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The *C. elegans* Protein EGL-1 Is Required for Programmed Cell Death and Interacts with the Bcl-2–like Protein CED-9

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Summary

Gain-of-function mutations in the *Caenorhabditis elegans* gene *egl-1* cause the HSN neurons to undergo programmed cell death. By contrast, a loss-of-function *egl-1* mutation prevents most if not all somatic programmed cell deaths. The *egl-1* gene negatively regulates the *ced-9* gene, which protects against cell death and is a member of the *bcl-2* family. The EGL-1 protein contains a nine amino acid region similar to the Bcl-2 homology region 3 (BH3) domain but does not contain a BH1, BH2, or BH4 domain, suggesting that EGL-1 may be a member of a family of cell death activators that includes the mammalian proteins Bik, Bid, Harakiri, and Bad. The EGL-1 and CED-9 proteins interact physically. We propose that EGL-1 activates programmed cell death by binding to and directly inhibiting the activity of CED-9, perhaps by releasing the cell death activator CED-4 from a CED-9/CED-4–containing protein complex.

Introduction

Programmed cell death is a physiological process that has been conserved through evolution (Ellis et al., 1991a; Raff, 1992). Genetic analyses of the cell death process in *Caenorhabditis elegans* have defined a genetic pathway for programmed cell death (reviewed by Horvitz et al., 1994). Mutations in three genes, *ced-9*, *ced-4*, and *ced-3* (*ced*, *c*ell-*d*eath abnormal), affect most if not all of the 131 somatic cell deaths that occur during the development of the *C. elegans* hermaphrodite (Sulston and Horvitz, 1977; Sulston et al., 1983). Loss-of-function (lf) mutations in *ced-3* or *ced-4* result in the survival of cells that normally die, indicating that these genes are required for the killing process (Ellis and Horvitz, 1986). *ced-9*, by contrast, is a negative regulator of programmed cell death. A gain-of-function (gf) mutation in *ced-9* prevents most, if not all, programmed cell deaths, and lf mutations in *ced-9* cause embryonic lethality as a consequence of ectopic cell death (Hengartner et al., 1992). This lethality is suppressed by lf mutations in *ced-3* or *ced-4*, indicating that *ced-3* and *ced-4* act downstream of or in parallel to *ced-9* (Hengartner et al., 1992). *ced-4* is likely to act upstream of *ced-3*, since cell death induced by *ced-4* overexpression is greatly reduced in the absence of *ced-3* activity (Shaham and Horvitz, 1996a). The *ced-9*, *ced-4*, and *ced-3* central cell death machinery is thought to be regulated by cell type–specific regulators, which include the cell death specification or *ces* genes *ces-1* and *ces-2*; these two genes specify the life-versus-death decisions of a subset of cells, including the sisters of the NSM neurons in the pharynx (Ellis and Horvitz, 1991).

The genetically established interactions among *ced-9*, *ced-4*, and *ced-3* may reflect direct physical interactions of the protein products of these genes. The CED-9 protein binds to the CED-4 protein (Chinnaiyan et al., 1997a; James et al., 1997; Ottilie et al., 1997a; Spector et al., 1997; Wu et al., 1997a), which in turn can bind to the CED-3 protein (Chinnaiyan et al., 1997a; Wu et al., 1997b). Furthermore, the interaction of CED-4 with CED-3 appears to lead to the activation of CED-3 and the initiation of cell death (Chinnaiyan et al., 1997b; Seshagiri and Miller, 1997; Wu et al., 1997b).

ced-9, *ced-4*, and *ced-3* have mammalian counterparts also shown to be involved in programmed cell death. *ced-9* encodes a protein structurally and functionally similar to the mammalian cell death inhibitor Bcl-2 (Hengartner and Horvitz, 1994b), the prototype of a family of Bcl-2–like molecules that act as regulators of cell death in mammals (reviewed by White, 1996; Rinkenberger and Korsmeyer, 1997). CED-3 is a member of a family of invertebrate and mammalian cysteine proteases, collectively called caspases, that are cell death effectors acting mainly downstream of Bcl-2–like cell death regulators (reviewed by Fraser and Evan, 1996; Nicholson and Thornberry, 1997). Finally, Apaf-1, a mammalian protein recently identified biochemically as essential for caspase-3 activation in an in vitro assay for programmed cell death (Zhou et al., 1997), is in part similar in sequence to the CED-4 protein (Yuan and Horvitz, 1992).

Recently, a new group of mammalian cell death activators has been identified. These proteins, which include Bik, Bid, Harakiri, and Bad, interact with Bcl-2–like proteins and can induce cell death when overexpressed (Boyd et al., 1995; Yang et al., 1995; Han et al., 1996a; Wang et al., 1996; Inohara et al., 1997; Kelekar et al., 1997; Ottilie et al., 1997b; Zha et al., 1997). The amino acid sequences of these cell death activators are dissimilar, except for a nine amino acid stretch similar to one of the four Bcl-2 homology (BH) domains, the BH3 domain, and particularly similar to the BH3 domain of the Bcl-2–like cell death activators Bax and Bak (Chittenden et al., 1995; Han et al., 1996b; Hunter and Parslow, 1996; Zha et al., 1996). As in the cases of Bax and Bak (Chittenden et al., 1995; Han et al., 1996b), the BH3 domains of this new group of cell death activators are important both for their interaction with Bcl-2–like molecules and for their ability to induce cell death (Wang et al., 1996; Inohara et al., 1997; Kelekar et al., 1997; Ottilie et al., 1997b; Zha et al., 1997).

In this paper, we report that the *C. elegans* gene *egl-1* (*egl*, *eg*g-*l*aying defective) encodes a protein that interacts with CED-9 and contains a region similar to the BH3 domains of BH3-containing cell death activators. gf mutations in *egl-1* cause the two hermaphrodite-specific neurons (HSNs), which are required for egg laying, to undergo programmed cell death inappropriately;

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Table 1. *n3082* Blocks Programmed Cell Deaths

Genotype	% Egg-Laying Defective (n)	% HSNs Surviving n = 40	No. Extra Cells/ Pharynx[a] n = 15
N2	1 (248)	100	0.0 ± 0.0
egl-1(n1084)	99 (166)	0	0.0 ± 0.3
egl-1(n1084)/+	71 (238)	27	ND
nDf42/+	1 (108)	92	0.9 ± 0.7
egl-1(n1084)/nDf42	68 (165)	42	ND
egl-1(n1084 n3082)	0 (113)	97	11.1 ± 1.5
egl-1(n1084 n3082)/egl-1(n1084)	66 (423)	30	ND
egl-1(n1084 n3082)/+	0 (256)	98	1.0 ± 1.5
egl-1(n1084 n3082)/nDf42	1 (88)	100	11.5 ± 1.5

The Egl phenotype and the presence of HSNs and extra cells in the anterior pharynx were scored as described in Experimental Procedures. The complete genotypes of the animals scored were, from top to bottom, as follows: N2 (wild-type), *egl-1(n1084)*, *egl-1(n1084) unc-76(e911)/++*, *nDf42/+*, *egl-1(n1084)/nDf42, egl-1(n1084 n3082), egl-1(n1084) unc-76(e911)/egl-1(n1084 n3082), egl-1(n1084 n3082)/unc-42(e270)* for the egg-laying phenotype and *egl-1(n1084 n3082)/unc-76(e911)* for the HSN and extra cells phenotype, *egl-1(n1084 n3082)/nDf42*. For comparison, *ced-3(n717)* animals have 10.7 ± 1.7 (n = 15), *ced-4(n1162)* animals 10.2 ± 1.5 (n = 15), and *ced-9(n1950)* animals 12.4 ± 1.9 (n = 15) extra cells in the anterior pharynx. ND, not determined.

[a] Numbers represent averages ± standard deviations.

these mutations were identified in screens for egg-laying defective (Egl) mutants (Trent et al., 1983). By isolating a dominant suppressor of the *egl-1* Egl phenotype, we identified an lf mutation in the *egl-1* gene. This mutation prevents not only the ectopic deaths of the HSNs but most if not all normally occurring programmed cell deaths, indicating that *egl-1* is a cell death activator and encodes a component of the general cell death machinery in *C. elegans*.

Results

An lf Mutation in *egl-1* Blocks Programmed Cell Death

Dominant mutations in the gene *egl-1* cause the HSNs to undergo programmed cell death (Trent et al., 1983; Ellis and Horvitz, 1986). This ectopic cell death results in an Egl phenotype, as exemplified for the *egl-1* allele *n1084* in Table 1. Hermaphrodites heterozygous for *nDf42*, a deficiency that deletes the *egl-1* locus, do not exhibit an Egl phenotype (Horvitz et al., 1994) (Table 1), indicating that the dominant mutations in *egl-1* are not null mutations but rather are likely to be gf mutations. To date, seven *egl-1*(gf) mutations have been identified (Trent et al., 1983; Desai and Horvitz, 1989; Horvitz et al., unpublished data).

To determine the lf phenotype of *egl-1*, we performed a screen for dominant suppressors of the *egl-1* Egl phenotype. Specifically, hermaphrodites homozygous for the mutation *egl-1(n1084)* were mutagenized with ethyl methanesulfonate (EMS), allowed to self-fertilize, and their F1 progeny were screened for rare non-Egl animals. Since 68% of *egl-1(n1084)/nDf42* heterozygous hermaphrodites were Egl (Table 1), we reasoned that if one copy of the *egl-1* gene in an *egl-1(n1084)* hermaphrodite were inactivated by an *egl-1* lf mutation, the resulting animal should have about a one-third probability of being non-Egl. We analyzed 30,000 mutagenized haploid genomes and isolated seven allelic suppressors that are closely linked to the *egl-1* locus on chromosome V. All seven suppressor mutations proved to be alleles of *egl-1* (B. C. and H. R. H., unpublished data) and therefore represent intragenic revertants in the *egl-1* gene. The seven mutations were not necessarily independent in origin, and all seven carry the same 5 bp deletion (see below). We assume that they derived from a single, possibly spontaneous, deletion event. The data presented below were therefore obtained from one representative isolate, which carries the allele *n3082*.

The *n3082* mutation is a semidominant suppressor of *egl-1(n1084)*: while 0% of hermaphrodites homozygous for *egl-1(n1084 n3082)* are Egl, 66% of hermaphrodites homozygous for *egl-1(n1084)* but heterozygous for *n3082* (i.e., of genotype *egl-1[n1084 n3082]/egl-1[n1084]*) are Egl (Table 1). Microscopic analyses using Nomarski optics revealed that the HSN neurons are present in *egl-1(n1084 n3082)* hermaphrodites, suggesting that the suppression of the Egl phenotype is caused by the suppression of the deaths of the HSNs (Table 1).

To determine the effect of the *n3082* mutation on cells that normally undergo programmed cell death in *C. elegans*, we determined the number of cells in the anterior pharynx of *egl-1(n1084 n3082)* animals at the third stage of larval development. Mutations that block programmed cell death in *C. elegans*, such as a gf mutation in *ced-9* or lf mutations in *ced-4* or *ced-3*, result in the presence of 11–12 extra cells in the anterior pharynx (Hengartner et al., 1992). *egl-1(n1084 n3082)* animals had on average 11.1 extra cells in the anterior pharynx (Table 1). This result indicates that the *n3082* mutation not only blocks the ectopic deaths of the HSNs in *egl-1(n1084)* animals but, like the *ced-9*(gf), *ced-4*(lf), and *ced-3*(lf) mutations, also causes a general block in programmed cell death, that is, a Ced phenotype.

This conclusion was supported by our analysis of *egl-1(n1084 n3082)* embryos for the presence of cell corpses in an engulfment-defective mutant background: a *ced-1* mutation blocks the engulfment of the cell corpses generated by programmed cell death and therefore allows a quantitative assessment of the number of programmed cell deaths that have occurred as of a particular stage of development (Ellis et al., 1991b). Embryos homozygous for *ced-1* or for *ced-1; egl-1(n1084)* had on average a total of 24.9 (n = 15) and 24.1 (n = 15) corpses at the 1 1/2-fold stage of embryogenesis, respectively. Homozygous *ced-1; egl-1(n1084 n3082)*

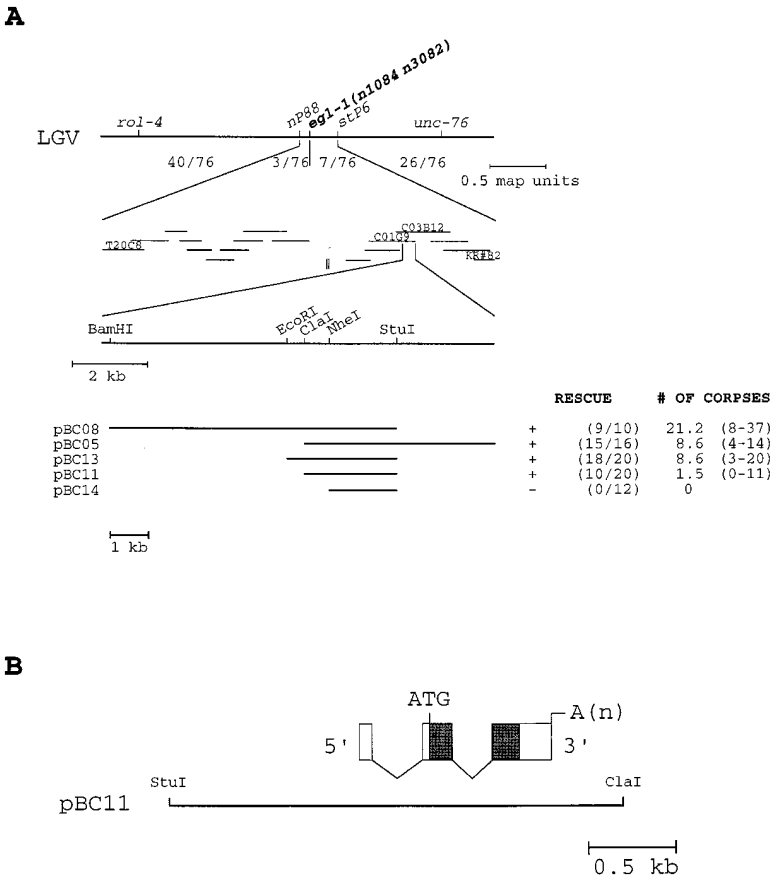

Figure 1. Localization of the *egl-1* Locus

(A) (Top) Genetic map. Genes and polymorphisms used for mapping the *egl-1* locus are indicated. Numbers below the genetic map represent the fraction of the 76 recombination events identified between *rol-4* and *unc-76* that occurred between particular loci. (Middle) Physical map of the interval between *nP88* and *stP6*. Cosmids tested for rescue are shown. The cosmids that detected *nP88* (T20C8) and *stP6* (KR#82), as well as the two rescuing cosmids (C01G9 and C03B12), are labeled.

(Bottom) Partial restriction map of the right end of cosmid C01G9 and *egl-1* rescue data obtained with various subclones. Transgenic animals carrying the indicated constructs as extrachromosomal arrays were generated, and rescue was scored as described in Experimental Procedures. The numbers of lines rescued and the total number of lines obtained are indicated in parentheses. The average numbers of corpses scored and the range of corpses (parentheses) for one representative line are also indicated (n = 15).

(B) Structure of the full-length *egl-1* transcript as determined by cDNA cloning, RT-PCR, and RACE. Coding exons are shaded. The direction of the gene is indicated by its 5′ and 3′ ends. (Note that the orientation of the *egl-1* locus has been reversed in [B]).

embryos, by contrast, contained only 0.1 corpses (n = 10) on average at this developmental stage.

The Ced phenotype of *egl-1(n1084 n3082)* animals is recessive: *egl-1(n1084 n3082)*/+ heterozygotes had only 1.0 extra cell (n = 15) on average in the anterior pharynx (Table 1). In addition, the Ced phenotype of animals heterozygous for *egl-1(n1084 n3082)* and *nDf42* (i.e., of genotype *egl-1[n1084 n3082]/nDf42*) was indistinguishable from that of *egl-1(n1084 n3082)* animals (Table 1), suggesting that *egl-1(n1084 n3082)* is a strong lf allele and that *egl-1* is required for programmed cell death in somatic tissues of *C. elegans*. Interestingly, cell death in the *C. elegans* germline did not seem to be affected by the *egl-1(n1084 n3082)* mutation (our unpublished observations).

Cloning of *egl-1*

To determine the molecular identity of *egl-1*, we cloned the gene by transformation rescue of the recessive Ced phenotype of *egl-1(n1084 n3082)* homozygotes. First, we used the Ced phenotype to map *egl-1(n1084 n3082)* to the right arm of chromosome V between *rol-4* and *unc-76* (data not shown), the interval to which *egl-1*(gf) mutations were mapped previously (Trent et al., 1983; Desai and Horvitz, 1989). We further mapped *egl-1(n1084 n3082)* between the restriction fragment length polymorphisms (RFLPs) *nP88* on cosmid T20C8 and *stP6* on cosmid KR#82 (Williams et al., 1992) (Figure 1A). Cosmids that map between *nP88* and *stP6* were introduced into *egl-1(n1084 n3082)* animals by germline transformation (Mello and Fire, 1995) and tested for their ability to rescue the Ced phenotype. Rescue was assayed by analyzing transgenic animals for the presence of cell corpses in embryos at the 1 1/2-fold stage of embryogenesis. To increase the sensitivity of the assay, the transgenic lines were established in a *ced-1* mutant background to allow the persistence of cell corpses. Two overlapping cosmids, C01G9 and C03B12, both almost completely rescued the Ced phenotype caused by the *n1084 n3082* allele (as many as an average of 23.4 corpses in embryos; n = 15). We narrowed this rescuing activity to a 7.8 kb subclone of C01G9 contained in plasmid pBC08 (21.2 corpses; n = 15) (Figure 1A). A 2.5 kb subclone of pBC08, pBC11, also rescued the Ced phenotype but much less efficiently (1.5 corpses on average with a range of 0–11). Additional deletions completely abolished the residual rescuing activity (Figure 1A).

Using this minimal rescuing fragment as a probe, we screened an embryonic cDNA library and isolated two clones. The same probe detected a single approximately 650 bp message of low abundance in poly(A)+ mRNA isolated from embryos and L1 larvae but not from later larval stages or young adults (data not shown). Using reverse transcriptase-polymerase chain reaction (RT-PCR) and anchored reverse-transcriptase PCR (RACE; rapid amplification of cDNA ends), we determined the complete structure and sequence of the transcript,

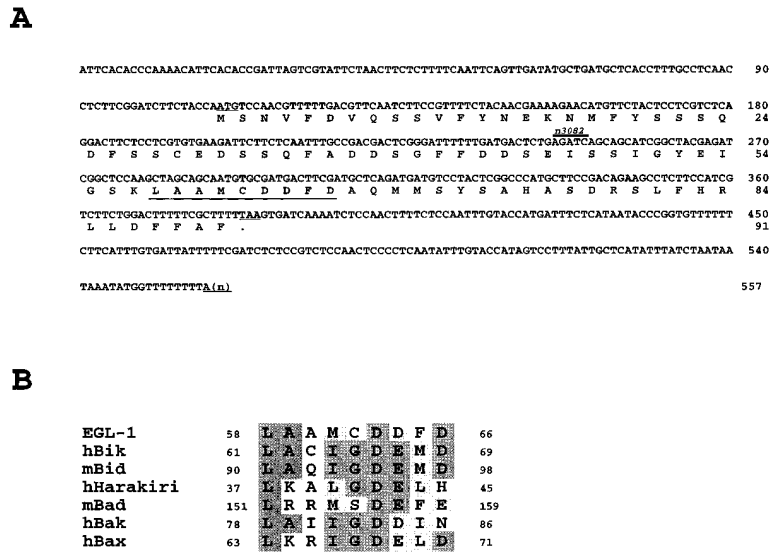

Figure 2. *egl-1* Sequence

(A) Sequences of the complete *egl-1* transcript and protein product. Amino acids underlined (58 to 66) constitute the BH3-like region. The five nucleotides deleted by the *n3082* mutation are indicated by the horizontal bar.

(B) Alignment of the EGL-1 BH3-like region with the BH3 domains of BH3-containing proteins (human Bik, accession number U34584; mouse Bid, U75506; human Harakiri, U76376; and mouse Bad, L37296) and the Bcl-2-like proteins Bak and Bax (human Bak, U23765; human Bax, L22474). Amino acids that are identical in at least four of the seven sequences and conserved amino acids are shaded in dark and light grey, respectively.

which has two coding exons and two potential initiator codons, of which we predict the more 3′ ATG to be used in vivo (Figure 1B and Figure 2A, data not shown). Frameshift mutations introduced into the genomic fragment contained in pBC13 confirmed that this transcript was responsible for the rescuing activity (data not shown). Furthermore, the expression of the predicted coding region under the control of a *C. elegans* heat-shock promoter (P_{hsp}; Mello and Fire, 1995) was sufficient to rescue the Ced phenotype of *ced-1; egl-1(n1084 n3082)* animals. Transgenic *ced-1; egl-1(n1084 n3082)* embryos carrying extrachromosomal arrays containing the P_{hsp} *egl-1* construct contained up to 31.9 corpses on average at the 1 1/2-fold stage after being subjected to heat shock (Table 2).

To confirm that the identified gene is *egl-1,* we determined the nucleotide sequence of the gene for each of the seven *egl-1*(gf) suppressors we had isolated. All seven alleles had an identical 5 bp deletion at the beginning of exon 2 (Figure 2A). This deletion is predicted to result in a frameshift leading to the generation of a truncated protein composed of the first half of the wild-type protein (45 amino acids) and a 16 amino acid C-terminal extension in a different reading frame.

egl-1 Encodes a Novel Protein with a Nine Amino Acid Region Similar to the BH3 Domain of Bcl-2–like and BH3-Containing Proteins

Searches of current nucleotide and protein databases using various BLAST programs identified no known sequences or proteins with significant similarity to the *egl-1* gene or the EGL-1 protein. *egl-1* therefore encodes a novel protein of 91 amino acids with no apparent hydrophobic stretches indicative of transmembrane domains (Figure 2A). Closer inspection of the EGL-1 sequence revealed a stretch of nine amino acids in the center of the protein (amino acids 58 to 66) with similarity to the BH3 domain of the family of Bcl-2–like and BH3-containing proteins (Figure 2B). Like the BH3 domain of Bcl-2–like and BH3-containing proteins, the BH3-like region of EGL-1 is predicted to adopt the structure of an amphipathic α-helix (our unpublished observations). The truncated EGL-1 protein predicted to be formed in *egl-1(n1084 n3082)* animals lacks this BH3-like region and the C terminus of the protein.

egl-1 Null Phenotype

The observation that animals homozygous for *egl-1(n1084 n3082)* have the same number of extra cells in the anterior pharynx as animals *trans*-heterozygous for *egl-1(n1084 n3082)* and *nDf42* (Table 1) suggests that the *n3082* mutation results in at least a severe loss of the normal function of the EGL-1 protein. Furthermore, others have observed that embryos homozygous for deficiencies that uncover loci to the left and right of the *egl-1* locus, and therefore that presumably delete *egl-1*, lack cell corpses (J. Rothman, personal communication) and contain up to 40% more nuclei than do wild-type embryos (M. Labouesse, personal communication), indicating that a total loss of the *egl-1* region results in a block in programmed cell death. We have observed that

Table 2. An *egl-1* cDNA Expressed under the Control of a *C. elegans* Heat-Shock Promoter Rescued the Ced Phenotype of *n3082* Animals

		Number of Corpses (Range)	
Transgene	Line	Mock	Heat Shock
P_{hsp}	A	0.0	0.0
P_{hsp}	B	0.0	0.0
P_{hsp} *egl-1*	A	0.3 (0–1)	30.8 (19–45)
P_{hsp} *egl-1*	B	0.1 (0–1)	31.9 (20–42)
P_{hsp} *egl-1*	C	0.1 (0–1)	26.2 (17–40)
P_{hsp} *egl-1*	D	0.3 (0–1)	28.6 (17–43)

Transgenic animals were generated as described in Experimental Procedures. Different lines carried independently derived extrachromosomal arrays of P_{hsp} (P_{hsp} A and B) or the P_{hsp} *egl-1* transgene (P_{hsp} *eg1-1* A–D). Heat shock was performed as follows. Transgenic adults were allowed to lay eggs for 1 hr and then were removed from the plate. The eggs were heat-shocked at 33°C for 45 min and scored for rescue at the 1 1/2-fold stage of embryogenesis. Data presented are averages and ranges of the number of cell corpses. For each experiment, 10–15 animals were scored.

Table 3. *n3082* Is Epistatic to an lf Mutation in the Cell Death–Specification Gene *ces-1*

Strain	% NSM Sister Survival (n = 50)
ces-1 (lf)	0
egl-1 (n1084 n3082)	88
ces-1 (lf); *egl-1 (n1084 n3082)*	86

NSM sister cells were scored as described in Experimental Procedures. The *ces-1* (lf) mutation used was *ces-1(n703 n1434)*.

embryos homozygous for deficiencies that fail to complement *egl-1(n1084 n3082)*, such as *nDf42*, *yDf8*, *zuDf2*, and *itDf2*, lack cell corpses at the comma stage of embryogenesis (n = 10–15) (a stage of development at which wild-type embryos have an average of about six corpses; n = 10) and therefore are cell death defective. By contrast, embryos homozygous for deficiencies that delete regions to the left or right of the *egl-1* locus, such as *lwDf3* or *yDf11*, have an average of 8.3 (n = 10) and 5.2 (n = 10) corpses, respectively. These observations are consistent with the hypothesis that the complete inactivation of the *egl-1* locus results in a block in programmed cell death. However, since *nDf42*, *yDf8*, *zuDf2*, and *itDf2* cause not only a Ced phenotype but also embryonic lethality, it is possible that *egl-1(n1084 n3082)* is not a null mutation and that a null allele of *egl-1* would result in some additional phenotypic abnormality, such as embryonic lethality.

egl-1 Acts Downstream of or in Parallel to the Genes Involved in Cell Death Specification

To determine at what stage in the well characterized *C. elegans* genetic pathway for programmed cell death *egl-1* acts, we performed epistasis analysis. We first constructed double mutants between *egl-1* and the cell death–specification gene *ces-1*, which acts downstream of the cell death–specification gene *ces-2* (Ellis and Horvitz, 1991). In animals carrying an lf mutation in *ces-1*, the sisters of the NSM neurons in the pharynx undergo programmed cell death (Ellis and Horvitz, 1991); these cells survive in *egl-1(n1084 n3082)* animals (Table 3). We found that the NSM sisters also survived in *ces-1(lf); egl-1(n1084 n3082)* double mutant animals, indicating that the *egl-1(n1084 n3082)* allele is epistatic to the *ces-1*(lf) mutation and suggesting that *egl-1* acts downstream of or in parallel to *ces-1*.

egl-1 Acts Upstream of or in Parallel to *ced-4* and *ced-3*

$P_{mec\text{-}7}$ is a *C. elegans* promoter that becomes activated in a small number of cells, including the six touch cells, neurons involved in mechanosensation (Hamelin et al., 1992). Like *ced-3* or *ced-4* cDNAs (Shaham and Horvitz, 1996a), an *egl-1* cDNA expressed under the control of $P_{mec\text{-}7}$ can induce the *C. elegans* touch cells (including the ALM neurons), which normally survive, to undergo programmed cell death (Table 4). We used this ectopic killing to determine the stage at which *egl-1* acts with respect to *ced-4* and *ced-3*.

In animals homozygous for integrated arrays of $P_{mec\text{-}7}$ *egl-1* ($P_{mec\text{-}7}$ *egl-1* A-C), only about 10% of the ALM neurons were present (Table 4). By contrast, almost all ALMs survived in animals either carrying an extrachromosomal array with the $P_{mec\text{-}7}$ vector (Table 4, $P_{mec\text{-}7}$ A and B) or homozygous for an integrated $P_{mec\text{-}7}$ *lacZ* fusion construct (Shaham and Horvitz, 1996a). The *egl-1*–induced killing of the ALMs was dependent on the number of copies of the *egl-1* gene: 50% of the ALMs survived in animals heterozygous for $P_{mec\text{-}7}$ *egl-1* C ($P_{mec\text{-}7}$ *egl-1* C/+). *egl-1*–induced killing was completely blocked by lf mutations in *ced-4* or *ced-3* (Table 4), confirming that *egl-1*–induced killing involved the known cell death pathway. That *egl-1* required the activities of *ced-3* and *ced-4* to induce killing indicates that *egl-1* functions upstream of or in parallel to *ced-4* and *ced-3*. This conclusion is supported by the observation that the *egl-1*(gf) phenotype, the ectopic deaths of the HSN neurons in hermaphrodites, is suppressed by lf mutations in either *ced-4* or *ced-3* (Trent et al., 1983; Ellis and Horvitz, 1986).

Table 4. *egl-1*–Induced Ectopic Killing Is Suppressed by Mutations that Block Programmed Cell Death

Transgene	% ALMs Surviving (n = 60)
$P_{mec\text{-}7}$ A	98
$P_{mec\text{-}7}$ B	100
$P_{mec\text{-}7}$ *egl-1* A	8
$P_{mec\text{-}7}$ *egl-1* B	9
$P_{mec\text{-}7}$ *egl-1* C	10
$P_{mec\text{-}7}$ *egl-1*C/+	50
$P_{mec\text{-}7}$ *egl-1* C; *ced-9* (gf)	98
$P_{mec\text{-}7}$ *egl-1* C; *ced-4* (lf)	97
$P_{mec\text{-}7}$ *egl-1* C; *ced-3* (lf)	98

Transgenic animals were generated and ALMs in transgenic L1 larvae scored as described in Experimental Procedures. Control lines $P_{mec\text{-}7}$ A and B contained extrachromosomal arrays of $P_{mec\text{-}7}$, and experimental lines contained $P_{mec\text{-}7}$ *egl-1* A–C integrated arrays of the transgene $P_{mec\text{-}7}$ *egl-1*. Mutations used were as follows: *ced-9* (gf) was *ced-9(n1950)*, *ced-4* (lf) was *ced-4(n1162)*, and *ced-3* (lf) was *ced-3(n717)*.

egl-1 Functions through *ced-9*

The lethality caused by lf mutations in the cell death inhibitor gene *ced-9* is suppressed by lf mutations in either *ced-4* or *ced-3*, genes thought to act downstream of *ced-9*. To determine where *egl-1* acts with respect to *ced-9*, we tested the viability of a *ced-9*(lf); *egl-1(n1084 n3082)* double mutant strain. We found that this strain was inviable, indicating that an *egl-1*(lf) mutation did not suppress *ced-9*(lf)–induced lethality. *egl-1* is therefore likely to act upstream of or in parallel to *ced-9*. *egl-1*–induced killing of the ALM neurons (see above) was suppressed by the *ced-9* gf mutation *n1950* (Table 4), supporting this conclusion.

To characterize the role of *ced-9* in *egl-1*–induced cell killing in more detail, we tested whether the block in cell death caused by the *egl-1(n1084 n3082)* allele is affected by a *ced-9*(lf) mutation. If *egl-1* acts through *ced-9*, the *egl-1(n1084 n3082)* mutation should prevent cell death only in the presence of a functional *ced-9* gene. On the other hand, if *egl-1* acts in parallel to *ced-9*, a *ced-9* (lf) mutation might not affect the ability of *egl-1(n1084 n3082)* to block cell death.

Because a *ced-9* null mutation, *ced-9*(0), is lethal (in both an *egl-1*[+] and *egl-1[n1084 n3082]* background), we could not directly compare the effects of a *ced-9*(0) mutation on cell death in *egl-1*(+) and *egl-1(n1084 n3082)* animals. Instead, we did this comparison in the background of the weak *ced-3* lf mutation *n2427*, which suppresses the lethality of a *ced-9*(0) mutation. The *ced-3(n2427)* mutation weakly inhibits programmed cell death and resulted in 1.6 ± 0.8 (average ± standard deviation, n = 15) extra cells in the anterior pharynx. In the background of the *ced-3(n2427)* mutation, as in a *ced-3*(+) background, the *egl-1(n1084 n3082)* allele resulted in 11.1 ± 1.7 extra cells (n = 15). Using a *ced-9*(0); *ced-3(n2427)* double mutant background, we assessed the death-inducing activity of *egl-1* in the absence of *ced-9* function. *ced-9*(0); *ced-3(n2427)* animals carrying the *egl-1*(+) allele had an average of 6.3 ± 2.9 extra cells in the anterior pharynx (n = 15). Mutant animals additionally carrying the *egl-1(n1084 n3082)* allele had an average of 7.0 ± 1.5 extra cells (n = 15). Thus, the presence of *egl-1* activity did not affect programmed cell death in a *ced-9*(0) animal, indicating that *ced-9* must be functional for *egl-1* to exert its effect on programmed cell death and hence that *egl-1* acts upstream of *ced-9*.

In the *ced-3(n2427)* background, *ced-9*(+) animals had 1.6 ± 0.8 extra cells, and *ced-9*(0) animals had 6.3 ± 2.9 extra cells in the anterior pharynx. This reduction in cell death associated with a reduction in *ced-9* activity in a *ced-3*(weak) background has been reported previously and has been interpreted as suggesting that *ced-9* might not only have a cell death protective activity but also a cell death promoting activity (Hengartner and Horvitz, 1994a).

EGL-1 and CED-9 Physically Interact

The mammalian BH3-containing proteins Bik, Bid, Harakiri, and Bad physically interact with members of the Bcl-2 family of proteins (Boyd et al., 1995; Yang et al., 1995; Han et al., 1996a; Wang et al., 1996; Inohara et al., 1997; Kelekar et al., 1997; Ottilie et al., 1997b; Zha et al., 1997). To determine whether the EGL-1 and CED-9 proteins can directly bind to each other, we first used a yeast two-hybrid system based on the DNA-binding and transactivation domain of the yeast GAL4 protein (Bai and Elledge, 1996). Clones transformed with either the EGL-1-GAL4 transactivation fusion or the CED-9-GAL4 DNA-binding domain fusion alone did not turn blue even after extended periods of incubation using a β-galactosidase–filter assay and had no detectable β-galactosidase activity when measured more quantitatively in liquid (0.2 ± 0.2 and 0.1 ± 0.1 units/OD600 ± standard deviations, respectively). By contrast, clones transformed with both fusions turned blue in fewer than 30 min and had 32.0 ± 2.7 units of β-galactosidase activity/OD600, suggesting that the EGL-1 protein and the CED-9 protein interacted in this system. We confirmed this result by examining the interaction of these two proteins in vitro. Glutathione S-transferase (GST)–tagged CED-9 (GST-CED-9) or GST alone was affinity purified and incubated with in vitro transcribed and translated ^{35}S-methionine–labeled tagged EGL-1 protein (^{35}S-S·TAG-EGL-1). GST-CED-9 or GST alone was then reisolated and analyzed for associated ^{35}S-S·TAG-EGL-1 by SDS-PAGE and autoradiography. As shown in Figure 3A, GST-CED-9 interacted with S·TAG-EGL-1 but not with any of the control proteins tested, such as S·TAG alone, luciferase, or the yeast protein SSN6. Furthermore, incubation of GST alone with S·TAG-EGL-1 did not result in binding, indicating that the observed binding is specific for the CED-9 and EGL-1 proteins.

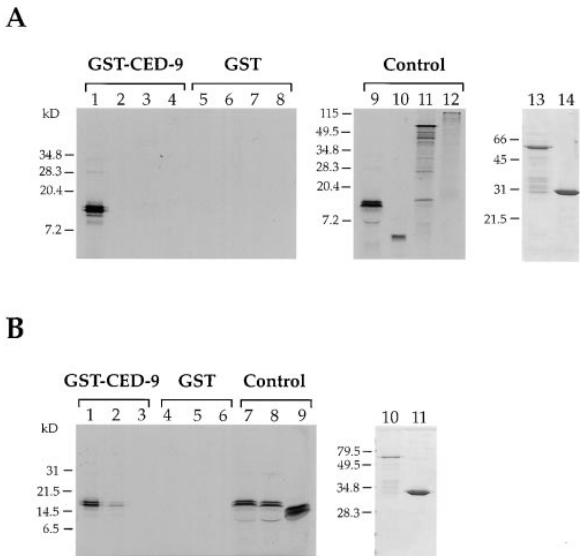

Figure 3. EGL-1 and CED-9 Interactions

(A) Wild-type CED-9 and EGL-1 proteins interact in vitro. ^{35}S-labeled S·TAG-EGL-1 (lanes 1 and 5; 14.5 kDa), S·TAG alone (lanes 2 and 6; 6.7 kDa), luciferase (lanes 3 and 7; 61 kDa), or SSN6 (lanes 4 and 8; 107 kDa) were incubated with GST-CED-9 (lanes 1–4; 60 kDa) or GST alone (lanes 5–8; 27 kDa) and analyzed for binding by SDS-PAGE and autoradiography as described in Experimental Procedures. Lanes 9–12 represent 10% of the input of S·TAG-EGL-1 (lane 9), S·TAG (lane 10), luciferase (lane 11), and SSN6 (lane 12). Lanes 13 and 14 represent 60% of GST and 40% of GST-CED-9 protein used per binding reaction (12% SDS-PAGE, stained with Coomassie blue).

(B) The interaction between CED-9 and EGL-1 depends on the BH3-like region of EGL-1. ^{35}S-labeled S·TAG-EGL-1 (lanes 1 and 4), S·TAG-EGL-1 with a deletion of the BH3-like region (S·TAG-EGL-1 ΔBH3) (lanes 2 and 5; 13.4 kDa), or S·TAG-EGL-1 protein encoded by the *egl-1(n1084 n3082)* allele (which causes a frameshift and premature termination) (S·TAG-EGL-1 trunc) (lanes 3 and 6; 11.2 kDa) were incubated with GST-CED-9 (lanes 1–3) or GST alone (lanes 4–6) and analyzed for binding. Lanes 7–9 represent 10% of the input of S·TAG-EGL-1 (lane 7), S·TAG-EGL-1 ΔBH3 (lane 8), or S·TAG-EGL-1 trunc (lane 9). Forty percent of the GST-CED-9 and GST proteins are shown in lanes 10 and 11 (15% SDS-PAGE, stained with Coomassie blue).

The BH3 domain is involved in the binding of BH3-containing proteins to members of the Bcl-2 family of proteins (Han et al., 1996b; Wang et al., 1996; Inohara et al., 1997; Kelekar et al., 1997; Ottilie et al., 1997b; Zha et al., 1997). To analyze the role of the BH3-like region of EGL-1 in the interaction between EGL-1 and CED-9, we tested mutant EGL-1 proteins in the in vitro binding assay. EGL-1 protein that lacks the nine residues similar to the BH3 domain of Bcl-2–like and BH3-containing proteins (S·TAG-EGL-1 ΔBH3) had a reduced ability to bind to GST-CED-9 (Figure 3B). The interaction of

S·TAG-EGL-1 with GST-CED-9 was completely eliminated by the introduction into the EGL-1 protein of the *egl-1*(lf) mutation *n3082* (S·TAG-EGL-1 trunc), which, as described above, is a 5 bp deletion predicted to result in the formation of a truncated EGL-1 protein lacking the putative BH3 domain and the C terminus of the protein. These results suggest that the BH3-like region of EGL-1 is required for efficient binding of EGL-1 to CED-9.

Discussion

Why Were *egl-1*(lf) Alleles Not Identified Previously?

By isolating a lf mutation in *egl-1*, we have identified a fourth component of the central cell death machinery in *C. elegans*. Previous screens that could have identified mutants with a general cell death block like that of *egl-1 (n1084 n3082)* yielded a gf mutation in *ced-9* and numerous lf mutations in *ced-3* and *ced-4* but no lf mutation in *egl-1* (Ellis and Horvitz, 1986; Hengartner et al., 1992; Horvitz et al., unpublished observations). The previous failure to isolate such mutations might be explained in a number of ways.

egl-1 is only about one-fifth the size of the average *C. elegans* gene and hence provides a small target. Nonetheless, a large number of EMS-mutagenized haploid genomes have been screened (we examined at least 45,000 such genomes; our unpublished results), and given that the average gene mutates to lf at a frequency of about 1 per 2,000 EMS-mutagenized haploid genomes (reviewed by Anderson, 1995), *egl-1* target size alone seems unlikely to be responsible. Perhaps the small *egl-1* gene contains no sites readily mutable by EMS, which predominantly induces GC-to-AT transition mutations and mutates different GC base pairs at very different frequencies (Coulondre and Miller, 1977).

Alternatively, as described above, the *n3082* mutation might not be a null mutation, and an *egl-1* null mutation might cause a phenotype in addition to the block in programmed cell death, such as embryonic lethality. The screens performed previously for mutants with a general cell death block would not have identified inviable cell death defective mutants. It is also conceivable that *n1084 n3082* is an altered-function (neomorphic) allele and results in an *egl-1* gf phenotype such that the novel mutant EGL-1 protein interferes with the pathway for programmed cell death. This possiblity seems unlikely for three reasons: gene-dosage experiments suggest that *n1084 n3082* is an lf allele; deficiencies that span *egl-1* when homozygous lead to a block in cell death; and the ectopic expression of wild-type *egl-1* can induce cell death.

Another possibility is that the *egl-1(n1084 n3082)* chromosome contains an additional mutation that in combination with this *egl-1* allele prevents programmed cell death. If so, based upon our analyses of the phenotype of *egl-1(n1084 n3082)*/deficiency heterozygotes, this second gene would have to be located within a region of less than 0.5 map units on the genetic map and about 1 Mb on the physical map (our unpublished observations).

We prefer the possibilities that either the *egl-1* gene is a poor target for EMS or that the *egl-1* null phenotype is not simply a block in cell death but includes other defects, such as embryonic lethality.

egl-1 Might Integrate Cell Death–Inducing Signals

The *egl-1* gene is the earliest acting component of the central cell death machinery identified in *C. elegans* so far. *egl-1* functions upstream of *ced-9*, *ced-4*, and *ced-3* and downstream of or in parallel to *ces-2* and *ces-1*, two *C. elegans* genes thought to be involved in specifying the cell death fate of a subset of cells, such as the sister cells of the pharyngeal NSM neurons (Figure 4A). It is possible that *egl-1* is a direct target of *ces-1* and/or of *ces-2*, which encodes a protein with a bZIP (basic region/leucine zipper) DNA-binding domain similar to that of the human hepatic leukemia factor (HLF) (Metzstein et al., 1996). gf mutations in *egl-1* lead to the deaths of the HSN neurons, which are required for egg laying and which normally survive in hermaphrodites but undergo programmed cell death in males (Sulston and Horvitz, 1977; Sulston et al., 1983). This phenotype might be caused by a defect in the regulation of *egl-1* activity by a *ces-1*– or *ces-2*–like factor that normally specifies the sexually dimorphic life-versus-death fate of the HSNs. The *egl-1* gene thus appears to act at the point of the cell death pathway at which signals that determine the cell death fate in *C. elegans* are integrated to specify which cells activate the process of programmed cell death.

EGL-1 May Act by Antagonizing CED-9

EGL-1 might be a new member of the BH3 only–containing group of cell death activators, which includes Bik, Bid, Harakiri, and Bad (Boyd et al., 1995; Yang et al., 1995; Han et al., 1996a; Wang et al., 1996; Inohara et al., 1997; Kelekar et al., 1997; Ottilie et al., 1997b; Zha et al., 1997). Various models have been proposed for how BH3 only–containing cell death activators induce cell death. In the cases of Bik, Harakiri, and Bad, for example, all of which bind to Bcl-2–like cell death inhibitors, deletions of, or mutations within the BH3 domains reduce or eliminate both their abilities to bind to Bcl-2–like cell death inhibitors and to induce cell death (Chittenden et al., 1995; Han et al., 1996a; Inohara et al., 1997; Kelekar et al., 1997; Zha et al., 1997). This result suggests that Bik, Harakiri, and Bad induce death by binding to and blocking Bcl-2–like cell death inhibitors. The situation appears different in the case of Bid, which binds to both the cell death inhibitor Bcl-2 and to the cell death activator Bax (Wang et al., 1996). Analysis of the BH3 domain of Bid resulted in the identification of mutations that block the ability of Bid to bind to Bcl-2 but do not affect its capacity to bind to Bax and to induce cell death, and vice versa. These data favor a model in which a Bid/Bax heterodimer is the functional cell death effector, which might be antagonized by the cell death inhibitor Bcl-2.

How might EGL-1 function to engage the cell death machinery and cause cell death in *C. elegans*? Our data indicate that EGL-1 requires the activities of CED-4 and of the caspase CED-3 to trigger cell death and that EGL-1 acts through the Bcl-2–like protein CED-9 (Figure

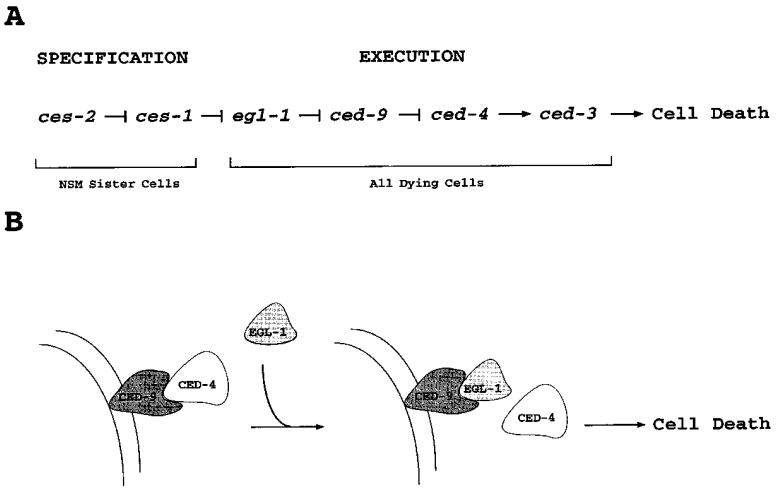

Figure 4. The Function of *egl-1* in Programmed Cell Death in *C. elegans*

(A) The genetic pathway for programmed cell death in *C. elegans*. *egl-1* appears to act between the cell death specification gene *ces-1* and the anti–cell death gene *ced-9*.

(B) Molecular model for the role of EGL-1 in initiating programmed cell death. EGL-1 displaces CED-4 from the membrane-bound cell death inhibitor CED-9, thereby allowing CED-4 to trigger downstream events required for the killing process.

4A). It has been suggested that CED-9 and CED-4 exist in a membrane-associated complex and that the association of CED-9 with CED-4 in this complex prevents CED-4 from activating the caspase CED-3 and initiating programmed cell death (reviewed by Jacobson, 1997; Vaux, 1997). We propose that EGL-1 triggers cell death by binding to CED-9. This interaction might result in the displacement of CED-4 from a membrane-associated complex, thereby allowing CED-4 to initiate cell death (Figure 4B). EGL-1 might therefore act to antagonize the ability of CED-9 to block CED-4.

This model is based on the following observations. First, EGL-1 interacts with CED-9, both in a two-hybrid system and in vitro. This interaction is abolished by the *egl-1*(lf) mutation *n3082*, which causes a Ced phenotype in vivo. These observations are consistent with the hypothesis that the ability of EGL-1 to bind to CED-9 is required for the ability of EGL-1 to induce killing in vivo. Second, it seems likely that CED-9 cannot bind to EGL-1 and CED-4 simultaneously. The BH1, BH2, and BH3 domains of Bcl-2 are required for Bcl-2 to interact with Bax (Yin et al., 1994; Hunter et al., 1996; Zha et al., 1996; Diaz et al., 1997), Bak (Chittenden et al., 1995), and Bad (Zha et al, 1997), and the BH1, BH2, and BH3 domains of Bcl-x_L are necessary for the interaction of Bcl-x_L with Bak (Chittenden et al., 1995) and Bad (Kelekar et al., 1997). Furthermore, when complexed with a Bak-derived BH3 peptide, the BH1, BH2, and BH3 domains of Bcl-x_L form an elongated hydrophobic pocket, which acts like a receptor that binds the Bak BH3 domain as a ligand (Muchmore et al., 1996; Sattler et al., 1997). Considering the overall sequence similarities among Bcl-2, Bcl-x_L, and CED-9 and the fact that EGL-1 contains a region similar to the BH3 domains of Bax, Bak, and Bad, it seems likely that the interaction between CED-9 and EGL-1 is mediated by the BH1, BH2, and BH3 domains of CED-9. The sequence requirement of CED-9 for its interaction with CED-4 has recently been analyzed using two-hybrid analysis (Ottilie et al., 1997a), and the results suggest that all four BH domains of CED-9 are necessary for CED-9 to bind efficiently to CED-4. Together, these observations suggest that the CED-9–binding sites for EGL-1 and CED-4 might be largely overlapping. That CED-4 binding to cell death inhibitors of the Bcl-2 family can be disrupted by the presence of BH3-containing cell death–activating proteins or peptides is indicated by the observation that the interaction between Bcl-x_L and CED-4 in vitro can be blocked by Bax, Bak, Bik, or a Bak-derived BH3 peptide (Chinnaiyan et al., 1997a; Ottilie et al., 1997a).

Our studies demonstrate that EGL-1 plays a major role in controlling programmed cell death in *C. elegans* by acting as a negative regulator of the Bcl-2–like cell death inhibitor CED-9. Although we believe that EGL-1 induces cell death by displacing CED-4 from CED-9, this model may be an oversimplification, since additional Bcl-2–like molecules might exist in *C. elegans*, an anti-apoptotic isoform of CED-4, called CED-4L, has been described (Shaham and Horvitz, 1996b), and CED-9 can act as a substrate for the CED-3 caspase (Xue and Horvitz, 1997). These considerations indicate that the control of programmed cell death in *C. elegans* may well be more complex.

Experimental Procedures

General Methods and Strains

C. elegans strains were maintained as described by Brenner (1974). The wild-type strain generally used was N2 (Bristol). The wild-type strain RW7000 (Bergerac) was used in conjunction with N2 for RFLP analysis. Mutations used in this study are listed below and are described by Riddle et al. (1997), except where noted otherwise. LGI: *ced-1(e1735)*, *ces-1(n703 n1434)* (Ellis and Horvitz, 1991). LGIII: *ced-4(n1162)*, *ced-9(n1950)*, *ced-9(n2812)* (Shaham and Horvitz, 1996b). LGIV: *ced-3(n717)*; *ced-3(n2427)* (M. O. Hengartner and H. R. H., unpublished results). LGV: *unc-42(e270)*, *rol-4(sc8)*, *egl-1(n1084)*, *egl-1(n1084 n3082)* (this study), *unc-76(e911)*. Deficiencies used were as follows: *nDf42* (M. O. Hengartner and H. R. H., unpublished results), *yDf8* and *yDf11* (DeLong et al., 1993), *itDf2* (K. Kemphues, personal communication), *zuDf2* (Zhu et al., 1997), and *lwDf3* (M. Moseley and J. Shaw, personal communication). Allele designations for the integrated lines are as follows: $P_{mec\text{-}7}$ *egl-1*A is *nIs94*; $P_{mec\text{-}7}$ *egl-1*B is *nIs95*; and $P_{mec\text{-}7}$ *egl-1*C is *nIs91*.

Mapping of *egl-1(n1084 n3082)*

Standard genetic techniques were used to map *egl-1(n1084 n3082)* to LGV between *rol-4* and *unc-76* (Brenner, 1974; Williams et al., 1992). Various cosmids in the *rol-4* to *unc-76* interval were tested for their abilities to detect RFLPs between N2 and RW7000. Cosmid T20C8 was found to detect a RFLP that we named *nP88* (EcoRI digest) (our unpublished result). The position of *n1084 n3082* with respect to *nP88* and *stP6* was determined as described by Ruvkun

et al. (1989). Briefly, recombinants for LGV between N2 and RW7000 were obtained by screening the progeny of *rol-4(sc8) egl-1(n1084 n3082) unc-76(e911)* (N2)/+ (RW7000) animals for Rol non-Unc and Unc non-Rol animals. Homozygous recombinants were scored for *n1084 n3082*, *nP88*, and *stP6*.

Transgenic Animals

Germline transformation experiments were performed as described by Mello and Fire (1995). For *egl-1(n1084 n3082)* rescue with genomic DNA, we injected *ced-1(e1735); egl-1(n1084 n3082) unc-76(e911)* animals with genomic DNA contained in cosmids or plasmids (1–10 μg/ml) and the coinjection marker p76-16B, which rescues *unc-76* (50 μg/ml) (Bloom and Horvitz, 1997). Non-Unc transgenic F1 animals were identified and lines established. For *egl-1(n1084 n3082)* rescue with the *egl-1* cDNA under the control of the *C. elegans* heat-shock promoters (P_{hsp}), we injected *ced-1(e1735); egl-1(n1084 n3082) unc-76(e911)* animals with the 276 bp cDNA transcriptionally fused to P_{hsp} in the vectors pPD49.78 and pPD49.83 (50 μg/ml) (Mello and Fire, 1995). For ectopic expression of the *egl-1* cDNA in the touch cells, the same cDNA was transcriptionally fused to the *C. elegans mec-7* promoter (P_{mec-7}) contained in pPD52.102 and injected into *unc-76(e911)* animals (50 μg/ml). Extrachromosomal arrays carrying the P_{mec-7}*egl-1* fusion and the p76-16B coinjection marker were integrated into the genome by irradiating transgenic animals with γ-rays or UV.

Molecular Analysis

Standard molecular biology protocols were used as described by Sambrook et al. (1989), unless otherwise noted. The sequences of genomic DNA around the *egl-1* locus were determined by the *C. elegans* Genome Consortium, and primers used throughout this study were designed based on this sequence. The 2.5 kb minimal rescuing fragment was used to screen 4.5×10^6 clones of an embryonic cDNA library (Okkema and Fire, 1994). The DNA sequences of the PCR-amplified inserts of the two clones identified (OE8, OE18) were determined using an automated ABI 373A DNA sequencer (Applied Biosystems). RT-PCR analysis was done using reverse transcription of embryonic poly(A)+ RNA, followed by PCR amplification. To determine the 5′ end of the *egl-1* transcript, we used the 5′ RACE system as described by the manufacturer (GIBCO-BRL) and embryonic poly(A)+ RNA. The sequences of mutant alleles were determined from PCR-amplified genomic DNA.

Microscopic Analysis of Mutant and Transgenic Animals

To analyze the Egl phenotype, L4 larvae were picked and visually scored 24 hr later for the accumulation of eggs inside their body using a dissecting microscope. For the analysis of the Ced phenotype or the presence of NSM sisters, HSNs, or ALMs, animals were anesthetized with 50 mM sodium azide and observed using Nomarski optics (Sulston and Horvitz, 1977). Extra cells in the anterior pharynx and NSM sisters were counted in L3 larvae as described (Hengartner et al., 1992; Ellis and Horvitz, 1991). The presence of HSNs and ALMs was determined in L1 larvae. For rescue experiments, the presence of corpses was analyzed in stage 1 1/2-fold embryos.

Yeast Two-Hybrid System

Full-length cDNAs of *ced-9* and *egl-1* were cloned into the vector pAS1 (*ced-9*) to create a CED-9-GAL4 DNA-binding domain fusion (Z. Zhou and H. R. H., unpublished results) or into pACT2 (*egl-1*) to create an EGL-1-GAL4 transactivation domain fusion (pAS1, pACT2; Bai and Elledge, 1996). Yeast strain Y190 (Bai and Elledge, 1996) was cotransformed with these two plasmids using standard protocols (Rose et al., 1990). Transformants were selected on SD medium lacking tryptophan and leucine (Rose et al., 1990). β-galactosidase activity was assayed on filters or in solution as described by Bai and Elledge (1996).

In Vitro Binding Assay

The ΔBH3 deletion and *n3082* mutation were introduced into the *egl-1* cDNA by primer-mediated mutagenesis as described (Higuchi, 1990). The sequences of all constructs generated by PCR amplification used throughout this study were confirmed by DNA sequencing. Wild-type and mutant *egl-1* cDNAs were cloned into vector pCITE-4a(+) (Novagen) such that the resulting fusion protein was tagged with the S·TAG (Novagen) and transcribed and translated in vitro in the presence of ^{35}S-methionine using a T7-based coupled reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega). For GST-CED-9, the *ced-9* cDNA was cloned into vector pGEX-4T-3 (Pharmacia), expressed in BL21(DE3) cells, and purified using glutathione sepharose as suggested by the manufacturer (Pharmacia). ^{35}S-labeled S·TAG-EGL-1 and purified GST-CED-9 were incubated for 30–60 min at 4°C in 500 μl binding buffer (142.5 mM KCl, 10 mM HEPES [pH 7.6], 5 mM $MgCl_2$, 1 mM EDTA, 0.25% IGEPAL CA-630/Sigma, 2.5 mg/ml BSA). Protein complexes were washed 3× with 1 ml binding buffer, 1× with 1 ml binding buffer without BSA, resuspended in 25 μl of 2× sample buffer, and analyzed by SDS-PAGE (15%) and autoradiography.

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