Proapoptotic activity of Caenorhabditis elegans CED-4 protein in Drosophila: Implicated mechanisms for caspase activation

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ABSTRACT CED-4 protein plays an important role in the induction of programmed cell death in Caenorhabditis elegans through the activation of caspases. However, the precise mechanisms by which it activates caspases remain unknown. To investigate, the conservation of CED-4 function in evolution, transgenic Drosophila lines that express CED-4 in the compound eye were generated. Ectopic expression of CED-4 in the eyes induced massive apoptotic cell death through caspase activation. An ATP-binding site (P-loop) mutation in CED-4 (K165R) causes a loss of function in its ability to activate Drosophila caspase, and an ATPase inhibitor blocks the CED-4-dependent caspase activity in Drosophila S2 cells. Immuno precipitation analysis showed that both CED-4 and CED-4 (K165R) bind directly to Drosophila caspase drICE, and the overexpression of CED-4 (K165R) inhibits CED-4-, ecdysone-, or cycloheximide-dependent caspase activation in S2 cells. Furthermore, CED-4 (K165R) partially prevented cell death induced by CED-4 in Drosophila compound eyes. Thus, CED-4 function is evolutionarily conserved in Drosophila, and the molecular mechanisms by which CED-4 activates caspases might require ATP binding and direct interaction with the caspases.

In the nematode Caenorhabditis elegans, three genes, ced-3, ced-4, and ced-9, play critical roles in the execution of programmed cell death (1–3). In a loss-of-function mutant of ced-9, 151 somatic cell deaths that occur during normal nematode development are completely blocked (4). Genetic studies in C. elegans have suggested that ced-4 acts to induce cell death upstream of or parallel to ced-3, which encodes a cytotoxic protease homologous to the mammalian caspase family, and that ced-9 exhibits its inhibitory effect partly through ced-4 (5, 6). CED-4 physically interacts with CED-9 and CED-3 (7–10) or with caspase-1, caspase-8, and mammalian caspase family, and that effect partly through to

However, the molecular mechanisms for initiating caspase activation remain poorly understood for organisms across the phylogenetic scale (11, 12, 17). In contrast to C. elegans, multiple caspases are involved in the execution of the cell death program in Drosophila (18–20). Drosophila contains at least three caspases, DCP-1, drICE, and DCP-2 (21–23), and apoptosis in Drosophila can be induced in response to various stimuli, as in mammalian cells (24). Therefore, we expect Drosophila to be an appropriate animal model for studying the activation mechanism of the caspase family in vivo. In addition to genetic studies, biochemical analyses, and death-inducing activities of cell-death genes can be performed in the Drosophila S2 cell line. We have reported that CED-9 inhibits CED-3-induced cell death in Drosophila S2 cells (25). This suggests that the molecular components that work through CED-9 prevent CED-3-induced cell death and that these components, including the CED-4 homologue, are conserved in Drosophila. A simple hypothesis is that the Drosophila homologue of CED-4 can directly activate caspases to execute the cell-death program, and that C. elegans CED-4 can mimic its effect in Drosophila. In the present study, we show that the function of CED-4 in Drosophila is evolutionarily conserved, and we performed further functional and biochemical analyses of CED-4. A P-loop mutation of CED-4 acts as an antiapoptotic molecule and prevents the caspase activation normally induced by CED-4 and by ecdysone. Our results strongly suggest that ATP is required for caspase activation and thus identify a unique molecular mechanism for caspase activation initiated by CED-4 that is evolutionarily conserved.

MATERIALS AND METHODS

Production of Transgenic Flies. The fragment containing the full-length CED-4 or CED-4 (K165R) coding region was ligated into pGMR (Glass Multimer Reporter) (26). The resulting plasmids pGMRced-4 and pGMRced-4 (K165R) were injected into w1118; Dr/TM6, Sb P[y+], Δ2–3 embryos as previously described (27). Several independent transformant lines showing similar phenotypes were obtained. Drosophila stocks used in this study were GMR-p35 (26), GMR-diap1 (28), and GMR-diap2 (28). Canton-S or white1118 was used as a wild-type strain.

Histology, Immunohistochemistry, and in Situ Caspase Detection. Flies were prepared for scanning electron microscopy as described (29). Semithin sections of adult heads were prepared as described (27). Acridine orange staining to detect dead cells was performed by methods previously described (30).

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Immunohistochemistry of eye discs was carried out essentially as described (31), except that discs were fixed in 4% paraformaldehyde in PBS. For anti-ELAV staining of third-instar eye discs, primary antibody (7E8A10) purchased from Developmental Studies Hybridoma Bank, Iowa City, was diluted 1:10 in 0.3% Triton X-100 in PBS. Cy3-labeled anti- rat IgG (1:100 dilution, Chemicon) was used as the secondary antibody.

In situ affinity labeling (32) of eye discs from third-instar larvae was performed by using a specific substrate for caspase-3-like protease (PhePheLys G6D2, OncolImmunin). Live discs were incubated in RPMI-1640 medium containing 10 μM substrate for 1 h at 37°C in a 5% CO2 incubator and washed with flow cytometry dilution buffer (OncolImmunin). Samples were mounted and examined with an Axioplan2 fluorescence microscope (Zeiss).

Expression Vectors. cDNAs encoding full-length CED-4, CED-3, and Drosophila homolog of inhibitor of apoptosis protein 2 DIA2 were cloned into pCasperR-hs (33) under the control of the hsp70 promoter. The coding sequences of dcp-1, drICE, or dcp-2 were amplified by reverse transcription–PCR (RT-PCR) from total RNA prepared from 8–18 h Drosophila embryos or S2 cells by using the following primers: dcp-1, 5′-TTGGATCCCATCTAGGACGAGTGCGTAACC-3′ and 5′-AAAGGATCCCAAGAATTCTCAGTCTGCCAATTCCCA-3′; dICE, 5′-AAGAAGATATCTGAGGACGACTAACAA-3′ and 5′-TTGGATAACTTCAAAACCCGTCGGTGGAGC-3′; dcp-2, 5′-AAAGGATCATGTCGTTGAAGACGCTCTACAGG-3′ and 5′-TTGGATCCTCTACAGCAGGAAGTGGAGATCAC-3′. Amplified fragments were inserted into pCasperR-hs. The construction of pCasperR-Hs-lucE, -p35, -reaper, and -ced-9 was previously described (24).

Cell Culture, Transfections, Cell-Death Assay, and Preparation of Cytoplasmic Lysates for Caspase Assay. S2 cells (34) were cultured, transfected, and used for the cell-death assay as described previously (25, 35). Cytoplasmic extracts of S2 cells were cultured, transfected, and used for the cell-death assay as described (24).

Affinity Labeling of Active Caspase, Immunoprecipitation, and Western Blotting. The affinity labeling was performed by using biotin-DEVD-amin (Biosyn Diagnostics) followed by the procedure described previously (37).

Immunoprecipitation was performed as previously described (38). S2 cells (1 × 10⁶ per 6 cm dish) were transiently transfected with His-tagged drCEnt (C211A) and/or dced-4, then heat shocked. At 24 h after heat shock, cells were harvested, packed by centrifugation, and lysed by 80 μl Nonidet P-40 lysis buffer (38) without DTT. ProBond Resin (50 μl) (Invitrogen) was blocked with control S2 cell lysates for 1 h at 4°C then incubated with cell lysates overnight at 4°C with rotation. The resin was washed five times in Nonidet P-40 lysis buffer. Aliquots of the total lysates and precipitates were separated by 12.5% SDS/PAGE, transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore) and then probed with anti-CED-4 or anti-drICE mouse antisera (described below). These antisera were used for Western blotting at a 1:500 dilution in 4% skim milk/Tris-buffered saline (pH 7.5)/150 mM NaCl/0.2% Tween 20. Horseradish peroxidase-conjugated secondary antibodies (antimouse IgG, EY Laboratories) were used at a 1:500 dilution, and signals were visualized by enhanced chemiluminescence (Amersham).

Antisera were produced by the following procedure. ORFs corresponding to full-length ced-4, dcp-1, and drICE were cloned into pRSET (Invitrogen) to allow the expression of His-tagged CED-4, DCP-1, and drICE in Escherichia coli. These constructs were transformed into BL21, and expression was induced by incubation in a 1,000-mI liquid culture with 1 mM IPTG at 37°C for 4 h. These proteins were separated by SDS/PAGE, the band corresponding to the His-tagged protein was excised, and the protein was electroeluted and used to immunize rats, mice, or rabbits.

RESULTS AND DISCUSSION

GMRced-4 Transgenic Flies Exhibit a “Rough-Eye” Phenotype. To investigate the ability of CED-4 to kill cells in Drosophila, we generated transgenic flies that misexpress CED-4 in the Drosophila compound eye. The eyes of transgenic flies carrying one copy of the GMRced-4 transgene showed roughened morphology and reduced size, compared with wild-type eyes (Fig. 1A and B). Consistent with this observation, the cellular structures of pigment cells as well as photoreceptor cells were hardly detectable in the GMRced-4 ommatidia (Fig. 1E), in contrast with the wild-type cells (Fig. 1D). Although the overexpression of ced-4 (one copy) was as effective as one copy of GMRp35 and GMRreaper and was less effective in reducing the size of eyes than was GMRhid (18, 39, 40), CED-4 affected both photoreceptor cells and pigment cells, suggesting that the effect of CED-4 on ommatidia development would be broad and would include a variety of cell types.

To analyze the mechanisms underlying excessive cell death in the eyes caused by CED-4 overexpression, we examined the effects of antiapoptotic genes on this process. Baculovirus p35, which inhibits a broad spectrum of caspases (41) and is known to prevent cell death in Drosophila (18–20, 26), was coexpressed with GMRced-4 in the eye. The size of the GMRced-4 eyes was significantly rescued by the coexpression of p35 (Fig. 1C). Sections through the eyes revealed that some of the photoreceptor and pigment cells survived until eclosion (Fig. 1F). These results imply that overexpression of CED-4 caused cell death through apoptotic pathways that include the activation of caspases. Overexpression of either DIA1 or DIA2, Drosophila homologues of the baculovirus inhibitor of apoptosis protein (IAP), have been shown to prevent apoptosis induced by rpr, hid, and argos in the eye (28, 42). Coexpression of DIA1 or DIA2 with CED-4 partially prevented the decrease in eye size seen in GMRced-4 flies, but these inhibitory effects were significantly less than observed with p35 (data not shown).

Ectopic Expression of CED-4 Induces Cell Death in the Drosophila Larval Eye Disc with Caspase Activation. The reduction in eye size seen in the GMRced-4 transgenic flies may have resulted from excess cell death in the eye disc. To examine this possibility, eye discs were stained with acridine orange to detect apoptotic cell death. In eye discs from wild-type third-instar larvae, a small number of dying cells was observed, mainly posterior to the morphogenetic furrow (Fig. 1G). In contrast, a greater number of apoptotic cells could be detected behind the furrow in the GMRced-4 transgenic larvae (Fig. 1H). We also examined the differentiation of photoreceptor cells by labeling larval eye discs with anti-ELAV antibody, a nuclear marker for all Drosophila neurons. There were fewer ELAV-positive photoreceptor cells and their arrangement was disordered in the posterior region of the GMRced-4 eye discs, where ectopic cell death occurred (Fig. 1H), compared with wild type (compare Fig. 1 J and K). Coexpression of p35 with CED-4 prevented ectopic cell death caused by CED-4 overexpression.
FIG. 1. (A–F) The phenotypes of GMRced-4 transgenic flies are partially blocked by p35. (A and D) Wild-type compound eye. (B and E) Ectopic expression of one copy of GMRced-4 causes a reduction of eye size and disordered cell arrangement in the eye. (C and F) The size of the GMRced-4-expressing eye was partially rescued by the coexpression of p35. (A–C) Scanning electron microscope images. (D–F) Thin sections of adult eyes. Scale bar, 10 μm. (G–O) Ectopic expression of GMRced-4 induces cell death and caspase activation in eye discs from third-instar larvae. In this figure, anterior is to the right and posterior is to the left. (G, J, M) Wild-type. (H, K, M) Third-instar eye disc from GMRced-4 larvae. (I, L, O) GMR-p35; GMRced-4. (G–I) Acridine orange staining (AO) to detect dead cells. Scale bar, 100 μm. (J–L) Immunostaining by using anti-ELAV antibody. Scale bars, 100 μm. (M–O) In situ affinity labeling (IAL) of active caspses in third-instar larval eye discs. The dotted line indicates the outer shape of the eye disc. Scale bars, 80 μm. In each experiment, immunohistochemical analysis revealed the expression of CED-4 in larval eye discs, and CED-4-positive cells were distributed in the posterior of the morphogenetic furrow (data not shown).
death and the decrease in ELAV-positive cells (Fig. 1A and L), suggesting that the eye phenotype of GMRced-4 is mostly caused by ectopic apoptosis induced by caspases.

The fact that the phenotypes of CED-4 transgenic flies were blocked by the coexpression of p35 raised the possibility that CED-4 activated the endogenous Drosophila caspases in the developing eyes. To investigate this hypothesis, we examined the active forms of caspases in larval eye discs by in situ affinity labeling, using a fluorescent substrate for the caspase-3-like protease. In contrast to wild type, large numbers of labeled cells were seen in the CED-4-expressing eye discs (Fig. 1M and N). Additionally, coincident with the results from the eye phenotype, the caspase activation detected in the eye discs expressing CED-4 was significantly inhibited by the coexpression of p35 (Fig. 1O). These results strongly indicated that CED-4 activated Drosophila caspase-3-like caspases, resulting in massive cell death in vivo and leading to the eye phenotypes of the GMRced-4 transgenic flies.

Although the increase of both caspase activity and apoptotic cells in the larval eye discs of CED-4 transgenic flies was completely blocked by the overexpression of p35, the inhibitory effect of p35 on the external morphology of compound eyes was less effective than against the activation of caspases and induction of cell death. To see whether this result could be modified by increasing the dosage of p35, we generated CED-4 transgenic flies that carried two copies of p35 (GMR-p35/GMR-p35;GMR-  

![](image)

**Fig. 2. Overexpression of CED-4 induces cell death and caspase activation in Drosophila S2 cells.** (A) Overexpression of CED-4 kills Drosophila S2 cells. Cells were transfected with ced-4. At 24 h after heat-shock treatment, cells were fixed and stained with Hoechst 33342. Open arrowheads indicate condensed nuclei. Scale Bar, 10 μm. (B) Quantification of cell death in Drosophila S2 cells. The data (mean ± SD) are the percentage of cells with condensed chromatin in the total number of cells counted. (C and D) DEVD- and YVAD-cleaving activities of the cytosplasmic lysates of S2 cells expressing various proteins or treated with 1 μM staurosporine. S2 cells were transfected with various plasmids, and lysates were prepared 24 h after heat-shock treatment.

**Overexpression of CED-4 Activates Endogenous Caspases in Drosophila S2 Cells.** To examine in more detail the role of CED-4 in activating the endogenous apoptotic pathway and inducing cell death in Drosophila, we analyzed the effect of CED-4 expression in the Drosophila S2 cell line. Transient expression of CED-4 induced cell death with rapid chromatin condensation and approximately 23.7% of the S2 cells underwent apoptosis because of the overexpression of ced-4 (Fig. 2A and B). Next we transfected ced-4 together with the antiapoptotic genes C. elegans ced-9, baculovirus caspase inhibitor p35, and diap2. In our transient transfection experiments, the overexpression of CED-4 most effectively prevented CED-4-induced cell death, and p35 inhibited apoptosis moderately. Transfection of diap2 could block rpr-induced cell death in S2 cells (data not shown) but not CED-4-induced cell death, indicating that the CED-4-induced cell death pathway does not include the mechanisms that are inhibited by DIAP2. These results suggested that an apoptotic pathway containing certain elements targeted by CED-4 is present in Drosophila S2 cells.

The caspase activities were measured in S2 cells expressing CED-4 by using two kinds of specific substrates that distinguish caspase-1 like proteases (Ac-YVAD-MCA) from caspase-3 like proteases Ac-DEVD-MCA. High DEVD- but not YVAD-cleaving activities were observed in the cytoplasmic lysates from ced-4-transfected S2 cells (Fig. 2C). These activities were effectively inhibited by the coexpression of p35 or ced-9 (Fig. 2D). In contrast, diap2 expression could not inhibit the activation of caspases by CED-4 (Fig. 2D), consistent with the results of the cell-death assay in S2 cells (Fig. 2B). In insect cells (SF21), Op-IAP and Cp-IAP cannot block active-form caspase-dependent cell death (45), and the IAP family also binds to RPR, HID, and GRIM (46), which are proapoptotic proteins known to activate caspases in Drosophila (18–20), suggesting that these IAPs act upstream of caspase activation for the inhibition of cell death. These data suggest that CED-4 could enhance the activation of endogenous caspase-3 like proteases in S2 cells, and the inhibitory effect of CED-9 on CED-4-induced caspase activation in S2 cells was consistent with the antiapoptotic function of CED-9 against CED-4 in C. elegans (5, 6).
the activation of drICE by CED-4, we first examined the functional domain of wild-type CED-4 using various mutants (Fig. 4A). All the CED-4-mutant constructs we tested exhibited reduced caspase-activating activity (Fig. 4B). Activation of DEVD-cleavage activity by CED-4 was reduced by the ATPase inhibitor 5′-p-fluorosulfonylbenzoyl adenosine (FSBA; 48, 49) and by a P-loop mutation, and caspase activity was recovered by the addition of ATP (Fig. 4B and C), indicating that binding to and hydrolysis of ATP at the P-loop motif in CED-4 is required for caspase activation. A similar result was reported in CED-4-induced CED-3 activation (13). It is noteworthy that the coexpression of a P-loop mutant, CED-4 (K165R), with the wild-type CED-4 effectively prevented CED-4 from inducing caspase activity (Fig. 4B). Subsequently, we examined whether drICE directly interacts with CED-4 in a manner similar to that reported for CED-3 and CED-4, which form a physical complex (7, 10). Immunoprecipitation analysis revealed that overexpressed CED-4 in S2 cells specifically binds to drICE (Fig. 4D). Interestingly, CED-4 (K165R) retained its binding activity to drICE, suggesting that CED-4 (K165R) seems to work as a dominant-negative inhibitor for the activation of caspases. These data imply that both a direct interaction between CED-4 and caspases and ATP-binding activity of CED-4 are required for caspase activation.

P-Loop Mutation of CED-4 Converts the Caspase Activator to a Suppressor. We generated a CED-4 (K165R) transgenic fly line (GMR-ced4K165R) using an eye-specific promoter. Western blot analysis of eye disc revealed that expression level of CED-4 (K165R) was five times lower than that of CED-4 (data not shown). GMR-ced4K165R flies exhibited normal eye morphology (Fig. 5A), indicating that the P-loop mutant lost its apoptosis-inducing activity in vivo. Interestingly, overexpression of CED-4 (K165R) moderately suppressed the CED-4-dependent reduction of eye size (Fig. 5B and C). Because CED-4 (K165R) also suppressed the CED-4-dependent caspase activation in S2 cells (Fig. 4B), the restoration of the eye phenotype in CED-4 transgenic flies by coexpression of CED-4 (K165R) is probably because of a decrease in caspase activities. Next, we were concerned about whether the cell-death pathways induced by various stimuli use CED-4 or its homologues for caspase activation in S2 cells. Treatment of S2 cells with cycloheximide (10 μg/ml), staurosporine (1 μM), or ecdisone (20-hydroxyecdysone, 10 μM) for 24 h induced rapid apoptosis and caspase activation (ref. 47 and unpublished data). Ecdisone is an endogenous steroid that regulates metamorphosis in Drosophila development and induces apoptosis by means of caspase activation, for example in salivary glands and the midgut, in which apoptotic cell deaths are inhibited by p35 (50). In our results, caspase activation provoked by cycloheximide and ecdisone in S2 cells were effectively blocked by overexpression of CED-4 (K165R) (Fig. 5D). These results suggest that CED-4 homologues of Drosophila exist in S2 cells. CED-4 (K165R) may inhibit their function through the heterodimerization, which is thought to be a potent mechanism for caspase activation in CED-4/Apaf-1 (51, 16) or by competing with Drosophila CED-4 homologues to bind caspases. In addition, the loss of ATP-binding ability by a P-loop mutation in CED-4 prevents it from activating procaspases; however, ATP binding ability may be not required for CED-4 to bind other components (e.g., drICE). Thus, the P-loop mutant may act as a “dominant negative” molecule.

In conclusion, our results indicate that CED-4 function is conserved in Drosophila. Our experiments using the CED-4
Fig. 5. A P-loop mutation of CED-4 converts the caspase activator to a suppressor. (A–C) The phenotypes of GMR-ced-4 transgenic flies are moderately inhibited by the ectopic expression of CED-4 (K165R). (A) GMR-ced4 (K165R)/+. (B) GMR-ced4+/+. (C) GMR-ced4 (K165R)/+;GMR-ced4+/+. (D) Overexpression of CED-4 (K165R) suppresses the caspase activation induced by cycloheximide (CHX), staurosporine (STS), and ecdysone (Ec). S2 cells were transfected with empty vector or caspase activation induced by cycloheximide (CHX), staurosporine (STS), and ecdysone (Ec). S2 cells were transfected with empty vector or caspase-9 (14, 15). Recent observations showed that ATP is required to execute the apo-
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