The Death Inhibitory Molecules CED-9 and CED-4L Use a Common Mechanism to Inhibit the CED-3 Death Protease*

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The apoptotic machinery of Caenorhabditis elegans includes three core interacting components: CED-3, CED-4, and CED-9. CED-3 is a death protease composed of a prodomain and a protease domain. CED-4 is a P-loop-containing, nucleotide-binding molecule that complexes with the single polypeptide zymogen form of CED-3, promoting its activation by autoprocessing. CED-9 blocks death by complexing with CED-4 and suppressing its ability to promote CED-3 activation. A naturally occurring alternatively spliced form of CED-4 that contains an insertion within the nucleotide-binding region (CED-4L) functions as a dominant negative inhibitor of CED-3 processing and attenuates cell death. Domain mapping studies revealed that distinct regions within CED-4 bind to the CED-3 prodomain and protease domain. Importantly, the CED-4 P-loop was involved in prodomain binding. Disruption of P-loop geometry because of mutation of a critical lysine (K165R) or insertional inactivation (CED-4L) abolished prodomain binding. Regardless, K165R and CED-4L still retained CED-3 binding through the protease domain but were unable to initiate CED-3 processing. Therefore, the CED-4 prodomain interaction is critical for triggering CED-4-mediated CED-3 processing. Underscoring the importance of this interaction was the finding that CED-9 contacted the P-loop and selectively inhibited its interaction with the CED-3 prodomain. These results provide a simple mechanism for how CED-9 functions to block CED-4-mediated CED-3 processing and cell death.

Tissue homeostasis in multicellular organisms depends upon appropriately regulated programmed cell death (1–4). Disruption of this physiologic process, termed apoptosis, contributes to the pathogenesis of several human diseases (5–7). Apoptosis is evolutionarily conserved and genetically regulated (8). The genetic dissection of developmental cell death in the nematode Caenorhabditis elegans has illuminated three core components of the cell death pathway: CED-3, CED-4, and CED-9 (9). Although CED-3 and CED-4 induce cell death, CED-9 is a negative regulator and inhibits apoptosis.

Mammalian counterparts to the worm components include the Bcl-2 family that is related to CED-9 (10, 11), the caspase family of proteases that are similar to CED-3 (12), and the recently identified Apaf-1 molecule that is like CED-4 (13–15). The interchangeability of death components between worm and man emphasize their conservation and suggest that they likely share a fundamentally similar mechanism of action. For example, CED-3 transfected into mammalian cells will effectively activate endogenous caspases leading to cell death (16). Conversely, ced-9 loss of function worm mutants can be partially complemented by human Bcl-2 (10). Although similar in outline, the mammalian pathway is complex in that each family has a number of distinct gene products. The Bcl-2 family, for example, has upwards of 16 members (17–21), some of which, like CED-9, function to inhibit cell death, whereas others promote cell death. Given that the basic mechanism of action is likely conserved, we have sought to understand how the core components of the worm death machine function as a means of illuminating the underlying biochemistry of mammalian cell death.

CED-4 functions by binding CED-3 and facilitating its proteolytic autoactivation from the zymogen form to the active dimeric species (16, 22–26). Although CED-4 itself is not a protease, it contains a phosphate-binding P-loop motif and a magnesium-binding site that is observed in nucleotide-binding proteins, including ATPases (26). Mutation of the P-loop motif inhibits the ability of CED-4 to activate CED-3 (16, 26). Additionally, CED-4 can be photoaffinity labeled by the ATP analogue 8-N3ATP (26). Further, the noncleavable ATP analogue FSBA blocks CED-4-mediated activation of CED-3 (26), indicating a requirement for ATP hydrolysis. Collectively, these data indicate that CED-4-mediated activation of CED-3 requires an intact nucleotide binding capability and can be extrapolated to suggest that CED-4 functions as an ATPase. Native CED-4 is capable of simultaneously binding the proapoptotic CED-3 zymogen and the anti-apoptotic CED-9 molecule to form a neutral ternary complex (16, 23–25, 27, 28). In this complex, CED-4 bound to CED-9 is still able to bind CED-3 but is unable to stimulate its processing. Intriguingly, CED-4L contains an in-frame 72-base pair insertion between the P-loop and Mg2+-binding site (29) that should disrupt the architecture of the nucleotide-binding domain. In keeping with this notion, CED-4L could not be affinity labeled using an azido-ATP analogue. A more comprehensive description of the binding regions involved was obtained by undertaking a deletional mapping study that unexpectedly provided mechanistic insight into how CED-4L and CED-9 abrogate cell death.

EXPERIMENTAL PROCEDURES

Cells and Transfections—Epitope-tagged expression constructs were made in pcDNA5 or pcDNA3.1/MycHisA (Invitrogen) using standard recombinant methods. 293T cells were transiently transfected with the indicated expression plasmids using the calcium phosphate method as described previously (16). The catalytic mutant (CED-3mt) was utilized as wild type CED-3 activated the apoptotic program upon overexpress-

1 The abbreviations used are: 8-N3ATP, 8-azidoadenosine-5’-triphosphate; FSBA, 5’-fluorosulfonyl benzoyladenosine; mAb, monoclonal antibody; HA, hemagglutinin.
2 D. Chaudhary and V. M. Dixit, unpublished observation.
FIG. 1. Mutational analysis of CED-4. A, schematic representation of CED-4 deletion mutants and their interactions. B, C, D, and E, CED-3 binds CED-4^{171-549}, CED-4^{1-152}, and CED-4^{K165R} but not CED-4^{269-549} in vivo. The catalytic mutant (CED-3mt) was utilized as wild type CED-3 activated the apoptotic program upon overexpression. 293T cells were transfected with the indicated tagged expression constructs as described under "Experimental Procedures." After 38 h, extracts were prepared and immunoprecipitated (IP) with a control mAb (C), Myc mAb (myc) (Boehringer Mannheim) for CED-4 proteins, or FLAG mAb (IBI Kodak) for CED-3 proteins. Upper insets show expression levels of FLAG-tagged CED-3. F, in vitro translated CED-3 binds CED-4, CED-4^{171-549}, CED-4^{1-152}, but not CED-4^{269-549}. 35S-Labeled CED-3 was prepared by coupled in vitro transcription translation and incubated with immunopurified CED-4 or CED-4 deletions or a control immunopurification.
Deletion of the P-loop Motif Disrupts CED-4-CED-3 Prodomain Binding—CED-4 deletion mutants (Myc-His or HA epitope-tagged) were co-expressed in 293T cells with FLAG epitope-tagged CED-3. The lysates were divided equally for immunoprecipitation with control or specific antibodies followed by Western blotting for CED-3 proteins co-precipitated with CED-3 (Fig. 1, A–E), this segment contained the CED-3-binding site. Further, because the N-terminal truncation CED-4~171–549 bound CED-3 (Fig. 1B), an interaction site could be localized to residues 171–269 of CED-4. However, because CED-4~152 also co-precipitated with CED-3 (Fig. 1D), two distinct CED-3 interaction domains must exist within the N-terminal half: residues 1–152 and 171–269.

**RESULTS AND DISCUSSION**

**Deletion of the P-loop Motif Disrupts CED-4-CED-3 Prodomain Binding**—CED-4 deletion mutants (Myc-His or HA epitope-tagged) were co-expressed in 293T cells with FLAG epitope-tagged CED-3. The lysates were divided equally for immunoprecipitation with control or specific antibodies followed by Western blot analysis for CED-3 and CED-3~mt. In vitro translation of 35S-labeled CED-3 or CED-3~mt was synthesized by in vitro transcription/translation (Promega) (30 min, 30 °C). Prolonged incubation of the in vitro translate triggers auto-processing of CED-3 (30) and was therefore avoided. The processing reactions were performed using 5 μl of radioactive lysis buffer and washed extensively in 500 mM NaCl buffer as described above and analyzed by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

**Subcellular Localization—Epitope expression constructs were transfected in 293T cells using calcium phosphate. The cells were analyzed 24 h following transfection. Briefly, cells were incubated with antibodies against the indicated epitopes for 1 h at 37 °C, followed by incubation with fluorescein isothiocyanate-conjugated (Sigma) or Cy3-conjugated secondary antibodies (Sigma) for 1 h at 37 °C. The cells were washed and mounted in Prolong Antifade (Molecular Probes) and analyzed by confocal microscopy (Bio-Rad MRC 600 scanning confocal microscope).

**In Vitro Processing of CED-3**—Extracts were prepared from 293T cells 36 h following transfection with the indicated expression constructs. Cells were incubated in cold hypotonic buffer containing protease inhibitors. The lysates were divided equally and immunoprecipitated with control or specific antibodies as indicated for 2 h at 4 °C. The beads were washed with lysis buffer (adjusted to 500 mM NaCl) three times and transferred to nitrocellulose. Subsequent protein immunoblotting was performed as described previously (16).

**In Vitro Binding Analysis**—CED-4 constructs were expressed in 293T cells and purified by immunoprecipitation and immobilization on protein G beads, followed by 2 h of incubation with 35S-labeled CED-3 at 4 °C. The lysates were divided equally and immunoprecipitated with control or specific antibodies as indicated for 2 h at 4 °C. The beads were washed with lysis buffer (adjusted to 500 mM NaCl) three times and transferred to nitrocellulose. Subsequent protein immunoblotting was performed as described previously (16).

**Co-immunoprecipitation**—Immunoprecipitations were performed 24–38 h following transfection essentially as described previously (16). Briefly, cells were harvested and lysed in 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and protease inhibitors). The lysates were divided equally and immunoprecipitated with control or specific antibodies as indicated for 2 h at 4 °C. The beads were washed with lysis buffer (adjusted to 500 mM NaCl) three times and transferred to nitrocellulose. Subsequent protein immunoblotting was performed as described previously (16).

**Terminal truncation CED-4~171–549 bound CED-3** (Fig. 1B), an interaction site could be localized to residues 171–269 of CED-4. However, because CED-4~152 also co-precipitated with CED-3 (Fig. 1D), two distinct CED-3 interaction domains must exist within the N-terminal half: residues 1–152 and 171–269. These domain interactions are also observed in an in vitro binding analysis of 35S-labeled CED-3 with CED-4~171–549 and CED-4~152 (Fig. 1F).

Because CED-4 binds to both the prodomain and the catalytic protease domain of CED-3 (16), these domains were inde-
pendently expressed to map the interaction sites. As shown in Fig. 2A, both the prodomain and the protease domain-bound CED-41–152 that contains the P-loop. Sequential N-terminal deletions of CED-4 did not result in loss of prodomain binding until the P-loop motif was eliminated (Figs. 1A and 2B). However, CED-4171–549 missing the P-loop still retained binding to the protease domain (Fig. 2B). Therefore, although the CED-3 prodomain required the presence of residues 152–171 containing the P-loop motif for binding to CED-4, the protease domain did not. Sequence upstream of the P-loop CED-41–232 also bound to the protease domain (Fig. 2C) and did not interact with the prodomain (Fig. 2C). Therefore, there exist two distinct protease domain-binding sites that lie upstream and downstream of the P-loop motif (residues 1–152 and 171–269). Taken together, these data do not substantiate the hypothesis that CED-4 binds CED-3 through a homophilic interaction involving potential caspase activation recruitment domains present within the CED-3 prodomain and N-terminal 100 residues of CED-4 (31, 32). Rather, CED-41–152 fails to bind the prodomain but instead interacts with the protease domain (Fig. 2C). This observation is in contrast to the mammalian counterparts Apaf-1 and caspase-9 (33, 34) that bind each other through N-terminal caspase activation recruitment domain motifs. Interestingly, CED-41–232 with the Mg2+–binding domain deleted retains CED-3 binding. Although Mg2+–binding motif is essential for CED-4 function, it is not essential for initial contact with the CED-3 prodomain. Intactness of the P-loop and the nucleotide-bound conformation may be required for CED-4 to promote the processing of CED-3 zymogen, following binding to the prodomain.

CED-4-mediated CED-3 Processing Requires the P-loop-Prodomain Interaction—Because both CED-4L (16) and the P-loop inactivating point mutant K165R (Fig. 1E) bound CED-3 but did not initiate its processing (26), we hypothesized that this was because of lack of the crucial P-loop-prodomain interaction. In such a scenario, the observed binding would be mediated exclusively through the protease domain. Consistent with this, CED-4K165R as well as CED-4L, bound the CED-3 protease domain but failed to interact with the CED-3 prodomain (Fig. 3A).
If the P-loop-prodomain binding is indeed crucial for triggering processing, it follows that interrupting this interaction by competing with free prodomain should attenuate CED-4-mediated CED-3 processing. As previously shown, in vitro translated 35S-labeled CED-3 is processed to active signature subunits (26) when incubated with extracts expressing native CED-4 (Fig. 3B) but not CED-4L. However, this processing was inhibited by extracts expressing the prodomain, confirming the relevance of the prodomain-P-loop interaction in initiating CED-3 processing.

CED-9 Selectively Disrupts the CED-3 Prodomain Binding to CED-4—In the presence of CED-9, a ternary complex is assembled with CED-4 still bound to CED-3 (Fig. 4A), but no longer able to trigger its processing (16, 22–26, 27, 28). To define the mechanism by which CED-9 accomplishes this, we asked if, in the CED-3-CED-4-CED-9 ternary complex, the crucial prodomain-P-loop interaction was disrupted. As anticipated, full-length CED-3 and CED-3 protease domain co-precipitated with CED-4 complexed to CED-9 (Fig. 4A), but CED-3 prodomain did not (Fig. 4A). This is consistent with CED-9 inhibiting binding of the P-loop to the prodomain. Such an inhibition could be the result of steric hindrance, especially if CED-9 also bound the P-loop (22). Consistent with this, CED-9 was found to bind native CED-4 but not CED-4<sup>K165R</sup>, the P-loop inactivating point mutant (Fig. 4B). Regardless, disruption of the P-loop-prodomain interaction within the ternary CED-3-CED-4-CED-9 complex likely accounts for the mechanism by which CED-9 neutralizes the ability of CED-4 to activate CED-3.

CED-3, CED-4, and CED-9 Form a Ternary Complex in Cells—To confirm that the three core components can indeed form a ternary complex in vivo consistent with the biochemical data (16) each component was expressed individually or together in 293T cells. The subcellular localization was determined by immunostaining and confocal microscopy. When expressed alone in mammalian cells, CED-9 displayed a compact granular pattern confined to the perinuclear region and membranes of intracellular organelles (presumably mitochondria) (Ref. 35; Fig. 5A). By contrast, CED-4 exhibited a diffuse cyttoplasmic labeling, whereas CED-3 stained a punctate cytoplasmic pattern reminiscent of bacterial inclusion bodies and suggesting insolubility (Fig. 5A). Although recent studies have shown that CED-9 can redistribute the subcellular localization of CED-4 from cytosolic to intracellular membranes (35), we show for the first time that CED-3-CED-4-CED-9 can assemble as a ternary complex at intracellular membranes (Fig. 5B).

Summary—It has recently been shown that CED-9 can serve as a substrate for the CED-3 protease and that a competitive inhibition mechanism may in part account for the cell death inhibitory activity of CED-9 (36). Although competitive inhibition may be part of the mechanism by which CED-9 exerts its antiapoptotic effect, our studies support an active role within the CED-3-CED-4-CED-9 ternary complex in which CED-9 suppresses CED-3 activation by selectively inhibiting the prodomain-P-loop interaction.

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REFERENCES
1. Chinnaiyan, A. M., and Dixit, V. M. (1996) <i>Cell</i> 86, 201–208
2. Shahnaz, S., and Horvitz, H. R. (1996) <i>Cell</i> 86, 201–208
3. Hugunin, M., Quintal, L. J., Mankovich, J. A., and Gayhur, T. (1996) <i>J. Biol. Chem.</i> 271, 3517–3522
4. Bauer, M. K., Wesselborg, S., and Schule-osthoff, K. (1997) <i>FEBS Lett.</i> 420, 256–258
5. Hofmann, K., Bucher, P., and Tschopp, J. (1997) <i>Trends Biochem. Sci.</i> 22, 155
6. Li, P., Nikhawand, D., Budhajide, I., Srinivasula, S. M., Ahmad, M., Alเสรม, E. S., and Wang, X. (1997) <i>Cell</i> 91, 479–489
7. Pan, G., O’Rourke, K., and Dixit, V. M. (1998) <i>J. Biol. Chem.</i> 273, 5841–5845
8. Wu, D., Wallen, H. D., and Nunez, G. (1997) <i>Cell</i> 86, 201–208
9. Shahnaz, S., and Horvitz, H. R. (1996) <i>Cell</i> 86, 201–208
10. Hugunin, M., Quintal, L. J., Mankovich, J. A., and Gayhur, T. (1996) <i>J. Biol. Chem.</i> 271, 3517–3522
11. Bauer, M. K., Wesselborg, S., and Schule-osthoff, K. (1997) <i>FEBS Lett.</i> 420, 256–258
12. Hofmann, K., Bucher, P., and Tschopp, J. (1997) <i>Trends Biochem. Sci.</i> 22, 155
13. Li, P., Nikhawand, D., Budhajide, I., Srinivasula, S. M., Ahmad, M., Alเสรม, E. S., and Wang, X. (1997) <i>Cell</i> 91, 479–489
14. Pan, G., O’Rourke, K., and Dixit, V. M. (1998) <i>J. Biol. Chem.</i> 273, 5841–5845
15. Wu, D., Wallen, H. D., and Nunez, G. (1997) <i>Cell</i> 86, 201–208
16. Xue, D., and Horvitz, H. R. (1997) <i>Nature</i> 390, 305–308