Essential Role of CED-4 Oligomerization in CED-3 Activation and Apoptosis

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Control of the activation of apoptosis is important both in development and in protection against cancer. In the classic genetic model *Caenorhabditis elegans*, the pro-apoptotic protein CED-4 activates the CED-3 caspase and is inhibited by the Bcl-2–like protein CED-9. Both processes are mediated by protein-protein interaction. Facilitating the proximity of CED-3 zymogen molecules was found to induce caspase activation and cell death. CED-4 protein oligomerized in cells and in vitro. This oligomerization induced CED-3 proximity and competed with CED-4:CED-9 interaction. Mutations that abolished CED-4 oligomerization inactivated its ability to activate CED-3. Thus, the mechanism of control is that CED-3 in CED-3:CED-4 complexes is activated by CED-4 oligomerization, which is inhibited by binding of CED-9 to CED-4.

Apoptosis, a process of cell suicide critical for development and tissue homeostasis of multicellular organisms, is controlled by an evolutionarily conserved program (*1, 2*). Genetic studies in the nematode *C. elegans* have identified three core components of the death machine (*2*). CED-4 activates the apoptotic protease CED-3, whereas CED-9 inhibits CED-4 function. Both of these interactions involve direct protein-protein contact in a ternary protein complex termed the apoptosome (*3–6*). How these interactions activate or inhibit cell death is unknown. CED-3 is a homolog of a family of mammalian cysteine protease (caspase) zymogens that, when activated, cleave various cellular proteins to execute apoptosis (*7*). CED-9 is a homolog of the mammalian Bcl-2 family of antiapoptotic proteins (*8*). Apaf-1, a mammalian homolog of CED-4 that activates pro-caspase-9, has also been identified (*9*).

Caspase zymogens that link to surface receptors are autoproteolytically converted to mature enzymes by the induced proximity of their protease domains (*10–12*). To test whether CED-3 can be activated in a similar way, we fused the CED-3 protease domain to three copies of the FK506-binding protein (Fkp) (Fig. 1A). Expression of this fusion protein, Fkp3–CED-3(205), in HeLa cells caused minimal cell death. However, oligomerization of the fusion protein by a dimeric ligand for Fkp, AP1510 (*13*), induced apoptosis in a dose-dependent and saturable manner, whereas addition of the monomeric, competitive Fkp ligand FK506 partially inhibited the effect (Fig. 1B). In a cell-free system, AP1510 induced in vitro–translated, ^{35}S-labeled Fkp3–CED-3(205) fusion protein to be cleaved, generating the mature CED-3 subunits, p17 and p15 (Fig. 1C). CED-3 processing was inhibited by FK506 or the caspase inhibitor z-DEVD (Fig. 1C), indicating that oligomerization and caspase activity are required for zymogen processing. These results imply that CED-3 can be activated by the induced proximity provided by drug-mediated protein aggregation.

Because CED-4 interacts with CED-3, we reasoned that CED-4 could induce CED-3 proximity and activation if CED-4 had the ability for homotypic oligomerization. This possibility was examined by coimmunoprecipitation assays in transfected human 293T cells expressing two differentially epitope-tagged CED-4 proteins. Myc–CED-4 specifically coimmunoprecipitated FLAG–CED-4, suggesting that CED-4 protein can oligomerize in cells (Fig. 2, A and B). Deletion analysis revealed that the CED-4 region consisting of amino acids 171 to 435 and mutants incorporating it, but not other parts of CED-4, interacted with full-length CED-4 (Fig. 2, A and B). The oligomerization domain of CED-4 is distinct from the NH_2-terminal CED-3–interaction domain (*3*), is smaller than the sequence required for CED-9 interaction (*14*), and is encompassed in a domain that is similar to mammalian Apaf-1 (*9*) (Fig. 2A). Deletion or mutation (K165R; Lys at position 165 mutated to Arg) of the putative adenosine triphosphate–binding P-loop (*15*) had no effect on CED-4 oligomerization. CED-4L, an antiapoptotic splice variant of CED-4 (*16*), also bound CED-4. In vitro–translated [^{35}S]CED-4 specifically bound to purified recombinant glutathione S-transferase (GST)–CED-4 fusion protein. Equal amounts of either in vitro–translated CED-3 or CED-4 bound with comparable efficiency

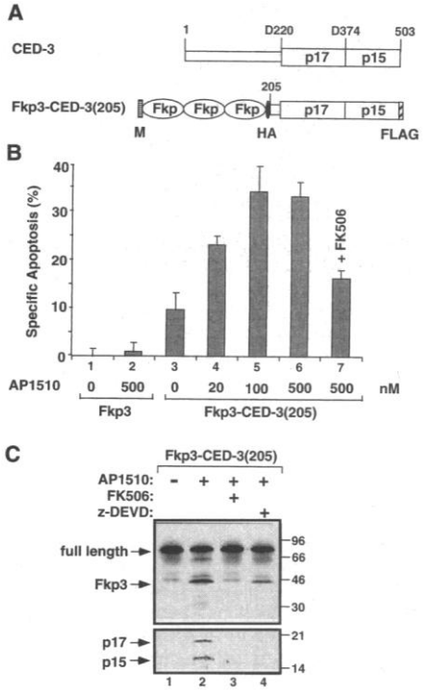

Fig. 1. Activation of the CED-3 protease by induced proximity. (**A**) Representation of CED-3 protein and Fkp fusion of the CED-3 protease domain. p17 and p15 indicate domains that form the mature CED-3 protein. D, aspartic acids at the cleavage sites for the generation of mature CED-3. M, c-Src myristylation signal. HA (hemagglutinin) and FLAG are epitope tags. (**B**) Oligomerization of Fkp–CED-3(205) enhanced its cell death activity. HeLa cells were transiently transfected with 0.15 μg of Fkp3 or Fkp3–CED-3(205) plasmid together with pCMV-lacZ (0.25 μg) (*23*). Twelve hours after transfection, AP1510 (Ariad Pharmaceuticals) at the indicated final concentration and FK506 at 50 nM were added to the cultures. Cells were stained for β-galactosidase expression 8 hours later and scored for specific apoptosis (*23*). Data (mean ± SD) were from at least three experiments, and in each experiment more than 300 blue cells were counted. (**C**) CED-3 processing induced by oligomerization. [^{35}S]methionine-labeled Fkp3–CED-3(205) was produced by coupled in vitro transcription and translation with TNT Reticulocyte Lysate System (Promega) for 30 min. The processing reaction was done with final drug concentrations of 500 nM AP1510, 200 nM FK506, or 1 μM Z-Asp-Glu-Val-Asp (z-DEVD, Enzyme System Products), (*10*). Reaction products were resolved by SDS-PAGE and detected by autoradiography. The bottom panel was exposed twice as long as the top panel. Molecular size standards (in kilodaltons) are shown on the right.

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to GST–CED-4, suggesting a similar affinity of the two interactions (Fig. 2C). The CED-4 oligomerization is probably direct, or at a minimum involves proteins found in reticulocyte lysates. Finally, the Apaf-1 region (amino acids 1 to 465) that is homologous to CED-4 and sufficient to activate pro–caspase-9 (*17*) could also homotypically oligomerize (Fig. 2D).

To examine whether homotypic CED-4 interactions could affect the trimeric CED-3:CED-4:CED-9 complex, we added the three components along with a truncated CED-4(171–549) that binds to CED-4 but not CED-9 or CED-3. Myc-tagged CED-4 provided the handle for immunoprecipitation. CED-4(171–549) competed with CED-9 but not CED-3 for interaction with Myc-CED-4 (Fig. 3A). CED-4(171–549) also inhibited the binding of a mammalian CED-9 homolog, Bcl-x_L (*3*, *17*), to CED-4 without affecting the CED-4:CED-3 interaction (Fig. 3A). These results indicate that CED-9 or Bcl-x_L binding to CED-4 is mutually exclusive with CED-4 oligomerization, and that CED-4 can simultaneously associate with itself and CED-3. Myc–CED-3 specifically coimmunoprecipitated with FLAG–CED-3 only in the presence of CED-4 (Fig. 3B), showing that CED-4 can induce the proximity of CED-3 molecules. In these experiments, the endogenous mammalian homologs did not seem to interfere with protein interactions, perhaps because of the higher expression level of the transfected proteins.

To assess the functional role of CED-4 oligomerization, we mutated amino acids within the CED-4 oligomerization domain that are conserved among CED-4, Apaf-1, and related plant *R* genes (*18*). Each CED-4 mutant (mut1 to mut5, Fig. 4A) expressed stable full-length protein but failed to oligomerize (Fig. 4B). Mutation 1 (D250A, D251A) is a loss-of-function mutant, but it can still bind CED-3 (*3*). Similarly, mutations 2 through 5 also can bind CED-3 (*19*), consistent with previous domain mapping experiments (*3*). We tested the pro-apoptotic activity of these CED-4 mutants in a transient transfection system in HeLa cells that accurately reflected the in vivo function of the apoptosome components. Expression of CED-3 or CED-4 alone had no cytotoxicity in HeLa cells, but their coexpression led to robust apoptosis in more than 60% of the cells (Fig. 4C). This apoptotic effect required the intrinsic caspase activity of CED-3 (Fig. 4C). Coexpression of antiapoptotic proteins CED-9 or Bcl-x_L completely inhibited the ability of CED-4 to activate CED-3–dependent death (Fig. 4C). None of the five CED-4 mutants were able to activate CED-3–dependent death (Fig. 4C). The correlation between the loss of CED-4 oligomerization and the loss of the ability to activate CED-3 indicates that CED-4 oligomerization may be essential for its pro-apoptotic function.

The present study suggests CED-4 oligomerization as a unifying mechanism in apoptosome function. Previous work demonstrated that CED-9 or Bcl-x_L, upon binding pro-apoptotic, BH3-containing ligands such as Bax, is released from the apoptosome (*3*). EGL-1, a *C. elegans* BH3-containing ligand functioning upstream of CED-9, is also thought to displace CED-9 from CED-4 (*20*). We propose that release from CED-9 allows CED-4 to oligomerize, bringing the associated CED-3 proteins into close proximity and facilitating subsequent autoproteolytic activa-

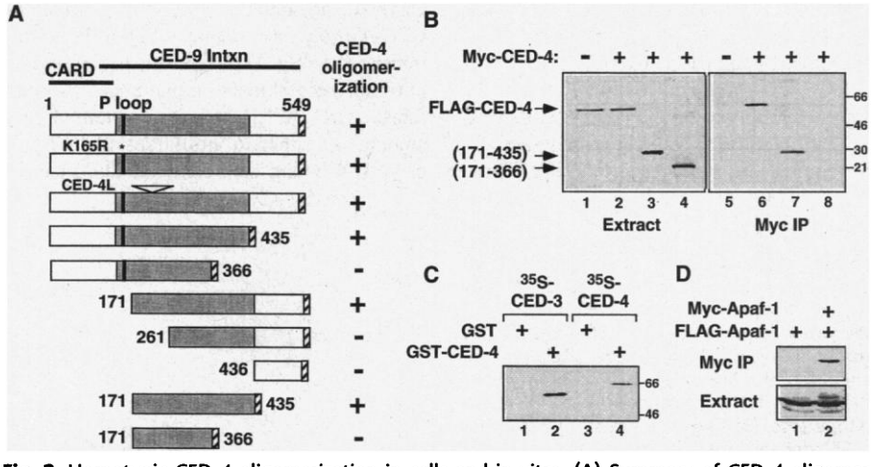

Fig. 2. Homotypic CED-4 oligomerization in cells and in vitro. **(A)** Summary of CED-4 oligomerization data. Amino acid boundaries of deletion mutants, CED-4 sequence that is similar to Apaf-1 (shaded box) (*9*), the nucleotide binding P-loop (solid vertical line) (*15*), the previously identified caspase activation and recruitment domain (CARD) (*18*), and the CED-9 interaction (Intxn) domain are indicated (*14*). CED-4L contains a 24–amino acid insert after amino acid 212 (*16*). Each CED-4 mutant (*23*) was FLAG-tagged at the COOH-terminus (hatched box) and tested for interaction with Myc–CED-4 by coimmunoprecipitation as illustrated in (B). **(B)** Representative data examining CED-4 self association. Human 293T cells were cotransfected with 2 μg each of vector (lanes 1 and 5) or Myc–CED-4 (lanes 2 to 4 and 6 to 8) and FLAG–CED-4 (lanes 1, 2, 5, and 6), FLAG–CED-4(171–435) (lanes 3 and 7), or FLAG–CED-4(171–366) (lanes 4 and 8) (*23*). Immunoprecipitation (IP) with anti-Myc conjugated to agarose beads (Santa Cruz Biotechnology) was performed as described (*24*). The bound proteins (right panel) or 5% of input IP extract (left panel) were resolved by SDS-PAGE and detected by immunoblotting with polyclonal FLAG antibody (Santa Cruz). Molecular size standards (in kilodaltons) are indicated on the right. **(C)** Interaction of GST–CED-4 with in vitro–translated CED-3 and CED-4. The ^{35}S-labeled CED-3 and CED-4 proteins were made with the TNT Reticulocyte Lysate System. The translation product (2 μl) was incubated with 1 μg of immobilized GST (lanes 1 and 3) or GST–CED-4 fusion protein (lanes 2 and 4) in 100 μl of IP-lysis buffer (*24*) for 2 hours at 4°C. After three washes with 500 μl of buffer, the bound [^{35}S]CED-3 or [^{35}S]CED-4 proteins were resolved by SDS-PAGE and detected by autoradiography. **(D)** Oligomerization of Apaf-1(1–465). Human 293T cells were transfected with 2.5 μg of FLAG–Apaf-1(1–465) plus 2.5 μg of vector (lane 1) or Myc–Apaf-1(1–465) (lane 2). Top: Cell lysates were immunoprecipitated with anti-Myc beads and immunoblotted with anti-FLAG as described in (B). Bottom: Expression of FLAG–Apaf-1(1–465) in the extracts.

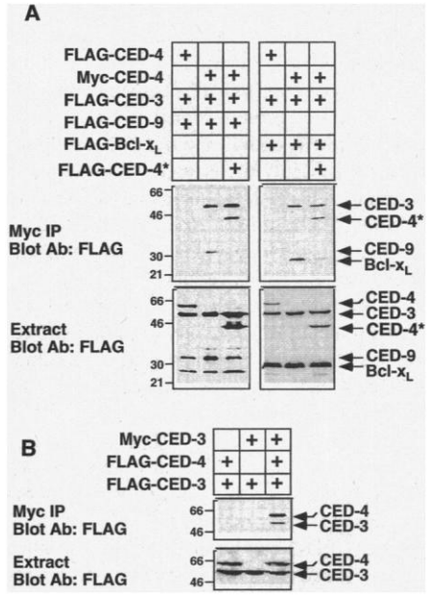

Fig. 3. **(A)** Effect of CED-4 oligomerization on the ternary CED-9:CED-4:CED-3 complex. FLAG–CED-3(C358S), FLAG–CED-9 or FLAG–Bcl-x_L and FLAG– or Myc–CED-4 (1 μg each) were cotransfected with pRK vector or FLAG–CED-4(171–549) (2 μg each) in 293T cells in the indicated combinations (*22*). CED-4*, CED-4(171–549). **(B)** CED-4 mediates CED-3:CED-3 interaction. Human 293T cells were cotransfected with Myc–CED-3(C358S) (2 μg), FLAG–CED-3(C358S) (1 μg), and FLAG–CED-4 (2 μg) in the indicated combinations. In the top panels of (A) and (B), cell lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-FLAG as in Fig. 2B. The bottom panels are immunoblots of 5% (A) or 7.5% (B) of IP input. Molecular size standards (in kilodaltons) are shown on the left.

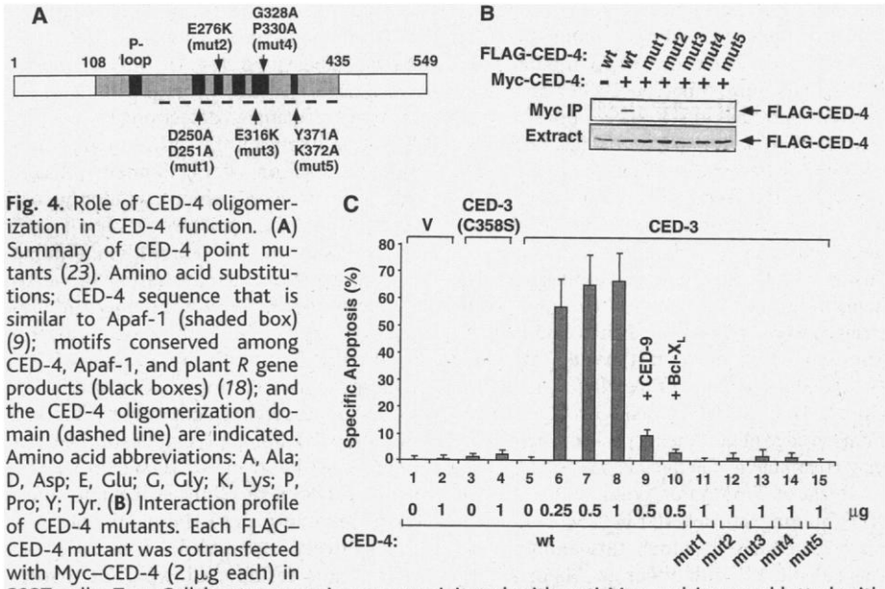

Fig. 4. Role of CED-4 oligomerization in CED-4 function. (**A**) Summary of CED-4 point mutants (*23*). Amino acid substitutions; CED-4 sequence that is similar to Apaf-1 (shaded box) (*9*); motifs conserved among CED-4, Apaf-1, and plant *R* gene products (black boxes) (*18*); and the CED-4 oligomerization domain (dashed line) are indicated. Amino acid abbreviations: A, Ala; D, Asp; E, Glu; G, Gly; K, Lys; P, Pro; Y; Tyr. (**B**) Interaction profile of CED-4 mutants. Each FLAG–CED-4 mutant was cotransfected with Myc–CED-4 (2 μg each) in 293T cells. Top: Cell lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-FLAG as in Fig. 2B. Bottom: Immunoblot of 5% of input IP extract. (**C**) Pro-apoptotic activity of CED-4 mutants. HeLa cells were transfected with indicated amount of CED-4 or CED-4 mutants; 50 ng of vector (lanes 1 and 2), CED-3(C358S) (lanes 3 and 4), or CED-3 (lanes 5 to 15); 1 μg of CED-9 (lane 9) or Bcl-x_L (lane 10); and 0.25 μg of pCMV-lacZ. Cells were stained 16 hours after transfection and scored for specific apoptosis (*25*). Data shown (mean ± SD) are from at least three independent experiments, and in each experiment more than 300 blue cells were counted; wt, wild type.

tion. Mammalian Apaf-1 can also oligomerize and may function in a similar way to activate pro–caspase-9. In this model, the activation of CED-9–binding activity of a pro-apoptotic protein upstream of the apoptosome is a key event and requires further investigation. Additional regulators, such as dATP and cytochrome c (*21*), may control apoptosome function through similar or distinct mechanisms.

Note added in proof: It was recently shown that Apaf-1 can form oligomers and may activate pro–caspase-9 molecules by oligomerizing them (*22*).

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23. The fusion protein pFkp3–CED-3(205), containing three copies of Fkp fused to CED-3 amino acids 205 to 503, was made in pRK5 as described (*10*). CED-3, CED-3(C358S), CED-4, CED-4 mutants, CED-9, Bcl-x_L, and Apaf-1(1–465) were each fused with a COOH-terminal FLAG epitope tag in pRK5. CED-3(C358S) was also fused to a COOH-terminal AU1 epitope tag in pRK5. Myc–CED-4 and Myc–CED-4 (K165R) were described (*3*). Myc–Apaf-1(1–465) and Myc–CED-3(C358S) were made in pcDNA3.1(-)/MycHis (Invitrogen) with a COOH-terminal Myc tag. Authenticity of each construct was confirmed by DNA sequencing.
24. The day before transfection, 2 × 10^6 293T cells [grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml), and glutamine (1 mM)] were plated per 60-mm dish. Twenty-four hours after transfection by the calcium phosphate method, cells were lysed in 300 μl of IP-lysis buffer [50 mM Hepes (pH 7.4), 1% NP-40, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol] supplemented with 1 mM phenylmethylsulfonyl fluoride and 1% aprotinin. Extracts (100 μl) were diluted 1:1 in IP-lysis buffer and immunoprecipitated with antibody for 3 hours at 4°C, washed with 600 μl of IP-lysis buffer, and resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
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Structure and Asn-Pro-Phe Binding Pocket of the Eps15 Homology Domain

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Eps15 homology (EH) domains are eukaryotic signaling modules that recognize proteins containing Asn-Pro-Phe (NPF) sequences. The structure of the central EH domain of Eps15 has been solved by heteronuclear magnetic resonance spectroscopy. The fold consists of a pair of EF hand motifs, the second of which binds tightly to calcium. The NPF peptide is bound in a hydrophobic pocket between two α helices, and binding is mediated by a critical aromatic interaction as revealed by structure-based mutagenesis. The fold is predicted to be highly conserved among 30 identified EH domains and provides a structural basis for defining EH-mediated events in protein trafficking and growth factor signaling.

Protein interaction domains such as Src homology domains 2 and 3 are devoted to the recruitment of ligands into multiprotein complexes (*1*). The recently discovered EH domain (*2*) is an interaction module that targets NPF-containing proteins such as RAB, NUMB (*3, 4*), clathrin assembly proteins (*5*), and synaptojanin (*6*). Proteins containing these EH domains mediate critical events in endocytosis (*7, 8*) and actin cytoskeletal organization (*8*), and they participate in signaling in conjunction with tyrosine kinases (*2, 9*)

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