Mutational analysis of *Caenorhabditis elegans* CED-4

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Abstract Much of our knowledge concerning the genetics that regulate cell death has come from the studies of cell death during the development of the nematode *Caenorhabditis elegans*. Of the 14 genes identified as components of nematode cell death pathways, two genes, ced-3 and ced-4, are required to promote cell death and a third, ced-9, blocks cell death. Recent studies show CED-4 to be an activator of CED-3 and CED-9 to be an inhibitor of CED-4. Two published sequence alignments suggest that CED-4 contains a death effector domain (DED), a protein sequence motif present in other death signaling proteins like Fadd and Flice; one study suggests a DED sequence similarity near the N-terminus while the other found sequence similarity near the C-terminus of CED-4. Using mutational analysis we have tested the functional significance of the conserved residues found within the putative DEDs of CED-4. Mutations in two conserved residues within the putative N-terminal DED of CED-4 affected its function, while mutations in the conserved residues within the putative C-terminal DED had no effect on CED-4 function. Our results do not support the presence of a DED in the C-terminus of CED-4 and suggest a potential role for the N-terminus in CED-4 function, possibly as a DED or as a CARD (caspase recruitment domain). We also found that CED-9 associated with all the CED-4 mutants and inhibited the activity of all the active-CED-4 mutants.

Key words: Apoptosis; Ced-4; Death effector domain; FADD; FLICE; Caspase activation

1. Introduction

Apoptosis or programmed cell death is known to play an important role in numerous biological processes [1–3]. Our primary understanding of the molecular machinery that promotes or prevents cell death has come from genetic analysis of the nematode *Caenorhabditis elegans* [4]. In nematodes, 14 genes that participate in cell death have been identified [4,5]. Three of these 14 genes, ced-3, ced-4, and ced-9, control the execution phase of cell death [6]. While CED-3 and CED-4 promote cell death, CED-9 opposes cell death. CED-3 is a member of the caspase family of cysteine proteases that are central components of the apoptotic pathway [7]. CED-4, a homolog of the recently cloned mammalian pro-apoptotic factor Apaf-1, promotes the activation of CED-3, and thus leads to the ultimate demise of the cell [8,9]. CED-9 is related to mammalian Bcl-2, an anti-apoptotic factor. CED-9 opposes cell death by associating with and blocking the activity of CED-4 [10–16]. An additional role for CED-9 as a CED-3 inhibitor has also been proposed [17].

In mammals the Fas receptor is a conduit for transmission of an external death-inducing signal known as FasL (Fas-ligand) [18–20]. Fas receptor, when occupied by FasL, recruits caspase-8 via an adaptor protein Fadd. The recruited caspase-8 is then activated leading to induction of apoptosis [18–20]. The interaction of caspase-8 and Fadd occurs through their death effector domains (DEDs), a protein motif present in the N-terminus of both Fadd and caspase-8 [21,22]. Recently, CED-4 was shown to interact with caspase-8, leading to speculation that CED-4 might function as an adaptor protein analogous to Fadd [10]. Two sequence alignments, one suggesting the presence of N-terminal DED in CED-4 and another, a C-terminal DED, have been published [23,24]. Sequence alignment studies of CED-4 by Peter et al. [25], based on other recently cloned DED containing proteins, suggest that the previously published alignments of CED-4 with DEDs might not be functionally significant. The functional relevance of these alignments has not been tested yet.

In this study, we have replaced, by site-directed mutagenesis, seven residues conserved in the CED-4/DED alignments of Bauer et al. [23] or Nagata [24] and assayed the effect of these mutations on the function of CED-4. We found that mutations in two conserved residues in the potential N-terminal DED affected the function of CED-4, while none of the four C-terminal DED mutants showed any significant reduction in their ability to promote apoptosis. All the functional mutants of CED-4 were inhibited by CED-9 and were found to associate with CED-9.

2. Materials and methods

2.1. Cells

The IPLB-Sf-21 cell line, originally derived from pupal ovaries of the fall army worm *Spodoptera frugiperda*, was maintained at 27°C in TC-100 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 0.26% tryptose broth [26].

2.2. Plasmid constructs and site-directed mutagenesis

All plasmids used in this study were derived by replacing the chloramphenicol acetyltransferase (CAT) gene in a previously described vector, phsp70PLVI+CAT [27], thereby placing all genes under *Drosophila hsp70* promoter control. This promoter is active in Sf-21 cells and can be induced further by heat shock, a procedure which, by itself, does not induce apoptosis in Sf-21 cells [27]. Plasmids containing epitope-tagged versions of wild type (wt) ced-3, ced-4 and ced-9 were described previously and are as active as untagged versions in apoptosis assays in Sf-21 cells [14]. While ced-3 contains an HA.11 tag as a C-terminal fusion, ced-4 and ced-9 contain N-terminally fused Flag and HA.11 tags, respectively [14]. All the ced-4 mutants were derived using splicing by overlap extension (SOE) using appropriate primers [28]. All the mutations were confirmed by sequencing. The mutated versions of ced-4 are in the same vector (phsp70PLVI+CAT) as wt Flag-ced-4 and contain a Flag tag as an N-terminal fusion. In ced-4 mutants L17A, L71A, L444A, leucines at positions 17, 71, and 444, respectively, were replaced with alanine, and in F454A, phenylalanine at position 454 was replaced with alanine. In E481A and

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E486A, an alanine replaced the glutamates at positions 481 and 486, respectively. The previously described ced-4 mutant K165M contains a lysine to methionine change at position 165 within the ATP/GTP-binding motif [14].

2.3. Viability assay

Sf-21 cells (0.5×10^6 per 35 mm diameter tissue culture dish) were transfected with indicated amounts of plasmid DNA using Lipofectin (Gibco BRL). Cells were heat-shocked in a 42°C water bath for 30 min beginning at 12–14 h post transfection. Viable cells excluding trypan blue were counted at 10–12 h after heat shock administration as previously described [27].

2.4. Multiple-sequence alignment

The regions in CED-4 predicted to contain a DED were aligned with other cellular and viral proteins known to contain DEDs. The sequence alignment was generated using the PileUp program of the Genetics Computer Group program package (GCG, Madison, WI) with a gap penalty of 3.0 and a gap extension penalty of 0.10. The aligned sequences were then used to generate the final shaded alignment using the Boxshade program version 3.0 (created by Kay Hofmann, Bioinformatics group, ISREC, Switzerland).

The accession numbers for the sequences used are Q13158 for Fadd, U58143 for Flice, U60519 for Mch4, AF009616 for Flame, U60315 for molluscum contagiosum virus (MCV 159L/160L), S55668 for equine herpesvirus-2 E8 (EHV-2), Z46385 for bovine herpesvirus E1.1 (BHV-4), Q01044 for herpesvirus saimiri gene 71(HVS), U90534 for Kaposi's sarcoma-associated herpesvirus orf K13 (HHV-8) and P30429 for Ced-4.

2.5. Immunoprecipitation

At 4 h after heat shock, cells were collected and lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 1 mM DTT) supplemented with protease inhibitor cocktail (Pharmingen, San Diego, CA). Soluble proteins were precipitated using anti-FLAG M2 affinity resin (Eastman Kodak, New Haven, CT), washed five times in NP-40 lysis buffer, and resolved on a 10% acrylamide gel by SDS-PAGE. The resolved proteins were electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA) and probed with monoclonal HA.11 anti-rabbit IgG to detect coprecipitating HA.11 epitope-tagged proteins. The expression of HA.11- or Flag-tagged proteins in the detergent soluble fraction was determined by Western blot analysis using either anti-HA.11 mouse monoclonal IgG or anti-Flag mouse monoclonal IgG and an appropriate secondary antibody conjugated to horseradish peroxidase.

3. Results and discussion

3.1. N-terminal CED-4 mutants are defective in function

Although two amino acid sequence alignments have predicted the presence of two different DEDs in CED-4 based on similarities to conserved death effector domains of Fadd and Flice (Fig. 1B,C) [23,24], the alignment of these two regions with additional, more recently identified DED-containing proteins suggests that their homology to DEDs is weak (Fig. 1A) [25]. To test the functional significance of these two

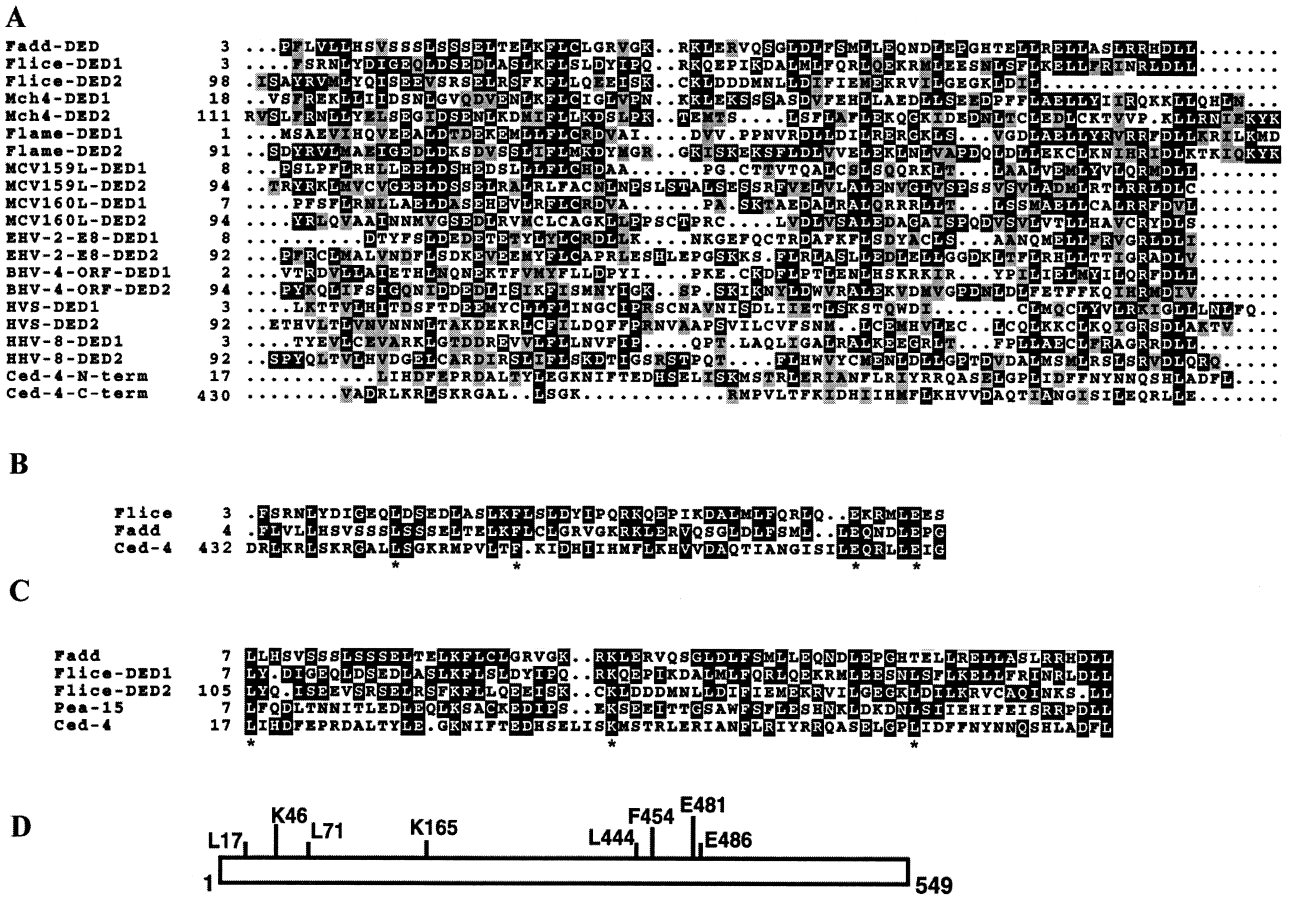

Fig. 1. A: Multiple sequence alignment of the putative DEDs of CED-4 with other DED-containing proteins. B: Sequence alignment of CED-4 with DEDs of Fadd and Flice according to Nagata [24]. C: Sequence alignment of CED-4 with DEDs of Fadd, Flice and Pea-15 as published by Bauer et al. [23]. In B and C the asterisks (*) indicate residues that were mutated. The output of Boxshade is shown in panels A, B and C. Boxshade uses a threshold function to identify identical and conserved residues within a position in the sequence alignment. The identical amino acid residues are enclosed in black boxes and similar amino acid residues are shown in shaded boxes. D: Schematic representation showing the residues that were mutated in CED-4.

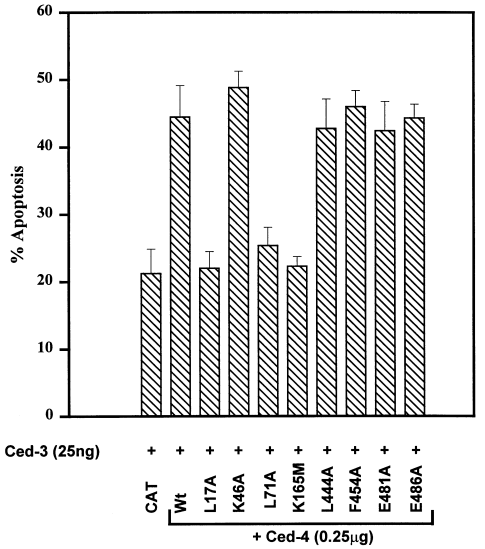

Fig. 2. Mutations in the amino-terminus of CED-4 affect its function. Sf-21 cells were transfected with plasmids expressing either CED-3 or CED-3 and CED-4 constructs as indicated. A plasmid expressing CAT was used to balance the amount of input DNA. At 12–14 h after transfection, expression was induced by heat shock, and cell viability was determined at 10–12 h after heat shock. The results indicate the viability of cells relative to cells transfected with CAT-expressing plasmid alone (not shown) set at 100%.

previously published alignments, we created point mutants in CED-4 at residues which are conserved within the putative DED alignments (Fig. 1B–D), and tested these mutants for function. Previously we have shown that CED-4 stimulates and increases the levels of CED-3-mediated apoptosis in Sf-21 insect cells [14]. We have used this assay to assess the function of the mutant CED-4 constructs. In brief, Sf-21 cells were transfected with plasmids expressing CED-3 or CED-3 and CED-4. Expression was induced by heat shocking and viable cells were quantitated 10–12 h after heat shock. Cells transfected with a plasmid expressing CAT served as the reference for 100% viability. The same plasmid was also used to balance the plasmid levels in cotransfection experiments.

In the absence of CED-4, expression of CED-3 induced apoptosis in about 21% of the cells (Fig. 2). Coexpression of wt CED-4 with CED-3 increased the levels of apoptotic cell death and about 45% of the cells were induced to undergo apoptosis under this condition (Fig. 2 and [14]). As expected, the previously reported null mutant of CED-4 K165M was unable to increase the levels of apoptosis induced by CED-3 (Fig. 2, [14]). Similarly, two mutants in the putative N-terminal DED, L17A and L71A, did not significantly increase the levels of CED-3-mediated apoptosis (Fig. 2), indicating that these two N-terminal residues of CED-4 are important for the activity of CED-4. Expression of all the CED-4 mutants was confirmed by Western blot analysis (see below). The other mutants of CED-4, the N-terminal K46A, and the C-terminal mutants L444A, F454A, E481A and E486A, were not impaired in their ability to augment the levels of CED-3-mediated apoptosis. The levels of apoptosis in cells expressing these mutants and CED-3 ranged from 44 to 49% and did not significantly differ from the 44% cell death observed in the presence of wt CED-4 and CED-3 (Fig. 2). None of the four C-terminal mutants of CED-4 was affected in function. The alignment of the C-terminus of CED-4 with the Flice and Fadd DEDs does not appear to be functionally important.

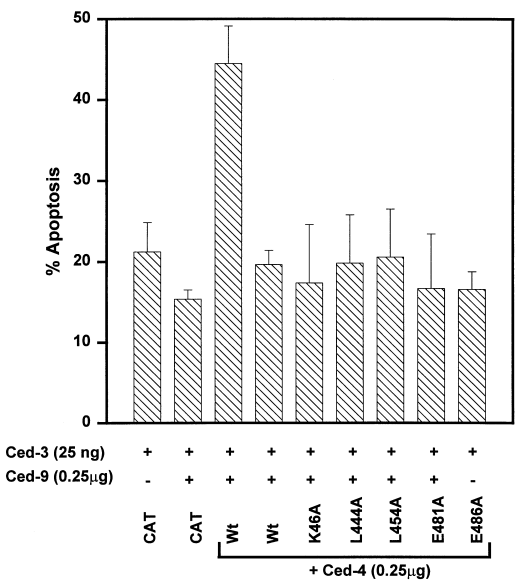

Fig. 3. CED-9 inhibits active CED-4 mutants. Sf-21 cells were transfected with plasmids expressing the various CED-4 mutants, CED-3 and CED-9 or CAT as indicated. The amount of input DNA was kept constant by the addition of a plasmid expressing CAT. At 12–14 h after transfection, the cells were heat-shocked to induce the expression from the transfected constructs. The number of viable trypan blue-excluding cells at 10–12 h after heat shock was used to calculate percent apoptosis. The results shown are relative to the viability of cells transfected with CAT-expressing plasmid set at 100%.

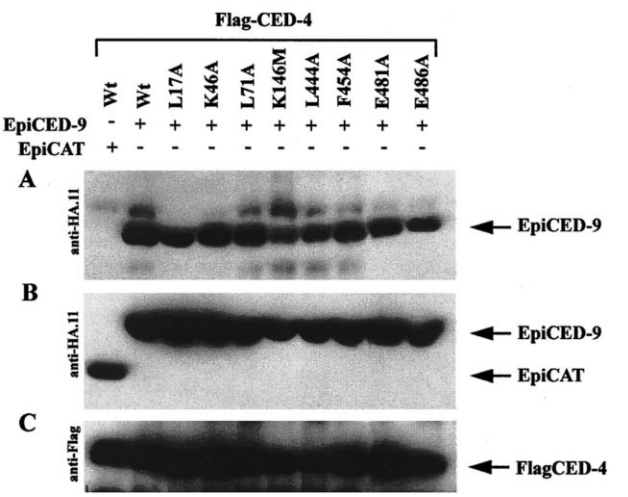

Fig. 4. CED-4 mutants interact with CED-9. A: Sf-21 cells were transfected with plasmids expressing Flag-tagged CED-4 wt or mutants and HA.11 (Epi-)tagged CED-9 or CAT driven by *Drosophila hsp70* promoter, as indicated. Cells were heat-shocked 14 h after transfection and cell lysates were prepared 4 h after heat shock induction. Flag-tagged proteins were immunoprecipitated using anti-Flag monoclonal antibody-coupled resin. Co-precipitating Epi-tagged proteins were then detected by Western analysis using anti-HA.11 monoclonal antibody. B: Expression of Epi-tagged proteins in cell lysates prior to immunoprecipitation. Expression was detected using anti-HA.11 monoclonal antibody. C: Expression of Flag-tagged CED-4 proteins. The same membrane as in A was probed with anti-Flag monoclonal antibody to detect expression of CED-4.

3.2. CED-9 binds and blocks CED-4 mutants

We and others have shown that CED-9 binds and blocks the activity of CED-4. Here we have tested the ability of CED-9 to block the activity of CED-4 mutants [10–16]. Consistent with our previous observation [14], CED-9 did not significantly affect CED-3-induced apoptosis when coexpressed with CED-3 (Fig. 3). Expression of CED-9 along with CED-3 and CED-4 blocks the stimulatory activity of CED-4, reducing the levels of apoptosis to 20% from 44% seen in the absence of CED-9 (Fig. 3). When expressed with the functional mutants of CED-4, CED-9 effectively blocked the activity of all the CED-4-mutants (Fig. 3).

We then tested if the ability of CED-9 to block CED-4 mutants coincided with the ability of the mutant CED-4 proteins to interact with CED-9 (Fig. 4). We coexpressed Flag-tagged CED-4 wt or mutants with HA.11 (Epi-)tagged CED-9 in Sf-21 cells. The Flag-tagged CED-4 proteins were immunoprecipitated using anti-Flag antibody. Coprecipitating HA.11-tagged (Epi) proteins were detected using an HA.11 antibody. We found that, as with wt CED-4, all the mutants of CED-4 were able to interact with CED-9, which is consistent with the ability of CED-9 to block the active CED-4 mutants (Fig. 4). The specificity of this interaction was confirmed by the inability of CED-4 to coprecipitate Epi-CAT (Fig. 4). The interaction between the mutant CED-4 proteins and CED-9 shows that the mutations in CED-4 did not alter the conformation of CED-4 so as to significantly alter the ability of CED-4 to interact with CED-9.

Although our functional analysis of CED-4 mutants does not support the proposal of a DED in the C-terminus of CED-4, the results do establish a role for the N-terminus of CED-4 in its function. Two of the three mutations in this region, the L17A and L71A mutations, eliminated the ability of CED-4 to increase the levels of CED-3-mediated apoptosis. The third mutation, K46A, had no effect on CED-4 function in this assay. However, this lysine residue is conserved only in the original alignments of CED-4 which was limited to the DEDs of Fadd, Flice and Pea-15 [23] and its relevance to DED activity has not been established. The variability of this region of DEDs and the non-conserved nature of this lysine are revealed in more extensive alignments with additional, recently identified DEDs (Fig. 1A). In contrast, the L17A and L71A mutations are well-conserved in both the original and extended alignments. The fact that they inactivate CED-4 indicates that they are functionally important but does not necessarily confirm the presence of a DED in this region. The region encompassing L17 and L71 has also been found to show some sequence similarity to another motif, the CARD (caspase recruitment domain), found in some caspases and other apoptosis-related proteins. L17 and, to a lesser extent, L71 appear to be conserved within this CARD alignment [29]. Our functional analysis does not distinguish if the region functions as a DED or a CARD, or has yet some other structural or biochemical function.

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