# Direct physical interaction between the *Caenorhabditis elegans* 'death proteins' CED-3 and CED-4

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**Abstract The two genes CED-4 and CED-3 (the nematode homologue of interleukin-1β converting enzyme, ICE) of *Caenorhabditis elegans* are implicated in the control of cell death, but the mechanism by which this occurs is unknown. Here we provide evidence that CED-3 and CED-4 both contain sequences with homology to a domain present in RAIDD and the prodomain of certain ICE-like proteases (caspases). This domain is known to establish an interaction between RAIDD and these caspases. Similarly, CED-4 was found to interact with CED-3. Thus, the activity of the death protease CED-3 appears to be controlled by CED-4 through a direct physical interaction.**



*Key words:* *Caenorhabditis elegans*; Apoptosis; Caspase; Interleukin-1β converting enzyme

## 1. Introduction

The apoptotic cell death program of most organisms includes as a central and irreversible step the proteolytic activation of a family of cysteine proteases (ICE/CED-3-like proteases/caspases) from their precursor molecules [1]. The absolute requirement of caspases for cell death is evolutionarily highly conserved. In the nematode *Caenorhabditis elegans*, for example, a caspase (CED-3) was found to be essential for programmed cell death [2]. Similarly, the nematode CED-9 is the structural and functional counterpart of the mammalian inhibitor of apoptosis Bcl-2 [3]. However, no mammalian homologue of the nematode cell death gene CED-4, which is essential for CED-3 function, has been found [4,5]. Although elegant genetic studies predict that CED-4 acts upstream of CED-3, the pathway which couples CED-4 to the protease CED-3 remains elusive.

The most direct signaling pathway leading to the activation of caspases in mammals is induced by death receptors. The members of this receptor family (Fas/Apo-1, TNF-R1, TRAMP/DR-3) relay the death signal via their cytoplasmic death domain (DD) [6,7]. This protein-protein interaction motif forms complexes with other death domain-containing proteins which act as adaptor proteins. Of particular interest is FADD, a bipartite molecule consisting of a DD and a death effector domain (DED) which complexes with the DED of the prodomain of the caspase FLICE, which then directly links the death receptors to caspases [8,9]. Another typical adaptor protein is RAIDD, which consists of two distinct building blocks [10]: a DD and a domain structurally homologous to the prodomain of two caspases, i.e. ICH-1 and CED-3. Similar to DD and DED, this novel protein-protein interaction motif (CARD, for Caspase-Recruitment Domain, see below) can establish a homophilic interaction (in this case between RAIDD and the prodomains of ICH-1 and CED-3), thereby theoretically translocating these caspases to the vicinity of death receptors.

## 2. Materials and methods

### 2.1. Construction of expression vectors

The tagged versions of CED-3 and CED-4 were prepared by cloning the corresponding cDNAs in a modified pCR3 vector (Invitrogen) containing a FLAG or a VSV epitope [11]. Proteins were tagged at the N-terminus.

### 2.2. Cell transfection, coimmunoprecipitation, and Western blot analysis

A 35 mm plate with $5\times10^5$ 293T cells was transfected by calcium phosphate precipitation with 5 μg of total DNA in the presence of 25 μM z-VAD (Bachem). Cells were harvested 24 h after transfection and lysed in 150 μl of lysis buffer (1% NP-40, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, supplemented with a protease inhibitor mixture). Postnuclear lysates were precleared for at least 1 h on Sepharose 6B (Pharmacia) and incubated with 5 μl of anti-FLAG agarose (Scientific Imaging Systems, Kodak) overnight at 4°C. Immune complexes were washed 4 times with lysis buffer, separated on a 12% SDS-polyacrylamide gel, and immunoblotted with anti-FLAG or anti-VSV (Sigma) monoclonal antibodies. Proteins were detected using the ECL kit (Amersham).

## 3. Results and discussion

In view of the high conservation of the death pathways, we reasoned that such a RAIDD-like adaptor molecule may also exist in nematodes and therefore screened the *C. elegans* sequence data base for the presence of DD and CARD containing molecules using the generalized profile method [12]. Even though the search for DD domains was negative, we identified with great significance a CARD motif located at the N-terminus of CED-4. The alignment of the CARD of CED-4 with those present in CED-3, RAIDD and ICH-1 shows that CARDs have 17–26% identical amino acid residues (Fig. 1). Moreover, phylogenetic analysis by the neighbor-joining method [13] revealed that the CARDs of CED-3 and CED-4 are more closely related to each other than to ICH-1 and RAIDD.

Since the CARD motif mediates the binding of the adaptor protein RAIDD to the caspase ICH-1, we investigated whether the CARDs of CED-3 and CED-4 were also able to interact with each other. HEK 293T cells were co-trans-

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Fig. 1. Amino acid sequence alignment of CED-3, CED-4, human RAIDD and human ICH-1. Sequence numbering starts at the N-terminus of the predicted mature protein. Positions with identical or similar residues in at least 50% of the sequences are represented in black and shaded boxes, respectively.

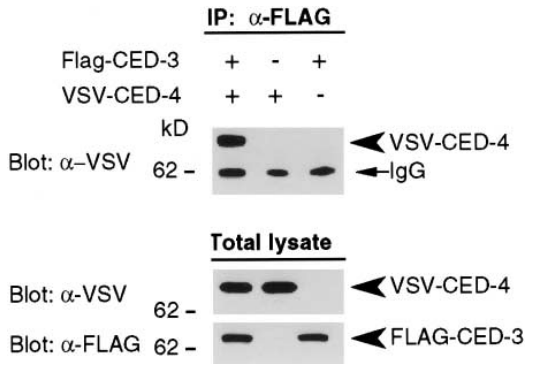


Fig. 2. Interaction of CED-4 with CED-3. 293T cells were transfected with FLAG-tagged CED-3 and VSV-tagged CED-4. CED-3 and CED-4 expression levels were assayed in cell lysates using antibodies to recognize the FLAG and the VSV epitope, respectively. The cleared lysates were immunoprecipitated with anti-FLAG antibodies (precipitating CED-3), and the presence of co-precipitating CED-4 was assessed by Western blot analysis using an anti-VSV antibody. In control experiments, 293T cells were transfected with either CED-3 or CED-4 expressing plasmids only.

fected with expression constructs encoding FLAG-tagged CED-3 and VSV-tagged CED-4. We found that full-length CED-4 is indeed associated with CED-3 (Fig. 2). Binding was also observed when immobilized CED-3 was incubated with $^{35}$S-labeled CED-4 (data not shown).

Taken together, CED-4 associates with the death protease CED-3 most likely through a homophilic CARD interaction similar to the binding observed between RAIDD and ICH-1. RAIDD acts as an adaptor protein recruiting the caspase to the TNFR-1 signaling complex via its DD. Future studies need to be undertaken to show whether in nematodes translocation of death proteases to receptor complexes is conserved, and whether the C-terminal sequence of CED-4 serves to make the link to such a receptor complex.

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