Journal of Cell Science 111, 667-673 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JCS1537

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

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Accepted 10 December, 1997: published on WWW 23 February 1998

SUMMARY

***ced-9*, a member of the *bcl-2* gene family in *Caenorhabditis elegans* plays a central roles in preventing cell death in worms. Overexpression of human *bcl-2* can partially prevent cell death in *C. elegans*. However, it remains to be elucidated whether *ced-9* can regulate cell death when expressed in other organisms. We demonstrated that the CED-9 protein is co-localized with BCL-2 in COS cells and *Drosophila* Schneider's L2 (SL2) cells, suggesting that the site of CED-9 action is located to specific cytoplasmic compartments. Overexpression of *ced-9* only poorly protected cells from the death induced by *ced-3* in HeLa cells, but *ced-9* significantly reduced the cell death induced by *ced-3* in *Drosophila* SL2 cells. Furthermore, apoptosis of SL2 cells that was induced by a *Drosophila* cell-death gene, *reaper*, was shown to be partially prevented by *ced-9*, *bcl-2* and *bcl-xL*. These results suggest that the signaling pathway that is required for the anti-apoptotic function of *bcl-2* family members, including *ced-9*, is conserved in *Drosophila* cells. In addition, SL2 cells provide a unique systems for dissecting the main machinery of cell death.**

Key words: *ced-3*, *ced-9*, *Drosophila*, *Caenorhabditis elegans*, Apoptosis, Caspase

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 (Sf-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 in 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% CO_2. SL2 cells (Schneider, 1972) were cultured in Schneider's insect medium (Sigma) with 10% heat-inactivated fetal bovine serum (FBS) and 5 mg of peptone/ml (Difco) at 27°C.

Plasmid construction

Both ends of the chimeric gene, *ced-3-lacZ* (Miura et al., 1993), were changed into *Xba*I sites by linker ligation. The fragment was cloned into pcDNA3 (Invitrogen) and the resulting construct was named pH53. pβactM10Z was previously described (Miura et al., 1993). *ced-3-lacZ* was also cloned into the *Xba*I site of pCaspeR-hs (Thummel and Pirrota, 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 deoxynucleotide 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′-CGGAATTCATGGCAGTGGCATTCTACA-3′,
Reverse: 5′-CCTCGAGTCATTGCGATGGCTTGCGATA-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 $MgCl_2$, 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 *Xho*I, blunt-ended with the Klenow fragment of DNA polymerase, and then digested with *Eco*RI. This *rpr* fragment was ligated into *Eco*RI-*Stu*I-digested pCaspeR-hs (Thummel and Pirrota, 1991), which we named pH75. pβactSTneoB-*bcl-2* was described previously (Gagliardini et al., 1994). pBabe/puro-*bcl-2* was described previously as pJ436 (Miura et al., 1993). The *bcl-2* fragment was isolated from pJ436. pJ436 was digested with *Sal*I, blunt-ended by Klenow, and then digested with *Eco*RI. The *bcl-2* fragment was cloned into pCaspeR-hs; we named this construct pH82. pβactSTneoB-*bcl-*X_L, and pcDNA3-T7-tag containing *crmA* were previously described as pM63 and pH38 (Hisahara et al., 1997). pM63 was digested with *Eco*RI and cloned into pCaspeR-hs; we named this construct pM134.
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′-TTGAATTCGAGATGACACGCTGCACGGCGG-3′,
M2: 5′-GGGAATTCGTTACTTCAAGCTGAACATCAT-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 *Eco*RI site of pBluescript. This construct was named pM61. The same vector containing the PCR product in the reverse orientation was named pM60. *ced-9* cDNA was cloned into the *Eco*RI site of pCaspeR-hs (named pM135). Both ends of the *ced-9* cDNA were changed to *Not*I sites by linker ligation and cloned into pBactSTneoB. The resulting plasmid was named pM64. The HA epitope-containing plasmid, pAS1-CYH2, was kindly provided by S. J. Elledge (Durfee et al., 1993). It was digested with *Bam*HI, and blunt ended using Klenow. The *ced-9*-containing plasmid, pM60, was digested with *Sma*I and *Hin*dIII, blunt ended using Klenow, and cloned into pAS1-CYH2; the resulting plasmid was named pI1 and used as a template. PCR was performed by using synthetic primers (prH3 and prH4) and their sequences were as follows:
prH3: 5′-TTCTCGAGCCATGGCTTACCCATACGAT-3′,
prH4: 5′-CCCTCGAGCCTTACTTCAAGCTGAACAT-3′.

The amplified fragment was cloned into the *Xho*I site of pcDNA3, and the resulting construct was named pH54. HA-tagged *ced-9* was isolated from pH54 by digesting with *Eco*RI and *Xba*I, and this fragment was cloned into pCaspeR-hs. The resulting plasmid was named pM133. A p35/pcDNA3 was kindly provided by V. M. Dixit (Beidler et al., 1995). A *p35 Eco*RI/*Xba*I fragment was isolated and cloned into pCaspeR-hs, and this construct was named pH76. *lacZ* fragment was isolated by digesting pactβgal′ (Miura et al., 1993) with *Nco*I/*Bam*HI, blunt ended by Klenow, and then cloned into pCaspeR-hs. The resulting control plasmid was named pCaslacZ.

Transfection and functional studies

COS and HeLa cells were seeded at a density of about 2.5×10^5 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, benzyloxycarbonyl-Asp-$CH_2OC(O)$-2,6,-dichlorobenzene (Z-Asp-CH_2-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. Apoptotic cells have a small, dense or split nucleus, whereas non-apoptotic cells have a large, round nucleus.

Production of anti-CED-9 antibody

The *ced-9* cDNA fragment was subcloned into the *Eco*RI 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, pβactSTneoB-*bcl-2* or pcDNA3-HA*ced-9* (pH54), were separately or simultaneously transfected with COS cells as described above. Two days after transfection, cells on coverslips were washed with 1× PBS three times, fixed in 4% paraformaldehyde/1× PBS for 10 minutes, and permeabilized in 0.1% Triton X-100/1× 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

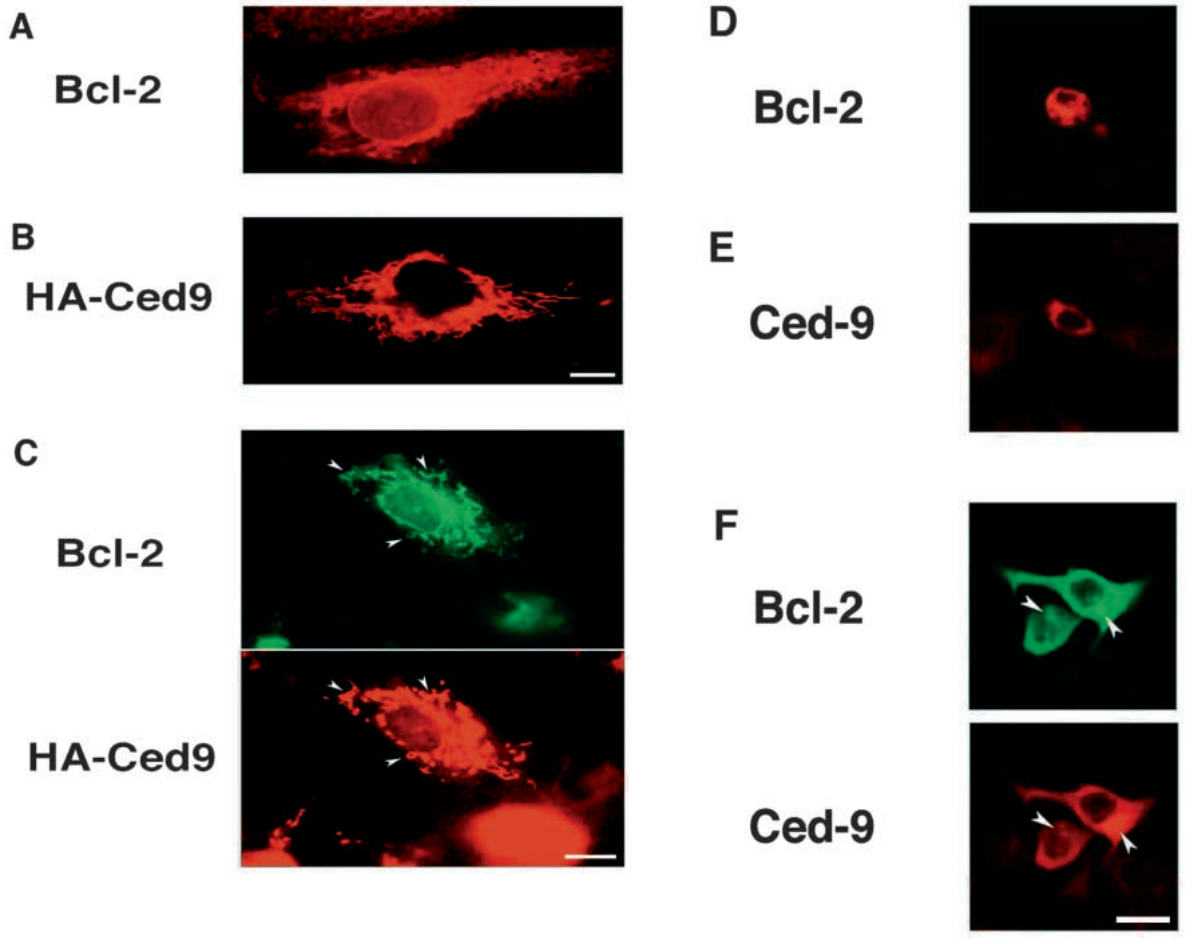

Fig. 1. Co-localization of CED-9 with BCL-2 in COS and SL2 cells. (A) *bcl-2* was transfected into COS cells. Cells were labeled with anti-BCL-2 monoclonal antibody. (B) HA-taggedced-9 was transfected and cells were labeled with anti-HA monoclonal antibody. (C) COS cells were transfected with a combination 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).

three times. Samples were incubated with Cy3-labeled anti-mouse IgG (1:100 dilution, Chemicon) for 1 hour and washed in PBS five times. Then they were incubated with FITC-labeled anti-BCL-2 antibody (1:50, Dako) at room temperature and washed with PBS three times. Samples were mounted (PermaFluor^{TM} Aqueous Mounting Medium, Immunon) and examined with a Leica fluorescence microscope (LeitzDMRD). SL2 cells were separately or simultaneously transfected with pCaspeR-hs-*bcl-2* (pH82) and pCaspeR-hs-*ced-9* (pM135). Cells were fixed and stained using the anti-CED-9 antibody (1:300) or FITC-labeled anti-BCL-2 antibody as described for COS cells.

RESULTS

CED-9 co-localizes with BCL-2 in COS cells and *Drosophila* SL2 cells

Evidence is accumulating to suggest that BCL-2 family proteins must be associated with or inserted into intracellular membranes to function. A carboxy-terminal hydrophobic region of BCL-2 is responsible for its localization to the outer mitochondrial membrane, endoplasmic reticulum, and the outer nuclear membrane (Krajewski et al., 1993; Jacobson et al.,1993). This hydrophobic region is conserved in CED-9. To examine the cellular localization of CED-9, which should be crucial for its function, HA-tagged *ced*-9 was transfected into COS cells (Fig. 1B). The transfected cells were labeled with anti-HA monoclonal antibody to reveal the localization of CED-9. The labelling pattern was granular and perinuclear, similar to that of BCL-2 (Fig. 1A). We then co-transfected COS cells with *bcl-2* and HA-tagged *ced*-9 expression vectors. Double labeling clearly showed that the Cy3-labeled HA-CED-9 was coincident with the FITC-labeled BCL-2 (Fig. 1C). These results suggest that the localization of CED-9 is quite similar to that of BCL-2 in COS cells. These results confirmed the observation that transfected HA-CED-9 is localized to membranes of intracellular organelles and the perinuclear region in 293T cells (Wu et al., 1997a). We then examined the localization of BCL-2 and CED-9 in SL2 cells. The labelling pattern was granular and perinuclear, similar to that for COS cells (Fig. 1D,E) and, as shown in Fig. 1F, we observed the co-localization of CED-9 and BCL-2 in SL2 cells as well.

Function of *ced*-9 against *ced-3*-induced cell death in mammalian cells

Overexpression of *ced-3* kills Rat-1 cells and *ced-3*-mediated cell death is partially inhibited by *bcl-2* (Miura et al., 1993). If the biochemical pathway underlying *ced-9*'s suppression of *ced-3*-mediated cell death is conserved from worm to mammals, overexpression of *ced-9* should suppress *ced-3*-induced cell death. To examine this possibility, we performed a transient transfection assay to examine the effects of anti-apoptotic genes on *ced-3* induced cell death. As shown in Fig. 2A, approximately 70% of HeLa cells underwent apoptosis due to expression of *ced-3*. We transfected *ced-3* together with two anti-apoptotic genes, *p35* and *crmA*, which are known to prevent the cell death induced by caspase family proteases (Fig. 2A). We also treated cells with Z-Asp-CH_2-DCB, an aspartate-based inhibitor that can also prevent caspase-induced apoptosis (Dolle et al., 1994). *p35* is a baculovirus gene that is thought to prevent cell death by acting as a competitive inhibitor of the caspase family (Xue and Horvitz, 1995; Bump et al., 1995). The cowpox virus *crmA* gene encodes a 38 kDa serpin protein that can inhibit ICE activity and apoptosis (Miura et al., 1993; Komiyama et al., 1994). In our transient transfection experiments, *ced-3*-induced cell death was most effectively prevented by *p35* and Z-Asp-CH_2-DCB. *bcl-2*, *bcl-X_L*, and *crmA* inhibited apoptosis moderately. 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.*

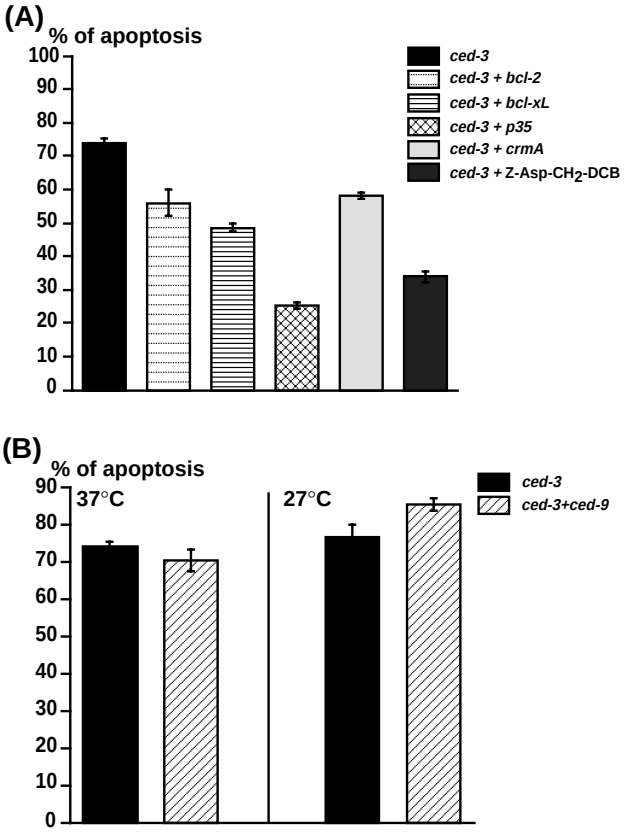

Fig. 2. Function of *ced-3* and *ced-9* in HeLa cells. (A) HeLa cells were transiently transfected with various combinations of *ced-3* and anti-apoptotic genes such as *bcl-2*, *bcl-X_L*, *p35*, and *crmA*. Cells were fixed 48 hours after transfection and stained with X-Gal for 5 hours. 40 μg/ml of Z-Asp-CH_2-DCB were administered 3 hours after transfection with *ced-3* and fixed at 24 hours after transfection. Each column indicates the percentage of small round blue cells in the total number of blue cells counted. The data were collected from three independent experiments and represented as mean ± s.e.m.
(B) Transfection with combinations of *ced-3* and *ced-9*. 37°C: Transfected cells were incubated at 37°C for about 24 hours before fixation. 27°C: Transfected cells were incubated at 27°C for about 24 hours before fixation. The data were collected from three independent experiments and represented as mean ± s.e.m.

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 caspases to initiate cell death, which can be suppressed by co-expression of *p35* (Grether et al., 1995; Chen et al., 1996; White et al., 1996; Pronk et al., 1996; Hay et al., 1995). To examine whether *ced-9* can block *ced-3*-induced apoptosis in *Drosophila*, we did a transient transfection assay using SL2 cells. We identified cells with a dense and fragmented nucleus, which are typical features of apoptosis. When *ced-3* was overexpressed in SL2 cells, approximately 70% of nuclei in β-galactosidase-positive cells were fragmented and condensed (Figs 3A, 4). To test the effects of anti-apoptotic genes against *ced-3*-induced cell death in SL2 cells, we constructed *bcl-xL*, *p35*, and *ced-9* expression plasmids, and each of them was co-transfected with *ced-3*. *ced-3*-induced cell death was effectively blocked by overexpression of *p35*. Notably, in SL2 cells, *ced-9* prevented *ced-3*-induced cell death more effectively than either *bcl-xL* or *bcl-2* did. These results suggest that the regulatory pathway involving *ced-9* and *ced-3* may be conserved from worm to *Drosophila*.

In *Drosophila*, overexpression of *rpr* results in cell death, putatively by activating caspase family proteins (White et al., 1996; Pronk et al., 1996; Hay et al., 1995). If the anti-apoptotic role of *ced-9* is conserved in *Drosophila*, *rpr*-mediated cell death can be suppressed by the *ced-9*/*bcl-2* gene family. *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-galacotosidase 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.

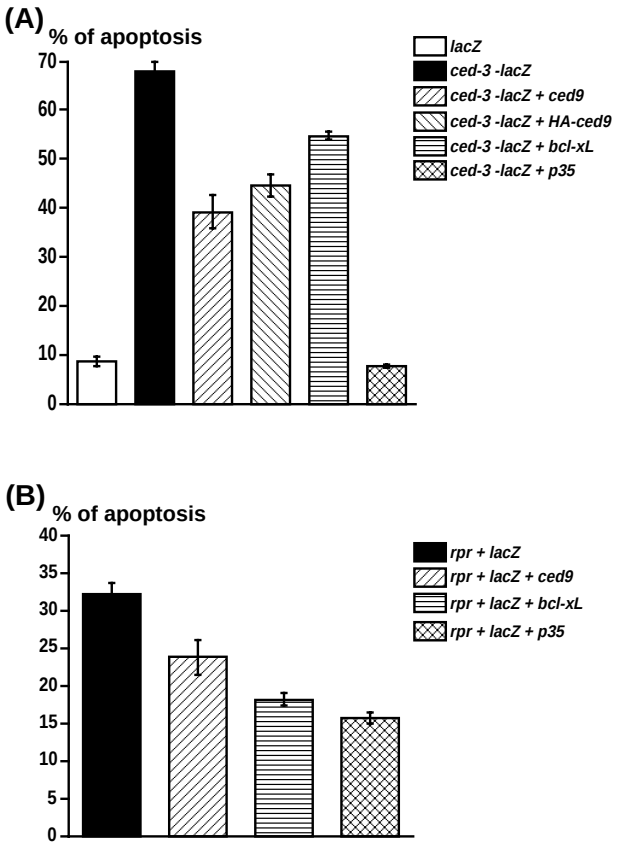

Fig. 3. Anti-apoptotic function of *ced-9* and *bcl-xL* in SL2 cells: cell death induced by *ced-3* and *rpr*. Cells were transfected with combination of *ced-3* and anti-apoptotic genes such as *ced-9*, HA-tagged *ced-9*, *bcl-xL*, and *p35* (A) and *rpr* and *ced-9*, *bcl-xL*, and *p35* (B). The *lacZ* expression construct was also transfected in all experiments. Each column indicates the percentage of blue cells with a fragmented nucleus in the total number of blue cells counted (see Fig. 4). The data were collected from three independent experiments and represented as mean ± s.e.m.

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

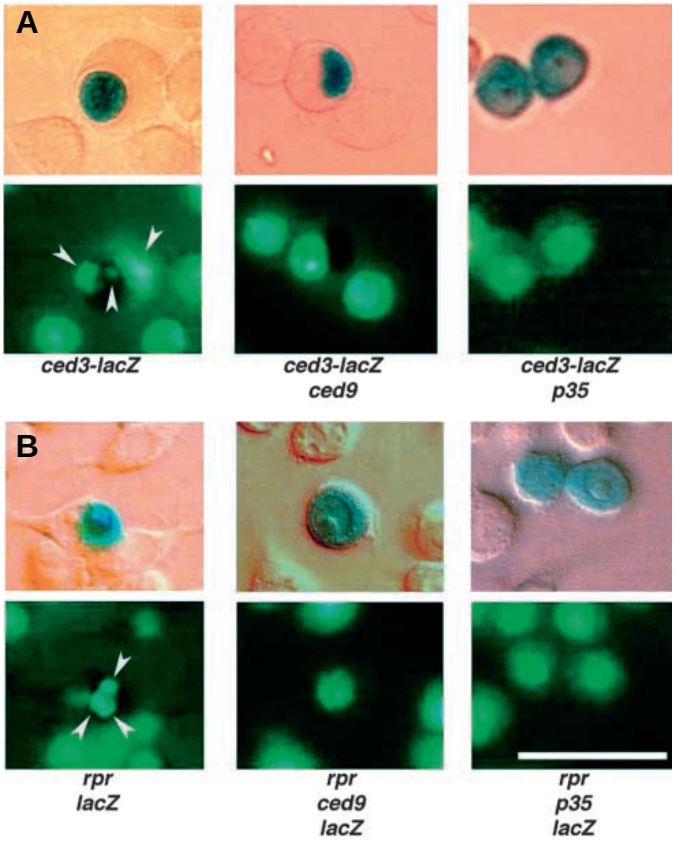

Fig. 4. *ced-3*- and *rpr*-induced apoptotic cell death in cultured SL2 cells. Cells were transfected with *ced-3* or *rpr* and anti-apoptotic genes. Overexpression of proteins was induced by heat shock after 24 hours of transfection. Cells were fixed 48 hours after transfection and stained with X-Gal (A) and Hoechst 33342 dye (B). The arrowheads indicate the fragmented nuclei of the apoptotic cells. Bar, 10 μm.

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-apotoic 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-4* 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.

We are grateful to Dr Nobuko Shiraiwa for *ced-3*-lacZ plasmid construction, Dr Kayoko Hamaguchi for pCaslacZ, and Ms Miyuki Matsuda at the Institute of Basic Medical Sciences, University of Tsukuba for pI1. We thank Dr Craig B. Thompson for providing us with the pSFFV-Neo-*bcl-X*$_L$ plasmid and Dr Vishva M. Dixit for providing us with the p35/pcDNA3 plasmid. This work is supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture to H.O. and M.M., and a research grant from Kyowa Hakko Kogyo Co., Ltd and a Grant-in-Aid from The Tokyo Biochemical Research Foundation to M.M. This work has been also supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST) to H.O.

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