News and Commentary

Identification of a 'genuine' mammalian homolog of nematodal CED-4: is the hunt over or do we need better guns?

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One of the most attractive molecules after which cell death researchers have been chasing in the last 14 years is a mammalian homolog of nematodal CED-4 that is regulated in exactly the same way as in the worm *Caenorhabditis elegans*. The reason for this hunt, both by genetic and biochemical approaches, is the still widely shared view that although higher eukaryotes have evolved more complex systems to regulate programmed cell death, the basic principle of how a CED-9-like survival factor regulates a CED-3-like caspase activity via a CED-4-like ATPase must have been conserved from worms to men. In the mid-1990s, the group of Xiaodong Wang appeared to temporarily end this hunt by isolating a mammalian CED-4 homolog, called Apaf-1 that acted as a dATP-binding protein and bound to and activated caspase-9. But the 'joy about the prey' did not last for long as it was later found that Apaf-1 did not bind any member of the CED-9/Bcl-2 family and required cytochrome *c* for its caspase-9-activating function, a process that was not needed for CED-4 activation in the worm. Later, a *Drosophila* homolog of CED-4/Apaf-1, called Dark was identified and shown to be required for caspase activation (Dronc) in the fly. However, neither cytochrome *c* nor the two known *Drosophila* CED-9/Bcl-2 homologs Debcl and Buffy have so far been found to bind to or directly regulate the activity of Dark. Based on these findings, it is about time to ask if we should give up the search for a 'genuine' mammalian CED-4 homolog because its regulation may have changed during evolution or if we should get better hunters with better guns on board to finally catch this enigmatic molecule.

The Simple Pathway in the Worm: a CED-4 Held in Check by the Survival Factor CED-9 Waiting to be Released by EGL-1 to Activate the Single Caspase CED-3

The molecular mechanism of programmed cell death has first been dissected during the hermaphrodite development of the nematode *C. elegans* where of the 1090 cells generated only 959 remain in the adult.[1] The process of cell death exhibits typical morphological features of apoptosis and follows a simple, linear pathway. All viable cells constitutively express the death regulatory proteins CED-4, CED-3 and CED-9.[2] CED-3 is a cytosolic caspase: a cysteine-containing protease that cleaves its substrates after aspartate residues.[3,4] The cells survive because CED-3 is synthesized as an auto-inhibited, inactive zymogen and its activator CED-4 is sequestered by the mitochondria-bound survival factor CED-9[5–8] (Figure 1A). When 131 cells are programmed to die, the CED-3 zymogen is activated by the adaptor molecule CED-4.[6,9–12] This occurs through the transcriptional upregulation of the proapoptotic protein EGL-1 which binds via a so-called BH3 domain to a hydrophobic pocket of CED-9 with high affinity, thereby disrupting the CED-4–CED-9 interaction[13–15] (Figure 1A). The released CED-4 undergoes tetramerization[16] and interacts with a N-terminal caspase recruitment domain (CARD)[17] with a similar motif in the prodomain of CED-3 (Figure 1A) to form a high molecular complex, called the apoptosome (Figure 1A). In this way, CED-4 acts like a scaffold protein to promote juxtaposition of adjacent CED-3 molecules, which then allows intermolecular cleavage and activation of the protease.[11] CED-4 belongs to the AAA + family of ATPases[18] but it does not show any detectable ATPase activity. It contains a nucleotide-dependent oligomerization domain (NBD) and constitutively binds ATP but probably uses the nucleotide for structural stability rather than for CED-4/CED-3 apoptosome formation.[16]

No other molecule appears to be required for the execution of programmed cell death during the nematodal development as all 131 cells (mostly neurons) survive in ced-4, ced-3 or egl-1 loss-of-function mutants.[2,13] On the other hand, loss of function of ced-9 leads to additional nematodal cell death.[18] This cell death is still CED-4 and CED-3 dependent indicating that the linear pathway controls yet other types of programmed cell death in the worm. Indeed, germ cell death either physiological or in response to DNA damage is similarly regulated by CED-9, CED-4 and CED-3 as during development with the exception that the upstream sensing or inducing molecules are different. For example, DNA-damage-induced cell death requires gene products involved in DNA repair and checkpoint control (hus-1, mrt-2 and rad-5)[19,20] as well as the *C. elegans* p53 homolog CEP-1[20,21] (Figure 1A). CEP-1 is thought to act as a direct transactivator of EGL-1 in response to DNA damage[21] indicating that also in this system programmed cell death is regulated by the EGL-1-mediated displacement of CED-4 from CED-9 with the consequent activation of the lethal proteolytic action of CED-3. An additional level of complexity has, however, recently been added by the finding that a second BH3-only protein, CED-13
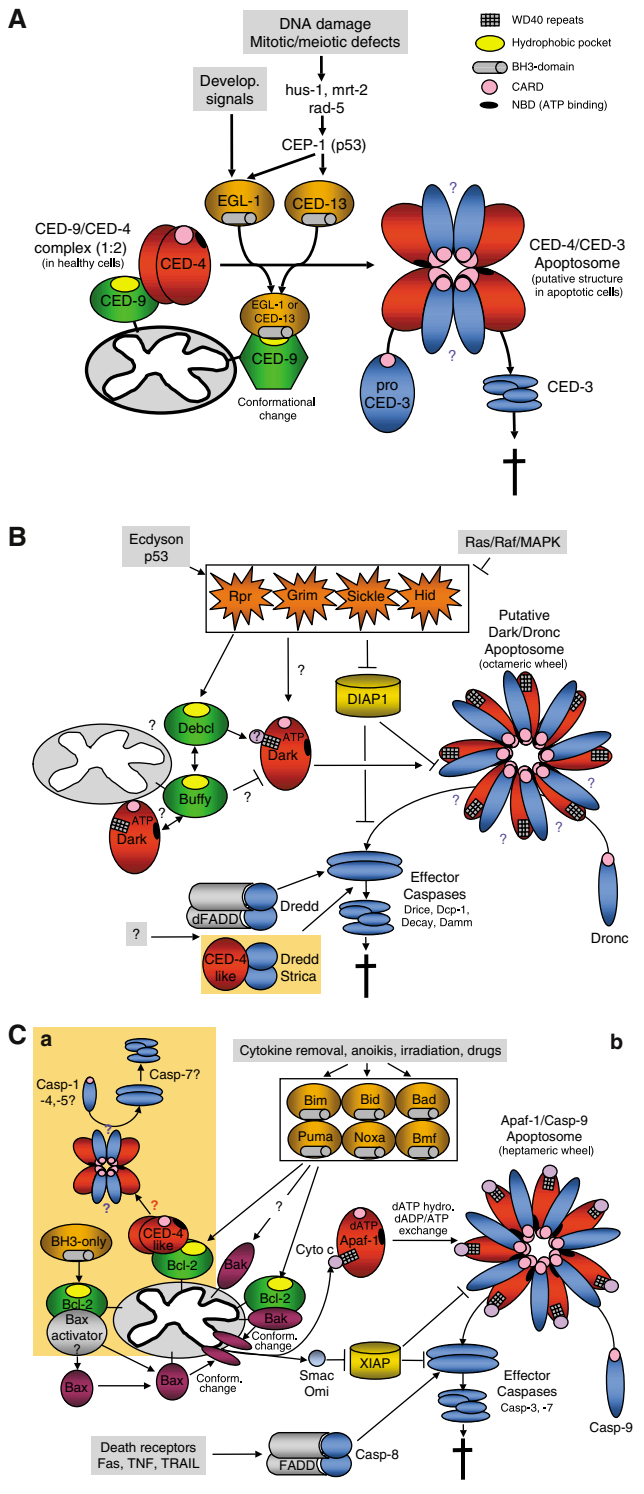
Figure 1 Comparison of the apoptotic systems in *C. elegans* (**A**), *Drosophila* (**B**) and mammals (**C**a and **C**b). Fundamental components of the apoptotic pathways are conserved between the species, but an increasing complexity from *C. elegans* to mammals is evident. Details are given in the text. The proteins that are homologous (by sequence or functionality) between the three organisms are depicted with the same color. The regions showing putative pathways mediated by a 'genuine' CED-4 homolog as well as a putative Bax activator (both sequestered by Bcl-2-like survival factors) are shaded in yellow (**B** and **C**a). Putative protein–protein interactions are indicated with question marks

seems to be required for DNA-damaged-induced apoptosis in somatic cells[22] (Figure 1A).

More Complex in the Fly: a Presumably Constantly Activated CED-4 Homolog Dark that is not Directly Regulated by CED-9/EGL-1 Homologs and Activates only a Subset of Initiator Caspases

In *Drosophila*, the core effectors of programmed cell death are also the caspases. But in contrast to *C. elegans*, there are seven identified in the fly genome. Based on their prodomain structure they are classified into initiator caspases (Dronc, Dredd and Strica) and effector caspases (Drice, Dcp-1, Decay and Damm)[23] (Figure 1B). Like CED-3, the initiator caspases contain large prodomains such as CARD or DED (death effector domains), which bind to the corresponding domain of a scaffold protein to facilitate proximity-induced caspase activation.[17] These initiator caspases do not, however, directly execute apoptosis but cleave and activate effector caspases with short prodomains (and hence unable to bind to and get activated by scaffold proteins) that then dismantle the cell by cleaving numerous substrates.[24] The major scaffold protein in flies is the CED-4 homolog Dark as it is required for many apoptotic responses.[25–28] After activation Dark forms an octameric, wheel-like structure[29] and, in analogy with the CED-4/CED-3 complex in *C. elegans*, constitutes a fly apoptosome (Figure 1B). Dark primarily activates the initiator caspase Dronc which is essential for programmed cell death during embryogenesis.[30] Active Dronc processes and activates the effector caspases Drice and Dcp-1 [31,32] (Figure 1B). No other homolog of CED-4 has yet been found in the *Drosophila* genome but because of the presence of other initiator caspases that require scaffold proteins for their activation, additional CED-4 homologs or, alternatively, different kinds of scaffold proteins must exist. Indeed, for Dredd, which most resembles human caspase-8, a CED-4-unrelated adaptor protein, called dFADD, was identified that also has an ortholog in mammals (FADD)[33] (Figure 1B). Thus, in flies, two or more death signaling pathways have evolved which use both CED-4-related and -unrelated scaffold proteins and a complex proteolytic network for caspase activation.

How is the CED-4 homolog Dark regulated in *Drosophila*? Like CED-4, Dark contains a N-terminal CARD domain and a NBD domain for ATP binding and presumably oligomerization[25–27,29] (Figure 1B). However, it additionally bears at its C-terminus so-called WD-40 repeats that are not present in CED-4 but occur in the mammalian CED-4 homolog Apaf-1.[34] Apaf-1 requires for its activation mitochondrially released cytochrome *c*, which specifically binds to the WD40 repeats to allow CARD domain exposure and oligomerization for caspase activation.[34,35] Both C-terminally deleted Apaf-1 and Dark are constitutively active indicating that the WD40 repeats serve the role of an autoinhibitory domain in both proteins.[25–27,35] However, although cytochrome *c* binds Dark *in vitro*, it only minimally stimulates Dark activity.[25–27] Moreover, cytochrome *c* is not released from mitochondria into the

cytoplasm during apoptosis of *Drosophila*[28,36,37] cells and although Dark ablation prevents most of insect cell apoptosis, cytochrome *c* elimination does not.[28,38] It therefore seems that Dark is either activated by another, yet unknown molecule or it has the potential to be constitutively active but is, like CED-4, restrained by CED-9-like survival factor.

Two homologs of CED-9 exist in *Drosophila*. Buffy/Borg-2 is a survival factor like CED-9 or mammalian Bcl-2.[39] Ablation of Buffy leads to ectopic apoptosis whereas Buffy overexpression results in the inhibition of developmental programmed cell death and γ-irradiation-induced apoptosis. Debcl (or dBorg-1, Drob-1, dBok) is a proapoptotic homolog of CED-9/Bcl-2 similar to Bax, Bak or Bok[40–43] (see below). Its ectopic overexpression in cultured cells and transgenic flies causes apoptosis, which is inhibited by the baculovirus caspase inhibitor p35 indicating that it is a proapoptotic protein that triggers downstream caspase activation.[40] As in mammals, where Bcl-2-like survival factors and Bax-like death factors can physically interact and block each others' activities (for a review see Borner[44]), Buffy genetically and physically interacts with Debcl to suppress Debcl-induced cell death[39] (Figure 1B). The question is how the two CED-9-like homologs impinge on the Dark/Dronc death machinery. Although both Buffy and Dark localize to the mitochondrial membrane analogous to CED-9 and CED-4,[25–27,39] no direct physical interaction has yet been reported. This is also the case for Debcl and Dark. However, reducing the dosage of Dark-suppressed Debcl-induced apoptosis in the *Drosophila* eye indicating that Dark and Debcl interact at least genetically.[40] Debcl does not trigger cytochrome *c* release in *Drosophila* cells as Bax and Bak do in mammals,[44] so it must activate Dark either directly or via another, yet unknown molecule (Figure 1B). In this context, Buffy would act as survival factor not by sequestering Dark, but by binding and neutralizing Debcl. Hence, in contrast to the situation in *C. elegans*, the CED-4 homolog Dark might be controlled by a proapoptotic CED-9-like activator rather than by a CED-9-like survival factor. This would also explain why no EGL-1 homolog has yet been found in *Drosophila* because no CED-9/CED-4-like complex has to be disrupted in apoptotic cells.

Another crucial difference in the regulation of programmed cell death between flies and worms is that insect caspase activities are mainly controlled by inhibitor of apoptosis proteins (IAPs) rather than by upstream activators (see review by Thomenius and Kornbluth in this issue,[45]). Why is then Dark needed for caspase activation? Two IAP homologs have been identified in *Drosophila*, DIAP1 and DIAP2[46,47] (Figure 1B). Virtually all *Drosophila* cells undergo rapid apoptosis in the absence of DIAP1 protein[48,49] indicating that DIAP1 is more important than DIAP2. Both, DIAP1 and DIAP2 directly inhibit caspase functions by binding of their conserved baculovirus IAP repeat (BIR) domain to the caspase active site, by promoting degradation of active caspase via the ubiquitin/proteasome system or by sequestering the caspase away from its substrates.[50] This means that in viable cells IAPs keep caspases constitutively in check and their ablation is thought to allow unrestrained caspase-dependent cell death.[51] Under physiological conditions, relieving caspases from DIAP inhibition is achieved by four proteins, called reaper

(rpr), hid, grim and sickle that are encoded in the same gene cluster (H99) (for a review see Thomenius and Kornbluth[45] in this issue). These proteins are transcriptionally upregulated in the dying cells during embryogenesis (e.g. by the fly hormone ecdyson) or other forms of programmed fly cell death and bind via a short N-terminal IAP-binding motif (BIM) to the BIR domains of DIAPs, thereby releasing caspases from DIAP inhibition[45] (Figure 1B). Importantly, if this regulation were sufficient for the full activation of caspases required for programmed cell death in *Drosophila*, it would make the need of an upstream activator such as Dark obsolete. However, cell deaths caused by both DIAP deletion and rpr, hid and/or grim overexpression are rescued by Dark mutations in development and in response to genotoxic stress[52] indicating that caspase-dependent cell death in flies still requires concurrent positive input from Dark and that rpr, hid and grim may be upstream activators of Dark in addition to their function as IAP inhibitiors (Figure 1B). As rpr-induced cell death was found to be blocked by Buffy[39] but did not affect Debcl-induced apoptosis,[40] it can be speculated that the Buffy/Debcl pair consists an important checkpoint between rpr, hid, grim and Dark (Figure 1B). Further work is needed to sort out how these molecules interact with each other to regulate programmed cell death in *Drosophila* but it is already clear that the regulation is more complex than in the worm.

Even More Complex in Mouse and Man: a CED-4 Homolog Apaf-1 that does not Physically Interact with CED-9 Homologs, Requires Mitochondrially Released Cytochrome *c* for its Activation and is not the only Scaffold Protein to Activate Caspases

The first mammalian homolog of CED-4 identified was Apaf-1.[52] Although two studies initially reported its interaction with the mammalian CED-9 homologs Bcl-2 and Bcl-x_L[53,54] this turned out to be an overexpression artefact that could not be reproduced with the endogenous proteins.[55–57] In fact, in contrast to Bcl-2 and Bcl-x_L, which mostly reside on mitochondrial and ER membranes,[44] Apaf-1 is a cytosolic protein.[55] Moreover, the binding of overexpressed Apaf-1 and Bcl-x_L could not be competed by BH3-peptides (own unpublished data). This should have been possible if Apaf-1 bound to Bcl-2 or Bcl-x_L in a similar way as CED-4 to CED-9. Thus, as for Dark in *Drosophila*, Apaf-1 is not directly controlled by CED-9/Bcl-2-like survival factors. Instead Apaf-1 is autoinhibited by an intramolecular interaction of its C-terminal WD40 repeats with two functional regions of the molecule, the nucleotide-binding and oligomerization domain (NBD) and the N-terminal CARD.[35,58] Thus, in contrast to CED-4, which lacks the WD40 repeats, Apaf-1 requires an activating molecule to change its conformation into an open state for caspase interaction. This molecule is cytochrome *c* that is released from the intermembrane space of mitochondria in response to many apoptotic stimuli[59] (Figure 1Cb). Upon binding to the WD40 repeats, cytochrome *c* induces the hydrolysis of dATP (at higher concentrations also ATP) to

dADP, which is immediately replaced by exogenous dATP.[60] If the nucleotide exchange does not happen, Apaf-1 forms an inactive aggregate. By contrast, upon dATP/dADP exchange Apaf-1 oligomerizes into a wheel-like heptamer, called the mammalian apoptosome[61] (Figure 1Cb). The central ring of the apoptosome is formed by the conjugation of seven CARD and NBD domains of Apaf-1, and each of the seven spikes extended from the central ring is made of 13 WD40 repeats bound to one cytochrome *c*. Apaf-1-mediated caspase activation is then accomplished through a cascade of caspases like in the fly. Caspase-9 functions as initiator caspase and caspases-3 and -7 as the downstream effector caspases[34,52,59] (Figure 1Cb). Via its N-terminal CARD domain caspase-9 binds to the central ring of the apoptosome and becomes activated.[61] In contrast to other procaspases, the caspase-9 zymogen can be activated without processing.[62] Notably, most of the active caspase-9 produced, whether processed or not, remains complexed with Apaf-1. Thus, Apaf-1 is not merely a scaffold for activating caspase-9; instead the Apaf-1-caspase-9 apoptosome acts as a ca. 1.0 MDa holoenzyme[63] that cleaves and activates caspase-3 and caspase-7, which in turn cleave cellular substrates leading to apoptosis.

How is the activity of Apaf-1 controlled by CED-9/Bcl-2 family members? The current view is that the regulation is distinct from that of CED-4 in worms and Dark in flies. In all three species, Bcl-2 family members primarily act on mitochondria but in different ways. In worms, CED-4 is sequestered by CED-9 until released by EGL-1 (Figure 1A); in flies, Dark is constitutively active or activated by an unknown molecule that is somehow controlled by Debcl or Buffy (Figure 1B); and in mammals antiapoptotic Bcl-2-like and proapoptotic Bax-like proteins control the release of apoptogenic factors such as cytochrome *c* from mitochondria (Figure 1C). Detailed reviews about the mode of action of mammalian Bcl-2 family members are presented in this issue. Briefly, in addition to the CED-9-like survival factors (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1) and the EGL-1-like death triggering BH3-only proteins (Bim, Bad, Bid, Bmf, Puma, Noxa, Nbk, Hrk) mammals have acquired a third subgroup of proapoptotic family members, called Bax, Bak and Bok (Figure 1Cb). It is known that Bax and Bak are directly responsible for the increased mitochondrial membrane permeability leading to the release of cytochrome *c* and other apoptogenic factors because cells from Bax/Bak double knockout cells are highly resistant to cytochrome *c* release and apoptosis in response to various stimuli.[64,65] By contrast, the molecular mechanism of this pore formation has remained obscure. Although it is now accepted that Bax and Bak change their conformation, oligomerize and stably insert into the outer mitochondrial membrane in response to apoptotic stimuli (Figure 1Cb), it is far from clear if they form protein pores *per se* or with the help of other proteins, or change the phospholipid structure and membrane topology to increase membrane permeability.[44,66] As in *C. elegans*, the BH3-only proteins are the sensors/ initiators of mammalian apoptosis signaling and the upstream activators of Bax/Bak.[65,66] Depending on the apoptotic stimulus, they are either transcriptionally induced (Bim, Puma, Noxa, like Egl-1) or post-translationally modified (Bid, Bim, Bad) and often change their subcellular localization (from the cytosol or cytoskeleton to mitochondria).[66] Based on the finding that the three-dimensional structure of Bax is closely related to that of Bcl-2, Bcl-x_L or CED-9,[15,67,68] it is conceivable to propose that the activated BH3-only proteins directly nestle into the hydrophobic pocket of Bax and Bak. However, instead of releasing a CED-4-like homolog, the BH3-only proteins would trigger conformational change and pore formation of Bax and Bak as had been proposed for tBid and Bim.[64–66] The problem is that the binding affinities between BH3-only proteins and Bax or Bak are in the low micromolar range and co-immunoprecipitations have been difficult to detect for the endogenous proteins so that direct BH3-only/Bax or -Bak interactions would only be transient (hit-and-run)[69] (Figure 1Cb). Alternatively, activated BH3-only proteins specifically interact with Bcl-2-like survival factors and displace (a) molecule(s) that activate(s) Bax and Bak (Figure 1Cb). This model has recently been supported by the finding that Bak is kept in check by interacting with Bcl-x_L and Mcl-1 on the mitochondrial membrane of healthy cells until displaced by particular BH3-only proteins in apoptotic cells.[70] The binding sites and affinities of such Bak/Bcl-x_L and Bak/Mcl-1 interactions have not yet been determined but it is unlikely that binding occurs through the hydrophobic pocket as the BH3-domain of Bak is not exposed in healthy cells.[44] As binding affinities of BH3-only proteins for Bcl-2, Bcl-x_L and Mcl-1 are high (100-fold higher than for Bax and Bak),[69] it is suggested that the favored binding of these activators to the hydrophobic pocket of Bcl-2 survival factors changes their conformation to release Bak, just as it has been recently proposed for the release of CED-4 from CED-9 by EGL-1[16] (see below). Hence, it can be speculated that during the evolution from worm to man, CED-4 was replaced by Bak and Apaf-1, still performing its function of activating a caspase was placed downstream of mitochondria. This model does, however, not take into account the activation of Bax. Bax is often a cytosolic protein that first needs translocation to mitochondria to act[44] (Figure 1Cb), and it does not interact with any antiapoptotic Bcl-2 family members under endogenous conditions (own unpublished data), except in nonionic detergent where its conformation is already changed.[71] So how can Bax be activated if it is not by a release from Bcl-2, Bcl-x_L or Mcl-1? Two scenarios can be envisaged (see review series in this issue). Particular BH3-only proteins such as tBid or Bim indeed directly activate Bax through a hit-and-run mechanism (Figure 1Cb). Or, Bcl-2-like survival factors have some unknown Bax activators bound, which are released in apoptotic cells upon BH3-only interaction (Figure 1Ca). In theory, such a factor could be a CED-4 homolog but it is difficult to imagine that the function of such a homolog has deviated to activate Bax instead of caspases in mammals. Moreover, caspases have only rarely been implicated upstream of Bax (except for caspase-2, see below).

The control of apoptosis by IAPs, as it is predominant in the fly, is also regulated by Bcl-2 family members in mammals. It turned out that rpr, hid and grim homologs are not transcriptionally induced in mammals, but reside in the intermembrane space of mitochondria from which they are released in apoptotic cells. These homologs, called Smac/DIABLO and Htr2A/Omi probably fulfill important survival functions in mitochondria, but when released specifically bind,

via their IBM motif to the BIR domains of IAPs[72–75] (Figure 1Cb). The most important IAP in mammals is XIAP.[76] XIAP serves two roles, to block fortuitously activated caspase-3 and -9 in healthy cells and to keep the two active caspases in check until displaced by Smac/DIABLO and Htr2A/Omi. However, whereas DIAP1 knockout flies show extensive apoptosis, deletion of XIAP has no effect (although this might be through a compensatory upregulation to two other IAPs, c-IAP-1 and c-IAP-2).[76] Obviously, maximal activation of mammalian caspases more depends on the input from upstream activators (such as cytochrome *c*/Apaf-1) than activation of fly caspases.

Another level of complexity present in mammals, but not in flies or worms, is the regulation of apoptosis by TNF-like death receptors (for review see Peter and Krammer[77]). Activated death receptors of the TNF family (TNF-receptor, Fas/CD95 or TRAIL-receptors) recruit, to their cytoplasmic side, a CED-4-unrelated scaffold protein, called FADD to activate initiator procaspase-8 (Figure 1Cb). The activation principle is the same as for other initiator caspases. FADD concentrates the procaspase-8 zymogen sufficiently for autocatalysis or allosteric activation without processing.[78] Active caspase-8 then cleaves and activates procaspase-3 (Figure 1Cb). This direct pathway (also called type I pathway) does not involve mitochondria and is not affected by pro- or anti-apoptoic members of the Bcl-2 family. Thus, a quite effective simple death signaling pathway has been maintained in mammals without the participation of any CED-4 or CED-9 homologs.

Do Mammalian Cells Require a 'Genuine' CED-4 Homolog? Arguments for and Against a Continuation of the Chase!

So far we may argue that mammalian and insect CED-4 homologs have kept the CED-3/caspase-activating function but changed the way how this function is regulated. Is this really true or may there still be a mammalian CED-4 homolog that is controlled by CED-9/Bcl-2 family members in exactly the same way as in the worm? We would like to make predictions based on structural, genetic and biochemical aspects that have been compiled about CED-4/CED-9 homolog interactions.

Structural aspect

Bcl-2 can prevent programmed cell death in *C. elegans*.[79] This finding may indicate that Bcl-2 has a binding site for CED-4. However, the most likely mechanism by which Bcl-2 blocks cell death in this case is by mobbing up the EGL-1, which is transcriptionally induced during development. Thus, EGL-1 is not available to displace CED-4 from CED-9 and the cells survive. So, although Bcl-2 can obviously replace one function of CED-9 (the binding of EGL-1), it has not yet been proven that it can also replace its second function (the binding of CED-4). For that purpose the function of Bcl-2 should be tested in a CED-9 loss-of-function (lf) mutant in *C. elegans* where CED-4 is free to activate CED-3. If Bcl-2 rescues such a mutant, it most likely sequesters CED-4. Bcl-2 and Bcl-x$_L$ have both been shown to interact with CED-4 when co-

overexpressed in mammalian cells or tested for interaction as recombinant proteins.[6–8,80] Deletions in the BH1/BH2 region (hydrophobic pocket) as well as in the N-terminus were found to reduce these interactions. Similarly, Bcl-2 and Bcl-x$_L$ interactions with Apaf-1 were initially reported in overexpressing systems.[53,54] However, we and others could not detect any significant interactions between the endogenous proteins,[55–57] and we should probably also question the existence of Bcl-2/CED-4 interactions until they are validated by clean biochemical and/or genetic studies. Would an interaction between Bcl-2 and CED-4 (or another mammalian CED-4 homolog) even be possible based on structural data? Recently, the three-dimensional structure of a CED-9/CED-4 complex has been reported[16] and compared to a previously published structure of the CED-9/EGL-1 complex.[15] In the complex with CED-9, CED-4 adopts a dimeric conformation with only one monomer bound to CED-9 (Figure 1A). As predicted from the previous study,[15] the CED-4 monomer binds to another surface patch on CED-9 than EGL-1. This patch primarily involves an N-terminal segment (aa67–79), the intervening loop between alpha helices 3 and 4 (aa143–147) and the alpha helix 6 (just before BH2). In the N-terminus, Asp67 and Asp79 accept a pair of charge-stabilized hydrogen bonds from Arg24 and Arg117 of CED-4, respectively. In alpha6, Arg211 donates two hydrogen bonds to Glu214 and Asp215 in CED-4 and Asn212 interacts with Glu52. The intervening alpha3–4 loop primarily forms van der Waals bonds with CED-4. Strikingly, none of these critical amino acids for CED-4 binding are conserved in Bcl-2, Bcl-x$_L$ or any other family member although there is relatively good conservation of amino acids important for for BH domain formation including the hydrophobic pocket (see Figure 1d in Muchmore et al.[68]). In particular, aa143–147 consists of an extra loop that is not present in Bcl-2 and Bcl-x$_L$, aa67–79 reside ahead of the N-terminal BH4 domain of mammalian survival factors and Arg211 and Asn212 are consistently replaced by Leu or Glu/His, respectively. This raises doubt about the conservation of a binding module for CED-9/CED-4 interactions between worms and mammals.

Although the CED-9 structure looks the same, irrespective of whether it is bound to CED-4 or not,[16] it is drastically changed upon binding the death-inducer EGL-1[15] (Figure 1A). Obviously, the EGL-1 inducer binds via its BH3 region to the hydrophobic pocket of CED-9 and thereby induces a conformational change in CED-9 to release CED-4.[16] The affinity of the EGL-1/CED-9 binding is the same as that of a BH3-only protein for Bcl-2 (8–10 nM)[69] and much higher than the affinity of the CED-4/CED-9 interaction (80 nM).[16] The latter is then further lowered by the EGL-1-induced conformational change, hence facilitating CED-4 release.[16] Although Ottilie et al.[80] have reported a displacement of CED-4 from Bcl-x$_L$ upon incubation with BH3 peptides, we were unable to reproduce this result (unpublished data). Thus, we believe that the binding of Bcl-2 to CED-4 is most likely nonspecific and cannot be relieved by a conformational change exerted by EGL-1 or another BH3-only protein. In fact, a very recent publication shows that only worm (EGL-1 and CED-13) but not mammalian BH3-only proteins can dissociate CED-4 from CED-9 *in vitro*.[81] It has also been difficult to detect any conformational change of Bcl-2 or Bcl-xL upon BH3-peptide

binding. This might be different for BH3-peptide binding to Bax and Bak (see above) but such cocrystals have not yet been produced. Thus, we speculate that the impact of BH3-only proteins on the structure of Bcl-2-like survival factors is much different from that observed with EGL-1 on CED-9.

Genetic aspect

It has been previously thought that stress-induced apoptosis regulated by the Bcl-2 protein family necessarily proceeds through mitochondrial disruption and activation of caspase-9 via Apaf-1.[82] However, several recent findings cast doubt that the caspase-9-Apaf-1-cytochrome *c* apoptosome is the only mediator of apoptosis. Whereas most mice lacking either Apaf-1[83,84] or caspase-9,[85,86] died near-birth with enlarged brains, owing to decreased apoptosis of neuronal precursors, some Apaf-1−/− mice survived and became healthy adults.[87] Moreover, although apoptosis of certain cell types lacking Apaf-1 or caspase-9 is retarded,[84–86] postmitotic neurons lacking Apaf-1 die normally,[88] cytokine-deprived mast cells lacking either gene do not clonogenically grow after cytokine re-addition[89] and lymphocytes from the same KO animals remain sensitive to diverse insults and die at near normal rates.[90] Strikingly, the cells dying in an apoptosome-independent manner still showed discernable caspase activity and caspase activation and apoptosis could be blocked by both Bcl-2 overexpression and caspase inhibitors.[90] The relevant effector caspase was identified as caspase-7 and caspases-1, -11 or -12 were implicated as initiators (although caspase-12 can be excluded because most humans do not produce the full-length enzyme, Saleh *et al.*[91]). As even cytochrome *c* release was retarded in the presence of caspase inhibition, this pointed towards a role of a caspase upstream or aside of mitochondria blockable by Bcl-2. Marsden *et al.*[90] thus proposed that Bcl-2 regulates as yet unidentified scaffold proteins that govern the activation of upstream initiator caspases, including caspase-1, -2 and -11 (Figure 1Ca). Once activated, the initiator caspase would then – directly or indirectly – mediate mitochondrial membrane disruption to activate the apoptosome, but could also directly activate caspase-7, independent of mitochondria (Figure 1Ca). What do we know about caspases-1, -2 and -11 activation (and caspases-4 and -5, the human orthologs of mouse caspase-11)? Are scaffold-activating proteins of these caspases known and if yes, do they show any homology to CED-4/Dark/Apaf-1? Caspase-2 has recently been reported to directly permeabilize mitochondria (even in the absence of its catalytic activity)[92] and to be required for stress/DNA-damage-induced apoptosis.[93] Activation of caspase-2 occurs in a complex, called the PIDDosome that contains the death domain containing protein PIDD, whose expression is induced by p53, and the adaptor protein RAIDD.[94] Although PIDD overexpression sensitized cells for genotoxic-induced apoptosis,[94] it is not yet clear whether PIDD and/or caspase-2 are really required for apoptosis rather than for other cellular responses to genotoxic stress.[95] Also, the role of caspase-2 as a mediator of DNA-damage-induced apoptosis upstream of apoptosis has come under scrutiny.[96] Neither PIDD nor RAIDD exhibit any sequence homology to CED-4 and thus it is unlikely that they are scaffold proteins sequestered by Bcl-2

survival factors in healthy cells. This is consistent with the report that Bcl-2 regulated apoptosis and cytochrome *c* release can occur independently of caspase-2.[97] Caspases-1 and -5 are activated by scaffold proteins, called NALPs.[98] These proteins contain NBD and CARD domains like CED-4/DARK/Apaf-1 but they seem to be mainly involved in regulating inflammatory responses (processing and secretion of IL-1β and IL-18) rather than apoptosis. Martinon *et al.*[99,100] reported two types of such NALP/caspase-1/-5-containing multiprotein complexes, called inflammasomes. The NALP1 inflammasome, composed of NALP1, the adaptor protein Pycard/ASC, caspase-1 and caspase-5 and the NALP2/3 inflammosome that contains, in addition to NALP2 or NALP3, the CARD-containing protein Cardinal, ASC and caspase-1. Similar to Toll-like receptors, the inflammosome is activated by pathogen-associated patterns (PAMPS) which specifically bind to a leucine-rich repeat (LRR) of the NALP proteins.[98] Fourteen NALP proteins have been identified in humans and it is thought that each of them may recognize another bacterial component to assemble an inflammasome-like structure that regulates IL-1β and IL-18 secretion.[98] Thus, the NALPs are unlikely to be CED-4 homologs that regulate apoptosis although it cannot be excluded that under extreme pathogenic stress conditions, they contribute to the apoptosis of host cells via caspases-1 and -5. Finally, three other proteins, Nod1, Nod2 and Ipaf-1 have been identified that bear the double CARD/NBD signature typcial for CED4-like proteins.[100,101] However, neither Nod1 nor Nod2 seem to activate caspases but may regulate the response of intracellular pathogens by linking LPS binding via their COOH-terminal LRR to the activation of NF-κB via an interaction with the CARD-bearing Ser/Thr protein kinase RICK.[101] On the other hand, Ipaf-1 (CARD12/CLAN) interacts specifically with caspase-1 and coexpression of a ligand-binding deficient version (LRR) of Ipaf-1 with procaspase-1 provoked its processing and induced apoptosis.[102] Thus, under stress or pathogen situation Ipaf-1/caspase-1 may act as a specialized apoptosome. It remains to be seen whether Bcl-2 survival factors regulate this apoptosome in any way.

Biochemical aspect

Our recent biochemical analysis revealed that Bcl-2 forms high molecular mass complexes of up to 300 kDa on both the outer mitochondrial and ER membranes of healthy monocytes and fibroblasts (own unpublished data). We identified Bak and small amounts of the BH3-only protein Bim as components of these complexes (Figure 1Cb). The molecular masses of these proteins are, however, too small (between 20 and 30 kDa) to be the only Bcl-2-binding partners. We therefore speculate that Bcl-2 and perhaps other family members bind a variety of so far unknown cellular proteins on intracellular membranes of healthy cells, forming a 'Bcl-osome' (Figure 1C). This would explain why Bcl-2 is such a potent survival factor when overexpressed and can regulate caspase-dependent and -independent forms of cell death, autophagy as well as entry into the cell cycle (see review series in this issue). Recently, the group of Stan Korsmeyer achieved the isolation of four novel binding partners of the BH3-only protein BAD by purifying the endogenous Bad

protein complex from mouse liver mitochondria over gel filtration columns and blue native gel electrophoresis.[103] By comparing mitochondrial extracts from the liver of Bad+/+ and Bad−/− mice, the specificity of the protein complex could be monitored at each step of the purification. We think that this ingenious procedure will provide us with new 'guns' to 'chase' after the binding partners of Bcl-2 family members that have so far been missing to completely understand the mode of action of these important life/death regulators. It is still possible that a 'genuine' mammalian homolog of nematodal CED-4 will turn up in the prey. Thus, stay tuned for the next couple of hunting seasons!

Acknowledgements

Note added in proof

Delivani et al.[104] recently reported that, although overexpression of CED-9 in mammalian cells can regulate mitochondrial fission/fusion dynamics, it cannot prevent cytochrome c release or apoptosis induced by various triggers. This finding suggests that, in mammalian cells, apoptosis does not depend on a CED-4 homolog that interacts with CED-9 as in *Caenorhabditis elegans*.

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