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A caspase homolog keeps CED-3 in check

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Abstract

Apoptosis is a highly conserved form of cell death that is essential for controlling cell numbers throughout the lifetime of an organism. In *Caenorhabditis elegans*, the final step in the apoptotic cascade is activation of the death-inducing protease CED-3. Until now, no direct negative regulators of CED-3 had been identified, so the mechanism for maintaining a proper life-death balance was unclear. Now, a new study identifies CSP-3 as an important negative regulator of CED-3 during *C. elegans* development.

Apoptosis: a conserved and highly regulated cell death program

The apoptotic cell death program is highly conserved from invertebrates to mammals and is mediated by a set of death-inducing proteases called caspases (cysteine-dependent aspartate-specific proteases), which cleave cellular substrates in a highly specific and regulated manner [1,2]. The importance of apoptosis in the development of multicellular organisms has been described particularly well for the nematode *Caenorhabditis elegans* (for a review, see Ref. [3]). Throughout *C. elegans* development, cell numbers are precisely controlled in the developing organs and virtually all adult worms contain precisely the same number of cells in each mature organ. Several mechanisms are in place to regulate this process, ensuring that the *C. elegans* executioner caspase CED-3 is triggered to execute apoptosis in cells that should die but is not mistakenly activated in cells that should survive. Now, a recent study by Geng *et al.* [4] provides a novel mechanism for keeping CED-3 inactive in cells that should not undergo apoptosis.

Apoptosis in *C. elegans* is broadly similar to apoptosis in higher organisms (Table 1) and CED-3 possesses substantial homology to mammalian caspase-3 and caspase-8 [5-7]. Like other caspases, CED-3 is synthesized as an inactive zymogen; dimerization and autoproteolysis generate the active components (the large and small subunits) from the N-terminal prodomain [8]. Inactive CED-3 monomers are brought together and activated by oligomerized CED-4, a process that is analogous to mammalian caspase-9 activation [9,10]. In species from flies to humans, caspase activation and proteolytic activity are subject to negative regulation by the inhibitor of apoptosis (IAP) protein family [11,12]. However, the two IAPs encoded in the *C. elegans* genome are thought to participate in cytokinesis, not apoptosis [13]. Given the similarities between *C. elegans* and mammalian apoptosis, the apparent absence of IAPs or other caspase inhibitors to keep CED-3 in check is puzzling. By contrast, in *Drosophila melanogaster*, the activity of IAPs, specifically DIAP1 (for *Drosophila* IAP1), is crucial in preventing uncontrolled caspase activity and apoptosis [14]. In mammals, another level of

caspase regulation is provided by a group of caspase-like decoy proteins [15]. Some of these proteins contain only a caspase-recruitment domain (CARD), whereas others resemble full-length caspases but lack the crucial catalytic cysteine residue. These decoy molecules can exert their anti-apoptotic effects by binding and sequestering procaspase zymogens or by competing with caspases for insertion into caspase-activating complexes. Until now, no caspase-like decoy proteins had been identified in *C. elegans* or any other non-mammals. Without IAP proteins or caspase-like decoys in *C. elegans*, it has been unclear how adequate CED-3 regulation could be achieved to prevent inappropriate apoptosis. The new report by Geng *et al.* [4] describes the identification of caspase homolog-3 (CSP-3), a *C. elegans* caspase-like decoy molecule that prevents inappropriate CED-3 activation and maintains the proper life-death balance during nematode development.

CSP-3: a caspase homolog that blocks apoptosis by inhibiting CED-3 activation

CSP-3 is a ubiquitously expressed cytoplasmic protein that mimics the CED-3 small subunit, binding to and sequestering the CED-3 zymogen, thus preventing inappropriate CED-3 dimerization and activation. The *csp-3* gene was originally identified as a *C. elegans* caspase-like gene, but it was not clear whether it encoded a functional component of a pro-death complex, a caspase-like decoy molecule or a protein of unrelated function [16]. Geng *et al.* [4] generated *csp-3* deletion alleles and found that, in animals harboring these deletions, some cells that are normally present in the mature anterior pharynx were missing. At a selection of developmental stages, *csp-3* mutant animals had increased apoptotic cell corpses, a phenotype that could be rescued by reintroducing the *csp-3* gene. Taken together, the missing cells in the adult anterior pharynx and the increase in apoptotic corpses during development indicated inappropriate activation of the apoptotic pathway in the *csp-3* mutant animals. Interestingly, CSP-3 overexpression in wild-type animals did not substantially increase the number of cells that survived development. Thus, it seems that CSP-3 might function as a negative regulator of apoptosis in cells that should survive; however, it does not block the appropriate induction of apoptosis in cells that should die.

Because *csp-3* is a caspase-like gene, it was not immediately obvious how it might block, rather than induce, apoptosis in developing *C. elegans*. To examine the mechanism by which CSP-3 prevents apoptosis, Geng *et al.* [4] employed a variety of clever biochemical approaches. By expressing CSP-3 together with CED-3 in bacteria, they demonstrated that CSP-3 binds the CED-3 zymogen *in vitro*. They then showed by immunoprecipitation experiments that CSP-3 physically interacts with CED-3 *in vivo* and *in vitro*. Deletion mapping studies showed that the CED-3 large subunit is sufficient to mediate the interaction with CSP-3, indicating that CSP-3 might mimic the CED-3 small subunit, thereby blocking CED-3 dimerization.

3D structural modeling and site-directed mutagenesis revealed a specific residue in CSP-3 that seems to be crucial for CED-3 binding. Substitution of Phe57 to aspartate drastically reduces the ability of CSP-3 to bind CED-3 and the F57D CSP-3 cannot rescue the *csp-3* null mutant phenotype. These findings indicate that CED-3 binding is required for the anti-apoptotic function of CSP-3 *in vivo*. However, how CSP-3-CED-3 binding might interfere with apoptosis was not entirely clear. CSP-3 binding to the CED-3 large subunit could potentially block CED-3 dimerization and activation or it could inhibit CED-3 enzymatic activity. To resolve this dilemma, Geng *et al.* [4] used an *in vitro* CED-3 autoactivation assay to assess the effect of CSP-3 on the formation of active CED-3. Recombinant wild-type CSP-3, but not the F57D mutant, efficiently blocked CED-3 zymogen autoactivation *in vitro*. However, CSP-3 could not block CED-4-induced CED-3 activation or inhibit CED-3 protease activity. Thus, CSP-3 seems to be a modulator of CED-3 activation rather than a true caspase inhibitor. In cells that should survive, in which no upstream pro-death signal is present, CSP-3 prevents inappropriate

CED-3 dimerization and autoactivation, whereas in cells that are fated to die, oligomerized CED-4 can override CSP-3 and effectively activate CED-3 to induce apoptosis.

Concluding remarks and evolutionary perspectives

Although functionally similar to IAPs in higher organisms, CSP-3 more closely resembles the caspase-like decoy molecules that have been identified in mammals (Figure 1). The identification of this decoy molecule in *C. elegans* is intriguing because, to our knowledge, no similar molecule has been identified in flies or other non-mammalian species. In mammals, several varieties of caspase-like decoy molecules have been identified. Some possess CARD domains only (e.g. CARD-only protein [COP], inhibitory CARD [INCA] and ICEBERG), whereas others, such as cellular FLICE (Fas-associated death domain-like interleukin-1 β converting enzyme)-inhibitory protein (c-FLIP) resemble full-length caspases but lack enzymatic activity [17-20]. The mechanism by which CSP-3 inhibits CED-3 activation seems to be distinct from those of all previously identified caspase-like decoys. Rather than binding CED-3 through a protein-protein interaction domain such as a CARD or death-effector domain (DED), CSP-3 mimics the CED-3 small subunit, binding and sequestering inactive CED-3 monomers to prevent CED-3 dimerization. Although a short c-FLIP splice variant has been described (c-FLIP_S), it comprises the N-terminal procaspase DED only, rather than the caspase-like domain [20]. Additionally, c-FLIP_S does not exert its anti-apoptotic effect by direct interaction with caspase-8, but rather by competing with the caspase-8 zymogen for association with caspase-activating complexes. The long splice variant of c-FLIP (c-FLIP_L) is similar to CSP-3 in that it binds caspase-8 directly but, rather than preventing caspase-8 activation entirely, it enables partial activation such that caspase-8 can cleave certain proteins, including c-FLIP_L itself, but not the effector caspase-3 [21]. Thus, despite its similarities to other caspase-like decoys, the precise mechanism by which CSP-3 blocks CED-3 activation seems to be unique.

All previously known caspase-like decoy proteins are restricted to mammals and several seem to be unique to primates [15,22]. The discovery of a caspase-like decoy in *C. elegans* raises several evolutionary possibilities. It is possible that these decoy molecules exist in all species that possess caspases and undergo apoptosis and that they simply have not yet been identified in other species. Alternatively, *csp-3* might have been lost in other species and caspase-like decoys reappeared in mammals in the form of c-FLIP, COP and others. A third possibility is that convergent evolution in nematodes and mammals accounts for this similarity, which is apparently missing in other non-mammalian species.

In any cell with caspase-like proteases that is capable of undergoing apoptosis, stochastic, inappropriate-proximity-induced dimerization and caspase autoactivation is possible. Several mechanisms have evolved to deal with this problem, ranging from modulators of upstream pro-death signals, to proteins that sequester monomeric procaspases, to enzymatic inhibitors. The apparent absence of caspase-like decoy molecules in *D. melanogaster* could explain the strict requirement for IAP-mediated apoptosis inhibition in fly development [14]. By contrast, in *C. elegans*, spontaneous, inappropriate CED-3 activation is effectively prevented by the presence of CSP-3 [4]. Thus, nematodes overcome the absence of IAPs or IAP-like proteins via a caspase-like decoy molecule. In mammals the picture is more complex, with several IAPs and several caspase-like molecules cooperating with pro-death proteins to achieve the proper life-death balance. It seems likely that in species ranging from nematode to human, the picture will become increasingly intricate as additional pro-apoptotic and anti-apoptotic regulators are identified and their roles are revealed.

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Figure 1.

Schematic representation of the similar roles of mammalian c-FLIP and *C. elegans* CSP-3. The mammalian decoy c-FLIP and the *C. elegans* decoy CSP-3 block apoptosis by distinct mechanisms. In mammalian cells, c-FLIP (blue) inhibits apoptosis in two ways: (i) by competing with procaspase-8 for insertion into an activating complex; or (ii) by binding and sequestering caspase-8 (yellow) to prevent full caspase-8 activation and cleavage of the downstream effector caspase-3 (green). In *C. elegans*, CSP-3 (blue) sequesters CED-3 monomers (yellow) to prevent inappropriate CED-3 dimerization, activation and apoptosis. However, oligomerized CED-4 (orange) can override CSP-3 and activate CED-3 in cells that are fated to die.

Principal proteins in *C. elegans* apoptosis and their mammalian homologs^a

Table 1

<i>C. elegans</i> protein	Function	Mammalian homolog	Refs
EGL-1 ^d	Liberate CED-4 from CED-9	BH3-only proteins	[23,24]
CED-9	Sequester CED-4 at the mitochondrial membrane	BCL-2	[9]
CED-4	Activate CED-3	APAF-1	[10]
CED-3	Cleave downstream targets to initiate apoptosis	Caspases	[5-8]
CSP-3	Sequester CED-3 zymogen monomers	Caspase-like decoys	[4]
None	Inhibit caspase activity	IAPs	[11,12]

^a Abbreviations: APAF-1, apoptotic protease-activating factor-1; BCL-2, B-cell lymphoma-2; BH3, Bcl-2 homology 3 domain; CED-9,-4,-3, cell death abnormality-9,-4,-3; EGL-1, egg-laying defective-1.