Programmed cell death (PCD)\(^1\) is a conserved mechanism of cellular demise that is critical for embryonic organ development and homeostasis in adult tissues (1, 2). In the nematode Caenorhabditis elegans, 131 of the 1090 somatic cells generated during development undergo PCD (3). Genetic analyses of the cell death process in the nematode have identified three genes that play critical roles in the induction and execution of PCD (4). The ced-9 gene protects cells that normally survive during worm development (5). ced-9 encodes a protein with significant homology to the mammalian Bcl-2 and Bcl-XL survival proteins (6). Two nematode genes, ced-3 and ced-4, are required for the execution of the cell death process (4). CED-3 is homologous to the mammalian interleukin-1β-converting enzyme, which is a member of a family of cysteine proteases (designated caspases) (7). CED-3 and related caspases are thought to act as executors of the nematode and mammalian PCD pathway (8). CED-4 also has a mammalian counterpart, Apaf-1 (9). Overexpression of ced-1 in nematode ALM neurons causes cell death that requires ced-3 activity for efficient killing, suggesting that ced-4 acts upstream of ced-3 (10).

Biochemical analyses of CED-3, CED-4, and CED-9 have provided important insight into the regulation of the central cell death machinery in the nematode. CED-9 interacts with CED-4 suggesting that CED-9 regulates cell death by binding to and inactivating CED-4 (11–13). Furthermore, CED-4 associates with CED-3 and promotes the proteolytic activation of CED-3, and this activation process is inhibited by CED-9 through a multimeric protein complex (13–15). Recent analyses of the mammalian counterparts have revealed physical associations of Bcl-XL, Apaf-1, and procaspase-9 (16, 17), suggesting that the regulation of the central cell death machinery is conserved through evolution from nematodes to humans.

In mammals, a family of proteins that belong to the Bcl-2 family including Bax (18), Bak (19–21), Bad (22), Bik/Nbk (23, 24), Bid (25), Hrk/DP5 (26, 27), Bim (28), Bok/Mtd (29, 30), and BNIPI3 (31) activate apoptosis. Structural and functional analyses have revealed that these pro-apoptotic proteins require the conserved BH3 region to interact with pro-survival Bcl-2/Bcl-XL/Mcl-1 and to activate apoptosis in transient assays (18–24). BH3-containing proteins most homologous to mammalian Bad, Bik/Nbk, Bid, and Hrk/DP5 (33). EGL-1 physically associates with CED-9, as determined by in vitro interaction analysis, and requires its BH3 domain for efficient binding to CED-9 (33),
suggesting that EGL-1 might act by binding to and inhibiting CED-9. However, the mechanism by which EGL-1 antagonizes the activity CED-9 remains unknown. In the present study, we have performed biochemical and functional analyses to assess the mechanism by which EGL-1 inhibits the activity of CED-9 and regulates CED-3 and CED-4.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The expression plasmids producing epitope-tagged CED-4, CED-3, and CED-9 have been described (14). The egl-1 gene was cloned by polymerase chain reaction using specific primers (5'-GGTGCAGGCAGCCATCTAGAATGCTCAAGCTAGCTCAGATGATGTCCTACTCGGCCCATGCTCAAAAGCTCTTAAAAA-3', 5'-GGTGAATTCGGGCCCGAGCTCTTAAAAAAGTTTATTCCATCGTCTTCTGGACTTTTTCGCTTTT-3'). The BH3 deletion mutant (∆BH3) was constructed by replacing XbaI and Apal fragment of the gene with a double strand oligonucleotide containing a deletion that correspond to amino acids 60–67 of EGL-1.

**Transfection, Immunoprecipitation, and Immunoblotting**—HEK293T cells (2 × 10^6/60-mm plate) were transiently transfected by lipofection with plasmids producing FLAG-EGL-1 (0.66 µg), Myc-CED-4 (0.66 µg in A or 0.33 µg in B), and HA-CED-9 (0.33 µg in A or 0.66 µg in B). In all cases 0.2 µg of pcDNA3-p35 was included, and pcDNA3 empty plasmid was added so that the total amount of DNA was 2 µg. 24 h after transfection, cell lysates were immunoprecipitated (IP) with anti-HA mAb and immunoblotted with anti-c-Myc, anti-HA, or anti-FLAG rabbit polyclonal antibody. Expression of proteins in total lysate is shown in the lower three panels. B, HEK293T cells were transfected as in A and lysates immunoprecipitated with anti-Myc mAb. Immunoprecipitates were immunoblotted as in A. Size markers are in kDa. The results shown are representative of at least three independent experiments.

**In Vitro Transcription and Translation of Proteins**—Coupled in vitro transcription and translation was carried out with the TNT system from Promega according to the manufacturer's instructions. Briefly, pcDNA3 plasmids producing FLAG-Egl-1 (wt and ∆BH3 mutant) and HA-CED-9 were transcribed with T7 polymerase and translated in the presence of [35S]methionine using a rabbit reticulocyte lysate.

**Cell Immunostaining and Confocal Microscopy**—HEK293 cells were cultured on poly-L-lysine-coated cover slides and transfected with the indicated plasmids (see figure legends) by lipofection with plasmids producing epitope tag antibodies (anti-FLAG M2 monoclonal antibody (mAb) from Sigma; anti-Flag-D-8 polyclonal antibody from Santa Cruz; anti-HA 12CA5 mAb from Boehringer Mannheim; anti-HA Y-11 polyclonal antibody from Santa Cruz Biotechnology; anti-AU1 mAb from Babco, and anti-c-Myc A-14 polyclonal antibody from Santa Cruz Biotechnology). Proteins were immunoprecipitated with protein A/G-Sepharose 4B (Zymed Laboratories Inc.), subjected to SDS-polyacrylamide gel electrophoresis, and immunoblotted with indicated antibodies. Proteins were detected by ECL (Amersham Pharmacia Biotech).

**Survival and Apoptosis Assays**—HEK293T cells or MEF were cultured in 24-well plates (5–7.5 × 10^4 cells/well) and transfected with the indicated plasmids (see figure legends) with LipofectAMINE (Life Technologies, Inc.) in triplicate. Each transfection mixture contained 20 ng of pcDNA3-β-galactosidase plasmid as reporter. Cell survival of transfected cells was determined by quantification of reporter gene activity as described (34). Briefly, 48 h after transfection cells were lysed in 200 µl of 1× Reporter lysis buffer (Promega), and 25–50 µl of each lysate was assayed for β-galactosidase activity in a reaction mixture containing 1 mg/ml o-nitrophenyl-β-D-galactopyranoside, 670 µM sodium phosphate, pH 7.5, 1 mM magnesium chloride, 45 µM β-mercaptoethanol. Reactions were incubated at 37 °C for 30–120 min, and product forma-
tion was monitored by reading optical density at a wavelength of 420 nm. Apoptotic morphology of transfected cells was determined 24 h after transfection by analysis of at least 100 cells expressing β-galactosidase as described (14).

RESULTS AND DISCUSSION

EGL-1 Binding to CED-9 Inhibits the Interaction of CED-4 with CED-9—Genetic studies have shown that egl-1 acts upstream of ced-9 and that the egl-1 product physically interacts with CED-9 in vitro (33). Because CED-9 binds to the adaptor molecule CED-4 and this activity appears critical for the anti-apoptotic function of CED-9 (11–15), we tested whether EGL-1 could affect the CED-9/CED-4 interaction. In these experiments, we transiently co-transfected HEK293T cells with expression plasmids producing HA-tagged CED-9, Flag-tagged EGL-1, and Myc-tagged CED-4. The interactions between these proteins were analyzed by immunoprecipitation and immunoblotting. As shown in Fig. 1A, immunoprecipitation of CED-9/CED-4 or CED-3. HEK293T cells (1.5 × 10^6/60-mm plate) were transiently transfected with plasmids producing FLAG-EGL-1 (1.5 µg) and HA-CED-4 (0.5 µg) or HA-CED-3 (0.5 µg) or HA-CED-9 (0.5 µg). In all cases 0.3 µg of pcDNA3-p35 was included, and pcDNA3 empty plasmid was added so that the total amount of DNA was 2.5 µg. 24 h after transfection, cell lysates were analyzed as in B. Size markers are in kDa. The asterisk indicates immunoglobulin heavy chain.

FIG. 3. CED-9 and EGL1 regulate the subcellular localization of CED-4. HEK293 cells (4 × 10^5/well, 6-well plate) were transiently transfected by lipofection with 0.5 µg of plasmids producing AU1-EGL-1 (A), HA-CED-9 (B), AU1-EGL-1 and HA-CED-9 (C), FLAG-CED-4 (D), FLAG-CED-4 and HA-CED-9 (E), or FLAG-CED-4, HA-CED-9 and AU1-EGL-1 (F). 24 h after transfection, cells were stained with mAb against the indicated molecules (lower quadrant of each panel). Samples were analyzed by confocal microscopy, and pictures of representative fields are shown. The experiment shown is one representative of at least three independent experiments.
the binding of CED-9 to CED-4. In contrast to wt EGL-1, transfection of a plasmid producing a mutant form of EGL-1 deficient in CED-9 binding due to deletion of 5 amino acids in the BH3 motif (EGL-1 \( \Delta BH3 \)) did not inhibit the association of CED-9 with CED-4 (Fig. 1A). Although EGL-1 binds to CED-9, EGL-1 might also inhibit the CED-4-CED-9 association by direct binding to CED-4. To test this possibility, we blotted CED-4 immunoprecipitates with anti-FLAG to determine whether EGL-1 was bound to CED-4. EGL-1 did not co-immunoprecipitate with CED-4 (Fig. 1B), confirming that CED-4 displacement from CED-9 complexes was due to CED-9 sequestration by EGL-1. These results indicate that EGL-1 binds to CED-9, an interaction that interferes with the ability of CED-9 to bind CED-4, releasing CED-4 from the death inhibitor.

CED-9 Regulates the Expression Levels of EGL-1—We have consistently found that EGL-1 expression in HEK293T cells is very low in the absence of CED-9. Furthermore, EGL-1 mutant (EGL-1 \( \Delta BH3 \)) did not significantly affect survival of HEK293T cells. HEK293T cells were transfected with 75–100 ng of plasmid producing wt or \( \Delta BH3 \) EGL-1 together with plasmid encoding \( \beta \)-galactosidase as a reporter. Cell survival was quantified 48 h after transfection as described under “Experimental Procedures.” We assigned a value of 100% to the survival of cells transfected with empty vector (dotted line) and normalized the rest of the samples as a percentage of that obtained with control plasmid. Represented is the average of 6 (EGL1 WT) or 4 (EGL1 \( \Delta BH3 \)) experiments performed in triplicate. Results are shown as the mean ± S.E. Differences in survival between cells transfected with control or EGL-1 plasmids were not statistically significant (\( p = 0.05 \)). B, EGL-1 inhibits the protective activity of CED-9 but not CED3/CED4-induced killing. HEK293T cells were transfected with 75–100 ng of each of the indicated plasmids, and cell survival was measured at 48 h as in A. Results from six independent experiments performed in triplicate are shown. Each dot represents the average of triplicate samples from a single experiment. The horizontal bar represents the mean of all the experiments shown. The difference in survival between cells transfected with CED-3/CED-4/CED-9 alone or in combination with wt EGL-1 was statistically significant (\( p = 0.05 \)). C, HEK293T cells were transfected with the indicated plasmids as in B together with a \( \beta \)-galactosidase reporter gene. The percentage of apoptosis was determined 24 h after transfection by scoring the number of apoptotic blue cells (\( \beta \)-galactosidase positive). Three independent experiments performed in triplicate are shown. Each dot represents the average of triplicate samples from a single experiment. The horizontal bars represent the mean of all the experiments shown. D, EGL-1 inhibits the protective activity of CED-9 but not CED3/CED4-induced killing in MEFs. Cells were transfected with 50–100 ng of each of the indicated plasmids, and cell survival was measured at 48 h as in A. Results from two independent experiments performed in triplicate are shown. Each dot represents the average of triplicate samples from a single experiment. The horizontal bars represent the mean of all the experiments shown.
The effect of CED-9 on EGL-1 expression was observed when co-transfected with a CED-9 plasmid (Fig. 2A). The presence of CED-9 was specific in that co-expression of CED-3 or CED-4 did not promote EGL-1 expression (Fig. 2B). Moreover, these results could not be explained by poor viability of cells transfected with CED-9, as expression of CED-9 did not induce cell death in the absence or presence of CED-9 (data not shown and Fig. 4).


tants deficient in CED-9 binding are expressed at lower levels than wt EGL-1 in the presence of CED-9. These observations suggested that EGL-1 expression might be regulated by its association CED-9. To test this hypothesis, we co-transfected HEK293T cells with constructs producing wt EGL-1 and CED-9 or control plasmids, and we analyzed EGL-1 expression by immunoblotting. In the absence of CED-9, no detectable expression of EGL-1 was observed (Fig. 2A). In contrast, EGL-1 was expressed in HEK293T cells when co-transfected with a CED-9 plasmid (Fig. 2A). The effect of CED-9 on EGL-1 expression was specific in that co-expression of CED-3 or CED-4 did not promote EGL-1 expression (Fig. 2B). Moreover, these results could not be explained by poor viability of cells transfected with CED-9, as expression of CED-1 did not induce cell death in the absence or presence of CED-9 (data not shown and Fig. 4).

To investigate further the role of CED-9 in the expression of EGL-1, we synthesized EGL-1 and CED-9 in a cell-free system. EGL-1 was synthesized and translated in reticulocyte lysate in the absence or presence of CED-9 (data not shown and Fig. 4). To test this hypothesis, we transiently transfected HEK293 cells with plasmids producing tagged EGL-1, CED-4, and CED-9, and we assessed the subcellular localization of the proteins by immunostaining and confocal microscopy. As previously reported (11), CED-9 displayed a granular, extra-nuclear staining pattern that is consistent with a localization to membranes of intracellular organelles such as mitochondria (Fig. 3B). In the majority of the cells, EGL-1 labeling was undetectable in the absence of CED-9 which is consistent with the results shown in Fig. 2. In cells in which EGL-1 expression was detected, the labeling pattern of EGL-1 was diffuse and cytoplasmic in the absence of CED-9 (Fig. 3A) which is consistent with the structure of EGL-1 that lacks a hydrophobic transmembrane tail (33). As previously reported (11), the labeling pattern of CED-4 was diffuse and cytoplasmic consistent with a cytosolic localization, but it was altered to a granular, cytoplasmic pattern in the presence of CED-9 (Fig. 3D and E). Significantly, expression of EGL-1 altered the distribution of CED-4 in cells that co-express CED-9 from an intracellular membrane pattern to a cytosolic pattern (Fig. 3F, compare E and F). Furthermore, the labeling pattern of EGL-1 changed from cytosolic to granular in the presence of CED-9.
(Fig. 3C), which is consistent with the interaction between CED-9 with EGL-1 observed in the same cells (Fig. 1). These results support the biochemical analysis shown in Fig. 1 that showed that EGL-1 inhibits the association of CED-9 with CED-4. Furthermore, they indicate that EGL-1 inhibits the ability of CED-9 to target CED-4 to intracellular membranes. In surviving cells of the worm, CED-4 might be sequestered at intracellular membranes and inactivated by CED-9, particularly if CED-9 is expressed in excess relative to CED-4. In cells that undergo PCD, EGL-1 might be induced or activated leading to the release of pro-apoptotic CED-4 from its inhibitor CED-9.

EGL-1 Blocks the Proteolytic Activity of CED-9 Against CED-4/CED-3-Mediated Apoptosis—In HEK293T cells, CED-4 enhances apoptosis induced by CED-3, an activity that is inhibited by CED-9 (14, 15). These results are in agreement with genetic and functional analyses of ced-3, ced-4, and ced-9 in C. elegans (4). Therefore, we used HEK293T cells to assess the ability of EGL-1 to regulate the protective activity of CED-9 against apoptosis promoted by CED-3 and CED-4. To test the function of EGL-1 in the mammalian model, HEK293T cells were co-transfected with a reporter plasmid expressing β-galactosidase with constructs producing EGL-1, CED-3, CED-4, CED-9, and control plasmid at different combinations. The cell killing activity was measured by a reduction in β-galactosidase activity (34) or by assessing the apoptotic morphology of β-galactosidase-positive cells (14). The results of six or three such independent experiments in which we measured cell survival or apoptosis are shown in Fig. 4, A and B. At the low concentrations of CED-3 and CED-4 plasmids used in these experiments, CED-3 or CED-4 had little effect on HEK293T survival. In contrast, co-expression of CED-3 and CED-4 dramatically reduced HEK293T cell survival, an effect that was reversed by CED-9 (Fig. 4B). Expression of EGL-1 had little effect by itself on HEK293T cell survival (Fig. 4A) and did not significantly alter cell survival when co-expressed with CED-3, CED-4, or CED-3 plus CED-4 (Fig. 4B and data not shown). However, the protective effect of CED-9 against CED-3/CED-4-mediated killing was completely abrogated by EGL-1 (Fig. 4B). The ability of EGL-1 to inhibit the protective activity of CED-9 was also observed when HEK293T cells were scored for apoptosis in three independent experiments (Fig. 4C). Furthermore, similar results were also observed when the effect of EGL-1 was assessed in MEF cells (Fig. 4D).

EGL-1 Promotes the Proteolytic Activation of CED-3—CED-3 associates with CED-3 and CED-9 and can form a ternary protein complex with CED-3 and CED-9 (13, 14). The inhibition of CED-9 binding to CED-4 by EGL-1 suggested that EGL-1 would promote the activation of CED-3, as CED-4 would be freed from its inhibitor CED-9. To test if EGL-1 could regulate the activation of CED-3, cellular lysates from HEK293T cells transfected with constructs producing CED-3, CED-4, CED-9, and EGL-1 were analyzed for CED-3 enzyme activity by immunoprecipitation and immunoblotting. As shown in Fig. 5, expression of CED-3 or CED-3 plus CED-4 resulted in significant processing of CED-3, as revealed by reduction in the amount of the 56-kDa proform of CED-3-FLAG protein and detection of the mature p13 and p15 products of CED-3 (Fig. 5). As described (14, 15), CED-9 inhibited processing of CED-3 promoted by CED-4 (Fig. 5A). Significantly, expression of wt EGL-1 inhibited the activity of CED-9 and promoted CED-3 processing, whereas an EGL-1 ΔBH3 mutant did not (Fig. 5A).

The results presented herein provide a mechanism to explain how EGL-1 promotes cell death through its binding to CED-9. The interaction of EGL-1 with CED-9 results in the release of CED-4 from CED-9/CED-4 complexes and translocation of CED-4 from intracellular membranes to the cytoplasm. In this model, EGL-1 promotes the release of CED-4 from its inhibitor CED-9, allowing the activation of CED-3 by CED-4 (Fig. 5B). Consistently, EGL-1 abrogated the CED-9-protective effect against apoptosis, but it did not have any significant effect on ced-3/ced-4-induced killing when assessed in the absence of CED-9. These results are in agreement with the genetic studies in C. elegans that showed that egl-1 acts upstream of ced-9 and that its pro-apoptotic activity is dependent on ced-9 function (33). The model of action of EGL-1 suggested by our results resembles that of its mammalian homologues such as Bad, Hrk, Bik, or Bid, whose pro-apoptotic effect is mediated at least in part through direct binding and inhibition of Bcl-2 or Bcl-XL (22–27). Moreover, it has been recently reported that binding of Bak, a pro-apoptotic member of the Bcl-2 family, to Bcl-XL prevents Bcl-XL binding to Apaf-1 (17), suggesting that the mechanism of action of these pro-apoptotic proteins has been conserved during evolution.

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Caenorhabditis elegans EGL-1 Disrupts the Interaction of CED-9 with CED-4 and Promotes CED-3 Activation
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