Caenorhabditis elegans anti-apoptotic gene ced-9 prevents ced-3-induced cell death in Drosophila cells

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INTRODUCTION

Programmed cell death plays a significant role in morphogenesis and histogenesis during animal development. The genetic pathway of programmed cell death has been most intensively studied in Caenorhabditis elegans (C. elegans). Three C. elegans genes play major roles in controlling the execution of the cell death program (Miura and Yuan, 1996). ced-3 and ced-4 are required for all programmed cell death in C. elegans. The ced-9 gene represses programmed cell death induced by ced-3 and ced-4 (Shaham and Horvitz, 1996a). Genetic studies of cell death in C. elegans favor the idea that ced-9 acts upstream of ced-3 (Shaham and Horvitz, 1996b), and that the anti-apoptotic function of the ced-9 gene product partly depends on the ced-4 product (Shaham and Horvitz, 1996a).

The amino acid sequence of CED-3 is homologous to the mammalian interleukin-1β converting enzyme (ICE), with 28% amino acid identity (Yuan et al., 1993). Overexpression of ice or ced-3 induces cell death in rat fibroblasts (Miura et al., 1993). So far, all caspase family members tested, including ced-3, can induce cell death when overexpressed in cultured cells. In Drosophila, at least two caspase family members (DCP-1 and drICE) have been reported (Song et al., 1997; Fraser and Evan, 1997). Both overexpression of drICE in Drosophila Schneider’s L2 (SL2) cells and of DCP-1 in HeLa cells causes apoptosis, suggesting the caspase family plays an evolutionarily conserved role across the phylogenetic scale.

In mammalian 293T cells and insect Spodoptera frugiperda IPLB-SF21 (Sp-21) cells, CED-4 stimulates CED-3-induced cell death, suggesting CED-4 promotes apoptosis in mammalian and insect cells (Chinnaiyan et al., 1997a,b; Seshagiri and Miller, 1997; Wu et al., 1997a,b). CED-4 was shown to facilitate CED-3 auto-activation in vitro (Chinnaiyan et al., 1997b), suggesting a biochemical role for CED-4 in the activation of CED-3. A physical association of CED-4 with both CED-3 and CED-9 has been reported (Chinnaiyan et al., 1997a: Wu et al., 1997a,b). CED-9 inhibits CED-4 activity by direct interaction (Seshagiri et al., 1997). These observations suggest the hypothesis that molecules functionally equivalent to CED-4 are present in cells where CED-9 can reduce CED-3-induced cell death.

ced-9 encodes a 280 amino acid protein that is 23% identical to the human BCL-2 proto-oncogene product (Hengartner and...
Horvitz, 1994). Overexpression of bcl-2 has been shown to protect cells from apoptosis in a number of systems including cell death induced in certain hematopoietic cell lines by cytokine deprivation and neuronal cell death induced by neurotrophic factor withdrawal (Yang and Korsmeyer, 1996). Overexpression of wild-type ced-9 in C. elegans not only prevents the ectopic cell death seen in the ced-9 (lf) mutant, but also many of the programmed cell deaths that are normally observed during C. elegans development (Hengartner and Horvitz, 1994). Whereas overexpression of human bcl-2 can partially prevent ectopic cell death in the ced-9 (lf) mutant as well as normal programmed cell death in C. elegans (Hengartner and Horvitz, 1994; Vaux et al., 1992), ced-9 function has not yet been tested in other organisms.

To understand the evolutionarily conserved cell death pathway, it is important to know if ced-9 functions to prevent ced-3-induced cell death in cells derived from other organisms such as mammals and Drosophila. In C. elegans, ced-4 is required for the anti-apoptotic function of ced-9 against ced-3-induced cell death. Thus, if the ced-9 gene product can prevent cell death induced by ced-3 in other organisms, it might suggest that the ced-9 gene product utilizes a ced-4 like gene activity in other organisms. To investigate this possibility, we performed a transient transfection assay to examine the protective effects of the ced-9 gene in mammalian and Drosophila cell lines. First we examined the cytoplasmic localization of CED-9 in COS cells and Drosophila SL2 cells, and found that CED-9 and BCL-2 were co-localized in the cytoplasm. Overexpression of ced-9 was only slightly effective in preventing ced-3-induced death of HeLa cells, but its protective ability was significant in Drosophila cells. Furthermore, ced-9, bcl-2 and bcl-xL partially protected cultured Drosophila cells from apoptosis induced by a Drosophila cell death gene, reaper (rpr). Our results suggest molecular components required for the anti-apoptotic function of ced-9 against ced-3 are conserved from worm to Drosophila.

MATeRIALS AND METHOds

Cell cultures

COS and HeLa cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 100 units of penicillin/ml and 0.1 mg of streptomycin/ml (IBL) at 37°C and 5% CO2. SL2 cells (Schneider, 1972) were cultured in Schneider’s insect medium (Sigma) with 10% heat-inactivated fetal calf serum. For the transfection experiments, we used the insect cell line SL2, which was kindly provided by Dr. S. J. Elledge (Durfee et al., 1993).

Plasmid construction

Both ends of the chimeric gene, ced-3-lacZ (Miura et al., 1993), were changed into XhoI sites by linker ligation. The fragment was cloned into pcDNA3 (Invitrogen) and the resulting construct was named pHS3. pβactM10Z was previously described (Miura et al., 1993), ced-3-lacZ was also cloned into the XhoI site of pCaspeR-hs (Thummel and Pirrotta, 1991); this construct was named pM132. pcDNA3 (Invitrogen) -ice-lacZ, named pA10, was previously described (Hisahara et al., 1997). The DNA fragment of reaper (rpr) was obtained by reverse transcription PCR (RT-PCR) as follows. Total RNA from 8-12 hour Drosophila melanogaster embryos was isolated using TRizol RNA Isolation reagent (Life Technologies) and purified to poly(A)+ RNA with Oligotex-dT30 (JSR). First strand cDNA was synthesized using 0.5 µg of RNA, 35 picomoles of random deoxyxynucleotide hexamers (Takara), first strand RT buffer (Gibco BRL), 0.1 M DTT (Gibco BRL), and 2.5 mM dNTPs (Gibco BRL) for 60 minutes at 37°C in the presence of 14 U RNase inhibitor (Promega) and 100 units MoMLV reverse-transcriptase (Promega) in a 20 µl reaction volume. The primer sequences used for RT-PCR are as follows and were based on the sequence data reported by White et al. (1994).

Forward: 5’-CGGAAATTCATGGCGATGGCATTTGCTA-3’,
Reverse: 5’-CTCTAGATCTCGGATCGTCTGCGATA-3’.

The following conditions were used for the PCR reaction: 1× PCR buffer (Gibco, BRL), 0.2 mM dNTPs, 25 picomoles of each primer, 1.5 mM MgCl2, and 1 unit of Taq DNA polymerase (Gibco, BRL) in a total volume of 50 µl. DNA was denatured at 94°C for 1.5 minutes, annealed at 50°C for 2.5 minutes, and elongated at 72°C for 2 minutes for 30 cycles. The resulting fragment was subcloned into pT7Blue T-vector (Novagen). This plasmid was digested with Xhol, blunt-ended with the Klenow fragment of DNA polymerase, and then digested with EcoRI. This rpr fragment was ligated into EcoRI-Stul-digested pCaspeR-hs (Thummel and Pirrotta, 1991), which we named pH75. pβactSTneoB-bcl-2 was described previously (Gaglardi et al., 1994). pBabe/puro-bcl-2 was described previously as pH436 (Miura et al., 1993). The bcl-2 fragment was isolated from pH436. pH436 was digested with Soti, blunt-ended with Klenow, and then digested with EcoRI. The bcl-2 fragment was cloned into pCaspeR-hs; we named this construct pH82. pβactSTneoB-hcl-XL and pcDNA3-T7-tag containing cermA were previously described as pH63 and pH38 (Hisahara et al., 1997). pH63 was digested with EcoRI and cloned into pCaspeR-hs; we named this construct pM134. pH75 has been shown to protect cultured cells from apoptosis induced by bcl-2 (Hasahara et al., 1997). pM63 was digested with EcoRI and cloned into pCaspeR-hs; we named this construct pH134. Total RNA was isolated from C. elegans using TRIsol (Life Technologies, Inc.) and full-length ced-9 cDNA was isolated by RT-PCR. First strand cDNA was synthesized as described above using C. elegans mRNA. The primers used for PCR to amplify ced-9 cDNA were: M1: 5’-TTGAAATTCGAGATGACGCGCTCAGGCGGGG-3’, M2: 5’-GGGAATTCGGTACTTCAAGCTGAACATCATCAT-3’. The PCR was performed by using pfu polymerase (Stratagene). The DNA was denatured at 94°C for 1.5 minutes, annealed at 55°C for 2.5 minutes, and elongated at 72°C for 2 minutes for 25 cycles. The PCR product was cloned into the EcoRI site of pBluescript. This construct was named pH61. The same vector containing the PCR product in the reverse orientation was named pH60. ced-9 cDNA was cloned into the EcoRI site of pCaspeR-hs (named pM135). Both ends of the ced-9 cDNA were changed to NotI sites by linker ligation and cloned into pBactSTneoB. The resulting plasmid was named pH64. The HA epitope-containing plasmid, pAS1-CYH2, was kindly provided by S. J. Elledge (Durfee et al., 1993). It was digested with BamHI, and blunt ended using Klenow. The ced-9-containing plasmid, pH60, was digested with Smal and HindIII, blunt ended using Klenow, and cloned into pAS1-CYH2; the resulting plasmid was named pH1 and used as a template. PCR was performed by using synthetic primers (pH3 and pH4) and their sequences were as follows: pH3: 5’-TTTCGAGAACATGGCTTACCATACGAT-3’, pH4: 5’-CCCTGCAGCCTTACCTTACCACTGAT-3’. The amplified fragment was cloned into the XhoI site of pcDNA3, and the resulting construct was named pH54. HA-tagged ced-9 was isolated from pH54 by digesting with EcoRI and XhoI, and this fragment was cloned into pCaspeR-hs. The resulting plasmid was named pH133. A p35/p53/ced-9 DNA was kindly provided by V. M. Dixit (Beidler et al., 1995). A p35 EcoRI/XhoI fragment was isolated and cloned into pCaspeR-hs, and this construct was named pH76. lacZ fragment was isolated by digesting pacBlgI (Miura et al., 1993) with Ncol/BamHI, blunt ended by Klenow, and then cloned into pCaspeR-hs. The resulting control plasmid was named pCas64Z.

Transfection and functional studies

COS and HeLa cells were seeded at a density of about 2.5×105 in 6-well plates the day before transfection. For each well, 0.3 µg each of expression vectors together with pcDNA3 (the total amount of DNA was 1 µg) were mixed with 8 µg of Lipofectamine reagent (Gibco BRL). Cells were incubated for 4 hours in serum-free medium containing the plasmid mixture, then cells were washed and incubated with serum-containing medium for 48 hours. Cells were fixed and
stained with X-Gal solution for 5 hours as described previously (Miura et al., 1993). A caspase-family protease inhibitor, benzylxycarbonyl-Asp-CH₂OC(0)-2,6,-dichlorobenzene (Z-Asp-CH₂-DCB; Dolle et al., 1994), at a final concentration of 40 μg/ml, was added 3 hours after the beginning of transfection. Cells were incubated for 24 hours and stained with X-Gal buffer. For transfecting SL2 cells, DNA and 4 μg of Cellfectin reagent (Gibco BRL) were mixed, added to cells in serum-free SFM medium (Life Technology) and incubated for 4 hours, then the cells were incubated with serum-containing medium. One day after transfection, the cells were heat-treated at 37°C for 30 minutes, and allowed to recover at 27°C for 30 minutes. This heat shock treatment was repeated twice. One day after heat-treatment, the cells were fixed and stained using an X-Gal solution and 10 μM Hoechst 33342 dye (Sigma). Samples were mounted using PermaFluor™ Aqueous Mounting Medium (Immunon) and the X-Gal positive cells were examined with a Leica fluorescence microscope DMRD.

**Production of anti-CED-9 antibody**

The *ced-9* cDNA fragment was subcloned into the EcoRI site of pGEX-4T-1 (Pharmacia). The resulting plasmid was named pK3. The plasmid was transformed into DH5α, and incubated overnight. Bacterial cells were diluted 1:10 into LA medium and incubated for 2 hours, then IPTG was added to a concentration of 0.1 mM. Cells were incubated for 3 hours at 37°C, then collected by centrifugation. Bacterial cells were resuspended in SDS-sample buffer and proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following Coomassie brilliant blue (CBB) staining, the GST-CED-9 band was cut out of the gel. Proteins were electroeluted from the gel slices. Antigen emulsion was made from the protein (100 μg) and complete Freund adjuvant (Sigma) using an ultrasonicator. Female 8-week-old BALB/c mice were immunized subcutaneously. Booster immunizations were given 2 and 5 weeks later, and blood was collected 1 week after the final booster immunization.

**Immunocytochemistry**

Two constructs, pcDNA3-bcl-2-HA and pcDNA3-HAced-9 (pH54), were separately or simultaneously transfected with COS cells as described above. Two days after transfection, cells on coverslips were washed with 1x PBS three times, fixed in 4% paraformaldehyde/1x PBS for 10 minutes, and permeabilized in 0.1% Triton X-100/1x PBS for 10 minutes at room temperature. Cells were blocked with 4% normal goat serum in PBS (blocking buffer) for 10 minutes at room temperature. Then they were incubated with monoclonal anti-HA antibody (12CA5, 1:200, BABCO) for an hour at room temperature and washed with PBS.

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**Fig. 1.** Co-localization of CED-9 with BCL-2 in COS and SL2 cells. (A) bcl-2 was transfected into COS cells. Cells were labeled with anti-BCL-2 monoclonal antibody. (B) HA-taggedced-9 was transfected and cells were labeled with anti-HA monoclonal antibody. (C) COS cells were transfected with a combination of bcl-2 and HA-tagged ced-9. Transfection and fixation are described in Materials and Methods. Cells were stained with anti-BCL-2 and anti-HA antibody. The arrowheads indicate spots where CED-9 co-localized with BCL-2. (D) bcl-2 was transfected into SL2 cells. Cells were labeled with anti-BCL-2 monoclonal antibody. (E) ced-9 was transfected and cells were labeled with anti-CED-9 polyclonal antibody. (F) SL2 cells were transfected with a combination of bcl-2 and ced-9. Cells were stained with anti-BCL-2 and anti-CED-9 antibodies. The arrowheads indicate spots where CED-9 co-localized with BCL-2. Bars: 10 μm (in A for A,B; in F for D,E,F).
transient transfection experiments, ced-3-induced cell death was most effectively prevented by p35 and Z-Asp-CH₂-DCB, bcl-2, bcl-X₁, and crmA. These data are in agreement with previous results that show that p35 can reduce the number of programmed cell deaths in C. elegans most effectively (Hengartner and Horvitz, 1994; Vaux et al., 1992; Sugimoto et al., 1994; Xue and Horvitz, 1995). These results suggested our transient transfection assay would be suitable to examine the function of CED-9.

To examine the function of ced-9 in suppressing ced-3-induced apoptosis in mammalian cells, we performed a transient transfection assay as described above (Fig. 2A). We could not detect significant anti- or pro-apoptotic function of ced-9 on ced-3-induced cell death in HeLa cells. We also examined the function of ced-9 by using a neuronal cell line, NG108-15, and obtained the same results as in HeLa cells (data not shown).

It is possible that ced-9 cannot exert its normal function at 37°C because 37°C is a non-permissive temperature in C.
elegans. To eliminate this possibility, we did the same transient assay at 27°C. Even in this condition, ced-9 still failed to prevent ced-3-induced cell death in these cell lines (Fig. 2B). These results suggest that ced-9 cannot prevent ced-3-induced cell death in mammalian cells.

**ced-9 prevents Drosophila SL2 cell death induced by ced-3 and reaper**

Genetic studies of cell death in *Drosophila* revealed three genes, reaper (rpr), head involution defective (hid), and grim, which appear to play key roles in regulating apoptosis (White et al., 1994; Grether et al., 1995; Chen et al., 1996). All three genes have pro-apoptotic functions and putatively activate caspase family proteins (White et al., 1996). For example, ced-3 and reaper can be suppressed by the anti-apoptotic gene *ced-9*, which may be conserved from worm to mammals (Nguyen et al., 1994; Tanaka et al., 1993). It has been shown that overexpression of *ced-9* RNA results in the prevention of both *rpr*- and *hid*-induced cell death in mammalian cells.

In *Drosophila*, overexpression of *rpr* results in cell death, putatively by activating caspase family proteins (White et al., 1994; Grether et al., 1995; Chen et al., 1996). If the anti-apoptotic gene *ced-9* is conserved in *Drosophila*, *rpr*-mediated cell death can be suppressed by the *ced-9* RNAi construct. *rpr* was transfected together with lacZ and *ced-9* or *bcl-xL* genes, then the heat shock treatments were delivered 4 times. Cells were fixed and stained with X-Gal solution and Hoechst 33342 dye 1 day after heat shock. Overexpression of *rpr* induces 32% of apoptosis among β-galactosidase-positive cells. *rpr*-induced apoptosis was blocked by *p35* (40% inhibition) (Figs 3B, 4). Both *bcl-xL* and *ced-9* can partially suppress the *rpr*-induced apoptosis of SL2 (25% and 37% inhibition, respectively). Thus, taken together, *ced-9* and *bcl-xL* have an inhibitory effect against not only *ced-3*-induced cell death but also *rpr*-induced apoptosis in SL2 cells.

**DISCUSSION**

In this report, we have characterized the functions of the *ced-9* gene to determine if they could have regulatory roles in cell death in an evolutionarily conserved manner. First we examined the localization of *CED-9* in COS and SL2 cells, and showed that *CED-9* co-localized with *BCL-2* in both cell lines. The biochemical mechanism by which *BCL-2* blocks apoptosis remains unclear. However, recent studies suggest that the localization of *BCL-2* to mitochondria is crucial to prevent cell death (Tanaka et al., 1993). *BCL-2* mutants lacking the C-terminal transmembrane domain inefficiently associate with mitochondria and are defective in anti-apoptotic function (Nguyen et al., 1994; Tanaka et al., 1993). It has been shown that targeting *BCL-2* to mitochondria by substituting a transmembrane domain of the yeast outer-mitochondrial membrane protein Mas70p restored full anti-apoptotic function (Nguyen et al., 1994). A C-terminal transmembrane domain is also found in *CED-9*, suggesting *CED-9* may localize to mitochondria in a similar way as *BCL-2*. Most members of the *BCL-2* family contain two evolutionarily conserved domains, termed BH1 (*BCL-2* homology domain 1) and BH2 (Yin et al., 1994). In addition, an N-terminal domain, termed the BH4 domain, is conserved in anti-apoptotic members of the *BCL-2* family including *CED-9*, but not in pro-apoptotic members of the *BCL-2* family such as BAX, BAK, and BAD (Zha et al., 1996). It has been shown that BCL-2 can target Raf-1 to mitochondria, through an interaction between the BH4 domain of BCL-2 and the catalytic domain of Raf-1. Translocation of Raf-1 may alter its substrate preference, thus promoting cell survival (Wang et al., 1996). Such regulatory mechanisms might be involved in the anti-apoptotic function of the *ced-9* gene product. Another important biochemical property of *BCL-xL* and *BCL-2* is their pore-forming activity which has properties similar to those of the diphtheria toxins. These anti-apoptotic proteins may form channels in mitochondrial lipid membranes and regulate the

![Graph showing % apoptosis](image)
membrane permeability (Minn et al., 1997; Schendel et al., 1997). In dying cells, AIF (apoptosis-inducing factor) and cytochrome c are released from mitochondria into the cytoplasm (Susin et al., 1996; Yang et al., 1997). Cytochrome c can rapidly induce nuclear apoptotic change and DEVD-specific caspase activation (Kluck et al., 1997). This cytochrome c release can be effectively prevented by bcl-2, supporting the idea that this family works at the mitochondria to prevent cell death. However, it is not clear yet whether the channel-forming activity of the bcl-2 gene family is absolutely required for its anti-apoptotic functions, and whether CED-9 can form channels or not.

ced-9's similarity in structure and subcellular localization to bcl-2 led us to test the possibility that ced-9 might function as an anti-apoptotic gene in mammals. However, overexpression of ced-9 could not prevent cell death induced by ced-3 in mammalian cells. This lack of anti-apoptotic function in mammalian cells suggests that ced-9 requires other molecules to exert its anti-apoptotic functions. In mammals, the functions of BCL-2 are modulated by heterodimerization with proteins such as BAX or BAD, other BCL-2 family members (Yang and Korsmeyer, 1996). Non-BCL-2 family proteins such as BAG-1 (Takayama et al., 1995) and calcineurin (Shibasaki et al., 1997) also can bind to BCL-2 and regulate apoptosis. In C. elegans, the non-BCL-2 family gene product CED-4 is essential for programmed cell death (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992). In C. elegans, ced-9's suppression of cell killing due to ced-3 overexpression is partly mediated via ced-4 activity. CED-9 and CED-4 regulate the activation of CED-3 through physical interactions (Chinnaiyan et al., 1997a; Wu et al., 1997b). Recently Apaf-1, which participates in the cytochrome c-dependent activation of caspase-3 in vitro, was cloned (Zou et al., 1997). Part of the sequence shows significant similarities with the gene encoding CED-4. Whether CED-3 and CED-9 physically bind to Apaf-1 has not yet been tested. It is possible that CED-9 cannot exert its function through the mammalian homolog of CED-4.

A genetic approach has been taken to screen a large fraction of the chromosomal regions that are involved in the execution of the cell death programe in Drosophila. Chromosomal region 75C1,2 contains at least three pro-apoptotic genes, rpr, hid, and grim (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Overexpression of only one of these genes can induce cell death. All three may activate caspases that are inhibited by p35, suggesting that regulation of caspase activity is crucial for cell death in Drosophila (Grether et al., 1995; Chen et al., 1996; White et al., 1996; Pronk et al., 1996; Hay et al., 1995). We tested the ability of members of the bcl-2 family to prevent cell death induced by ced-3 or rpr in Drosophila cultured cells. In the present study, we showed that ced-9 prevented the ced-3-induced cell death more efficiently than bcl-xL, in Drosophila cells. Recently Seshagiri et al. (1997) reported that ced-9 will not prevent cell death in SF-21 cells if ced-3 is used alone to induce apoptosis but does work if ced-3 and ced-4 are used together. This result may be due to differences in the cell lines. On the other hand, rpr induced cell death can be prevented more efficiently by bcl-2 and bcl-xL than by ced-9. In SL2 cells, therefore, rpr may activate several caspase family proteases simultaneously. Certain stimuli of cell death result in the activation of several caspases (Takahashi and Earnshaw, 1996). The anti-apoptotic function of ced-9 may be restricted to ced-3-induced cell death. These results suggest that regulatory components required for ced-9 function, most likely a CED-4 homolog, might be present in SL2 cells. Our results highlight the conservation of ced-9 gene function in Drosophila cells. In addition, SL2 cells offer a unique and useful assay system for identifying additional genes, such as the ced-9 homolog, that are important in ced-9 regulation of ced-3-induced cell death, and for biochemical analysis of the evolutionarily conserved cell death machinery.

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