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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*(lf) mutations or overexpression of the wild-type *ced-3* or *ced-4* genes can cause cells that normally survive instead 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*(lf) 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-1β-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 (TTNNAG; 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 P_{mec-7}. No ectopic cell deaths were observed (data not shown). We also 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*(lf) mutation. *ced-9*(lf) homozygous animals derived from *ced-9*(lf)/+ 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*(lf) 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 the *ced-4S*-specific splice-acceptor site 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

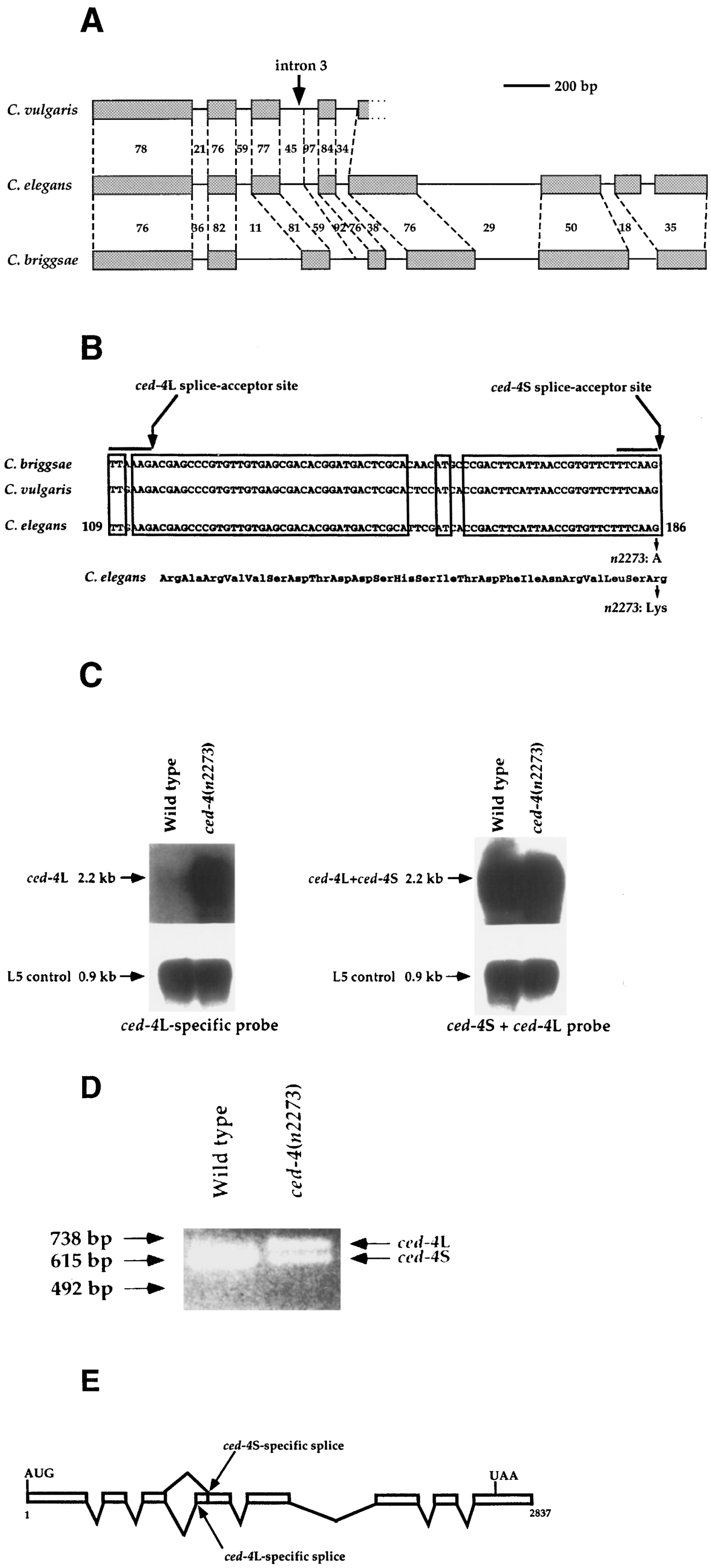

Figure 1. *ced-4* Encodes Two Alternatively Spliced Transcripts

(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 putative 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).

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

Transgene	Heat Shock	Number of Extra Cells (n)	Range
hsp–ced-4L-1	+	7.0 ± 0.9 (21)	0–13
hsp–ced-4L-1	−	0.07 ± 0.07 (15)	0–1
hsp–ced-4L-2	+	10 ± 0.6 (15)	5–14
hsp–ced-4L-2	−	0.2 ± 0.1 (15)	0–1
hsp–ced-4L-3	+	7.7 ± 0.7 (15)	4–12
hsp–ced-4L-3	−	0.07 ± 0.07 (15)	0–1

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 2 hr at 20°C, subjected to a 60 min heat shock at 33°C, allowed to lay eggs for an additional 2 hr, and removed from the petri plate. Hatched L4 roller larvae were scored for extra cells in the anterior pharynx as previously described (Hengartner et al., 1992). Number of extra cells, average number of extra cells ± standard error of the mean. n, number of animals observed. Range, range of extra cells seen in individual animals.

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

Genotype	Percent Viable Progeny (n)
ced-4(n2273)	98 (707)
ced-9(n1653)	99.6 (926)
ced-4(n2273) ced-9(n1653)	0.3 (532)
ced-4(n2273) ced-9(n1653); ced-3(n2427)	98 (651)
ced-4(n2273) ced-9(n1653); ced-3(n2438)	97 (583)

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.

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(n2273)* 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(n2273)* 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 2. Overexpression of *ced-4L* Can Rescue *ced-9*(lf) Animals from Lethality

	Number of Progeny per 20 Animals		
Transgene	*ced-9(n1950n2161)*	*ced-9(n1950n2077)*	*ced-9(n2812)*
$P_{dpy\text{-}30}$*ced-4L*	192 ± 24	67 ± 30	11 ± 3
$P_{dpy\text{-}30}$*ced-4L*(frameshift)	0	0	ND
None	0	0	0

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 4. *ced-4L* Might Be Negatively Regulated by *ced-9*

Genotype	Number of Extra Cells (n)
Wild type	0.1 ± 0.4 (40)
ced-9(n1653)	0.3 ± 0.5 (15)
ced-4(n2273)	2.9 ± 0.4 (15)
ced-9 enhances cell survival in *ced-3* (weak) mutants	
ced-3(n2427)	1.2 ± 0.2 (19)
ced-3(n2438)	2.1 ± 0.4 (10)
ced-9(n1653); ced-3(n2427)	7.4 ± 0.5 (15)*
ced-9(n1653); ced-3(n2438)	8.2 ± 0.4 (15)*
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)	11.9 ± 0.3 (15)
ced-4(n2273) ced-9(n1653); ced-3(n2427)	8.7 ± 0.4 (15)**
ced-4(n2273) ced-9(n1653); 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, $p < 0.04$. 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-3(n2427* or *n2438)* 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 is required for BCL-2 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 not of 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

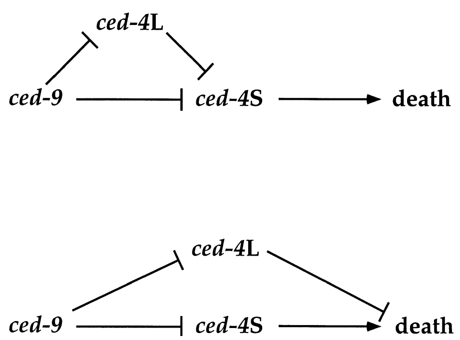

Figure 2. Alternative Models for the Regulation of Programmed Cell Death in C. elegans

The *ced-9* gene product can inhibit the functions of both the *ced-4S* and the *ced-4L* products. *ced-4S* promotes programmed cell death, and *ced-4L* prevents programmed cell death. *ced-9* can prevent cell death by inhibiting *ced-4S* and can promote cell death by inhibiting *ced-4L*. *ced-4L* inhibits cell death either by inhibiting *ced-4S* activity (above) or by interfering with a consequence of *ced-4S* action (below). Pointed and blunt arrows indicate positive and negative regulation, respectively.

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, $bcl\text{-}x_L$, can protect cells from cell death, and the shorter transcript, $bcl\text{-}x_S$, can accelerate cell death. *Ich-1*, a *ced-3/Ice*-like protease, also encodes two transcripts (Wang et al., 1994). $Ich\text{-}1_L$ encodes a full-length protease that can cause apoptosis, whereas the truncated $Ich\text{-}1_S$ product presumably encodes an inactive protease that can prevent apoptosis. Of these four transcripts, only $bcl\text{-}x_L$ 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 variety 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 SmaI. 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. P_{dpy-30}*ced-4L*: plasmid pS235, which contains the *dpy-30* promoter, was digested with NheI and SmaI and ligated to the full-length *ced-4L* insert as described above. P_{dpy-30}*ced-4L*(frameshift): plasmid P_{dpy-30}*ced-4L* was digested with BamHI, and the overhangs were filled with Klenow and religated. The resulting frameshift mutation should result in truncation of CED-4L at amino acid 165.

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. F1 animals segregating Rol progeny were established as lines containing extrachromosomal arrays (Way and Chalfie, 1988).

Isolation and Sequence Analysis of *ced-4* Homologs

Methods were as described by Sambrook et al. (1989). C. briggsae and C. vulgaris *ced-4* genes were cloned by low stringency hybridization from phage libraries provided by C. Link. Phage DNA was isolated and clones were digested with EcoRI or HindIII and analyzed using Southern blots. Blots were probed with a *ced-4S* cDNA labeled by random priming, and positive bands were excised and ligated to a pBluescript SK(+) vector (Stratagene, La Jolla, CA) digested with EcoRI or HindIII. Insert sequences were determined by shotgun sequencing (Sulston et al., 1992) using an ABI 373A sequencer.

Northern Blot Hybridizations and RT–PCR

Methods were as described by Sambrook et al. (1989) unless otherwise indicated. RNA was prepared from wild-type or *ced-4(n2273)* animals using the Fast-Track poly(A)$^+$ RNA isolation system (Invitrogen, San Diego, CA) and according to the instructions of the manufacturer. The *ced-4S* probe was prepared as above. The 72 bp intron probe was prepared by amplifying the genomic *ced-4* clone C10D8-5 (Yuan and Horvitz, 1992) with primers *ced-4I* (5′-ACGAGCCCGTGTTGTGAGCGAC-3′) and *ced-4G* (5′-TTGAAAG AACACGGTTAATGAAG-3′) located at the beginning and end of the sequence shown in Figure 1B, respectively, in the presence of ^{32}P-labeled dATP. A probe for RNAs that correspond to the L5 ribosomal protein probe (M. Koelle, unpublished data) was prepared by random priming. RT–PCR was performed as follows: primers *ced-4F* (5′-TCGACGAGATGTGTGATTTAG-3′) in exon 1 and *ced-4D* (5′-GTT TTCGGTTCACAAGACTTG-3′) in exon 6 were used to generate cDNAs and amplify products from wild-type and *ced-4(n2273)* RNAs prepared as described above using the rTth RNA amplification kit (Perkin Elmer Cetus, Norwalk, CT) according to the instructions of the manufacturer. The DNA in each band was purified using β-agarase (New England Biolabs, Beverly, MA), ligated to pBluescript SK(+) (Stratagene), and transformed into E. coli. Plasmid DNA from each of 20 colonies resulting from each ligation was purified, and insert sequences were determined using an ABI 373A sequencer.

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