Interaction and Regulation of the Caenorhabditis elegans Death Protease CED-3 by CED-4 and CED-9*

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In the nematode Caenorhabditis elegans, three genes, ced-3, ced-4, and ced-9, play critical roles in the induction and execution of the death pathway. Genetic studies have suggested that ced-9 controls programmed cell death by regulating ced-4 and ced-3. However, the mechanism by which CED-9 controls the activities of CED-4 and the cysteine protease CED-3, the effector arm of the cell-death pathway, remains poorly understood. Immunoprecipitation analysis demonstrates that CED-9 forms a multimeric protein complex with CED-4 and CED-3 in vivo. Expression of wild-type CED-4 promotes the ability of CED-3 to induce apoptosis in mammalian cells, which is inhibited by CED-9. The pro-apoptotic activity of CED-4 requires the expression of a functional CED-3 protease. Significantly, loss-of-function CED-4 mutants are impaired in their ability to promote CED-3-mediated apoptosis. Expression of CED-3 enhances the proteolytic activation of CED-3. We also show that CED-9 inhibits the formation of p13 and p15, two cleavage products of CED-3 associated with its proteolytic activation in vivo. Moreover, CED-9 inhibits the enzymatic activity of CED-3 promoted by CED-4. Thus, these results provide evidence that CED-4 and CED-9 regulate the activity of CED-3 through physical interactions, which may provide a molecular basis for the control of programmed cell death in C. elegans.

Programmed cell death (PCD) is critical during organ development and tissue homeostasis (1). In the nematode Caenorhabditis elegans, 131 of the 1090 somatic cells generated during development undergo PCD (2). Three genes have been identified in the nematode that play critical roles in the induction and execution of PCD (3). The ced-3 gene protects cells that normally survive from PCD during worm development (4, 5). ced-9 encodes a protein with significant homology to the mammalian Bcl-2 and Bcl-XL survival proteins (4, 5). Furthermore, Bcl-2 can partially substitute for ced-9 in C. elegans, suggesting that bel-2 is a homolog of ced-9 (6, 7). In contrast, two nematode genes, ced-3 and ced-4, are required for the execution of the cell death program. Thus, loss-of-function mutations of ced-3 and ced-4 cause all 131 somatic cells that normally die to survive (3). The ced-3 product is homologous to the mammalian interleukin-1β-converting enzyme (ICE), which is a member of a family of cysteine proteases (designated caspases) (7, 8). CED-3 and related caspases are thought to act as effectors of the nematode and mammalian PCD pathway (7, 8).

Genetic experiments have suggested that ced-9 protects cells from undergoing PCD by preventing the death-promoting activity of ced-3 and ced-4 (4, 9). Consistent with genetic experiments in C. elegans, Bcl-2 and Bcl-XL, two mammalian homologs of CED-9, can inhibit the activation of ICE-like proteases and therefore appear to act upstream of the death proteases in the mammalian apoptotic pathway (10, 11). Over-expression of ced-4 in the nematode ALM neurons causes cell death that requires ced-3 activity for efficient killing, suggesting that ced-4 acts upstream of ced-3 (9). Furthermore, protection against ced-3-induced cell death by ced-9 requires ced-4 activity, suggesting that ced-9 controls ced-3 by acting at least in part through ced-4 (9). Although genetic analysis has been essential for the identification and initial characterization of the nematode PCD pathway, the biochemical basis by which CED-3, CED-4, and CED-9 regulate cell death has remained elusive. Recent studies, however, indicate that CED-9 interacts with CED-4 suggesting that CED-9 regulates cell death by binding to and inactivating CED-4 (12–14). In the present studies, we performed further functional and biochemical analyses of CED-3, CED-4, and CED-9 interactions using mammalian cells as a model system to gain insight into the regulation and molecular basis of the PCD pathway.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The expression plasmids producing epitope-tagged CED-3 and CED-9 have been described (13). Plasmids encoding Myc-tagged CED-4 mutants were generated by PCR amplification of ced-4 template DNA using 3′ primers that included the natural translation termination sequences as described (15). Subsequently, the ced-4 constructs were ligated into the KpnI site of pcDNAs (Invitrogen). An HA- or Flag-tagged ced-3 insert was constructed by introducing each epitope tag at the COOH terminus of CED-3 by PCR. Inserts to express mutant CED-3 proteins were generated by PCR amplification of ced-3 template using a 3′ primer that included a translation termination codon (D220 CED-3) or by two-step mutagenesis to generate G360S CED-3. The HA-, Flag-, or Myc-tagged inserts were ligated into the BamHI site of pcDNAs. Orientation of the inserts was determined by restriction mapping. Authenticity of all tagged constructs was confirmed by dideoxy sequencing.

Transfection, Immunoprecipitation, and Western Blot Analysis—Culture dishes containing 2–5 × 10⁶ human embryonic kidney 293T cells were transfected with the indicated amount (see figure legends) of plasmid DNA by the calcium phosphate method. The expression of HA-CED-9, Myc-CED-4, and Flag-tagged CED-3 was determined in total lysates by immunoblotting as described previously (16). For immunoprecipitations, cells from each dish were lysed in 1 ml of Nonidet
P-40 isotonic lysis buffer at 14–20 h after transfection, and soluble lysates were incubated with 1 μg/ml rabbit anti-Myc, anti-Flag, or anti-HA antibody (Santa Cruz) or normal rabbit IgG overnight at 4 °C with 5% (v/v) of protein A-Sepharose 4B (Zymed Laboratories). Immune complexes were centrifuged, washed with excess cold Nonidet P-40 isotonic lysis buffer at least four times, separated on a 15% SDS-polyacrylamide gel, and immunoblotted with monoclonal anti-HA (clone 12CA5, Boehringer Mannheim), monoclonal anti-Flag (clone M2, Kodak), or rabbit anti-Myc antibody (Santa Cruz). The proteins were detected using an enhanced chemiluminescence (ECL) system (Amersham Corp.).

Cleavage of Fluorogenic Substrate—293T cells (5 × 10⁶) in 100-mm plates were transiently co-transfected with 5 μg of pcDNA3-ced-4-Myc and/or pcDNA3-ced-3-Flag in the presence or absence of 5 μg of pcDNA3-HA-ced-9 by calcium phosphate method. The total amount of transfected plasmid DNA was always 15 μg. Rabbit anti-Myc or anti-HA immunoprecipitates were immunoblotted with anti-Flag antibody to detect Flag-tagged CED-3. In the bottom panel, immunoprecipitates were immunoblotted with anti-HA to detect CED-9 or anti-Myc to detect CED-4. B, CED-9 is immunoprecipitated with CED-3 through CED-4. Lysates were immunoprecipitated with rabbit anti-Flag, and immunoprecipitates were immunoblotted with anti-HA to detect the HA-CED-9 protein. In the bottom panel, immunoprecipitates were immunoblotted with anti-Flag to detect CED-3. The amount of plasmid DNA and number of transfected cells was identical to that described for panel A. Size markers are in kDa. IP, immunoprecipitation.

RESULTS AND DISCUSSION

**CED-4 Forms a Multimeric Protein Complex with CED-3 and CED-9 in Mammalian Cells—**Genetic studies have suggested that CED-4 regulates the activity of CED-3 in the *C. elegans* death pathway (9). To determine if CED-4 interacts with CED-3, we transiently co-transfected human 293T cells with expression plasmids producing Flag-tagged CED-3, HA-tagged CED-9, and Myc epitope-tagged CED-4. Because CED-3 induces apoptosis in mammalian cells, we used a catalytically inactive mutant of CED-3 (CED-3-Flag-G360S) to study the interaction of CED-3, CED-4, and CED-9. Immunoprecipitates were prepared with rabbit anti-Myc antibody and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblotting with a monoclonal antibody to Flag revealed that CED-4 co-immunoprecipitated CED-3 in the presence or absence of CED-9 (Fig. 1A). Furthermore, immunoprecipitation with anti-HA followed by immunoblotting with anti-Flag revealed that CED-9 co-immunoprecipitated CED-3 (Fig. 1A). To further verify these results, we performed reciprocal experiments. Immunoprecipitation of CED-3 with anti-Flag antibody co-immunoprecipitated CED-9 (Fig. 1B). CED-4 co-immunoprecipitated both CED-3 and CED-9 (Fig. 1A). More importantly, CED-3 was unable to interact with CED-9 in the absence of CED-4 (Fig. 1, A and B) indicating that CED-3 associates with CED-9 through CED-4. These results confirm observations recently reported by Chinnaiyan et al. (12).

**Interaction of CED-4 Mutants with CED-3 and CED-9—**To further characterize the specificity and functional significance of the interaction between CED-3 and CED-4, we determined the ability of CED-3 to associate with wild-type CED-4 and three natural CED-4 mutants that exhibit a loss of function phenotype in *C. elegans* (Fig. 2A). Two point mutations, n1948 and n1894, introduce a single amino acid change (Ile to Asn) at
position 258 and a stop codon at residue 401 of the CED-4 protein, respectively (15). We engineered another mutation, G328, to insert a stop codon at amino acid 328 to recapitulate the loss of function mutation n1416, which results from a Tc4 insertion at residue 328 of CED-4 (15). To assess the interaction of CED-3 with CED-4 proteins, 293T cells were transiently co-transfected with plasmids producing Flag-tagged CED-3 and Myc-tagged wild-type or mutant CED-4 proteins. Immunoprecipitation analysis revealed that CED-3 co-immunoprecipitated wild-type CED-4 (Fig. 2B), confirming our results presented in Fig. 1. Significantly, W401 and G328, two loss of function CED-4 mutants resulting from C-terminal truncations, retained their ability to associate with CED-3 although the binding of the CED-4 mutants, particularly G328 and I258N, was reduced when compared with wild-type CED-4 (Fig. 2D).

CED-4 Associates with the Prodomain of CED-3 and with a Loss of Function CED-3 Mutant—To further define the interaction of CED-3 with CED-4, the ability of CED-4 to interact with two CED-3 mutants was investigated. We engineered CED-3 D220 to express a truncated CED-3 mutant (residues 1–220) containing the prodomain of CED-3 and CED-3 G360S, a natural loss of function mutation (n2433) of CED-3 with a single amino acid change (Gly to Ser) at position 360 located in the conserved pentapeptide QACRG. p15 represents the major processed product of CED-3 associated with activation. The position of point mutations n1948 and n1894 of ced-4 with loss of function phenotypes are shown. Another CED-4 mutant, G328, that recapitulates the n1416 mutation is also shown. B, CED-3 interacts with wild-type CED-4, and W401 and G328 CED-4 mutants. 293T cells (2 × 10^6/60-mm plate) were transiently transfected with 3 μg of the indicated Myc or Flag-tagged expression plasmids. In the case of transfection with one plasmid, cells were cotransfected with 3 μg of empty vector so that the total amount of transfected plasmid DNA was always 6 μg. Anti-Flag or control antibody immunoprecipitates were immunoblotted with anti-Myc antibody to detect Myc-tagged CED-4 proteins. C, interaction of CED-3 with mutant I258N CED-4. Lysates were immunoprecipitated with anti-Flag or control antibody and immunoblotted with anti-Myc to detect the Myc-tagged CED-4 proteins. D, interaction of CED-9 with loss of function CED-4 mutants. Lysates were immunoprecipitated with anti-Myc or control antibody and immunoblotted with anti-HA antibody to detect HA-tagged CED-9 proteins. Aspecific protein band representing immunoglobulin heavy chain is indicated by an asterisk. In bottom panels, immunoprecipitates were immunoblotted with anti-Myc, or total lysates were immunoblotted with anti-HA. E, interaction of CED-4 with mutant CED-3 proteins. Lysates were immunoprecipitated with anti-Flag, anti-AU1, or control antibody and immunoblotted with anti-Myc antibody to detect Myc-tagged CED-4. Expression of Flag-CED-3-G360S and AU1-CED-3-D220 in total lysates is shown at the bottom. Size markers are in kDa. IP, immunoprecipitation.

CED-4 Promotes the Ability of CED-3 to Kill Mammalian Cells—Genetic analysis in C. elegans has suggested that CED-4 regulates the activity of CED-3 and requires CED-3 to activate cell death. To further explore the function of CED-4 in apoptosis, we determined if CED-4 could regulate the cell...
death-promoting ability of CED-3 in mammalian cells. To do
this, 293T cells were transiently transfected with expression
constructs producing CED-4, CED-3, and empty vector. Ex-
pression of CED-4 did not induce apoptosis in 293T cells above
the levels observed with control plasmid (Fig. 3A). Transfection
of CED-3 induced a modest but significant level of apoptosis in
293T cells (Fig. 3A). More importantly, the great majority of the
cells underwent apoptosis when co-transfected with CED-3 and
CED-4 (Fig. 3A), indicating that CED-4 potentiates the ability
of CED-3 to induce apoptosis. Significantly, expression of
CED-9 blocked the ability of CED-4 and CED-3 to induce apo-
ptosis (Fig. 3A). Together with genetic experiments in C. el-
egans (3, 9), these results suggest that CED-4 interacts with
and activates the death-promoting ability of CED-3, which can
be inhibited by the interaction of CED-9 with CED-4. To fur-
ther assess the function of CED-4, we tested the ability of loss
of function CED-4 mutants to promote CED-3-mediated apo-
ptosis. Functional analysis revealed that the loss of function
CED-4 mutants were defective in their ability to potentiate
CED-3-mediated apoptosis when compared with wild-type
CED-4 (Fig. 3A). The levels of wild-type and mutant W401
and G328 CED-4 proteins expressed in 293T cells were similar,
ruling out the trivial explanation that the differential ability of
these proteins to promote apoptosis is due to differences in
protein expression (Fig. 2 and data not shown). However, the
expression of the I258N CED-4 mutant is reduced and appears
unstable, which agrees with previous results (14).

A Functional CED-3 Protein Is Required for CED-4 to Pro-
mote Apoptosis—To further determine the mechanism by
which CED-4 potentiates CED-3-mediated apoptosis, we exam-
Values were statistically significant at all time points.

At the 6 h time point in activity of CED-3 in cells expressing CED-3, CED-3 plus CED-4, and CED-3 plus CED-4 plus CED-9. The values correspond to the results obtained at the 6 h time point in panel B after normalization for the same amount of CED-3 protein. All comparisons yielded a p value < 0.01. Normalized values were statistically significant at all time points.

Ced-9 inhibits the processing of CED-3 in mammalian cells and presumably in C. elegans. To assess if the survival protein CED-9 regulates the activation of the CED-3 protease in vivo, 293T cells (5 x 10^5/100-mm plate) were transiently transfected with 5 μg of plasmids producing Flag-tagged CED-3, Myc-tagged CED-4, and HA-tagged CED-9. In the case of transfection with one or two plasmids, cells were cotransfected with 5–10 μg of empty vector so that the total amount of transfected plasmid DNA was always 15 μg. At 14 h, CED-3 complexes were immunoprecipitated and concentrated with rabbit anti-Flag antibody and immunoblotted with anti-Flag antibody to detect Flag-tagged CED-3. Total amount of CED-3 protein was calculated by densitometry and the percent of mature CED-3 was determined whether the pro-apoptotic activity of CED-4 requires a “functional” CED-3 protease. In contrast to the killing observed with wild-type CED-3, CED-4 failed to promote apoptosis mediated by either a truncated CED-3 mutant containing the prodomain of CED-3 or G360S CED-3, a natural loss of function CED-3 mutant with a single amino acid change (Gly to Ser) at position 360 located in the conserved active pentapeptide QACRG of CED-3 (Fig. 3B). Because the CED-3 (G360S) mutant protein is known to be inactive as a cysteine protease in vitro (17), we conclude that the protease activity of CED-3 is required for CED-4 to promote apoptosis in mammalian cells. These results are consistent with genetic studies which have shown that the CED-3 protease activity is crucial for the death-promoting function of CED-4 in C. elegans (9).

CED-4 Enhances the Proteolytic Activity of CED-3 and CED-9 Inhibits the Processing of CED-3 in Mammalian Cells—To assess if the survival protein CED-9 regulates the activation of the CED-3 protease in vivo, cellular lysates from 293T cells transiently transfected with plasmids expressing CED-3, CED-4, and CED-9 were analyzed for the presence of p13 and p15, two cleavage products of CED-3 associated with the mature protease (17, 18). As shown in Fig. 4A, expression of CED-3 resulted in partial processing of the immature 56-kDa CED-3-FLAG protein as determined by the detection of the mature p13 and p15 products of CED-3 (17). Significantly, processing of CED-3 increased dramatically in the presence of CED-4 when compared with cells expressing CED-3 alone (Fig. 4A). Moreover, expression of CED-9 inhibited the processing of CED-3 in cells transiently transfected with CED-3 plus CED-4 as determined by the absence of the p13 and p15 proteolytic products of CED-3 (Fig. 4A). To verify these results, we determined the caspase enzymatic activity of CED-3 in lysates from cells transiently transfected with CED-3, CED-3 plus CED-4, and CED-3 plus CED-4 plus CED-9. To measure the protease activity associated with CED-3, aliquots of the same cellular lysates used in Fig. 4B were incubated with anti-Flag antibody to immunoprecipitate CED-3 protein complexes, and the immunoprecipitates were assayed for enzymatic activity using the Ac-DEVD-AMC fluorogenic substrate (19). In agreement with results shown in Fig. 4A, lysates from cells expressing CED-3 or CED-3 plus CED-4 exhibited significant enzymatic activity when compared with control lysates (Fig. 4B). Importantly, the caspase activity of CED-3 from cells expressing CED-3 and CED-4 was inhibited by CED-9 (Fig. 4B). In control experiments, there was no significant protease activity when the same cellular lysates were immunoprecipitated with control antibody (data not shown). Because the cellular expression of CED-3 was diminished in the presence of CED-4 and enhanced in the presence of CED-9 when compared with cells expressing CED-3 alone (Fig. 4), we adjusted the values of enzymatic activity obtained with the Ac-DEVD-AMC substrate to normalize for the amount of CED-3 protein (immature plus mature). After normalization, we found that the enzymatic activity of CED-3 was enhanced significantly by co-expression of CED-4 (Fig. 4C). In contrast, CED-9 inhibited the catalytic activity of CED-3 in the presence of CED-4 (Fig. 4C).

These results demonstrate that CED-4 interacts with both CED-3 and CED-9 in mammalian cells and presumably in C. elegans cells. Our studies confirmed and extended the reported interaction of CED-4 with CED-3 and CED-9 (14). However,
our functional results differ from those recently reported (14), which demonstrated that CED-4 was capable of inducing apoptosis in C. elegans cells in even the absence of CED-3. In addition to 293T cells, we found that expression of CED-4 failed to kill human MCF-7 breast carcinoma cells and COS-7 monkey cells. In agreement with our results, another group has found that expression of CED-4 fails to kill Rat-1 and HeLa cells. Thus, we do not have an explanation to account for the discrepancy in the results. In our studies, CED-4 functioned only to promote apoptosis in mammalian cells, which was dependent upon the expression of enzymatically active CED-3. These observations support previous genetic experiments in C. elegans in which efficient killing by CED-4 was shown to be dependent upon CED-3 activity (9). Together with the results from other investigators (14), these studies argue that CED-4 promotes cell death by interacting with and activating CED-3. However, the mechanism by which CED-4 activates CED-3 is not sufficient for death-promoting function CED-4 mutants retained their ability to associate with CED-3, our studies suggest that the interaction between CED-4 and CED-3 is not sufficient for death-promoting function of CED-4. Consistent with the analysis in C. elegans, the loss of function CED-4 mutants were impaired in their ability to potentiate CED-3-mediated apoptosis in mammalian cells. Expression of CED-9 inhibited the killing activity of CED-4 plus CED-3, and in another study, it inhibited killing induced by CED-4 alone (14). Because CED-9 can form a protein complex with CED-3 through CED-4, the protective function of CED-9 could be mediated by preventing the activation of CED-3 through CED-4. Indeed, we show for the first time that CED-4 enhances the processing and conversion of CED-3 into the active CED-3 form in vivo. Furthermore, we show that CED-9 inhibits the proteolytic activation of CED-3 in cells that co-express CED-4. Since a C. elegans cell culture is not available, the biochemical analysis of the death regulators CED-3, CED-4, and CED-9 was performed in mammalian cells. The biochemical results that we have obtained with CED-3, CED-4 and CED-9 are consistent with the genetic analysis in C. elegans (9). However, we cannot formally rule out that these worm proteins behave differently in C. elegans cells. These studies predict that Bcl-2 and Bcl-XL, the mammalian CED-9 homologs, may regulate apoptosis by interacting with caspases, the cysteine proteases of the ICE/CED-3 family through a mammalian CED-4 counterpart.

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