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News and Commentary

A cytochrome *c*-free fly apoptosome

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The canonical caspase activation pathway, as first elucidated in the worm *Caenorhabditis elegans*, is largely conserved in metazoans. Recent structural and biochemical data demonstrate that in *C. elegans*, the caspase CED-3 is activated by a tetrameric CED-4 complex (apoptosome) that is prevented from assembling by interaction with the Bcl-2-like protein CED-9 until the BH-3-only protein EGL-1 sequesters CED-9 away.[1] CED-4 and its orthologues thus facilitate the activation of apical caspases (CED-3 and its orthologues) and provide a key regulatory step in the initial activation of caspases. The formation of the caspase-activating apoptosome in mammals is well documented[2–4] and a recent publication from Christopher Akey's laboratory now reports the assembly and three-dimensional structure of a *Drosophila* apoptosome.[5]

CED-4 is a member of the P-loop ATPase family that includes Apaf-1 in mammals and ARK (Dark/dApaf-1/Hac-1) in *Drosophila*.[6] CED-4, ARK and Apaf-1 exhibit conservation at the N-terminal region, which comprises a caspase recruitment domain (CARD) followed by a nucleotide-binding/oligomerisation domain (NOD) (which consists of a nucleotide-binding domain, NBD; conserved helical domain, HD1 and winged helix domain, WHD) and a second conserved helical domain (HD2).[1–5] The fly ARK and mammalian Apaf-1 share greater similarity to each other than either with CED-4. In addition to the domains shared with CED-4, the ARK and Apaf-1 proteins contain 14 and 13 WD40 repeats, respectively, at their C-terminus, which act as essential regulatory regions for apoptosome function (Figure 1a).[1–5]

In mammalian cells, the mitochondrial respiratory protein cytochrome *c* is released into the cytosol during apoptosis and assists the formation of an oligomeric Apaf-1 apoptosome.[2–4,6] Biochemical studies and the three dimensional structure of Apaf-1 apoptosome indicate that dATP is bound to the CED4 homology region and the binding of cytochrome *c* promotes Apaf-1 activation and the formation of an oligomer comprised of seven Apaf-1 molecules.[2–4] In the final step, procaspase-9 is recruited to the active apoptosome by binding to the exposed Apaf-1 CARD. This apoptosome activates caspase-9, which can then activate downstream caspases (caspase-3 and -7) to execute cell death.[2–4]

In *C. elegans*, CED-4 is retained in an inactive dimer conformation by CED-9.[1] During apoptosis, EGL-1 competitively binds to CED-9, thereby releasing CED-4. CED-4 can then oligomerise to form a tetramer, which can then induce CED-3 activation.[1] The models of CED-4 and Apaf-1 define the CARD ring as the active centre of the apoptosome.[1,3] However, in contrast to CED-4, the regulatory region of the human apoptosome organises into two β-propellers, which are packed nearly perpendicular to each other, with a single cytochrome *c* molecule between them.[2,3] The stoichiometry of cytochrome *c* to Apaf-1 is 1 : 1. Although both ATP and dATP can support apoptosome activation, recombinant Apaf-1 purified from SF21 cells was found to be associated with dATP.[4] Interestingly, if Apaf-1 is preincubated with cytochrome *c* in the absence of dATP, this induces permanent structural changes in Apaf-1 that renders it inactive. Therefore, a preformed Apaf-1/dATP complex must be present *in vivo* before binding of cytochrome *c* in order for an active apoptosome to assemble and to induce caspase activation.[5] In contrast to Apaf-1, although ATP is bound to the CED-4 complex, the CED-4 tetramer does not exhibit ATPase activity and ATP hydrolysis is not essential for CED-3 activation *in vitro*.[1] Furthermore, as a consequence of CED-4 lacking a C-terminal WD40 regulatory region, it does not require cytochrome *c* for its activity. Therefore in the worm, the CED-4 apoptosome is a tetrameric wheel that activates CED-3 without the requirement for any additional molecules and dATP may simply have a structural role in CED-4 complex formation.[1]

Similar to Apaf-1, the WD40 repeat region in *Drosophila* ARK was predicted to function as a regulatory region and bind cytochrome *c*. Previous studies have found that unlike in mammalian cells, cytochrome *c* is not released from mitochondria in *Drosophila* cells during stress-induced apoptosis.[7,8] Consistent with this observation, biochemical and RNA interference studies have shown that cytochrome *c* is not involved in caspase activation and stress-induced apoptosis in the fly.[9,10] Furthermore, recent cell free studies show clearly that mitochondrial factors do not have a role in caspase activation, and mitochondrial fractions are unable to influence caspase activity.[11]

The work from the Akey laboratory[5] provides the cryo-EM structure of ARK complex at 18.8 Å resolution. Similar to Apaf-1, ARK forms a wheel-like oligomer but is comprised of eight molecules (Figure 1). The *in vitro* assembly of the ARK complex strictly requires dATP, at a 10-fold concentration required for Apaf-1 assembly.[5] The WD40 motifs in ARK, as with its human counterpart, assemble into β-sheet structures to form the β-propellers. This β-propeller regulatory region is slightly larger compared to Apaf-1. The extra molecule in the ARK apoptosome forces a more tightly packed conformation with the NBD contacting the β-propeller regulatory region, the region which binds cytochrome *c* in Apaf-1. Importantly, cytochrome *c* could not form a stable complex with ARK; in fact, other than the requirement for an excess of dATP, an additional activator was not necessary for the assembly of this ARK complex *in vitro*.[5] Interestingly, although the β-propeller

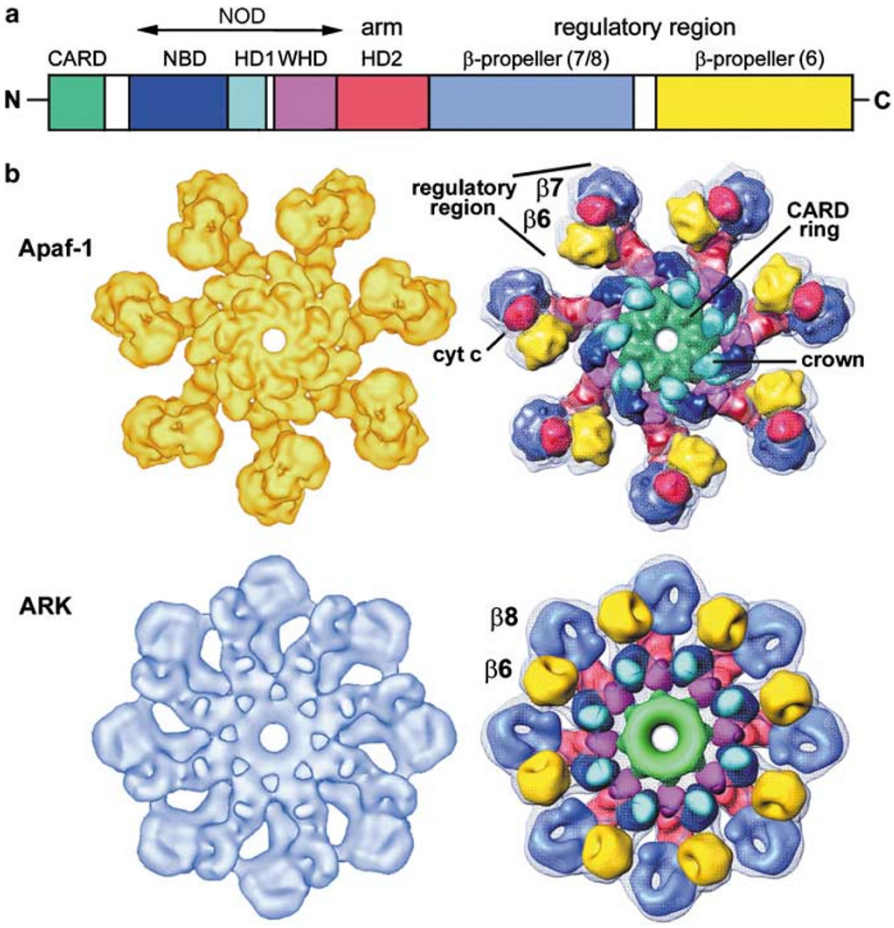

Figure 1 The three-dimensional structures of the Apaf-1 and ARK apoptosomes. (**a**) Structural domains of Apaf-1 and ARK are indicated as a linear schematic. The two β-propellers domains are comprised of two clusters of seven and six WD40 motifs in Apaf-1, or eight and six WD40 motifs in ARK. (**b**) Top view of the Apaf-1 and ARK apoptosome. Images of EM surface maps (left) and domain models (right) provided by CW Akey (Boston University) are shown. The different domains are colour coded as in (**a**). The position of cytochrome *c* bound between the two β-propellers in Apaf-1 is indicated. Note the different positions of the NBD and HD1 in ARK

region does not interact with cytochrome *c*, there is sufficient space to accommodate a small molecule. This suggests that the β-propellers may have preference for an alternative molecule and like Apaf-1, ARK may still be able to interact with an additional protein to facilitate its activation. The identification of such an ARK-activating factor, if it exists, will be crucial to understand the caspase activation mechanisms in the fly.

Interestingly, the structure of the ARK complex revealed that it comprised two layers of ring-like structures with a calculated mass of 2.5 MDa.[5] The helix domain (HD1) and NOD mediate a connection between two ARK rings, enabling face-to-face association for the formation of a double apoptosome. This indicates that the ARK apoptosome assembles as a dimeric ring structure, but it is unclear whether the two-ring apoptosome also forms *in vivo*. It is possible that the ARK ring dimer is preformed in cells but is inactive in this conformation. The model predicts that the initiator caspase DRONC interacts with the CARD ring in the central hub, between the two ARK apoptosomes in the double ring. However, the structure of this double ring suggests that the binding sites for DRONC are inaccessible, indicating that this structure may be inactive. This gives rise to the possibility that during apoptosis signalling, the ARK apoptosome changes conformation to allow DRONC interaction and activation. Recently published biochemical and structural data show that DRONC zymogen requires autocatalytic cleavage followed by dimerisation for activation.[12] Although it is well established that DRONC activation requires ARK, the structural studies on ARK and DRONC do not directly demonstrate how an ARK apoptosome facilitates DRONC activation. Without the analysis of the ARK apoptosome bound to DRONC, it remains unclear whether the double ring structure acts as the active apoptosome or whether the active apoptosome, like Apaf-1, is a single ring-like structure.

In a genetic screen to identify suppressors of the GMR-*hid*-induced eye ablation phenotype, Andreas Bergmann and co-workers have recently identified a large number of *ark* point mutants.[13] The analyses of these mutants provide some interesting clues about the functions of various ARK domains. Most of the missense mutations were clustered in the HD1 region of the NOD, and each affected ARK function to varying degrees.[13] Given that HD1 is important for the connection between the two ARK apoptosomes, it is possible that these mutants affect formation of the ARK double ring structure,

thus supporting the possibility that the ARK double apoptosome exists *in vivo*. Although not known whether the double ring structure is the active apoptosome, two conclusions can be drawn from the findings that HD1 is essential for ARK apoptotic activity: (a) if the double ring is inactive *in vivo*, the HD1 mutants may retain the double apoptosome in an inactive conformation, thereby preventing DRONC from binding, or (b) if the double ring is an active apoptosome *in vivo*, mutations in HD1 may block the formation of an active double apoptosome.

The deletion of the WD40 region generates a constitutively active Apaf-1, suggesting that the WD40 region acts as a negative regulator of Apaf-1 activation, by maintaining an inactive conformation in the absence of cytochrome *c*.[2–4] Binding of cytochrome *c* overcomes this inhibition, thereby allowing the hydrolysis of Apaf-1-bound dATP to ADP.[2–4] A similar function for the WD40 of ARK had been proposed, but interestingly, mutations affecting the WD40 region in ARK block developmental cell death, indicating that the WD40 region is a positive regulator of ARK function.[13,14] Given that the ARK apoptosome comprises eight molecules, it is possible that the WD40 region is required for stabilisation of the more compact apoptosome structure. Interestingly, ARK also comprises an extra 180 residues at its C-terminus following the WD40 repeat clusters, which is not found in Apaf-1 and truncation of this region also affects ARK-mediated cell death.[13] These findings indicate that the C-terminus is required for ARK activity and possibly provides structural integrity to the apoptosome complex.

The analysis of the structure of the ARK apoptosome by Yu *et al*.[5] is intriguing as it provides an informative scaffold to understand the mechanisms of ARK function and importantly illustrates the evolutionary changes in the requirements for activation of the apoptotic machinery in the fly. The structure of ARK also goes some way in explaining why cytochrome *c* cannot enhance caspase activation and why cytochrome *c* ablation does not affect apoptosis in *Drosophila* cells.[9–11]

The only known function of cytochrome *c* in mammalian apoptosis is in Apaf-1 activation and apoptosome formation. As discussed above, the biochemical, structural and RNAi-mediated knockdown studies now provide strong evidence that cytochrome *c* does not have an equivalent function in ARK activation and cell death in *Drosophila*. It is also apparent from recent studies that, despite the similarity in their structures, Apaf-1 and ARK apoptosomes are regulated in distinct manners and the function of the WD40 region is different in the two proteins. Intriguingly, a mutation in one of the *Drosophila* cytochrome *c* genes *cyt-c-d* is reported to abolish caspase activation in spermatids, suggesting a potential role for cytochrome *c* in caspase activation in this tissue.[15] Although cytochrome *c* does not interact with the ARK apoptosome, it is curious to know whether it can function in fly cell death in an apoptosome-independent manner. However, in the absence of biochemical and molecular studies, it remains unresolved if cytochrome *c* plays a role in ARK (and caspase) activation specifically in some tissues or whether the decrease in caspase activity seen in *cyt-c-d* mutant spermatids is an indirect effect.

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