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Mitochondria, Bcl-2 family proteins and apoptosomes: of worms, flies and men

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1. ABSTRACT

Initiator caspases are activated within specialized complexes, one of which is the apoptosome. The apoptosome is always constituted by at least an initiator caspase and a caspase activator. Apoptosome activation enables maturation of the associated caspase and constitutes a key step for cell fate. This activating complex is found throughout metazoans but its composition and regulation seem slightly different from one species to another. This review focuses on the composition and activation of the apoptosome in different species and details the role of mitochondrial factors and Bcl-2 family members in this activation.

2. INTRODUCTION

Apoptosis is a programmed cell death process necessary for metazoan survival. It shapes tissues throughout development and is critical all along adulthood for organ size maintenance or elimination of “harmful” cells. During normal development of the worm *C. elegans*, 131 cells are eliminated by apoptosis in the hermaphrodite (1). Genetic screens performed in this organism have allowed the discovery of a genetic control of apoptosis and elucidation of the signalling cascade leading to cell death (for review see (2)). At the heart of this pathway is CED-3. CED-3 encodes a caspase -for cysteine aspartase- whose activation is under the control of the caspase activator

CED-4. Activated CED-3 will then cleave cellular components leading to cell destruction. Dying cells are engulfed and eliminated by their neighbours. The complex composed of CED-4 and CED-3 is called apoptosome. Its formation and activation constitutes a key event for cell death execution in worm in a step controlled by Bcl-2 family members.

An apoptosome also exists in mammals and Drosophila. Containing homologues of CED-3 and CED-4, its activation is a key event for apoptosis execution in these species as well and is tightly regulated by various factors including Bcl-2 family members. Nevertheless, this apoptosome is different to some extent from one species to another both in its composition and regulation.

3. INITIATOR CASPASES

Apoptosis induction usually leads to caspases activation even though not all of caspase activities are linked to cell death commitment and apoptosis can proceed in some instances without caspase activity (3). Caspases are homologues of the nematode protein CED-3. These cysteine aspartases are produced as more or less inactive precursors called zymogens, and composed of a prodomain in the aminoterminal region, a large subunit domain and a small carboxyterminal subunit domain. Their full activation requires cleavages (autocatalyzed or not) that lead to the separation of their three different domains (prodomain, large and small subunit domains). They are active as heterotetramers regrouping two large and two small subunits. Caspases can be classified into two subgroups: the first one is constituted by effector caspases which present a short prodomain and whose activating cleavage is performed by other proteases (such as caspases or calpain). The second group is constituted by initiator or apical caspases, which present a long prodomain carrying a protein/protein interaction motif dubbed ”death domain” (4). This motif can either be a CARD (Caspase Activation and Recruitment Domain) or a DED (Death Effector Domain) domain. Initiator caspases are characterized by their ability to autoactivate within specialized complexes (5-7).

In mammals, two main pathways lead to caspases activation during apoptosis. The first one involves transmembrane receptors at the plasma membrane and is thus termed extrinsic pathway. The second one, which is more similar to the worm death pathway, is termed intrinsic or mitochondrial pathway and places mitochondria at the core of the signalling cascade. As in *C. elegans*, this second pathway is tightly regulated by Bcl-2 family members and relies on regulation of apoptosome activation.

In *Drosophila*, extrinsic and intrinsic apoptotic pathways have also been described. However, the extrinsic pathway seems to regulate immunity, and its role in apoptosis regulation remains anecdotal (8-13). Therefore, *Drosophila* apoptosis signalling mainly relies on the mitochondrial pathway.

3.1. The nematode case : a unique apoptotic caspase

CED-3, as any other caspase, is produced as an inactive precursor, whose full activity necessitates activating cleavages. As an initiator caspase, CED-3 is able to autocatalyze its own cleavage (14). These activating cleavages occurring after aspartate residues in position 221 and 374 can be artificially promoted by forced dimerization. However, the caspase activator CED-4 is essential *in vivo* for the oligomerization that will allow CED-3 activation (15, 16). As CED-3 is the only apoptotic caspase in the worm *C. elegans*, it plays a central role in apoptosis execution in this organism (17, 18). Indeed, it plays the role both of an initiator and an effector caspase. CED-3 presents in its aminoterminal region a CARD-carrying prodomain, which makes it an initiator caspase, but it also possesses features of effector caspases such as its affinity for DEVD target sequences (19).

3.2. Mammalian caspase 9

In mammals, there are five initiator caspases involved in apoptosis that can be activated either by the extrinsic or the intrinsic pathways (5, 6). Concerning the mitochondrial pathway, the initiator caspase 9 is a key regulator. As a matter of fact, mice that are deficient for caspase 9 exhibit developmental defects due to a lack of apoptosis. These knock-out mice defects are particularly severe in the central nervous system, which could be explained by the massive apoptosis required for modelling this tissue (20, 21). The lack of caspase 9 is probably viable because of caspases redundancy and the existence of alternative cell death pathways.

Caspase 9 aminoterminal part is a long CARD-carrying prodomain, which is cleaved during activation. Another activating cleavage generates the large and small subunits. Caspase 9 presents a long flexible domain divided between the large and the small subunits, allowing formation of an active site within the proform -and thus conferring some activity- in absence of any cleavage. Nonetheless, caspase 9 zymogen activity is low unless it is activated and part of the apoptosome where its activity increases by 2000 times (22-24).

3.3. The only Drosophila CARD-carrying caspase : Dronc

Dronc is the most studied caspase in *Drosophila* and appears to be the main apical caspase involved in apoptosis. Its expression is regulated throughout development, and in particular during metamorphosis when ecdysone stimulates its transcription, thus leading to larval structures elimination (25-28).

Dronc is necessary for most apoptotic processes in *Drosophila* (29-31). Indeed, loss of function *dronc* mutants do not survive beyond embryonic stage without maternal contribution (i.e. proteins and mRNAs stocked in the oocyte that will allow a proper development until transcription starts at the maternal to zygotic transition). In addition, these embryos present developmental apoptosis defects probably responsible for the observed lethality. Maternal contribution allows survival until pupal stage and some adult escapers are even able to survive up to three days after hatching. These escapers

present phenotypes that are reminiscent of developmental apoptosis defects such as rough eyes or opaque and curved wings (31). Indeed, these phenotypes are similar to those observed in mutants of other apoptotic regulators such as the caspase activator homologue of *apaf-1*, *dapaf-1/dark* (31-33). Lack of *dronc* induces more severe phenotypes in *Drosophila* than the loss of caspase 9 in mammals. This could be explained by the absence of redundant caspases in fly and most of all by the absence of alternative pathways for caspases activation, making Dronc, the key element to efficiently regulate developmental apoptosis. In addition, Dronc is required for irradiation-induced apoptosis. Indeed, mutants deficient for *dronc* exhibit a strong apoptosis defect in response to X-ray irradiation (30).

As for any initiator caspase, Dronc aminoterminal region is a long prodomain. This prodomain carries a CARD motif, which mediates Dronc recruitment in an activating platform, as in the case of caspase 9. Moreover, it is noteworthy that Dronc is the only *Drosophila* caspase carrying this type of domain, making it the fly counterpart of mammalian caspase 9. As such, its activation relies on a caspase activator, Dark/Dapaf-1, homologue of mammalian Apaf-1 and nematode CED-4 caspase activators. Dark/Dapaf-1 also carries a CARD domain, which interacts with Dronc's CARD domain to form the fly apoptosome.

Dronc originality mainly resides in its ability to cleave not only after an aspartate residue (as any caspase) but also after a glutamate (34). As an apical caspase, Dronc is able to autoactivate and catalyze its own cleavage after a glutamate in position 352 (TQTE motif). This autocatalyzed cleavage occurs between the large and small subunit domains to generate an intermediate referred as Pr1 (proform 1) and is thought to take place within the apoptosome (composed at least of Dark/Dapaf-1 and Dronc) (34). A second cleavage leading to the separation of the CARD-carrying prodomain from the large subunit is then performed by the main *Drosophila* effector caspase, Drice, after an aspartate residue located in position 135.

Until recently, Dronc's cleavage, and more specifically the autocatalyzed cleavage occurring after glutamate in position 352, used to be considered crucial for Dronc activity (34). However, a recent study using an uncleavable Dronc protein suggests that Dronc -as mammalian caspase 9- can exhibit some activity as a zymogen. A major difference remains between Dronc and caspase 9 with caspase 9 activity being increased 2000 fold when part of the apoptosome. In contrast, the *Drosophila* Dronc initiator caspase appears almost fully active without cleavage as its activity is only 1.5-fold increased after processing (36). In addition, E352 was thought to be a crucial cleavage site for Dronc activation as the E352A mutation impairs Dronc ability to cleave an artificial IETD-afc substrate *in vitro*. Nonetheless, this cleavage does not seem necessary for Dronc pro-apoptotic activity in cultured cells (35, 36).

4. APICAL CASPASES ACTIVATION IN THE MITOCHONDRIAL PATHWAY-ROLE OF THE APOPTOSOME

The term apoptosome has initially been used to describe the mammalian complex allowing caspase 9 activation and composed of caspase 9, Apaf-1 and cytochrome c. Afterwards, this designation has been extended to other species to describe complexes allowing caspase activation through use of an homologue of Apaf-1. Ecdysozoan homologues (i.e. fly and nematode homologues) of Apaf-1 do not appear to be orthologues of Apaf-1 but to have an ancient ancestor gene that would have been duplicated into multiple paralogues still present in species representing many phyla including *Chordata*, *Cnidaria* and *Echinodermata*. As a consequence of a probable different gene loss among the paralogues that happened between mammals ancestors and *Ecdysozoa* ancestors, Apaf-1 and its ecdysozoan homologues are quite different in sequence (37).

4.1. The simplest model: the worm apoptosome

In living cells, CED-3 activation is prevented by sequestration of CED-4 by CED-9 at the mitochondrial outer membrane. Activation of the cell death program triggers production of EGL-1, which competes with CED-4 for CED-9 interaction (Figure 1). By this means, EGL-1 permits the release of CED-4 from CED-9 stranglehold, thus allowing its dimerization and its translocation near the nucleus where it probably interacts with the nuclear envelop protein SUN-1 (38, 39). Indeed, the physical interaction between CED-4 and CED-3 occurs in the perinuclear region. At this stage, CED-4 dimers oligomerize to form a homotetramer (Figure 2C) onto which CED-3 is activated. This multimeric structure represents the worm apoptosome.

4.2. Caspase 9 activation in mammals: the apoptosome

In mammals, the so-called intrinsic or mitochondrial pathway leads to caspase 9 activation within a macromolecular complex called apoptosome. This complex brings into play Apaf-1, an adaptor molecule, which is the caspase activator homologue to nematode's CED-4. This adaptor carries a CARD domain, allowing its interaction with caspase 9 CARD domain, and regulator WD40 motifs that bind cytochrome c as a co-factor. Cytochrome c usually resides in the mitochondrial intermembrane space where it is involved in oxidative phosphorylation. During apoptosis, the mitochondrial outer membrane is permeabilized leading to the release in the cytosol of apoptogenic factors such as AIF, Smac/Diablo, Omi/HtrA2 or cytochrome c. Cytosolic cytochrome c is then able to bind the WD40 motifs of Apaf-1, changing Apaf-1 conformation in an ATP-dependent manner, and notably leading to the exposure of its CARD domain. Exposed CARD domains of Apaf-1 will interact to form an oligomer of seven Apaf-1 molecules arranged in a wheel-like structure (Figure 2A). Caspase 9 proteins will then be recruited onto this structure to form a mature apoptosome within which they will be activated by cleavage (40, 41).

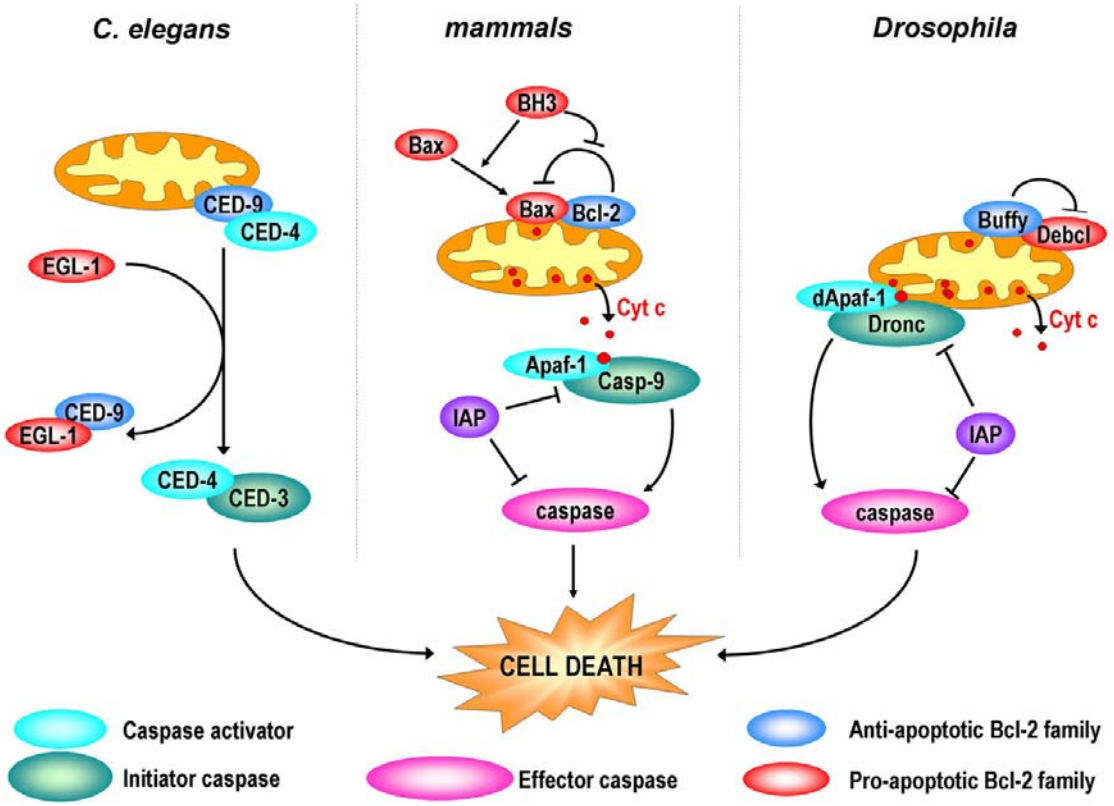

Figure 1. Main regulators of the mitochondrial apoptotic pathway in *C. elegans*, *Drosophila* and mammals. In all these species, this apoptotic pathway relies on apoptosome formation and activation. The apoptosome contains at least an oligomer of caspase activator (turquoise) and several copies of a CARD-carrying initiator caspase (green). In mammals, cytochrome c (red) is an additional co-factor required for apoptosome formation. In these three species, Bcl-2 family members, which are either pro-apoptotic (red) or anti-apoptotic (blue) regulate each other at the level of the mitochondria and are involved in regulation of apoptosome activation. Nonetheless, their mode of action differs: in the nematode, the anti-apoptotic protein CED-9 prevents apoptosome formation by direct binding to CED-4, and EGL-1 promotes apoptosome formation by releasing CED-4 from CED-9. In mammals, proteins of the Bcl-2 family either promote (such as Bax or BH3-only members) or inhibit (such as Bcl-2) release of apoptogenic factors from the mitochondrial intermembrane space to the cytosol, one of which being the Apaf-1 co-factor, cytochrome c. In *Drosophila*, Bcl-2 proteins are also mitochondrial and the pro-apoptotic Debcl induces an apoptosome-dependent cell death. Debcl has not been involved in cytochrome c release and cytochrome c does not seem to be necessary for apoptosome activation. Although its mode of action remains elusive, Debcl seems to act through different mechanisms than worm or mammalian Bcl-2 family members. In mammals and *Drosophila*, apoptosome activity can be limited by IAPs (purple).

Even if an activation of the apoptosome independent from cytochrome c has been described in thymocytes (42), this is not typical in mammals. In addition, even if mammalian apoptosome activity depends on cytosolic cytochrome c availability, it is also regulated by many other proteins. These proteins, without being constitutive components of the apoptosome, are able to interact with it and subsequently modulate its activity, either facilitating or on the contrary impairing apoptosome activity (for review, see (43)). For instance, IAPs are well known for their ability to inhibit, through physical interaction and thanks to their ubiquitin ligase activity, both free and apoptosome-linked caspases (Figure 1) (44-47). However, IAPs are far from being the only apoptosome-interacting proteins. As a matter of fact, JNK interaction with the pre-apoptosome also inhibits its activation by delaying caspase 9 recruitment, and thus impairing caspase 9 activation (48). On the contrary, proteins such as histone H1.2 or HCA66 (Hepatocellular Carcinoma Antigen 66) promote apoptosome activation. Indeed, HCA66 has been shown to bind Apaf-1 through its CED-4 domain (the region in between the CARD and WD40 domains), to favour caspase 9 recruitment and activation within the apoptosome (49). Concerning H1.2, this histone has been proposed to form a complex together with Apaf-1, caspase 9 and cytochrome c (50) and has been shown to promote cleavage of both effector caspases 3 and 7 in response to DNA damage following UV-irradiation in a caspase 9/Apaf-1 dependent manner. Nevertheless, it remains to be elucidated whether the possible H1.2 interaction with the apoptosome promotes assembly of the pro-apoptotic complex or whether this interaction stimulates activity of an already assembled apoptosome. Hence, it is enticing to envision that some of these proteins could activate the apoptosome in the absence of free cytochrome c.

4.3. The most controversial: the Drosophila apoptosome

In *Drosophila*, as in mammals or nematode, an apoptosome also exists within which a CED-4/Apaf-1 homologue, named Dark/Dapaf-1, is the adaptor molecule. This complex is composed of at least Dapaf-1 and the only CARD-carrying *Drosophila* caspase, Dronc. This apoptosome enables an autocatalyzed cleavage of Dronc and is involved in most of apoptotic processes in the fly (*i.e.* both developmental and stress-induced apoptosis) (29-31). Differently than in mammals, the role of cytochrome c in this activation remains controversial in *Drosophila*. Indeed, until very recently, cytochrome c release had never been demonstrated in *Drosophila*. Nonetheless, several studies tended to involve cytochrome c in regulation of *Drosophila* apoptosis. In 1999, the use of an antibody raised against cytochrome c showed that accessibility of an epitope on cytochrome c varies during apoptosis induction. This work raised the possibility that even if cytochrome c could not be released from the mitochondrial intermembrane space, a conformational change could regulate its interaction with the apoptosome (51). In addition, recent studies performed using a model of developmental programmed cell death (*Drosophila* retina) have shown that, in this system, cytochrome c is not essential for apoptosis execution but participates in the process, enabling a timely apoptosis execution. Indeed, retina deficient for *cytochrome c* exhibit a delay in execution of developmental apoptotic processes (52). Very recently, another study performed on S2 cells (an embryonic macrophage *Drosophila* cell line) has even more complicated our vision of cytochrome c role in the regulation of apoptosis in *Drosophila* (53). In this system, cytochrome c release has been described without linking cytosolic cytochrome c to apoptosis regulation. Moreover, cytochrome c release has only been observed under certain conditions. Indeed, *rpr*-, *hid*- or X-ray-induced apoptosis may trigger cytochrome c release. This release can be inhibited by the pan-caspase inhibitor z-VAD, suggesting that cytochrome c relocation requires caspase activity, thus placing mitochondria downstream of caspase activation. However, even though caspase activity seems necessary for this release, it is not sufficient. Indeed, apoptosis induction and subsequent caspase activation by actinomycin D, UV light or RNAi mediated *diap1* inactivation does not lead to cytochrome c relocation. Therefore, this last study makes a possible role of cytochrome c in *Drosophila* apoptosis more unlikely. As in mammals, cytochrome c could be released in some conditions but unlike mammalian cytochrome c, its relocation wouldn't have any regulatory role in caspase activation. This release would only be a collateral effect and could be part of a positive feedback loop.

Some data tend to eliminate any implication of cytochrome c in the regulation of apoptotic processes in *Drosophila* or at least in the apoptosome activation. This is the case of a study carried out both in cultured *Drosophila* cells and *in vivo*. This work relies on cytochrome c gene inactivation either by RNAi or using loss of function mutants. Dorstyn *et al.* noticed that a lack of cytochrome c does not impair caspase activation neither in cultured cells nor *in vivo* (54). In addition, a comparative structural study of *C. elegans*, *Drosophila* or mammalian apoptosomes also brings arguments against a direct interaction of cytochrome c with *Drosophila* apoptosome. This study shows that Dapaf-1 oligomerizes as an octamer while mammalian Apaf-1 forms a heptamer, suggesting that because of its compact structure the *Drosophila* apoptosome would not be wide enough for cytochrome c binding (Figure 2B). Furthermore, cytochrome c has never been found associated to the fly apoptosome. Hence, cytochrome c does not appear necessary for *Drosophila* apoptosome assembly (55, 56).

All these contradictory studies suggest that cytochrome c could in some particular cases, be involved in apoptosome activation and thus caspase activation. So far, in contrast to what can be observed in mammals, cytochrome c does not have a critical role in the apoptotic cascade of *Drosophila*. Nevertheless, the *Drosophila* apoptosome is far from being as understood as the mammalian one, and Dapaf-1 does not seem sufficient to fully activate Dronc (36). It is thus possible that still unidentified proteins can interact with the fly apoptosome and regulate its activity as observed in mammals. It is thus tempting to envision that *Drosophila* apoptosome could be at least partly regulated in the same manner. Identification of Dapaf-1 WD40 domain binding proteins would be of great interest to enlighten this question.

If *Drosophila* cytochrome c has only a minor role in regulating apoptosis, what about the mitochondrion? Indeed, although its role is still unclear, the mitochondrion is very likely to have a key role in apoptosis regulation in *Drosophila*. If mammalian mitochondria release apoptogenic factors in the cytosol, description of mitochondrial outer membrane permeabilization in *Drosophila* is only very recent and only two factors have been described to be released from mitochondria. These factors are cytochrome c (although its role in apoptosis remains to be elucidated) and dOmi (53, 57, 58). Indeed, two groups have recently shown that *Drosophila* genome encodes a mammalian Omi/HtrA2 homologue. In addition, both indicate that this serine protease is released from the intermembrane space during apoptosis (even though the final destination of this regulator remains unclear) (57, 58). In any case, it appears that a large number of *Drosophila* apoptosis regulators share a mitochondrial location during apoptosis. It is the case for members of the Bcl-2 family (*i.e.* Debcl and Buffy), which are constitutively located at the mitochondria (Figure 1), and caspases such as Dronc and Drice which have been found associated to mitochondria in some subcellular fractionation experiments (59). Localization of these caspases allows considering the existence of an apoptosome, which could be located around mitochondria. Other key regulators of apoptosis in *Drosophila* can be found at the mitochondria (or in its neighbourhood). These are the pro-apoptotic proteins Reaper, Hid and Grim (53, 60-62). In addition, several studies indicate that during *Drosophila* apoptosis, an early loss of mitochondrial membrane potential can be detected, similar to what is observed in mammals (53, 63, 64).

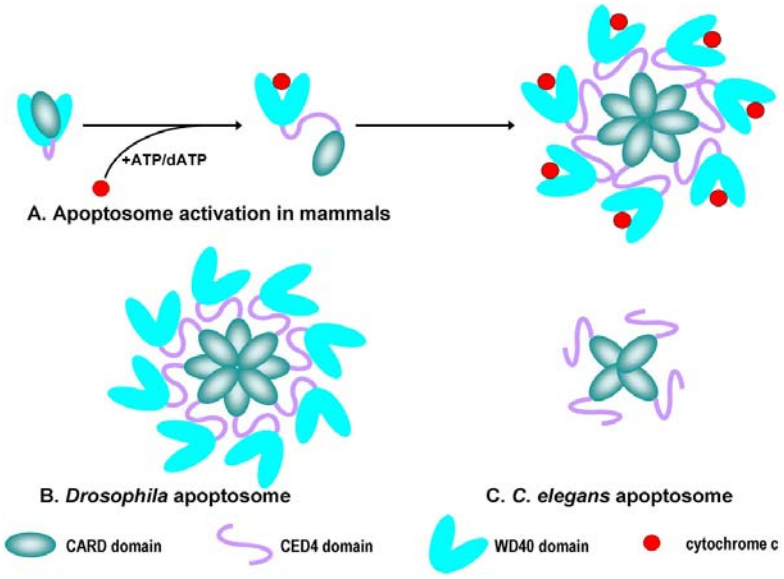

Figure 2. Apoptosome formation. In mammals (A), cytochrome c (red) binding induces an ATP-dependent conformational change of Apaf-1 leading to exposure of the CARD domain. At this stage, CARD domains of seven Apaf-1 molecules can interact to form a wheel-like heptameric structure on which procaspase 9 will be recruited by CARD/CARD interaction between procaspase and Apaf-1 molecules to be activated. In *Drosophila* (B) and *C. elegans* (C), there is also an oligomerization of the caspase activator (CED-4 and Dapaf-1 respectively) but instead of being based on an Apaf-1 heptamer, the apoptosome is based on a tetramer (*C. elegans*) or an octamer (*Drosophila*) of Apaf-1 homologues.

All these arguments suggest that the mitochondrion has an important place in the *Drosophila* apoptotic cascade, even though its role remains to be elucidated,.

5. BCL-2 FAMILY MEMBERS AND APOPTOSOME ACTIVATION

Apoptosome activation is tightly controlled by Bcl-2 family members in the worm, fly and mammals. Nevertheless, their mode of action is clearly different in each of these species. Members of the Bcl-2 family are characterized by their BH (Bcl-2 Homology) domains, that are the only regions conserved between members of this family. Three groups can be distinguished within this family. The first class is represented by anti-apoptotic members, which usually carry 4 BH domains. The two other classes contain the pro-apoptotic members of the Bcl-2 family. They are either multidomain members possessing BH1, BH2 and BH3 domains, or the so-called "BH3-only" pro-apoptotic members, which only possess a BH3 domain (65, 66). BH domains of Bcl-2 family proteins enable physical interaction between members of this family to form homo- or heterodimers. Indeed, BH1-3 domains are able to form a hydrophobic pocket where the BH3 domain of another Bcl-2 family member can dock.

5.1. The direct model: *C. elegans* Bcl-2 family members

Three members of the Bcl-2 family have been described in the genome of the nematode *C. elegans*. The first one is called CED-9 and possesses an anti-apoptotic activity. Its protective role is mediated by a direct physical interaction between CED-9 and the caspase activator CED-4. This mode of action is strikingly different from what happens in mammalian mitochondrial apoptosis. Indeed, mammalian Bcl-2 family members mainly regulate apoptosome activity by controlling availability of the co-factor cytochrome c. On the contrary, the worm control of the apoptosome activity is more direct, and release of factors from the mitochondrial intermembrane space seems to be only involved in an amplification feedback loop occurring after CED-3 activation (67).

In living cells, CED-9 directly interacts with CED-4. This physical interaction impairs CED-4 availability for CED-3 caspase activation through sequestration at the mitochondria. Apoptosis induction in the worm is associated to transcriptional activation of EGL-1. EGL-1 is the second member of the Bcl-2 family that can be found in the worm genome and is a BH3-only protein. EGL-1 BH3 domain enables its physical direct interaction with the anti-apoptotic Bcl-2 family member CED-9. This interaction between EGL-1 and CED-9 leads to the apoptosome activation.

A third member of the Bcl-2 family, CED-13, has been described in *C. elegans*. This other BH3-only protein does not seem to be required for developmental apoptosis but is thought to facilitate apoptosis in germline cells in response to DNA damage (68).

5.2. Permeabilization function: mammalian Bcl-2 family members

In mammals, mitochondrial events such as release of apoptogenic factors to the cytosol are kept under tight control by proteins of the Bcl-2 family that counts about twenty members (69). Caspase 9 activation in the

apoptosome requires release of cytochrome c from the mitochondrial intermembrane space to the cytosol and is therefore subjected to this control.

Members of the Bcl-2 family act mainly at the level of the mitochondria where they control outer membrane permeabilization. Two major models that are not mutually exclusive have been proposed to explain the Bcl-2 family proteins control of the mitochondrial outer membrane permeabilization (69, 70). The first one relies on the formation of channels formed by multidomain proteins of the Bcl-2 family, such as Bak and Bax, potentially after activation by a BH3-only protein. The way anti-apoptotic proteins of this family counteract formation of the channel remains uncertain, but their inhibitory activity has been proposed to be achieved by direct interaction with multidomain or BH3-only pro-apoptotic members of the family. The second model involves a regulation of the opening of the PTP (Permeability Transition Pore), a macromolecular channel which includes ANT, VDAC, cyclophilin D and other variable components. *In vitro* experiments as well as experiments on isolated mitochondria indicate that both anti-apoptotic and pro-apoptotic Bcl-2 family proteins could regulate the PTP opening. In both models, anti-apoptotic members impair release of apoptogenic factors in the cytosol while pro-apoptotic ones favour this relocation. In mammals, they constitute key regulators of the mitochondrial apoptotic pathway, and mice deficient for both Bax and Bak multidomain pro-apoptotic members exhibit a large number of developmental defects associated to a lack of apoptosis (71-73).

5.3. The least understood: Drosophila Bcl-2 family members

If in mammals the numerous members of the Bcl-2 family are well known for their important role at the level of mitochondria in apoptosis regulation, *Drosophila* members of this family remained unknown for a long time. Bcl-2 and Bax mammalian proteins have been proved to be functional in Drosophila and Bcl-2 is able to inhibit developmental or irradiated-induced cell death as well as *rpr*- and *bax*-induced apoptosis, which both involve mitochondrial events (63, 74). So far, two members of the Bcl-2 family have been identified. The first one is a pro-apoptotic multidomain member called Debcl (75-77), and the second is the anti-apoptotic Buffy (78). Debcl and Bax-induced cell death in *Drosophila* share common regulators but are not fully equivalent, since not all modifiers of *bax*-induced phenotypes mitigate *debcl*-induced apoptosis (J. Colin unpublished results).

Buffy and Debcl expression patterns correlate and these proteins can be co-immunoprecipitated in cellular extracts. Turning down the level of Buffy by RNAi treatment strongly increases embryonic developmental apoptosis and genetic studies suggest that Buffy acts upstream of Dronc activation (78). On the opposite, Debcl-induced apoptosis triggers caspase activation and requires a functional apoptosome. Moreover, *debcl* inactivation experiments by RNAi in embryos have shown a decreased apoptosis, thus supporting a role for *debcl* in the regulation of developmental apoptosis (74). Recently, loss of function mutants of *debcl and buffy have* been generated (79). Surprisingly, this work showed that null mutants of *debcl* or *buffy* are viable, fertile and do not present any remarkable phenotype that could be linked to apoptosis defects. These data can appear contradictory with previous experiments using RNAi. However, their difference could be explained by the existence of a mechanism of compensation occurring during development to compensate for *debcl* or *buffy* loss. In this case, the developmental stage chosen for observation would explain the different interpretations. Mutant phenotypes were observed in adults, leaving the whole period of development to correct defects induced by *debcl* or *buffy* loss, whereas in the case of RNAi-mediated inactivation, observations were made in embryos, *i.e.* a few hours after gene inactivation. As a consequence, these data are not sufficient to exclude a possible -albeit not crucial- role for Debcl and Buffy in developmental apoptosis. In addition, $debcl^{E26}$ mutant embryos display a lack of irradiation-induced apoptosis which implicates *debcl* in stress-induced cell death rather than in developmental processes (79). Similarly, *buffy* overexpression function protects from irradiation-induced cell death (79).

Debcl presents a membrane anchor in its carboxyterminal region. It has been shown that this domain is necessary for its mitochondrial localization, and most of all for its pro-apoptotic function, as deleting this region impairs *debcl* cell-killing activity (77). Buffy has been shown to be constitutively localised at the mitochondria in the *Drosophila* embryo (76), but in BG2 cells (derived from the larval brain) Buffy seems to localise at the endoplasmic reticulum and to be pro-apoptotic (80). Whether this switch in activity is associated to the cell type or to modifications of the protein is unclear. It relates to a study of Debcl, Buffy and polyglutamine genetic interactions in the fly nervous system (81). Indeed, Buffy appears to potentiate polyglutamine-induced toxicity in a model for neurodegenerative polyglutamine diseases, while Debcl decreases this toxicity. In this work, Senoo-Matsuda *et al.* also show that decreasing Debcl synthesis or over-expressing Buffy in *Drosophila* neurons induces an alteration of mitochondrial energy metabolism leading to ATP depletion and lifespan shortening. This study clearly shows that Debcl plays a role outside of cell death regulation and more specifically in neuronal energetic maintenance. This role could involve the control of mitochondrial reactive oxygen species production or accumulation that have been proved to be important regulators of mitochondrial homeostasis and cell death (82).

Nonetheless, the exact role of Debcl and Buffy on mitochondria and the way these proteins regulate the apoptosome is far from being understood. Moreover, while it has been recently shown that cytochrome c (and dOmi) could be released from the mitochondrial intermembrane space to the cytosol in some conditions, fly Bcl-2 family members have not yet been involved in this process (53, 57, 58).

6. CONCLUDING REMARKS

Apoptosome activation is a key step in apoptosis regulation in worm, *Drosophila* or mammals but mechanisms of control of its activity differ between these

species. In fact, in mammals, apoptosome activity is mainly regulated by cytochrome c availability, its release from the mitochondrial intermembrane space being tightly regulated by members of the Bcl-2 family. In other words, this apoptosome is regulated by control of its activation in response to mitochondrial outer membrane permeabilization. In contrast, the *Drosophila* apoptosome activity, although supposed to be located at or nearby mitochondria, has been shown to be mainly regulated by modulating its inhibition by IAP proteins. In fact, apoptosis induction leads to the release of apoptosome from Diap1 mediated inhibition (83). In this process of apoptosome activation, cytochrome c is clearly not crucial. In addition, Bcl-2 family members do not seem to be key regulators of developmental apoptosis and are rather thought to regulate stress-induced cell death. Moreover, the way this protein family regulates the *Drosophila* apoptosome is still unclear. Nevertheless, it seems that the apoptotic cascade is inverted between flies and worm/mammals. Indeed, contrarily to what happens in these last organisms, where apoptosis regulators are relocated from mitochondria to the cytosol, it seems that *Drosophila* apoptosis regulators use an opposite relocation to concentrate at or around mitochondria during apoptosis. In the nematode, it seems that a third mode of apoptosome control has been selected. Once more, Bcl-2 family members are important for regulation of apoptosome activity but this regulation, although involving CED-4 release from mitochondria-bound CED-9, occurs directly without involving mitochondrial membrane permeabilization as a decisive step. Taken together, studies of apoptosome activation in these different species show that the way Bcl-2 family proteins bound to mitochondria regulate caspases activity has evolved during evolution. One interesting point would be to determine whether a function of these proteins independent from cell death regulation processes is conserved throughout evolution.

7. ACKNOWLEDGEMENTS

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Abbreviations: CARD: caspase activation and recruitment domain; DED: death effector domain; Dronc: *Drosophila* nedd-2 like caspase; CED: cell death; EGL-1:egg-laying; Bcl-2: B-cell lymphoma 2; caspase: cysteine aspartase, IAP: inhibitor of apoptosis protein; BH: Bcl-2 homology; Pr1: proform 1, HCA66: hepatocellular carcinoma antigen 66, JNK: jun N-terminal kinase, PTP: permeability transition pore, ANT: adenine nucleotide translocase, VDAC: voltage-dependent anion channel

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