 mediates BCL-2±BAX interaction and is required and ced-4(n2273) also results in a reduction but not a total number of extra cells, average number of extra cells 6, error of the mean. n, number of animals observed. Single asterisk, a conserved glycine to a glutamic acid in the BH1 domain reduces cell survival caused by ced-4(n2273) prevents ced-9 enhancement of cell survival ced-4(n2273); ced-3(n2427) 10.1 ± 0.3 (15)*** ced-4(n2273); ced-3(n2438) 10.9 ± 0.3 (15)*** ced-4(n2273) reduces cell survival caused by ced-9(n1950) ced-9(n1950) 12.5 ± 0.2 (15) ced-4(n2273); ced-9(n1950) 10.1 ± 0.4 (14)*** In the top section are our reference data for wild-type, ced-9(n1653), and ced-4(n2273) animals. Extra cells were scored as in Table 1. Number of extra cells, average number of extra cells ± standard error of the mean. n, number of animals observed. Single asterisk, unpaired Student’s t test p < 0.001; double asterisk, p < 0.01; triple asterisk, p < 0.001. See text for relevant comparisons. ced-4(n2273) and ced-4(n2273)/ced-4(null) animals were dead. These results support the hypothesis that ced-4(n2273) also results in a reduction but not a total loss of ced-4L activity. ced-9 Inhibits Both Protective and Killer ced-4 Activities Previous studies have indicated that the cell-killing function of ced-4, which we now attribute to ced-4S, is inhibited by ced-9 (Hengartner et al., 1992; Shaham and Horvitz, 1996). How does ced-4L interact with ced-9 and ced-4S? First, as described above, overexpression of ced-4L can rescue the lethality caused by a loss of ced-9 function, suggesting that ced-4L functions downstream of or in parallel to ced-9. Second, ced-9 can inhibit the killing of ALM neurons caused by ced-4S overexpression in animals homozygous for the ced-4(n1162) allele and thus lacking all ced-4L function (Shaham and Horvitz, 1996), suggesting that ced-9 does not require ced-4L function to inhibit ced-4S function. Third, ced-9 appears to inhibit not only the activity of ced-4S, but also the activity of ced-4L. Specifically, Hengartner and Horvitz (1994a) reported that ced-9 has not only a death-preventing but also a death-promoting function: mutations that decrease ced-9 activity lead to enhanced rather than diminished cell survival in mutants slightly reduced in ced-3 function. For example, as we show in Table 4, ced-9(n1653); ced-3(n2427) animals contained an average of 7.4 ± 0.5 extra cells in the anterior pharynx, whereas ced-3(n2427) animals contained only 1.2 ± 0.2 extra cells. Does this death-promoting ced-9 function require ced-4L activity? To answer this question, we determined whether the ced-4(n2273) mutation, which presumably reduces ced-4L function, affects this death-promoting activity. We found that ced-4(n2273) ced-9(n1653); ced-3(n2427) or n2438) triple mutants had slightly fewer, rather than more, extra cells when compared with ced-4(n2273) ced-9(n1653); ced-3(n2427) double mutants, respectively (Table 4). For example, ced-4(n2273) ced-9(n1653); ced-3(n2427) animals contained on average 8.7 ± 0.4 extra cells in the anterior pharynx, whereas ced-4(n2273) ced-3(n2427) animals contained 10.1 ± 0.3 extra cells. These results suggest that ced-4(n2273) (which is defective in ced-4L function) prevented the ability of ced-9(n1653) to enhance cell survival and indicate that ced-9 can cause cell death by inhibiting the activity of ced-4L. That animals carrying the ced-3(n2433), ced-4(n1162), and ced-9(n1950) mutations contain on average 12.4, 12.2, and 12.5 (Table 4) extra cells, respectively, in the anterior pharynx suggests that if ced-9(n1653) could enhance cell survival in ced-4(n2273); ced-3(weak) animals, we would have been able to detect this enhancement. n1950, a Death-Preventing ced-9 Allele, Might Be Unable to Inhibit ced-4L The ced-9(n1950) allele dominantly inhibits programmed cell death (Hengartner and Horvitz, 1994a) and changes a conserved glycine to a glutamic acid in the BH1 domain of the CED-9 protein. The equivalent domain in BCL-2 mediates BCL-2–BAX interaction and changes to prevent cell death (Yin et al., 1994). ced-9(n1950) was proposed to activate the CED-9 protein (Hengartner and Horvitz, 1994a); however, a similar change in BCL-2 surprisingly resulted in loss of activity in both mammalian cells and in C. elegans (Yin et al., 1994; Hengartner and Horvitz, 1994a). We observed that ced-4(n2273) ced-9(n1950) double mutants contained fewer extra cells than did ced-9(n1950) animals (Table 4). That ced-4(n2273), which is defective in ced-4L function, reduces the extent of cell survival caused by ced-9(n1950) suggests that ced-9(n1950) causes cell survival at least in part as a result of an increase in ced-4L activity. We propose, therefore, that ced-9(n1950) can inhibit ced-4S, but not ced-4L. If so, the ced-9(n1950) phenotype would be a consequence of not the activation of CED-9, but rather of the loss of the inhibitory action of CED-9 on CED-4L. Consistent with this observation, Hengartner et al. (1992) showed that ced-9 product is maternally contributed to the embryo. If ced-9(n1950) resulted in increased ced-9 protective activity, genotypically wild-type self-progeny of ced-9(n1950)/+ parents might well have extra surviving cells. However, such progeny do not have extra surviving cells (Hengartner et al., 1992), as would be expected if the n1950 allele instead lacked a killing activity. Discussion Our results suggest that ced-4 encodes two opposing cell death functions mediated by two alternatively transcribed ced-4 mRNAs, ced-4L and ced-4S. Our experiments indicate that alternative splicing is normally used in vivo to regulate programmed cell death. Two mammalian genes have been shown to encode alternatively spliced products that have opposing cell death functions when overexpressed, although in neither case
have opposing normal in vivo activities been demonstrated. bcl-x, a bcl-2-like gene, encodes two transcripts (Boise et al., 1993). As in the case of ced-4, the longer bcl-x transcript, bcl-xL, can protect cells from cell death, and the shorter transcript, bcl-xS, can accelerate cell death. Ich-1, a ced-3/ice-like protease, also encodes two transcripts (Wang et al., 1994). Ich-1fs, encodes a full-length protease that can cause apoptosis, whereas the truncated Ich-1S product presumably encodes an inactive protease that can prevent apoptosis. Of these four transcripts, only bcl-xS has been shown normally to play a role in vivo. The observation that three classes of cell death genes apparently unrelated by primary sequence (ced-4, bcl-x, and Ich-1) are all alternatively spliced suggests that factors involved in RNA splicing may regulate programmed cell death by the coordinate differential splicing of a number of cell death–related primary transcripts.

Two alternative models for the genetic pathway for programmed cell death in C. elegans (Figure 2) are consistent with our observations that ced-4L acts downstream of or in parallel to ced-9, that ced-9 can inhibit ced-4S-induced killing in the absence of ced-4L (Shaham and Horvitz, 1996), and that ced-9 can inhibit both ced-4L and ced-4S. In both models, ced-9 negatively regulates ced-4L and ced-4S, and the antagonistic activities of ced-4L and ced-4S compete to determine whether a cell lives or dies. Since ced-4 null mutants, which lack both ced-4L and ced-4S transcripts, are viable animals containing many extra cells (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992; S. S. and H. R. H., unpublished data), ced-4S may well function downstream of ced-4L. However, our results do not reveal whether ced-4L inhibits programmed cell death by preventing ced-4S activity directly or by interfering with a consequence of ced-4S action, as indicated by the alternative models shown in Figure 2.

How might ced-4L, ced-4S, and ced-9 interact to regulate programmed cell death? One possibility is that ced-9 preferentially inhibits ced-4S in cells that normally live and preferentially inhibits ced-4L in cells that normally die. Differential inhibition of ced-4S or ced-4L in different cells by ced-9 could reflect differences in the splicing pattern of ced-4 between cells that normally die and those that normally live. Specifically, ced-4S could be produced at lower levels compared with ced-4L in cells that normally live and at higher levels compared with ced-4L in cells that normally die.

The models presented in Figure 2 offer a possible explanation for the effect of the ced-9(n1950) mutant, which causes a Gly-169 to glutamate substitution and results in extra cell survival (Hengartner and Horvitz, 1994a): rather than activating the CED-9 protein as originally suggested, this mutation might specifically inactivate the ability of the CED-9 protein to inhibit CED-4L, thereby allowing CED-4L to prevent cell death and causing increased cell survival. A glycine to glutamate mutation at the identical site of the BH1 domain of the BCL-2 protein inactivates BCL-2, apparently because the protein loses the ability to form heterodimers with other BCL-2 family members (Yin et al., 1994). We propose that the n1950 mutation similarly results in loss of a protein–protein interaction, i.e., of an interaction between CED-9 and CED-4L or between CED-9 and a protein that mediates CED-4L activity. In the case of BCL-2, this loss of interaction results in inability to protect against cell death, whereas in the case of CED-9 this loss of interaction results in inability to cause cell death. Alternatively, CED-9 might regulate other aspects of ced-4 function such as alternative splicing, transcription, or biochemical activity. Since mutations that give rise to excess cell survival may result in malignant growth (Vaux et al., 1988), it is possible that dominant oncogenes exist that encode BCL-2 family members that cannot inhibit a CED-4L functional counterpart. Such a mechanism for oncogenesis would contrast with the dominant induction of cancerous growth caused by overexpression of a BCL-2-like protective protein, as seen in follicular lymphomas (Yunis et al., 1987).

Another feature of the models presented in Figure 2 is that they suggest that ced-9 can act either to prevent or to promote cell death depending on its relative effects on ced-4S and ced-4L. Thus, a single cell-death protein can mediate both survival and killing. Two mammalian bcl-2 family members, Bax and Bak, as well as bcl-2 itself might be bifunctional in this way. A mutation in the mouse Bax gene was found recently to result in either hypoplasia or hyperplasia, depending on the tissue examined (Knudson et al., 1995). This result suggests that Bax might be able either to promote or to prevent cell death. Similarly, Bak accelerates cell death in a number of cell lines (Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995) and prevents cell death in others (Kiefer et al., 1995). Finally, bcl-2 can both potentiate and prevent cell death in response to different cell death stimuli (Cortazzo and Schor, 1996). We suggest that bcl-2, Bax, Bak, and other mammalian bcl-2/ced-9 family members can both promote and prevent programmed cell death by interacting with mammalian functional counterparts of CED-4S and CED-4L, which might differ in distribution among distinct cell and tissue types.
Experimental Procedures

General Methods and Strains

We cultured C. elegans as described by Brenner (1974). All strains were grown at 20°C. The wild-type strain used was C. elegans strain Bristol strain N2. Genetic nomenclature follows the standard C. elegans system (Horvitz et al., 1979). The following mutants were used: egl-1(n487) (Trent et al., 1983); ced-4(n1162) (Ellis and Horvitz, 1986); ced-4(n2273) (Yuan and Horvitz, 1992); ced-9(n1653, n1950, n1950 n2161, n1950 n2077) (Hengartner et al., 1992); ced-3(n2427, n2438) (Hengartner and Horvitz, 1994a); ced-9(n2812) (S. S. and H. R. H., unpublished data).

Plasmid Constructions

Plasmids were constructed as follows. hsp-ced-4L: plasmid pS241, which contains a full-length ced-4L cDNA, was digested with SpeI and BstNI. The insert was ligated either to the heat-shock vector pPD49.79 or to pPD49.83 (A. Fire, personal communication) digested with NheI and EcoRV. Plasmid pS241 was digested with EcoRI or HindIII and insert sequences were determined logue by interaction with adenovirus E1B 19K. Nature

Northern Blot Hybridizations and RT±PCR

by shotgun sequencing (Sulston et al., 1992) using an ABI 373A Faucheu, C., Diu, A., Chan, A.W., Blanchet, A.M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R.A., and Lippke, J.A. (1995). digested with EcoRI or HindIII. Insert sequences were determined by random priming. RT±PCR was performed as follows: primers ced-4±f (5’-AACACGGTTAATGAAG-3’) and ced-4±r (5’-TTGAAAG AACAGGTTAATGAAG-3’) located at the beginning and end of the sequence shown in Figure 1B, respectively, in the presence of 32P-labeled dATP. A probe for RNAs that correspond to the L5 ribosomal RNA was prepared from total RNA by Northern blotting and hybridization under conditions of high stringency.

Germline Transformation

Our procedure for microinjection and germline transformation followed that of Fire (1986) and Mello et al. (1991). DNA for injections was purified using a Qiagen system according to the instructions of the manufacturer (Qiagen, Chatsworth, CA). The concentrations of all plasmids used for injections were between 50–100 μg/ml. All constructs were coinjected with the pRF4 plasmid, which contains the rol-6(su1006) allele as a dominant marker. Approximately 30 animals were injected in each experiment, and 50–100 F1 Rol animals were picked onto separate plates. RT±PCR was performed as follows: primers ced-4±f and ced-4±r (frameshift): plasmid pS241 digested with BamHI, and the overhangs were filled with Klenow and religated. T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74, 597–606.

References


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