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News and Commentary

Apaf1 is no longer *single*

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The lower eukaryotes seem to get rid of the troublesome choice between life and death with a limited set of genes. In the nematode *C. elegans*, for example, the 'death' functional network involves four genes at its core: the pro-apoptotic factors *egl-1* (*egl*, egg laying defective), *ced-3* and *ced-4* (*ced*, cell death abnormal), and *ced-9*, which can inhibit the action of *ced-3* and *ced-4* (reviewed in[1]). By contrast, in mammalians a family of genes exists for almost each gene identified in the worm. However, until just recently, the docking protein Apaf1 that plays a pivotal role in apoptosis, mediating the transduction and amplification of the signal from mitochondria to activate caspases (Figure 1B), was the only protein showing this function.[2] Recent studies[3–5] have now identified new members of the Apaf1 family.

Three groups have recently reported the identification of a fly *ced-4/Apaf1* homolog[6,7] (and reviewed in[8]). These groups have named the gene *dapaf-1*, *dark* and *hac-1*, respectively; in the on-line *Drosophila* database the gene is called *ark* (*a*paf1-*r*elated *k*iller). These studies demonstrated that the mechanisms of apoptosome functions in flies are very likely to be the same as those that occur in worms and mammals.

Several authors have hypothesized, in the last 2 years, the existence of other *Apaf1*-like genes in vertebrates. Due to the evolutionary conservation of the general mitochondrial apoptotic pathway, with the *Bcl-2* family being the homolog of *ced-9* and the caspases playing *ced-3* role in higher eukaryotes, it seems reasonable to expect a large gene family as the mammalian counterpart of *ced-4*. Only very recently, some proteins have been identified and related to *Apaf1* in terms of function and sequence (Figure 1A). The first attempt to isolate an Apaf1-like protein by Imai and collaborators resulted in the identification of FLICE-associated huge protein (FLASH),[9] characterized by a domain similar to the Apaf1 ced-4-like domain (or nucleotide-binding domain, NBD), and a DED-recruiting domain (DRD). The DRD was shown to interact with a death-effector domain in caspase-8 (Casp8). FLASH is therefore necessary for the activation of Casp8 in CD95/Fas-mediated apoptosis.[9] Unfortunately, Koonin and colleagues have brought strong and convincing arguments against the structural similarity between *APAF1* and *FLASH*, supporting them by phylogenetic evidences.[10]

The second protein to be related to Apaf1 was the nucleotide oligomerization domain-containing protein 1 (Nod1)/CARD4 protein, identified by Inohara and collaborators[11] and Bertin and collaborators[12] searching for CARD-like domains on EST private databases. Nod1/CARD4 possesses a caspase-recruitment domain (CARD), an NBD and, instead of the Apaf1 WD-40 protein–protein interaction domain, several leucine-rich repeats (LRRs). This domain enables Nod1 to bind and regulate the CARD-containing kinase RICK and, in turn, to activate nuclear factor kappaB (NF-κB), as shown in Figure 1C. Another family member, Nod2, closely followed Nod1.[3] Nod2, found in the genomic database, searching for Nod1 homologs, is composed of two NH2-terminal CARDs, an NBD and multiple COOH-terminal LRRs. Nod1 and Apaf1 were shown to be broadly expressed in embryonic and adult tissues. By contrast, the expression of Nod2 is highly restricted to monocytes. In general terms, the innovations in apoptotic and related cytokine signals in vertebrates could have been linked to the evolution of the vertebrate immune system. Here different cell types could claim for more specialized regulatory pathways. Aravind and colleagues have extensively discussed the vastly increased complexity of the NBD-containing factors in vertebrates.[13] As it was reported for plant disease resistant R proteins (reviewed in[14]), the LRRs of Nod1 and Nod2 are required for lipopolysaccharides (LPS)-induced NF-κB activation.[15] These findings suggest that Nod1 and Nod2 are the mammalian counterparts of plant R gene products that function as receptors for pathogen components derived from invading bacteria. Nod1 and Nod2 constitute a subfamily of Apaf1-like proteins that function through RICK and the IKK complex to activate a NF-κB signaling pathway (Figure 1C). In other words, *Apaf1* and the *Nods* genes are structural but not functional *relatives*.

Chu and colleagues[4] and Hlaing and colleagues[5] independently reported in *J. Biol. Chem*. the amino acid sequence of a protein, named Defcap (Death effector filament forming ced-4-like apoptosis protein) and Nac (NBD and CARD), respectively. Nac/Defcap was once again found by homology search for CARD containing proteins. Nac/Defcap contains a CARD domain and a putative NBD domain, similar to Apaf1 and the Nods, but surprisingly placed at its carboxyl- rather than amino-terminus. Like the Nods, but different from Apaf1, Nac/Defcap contains a putative regulatory domain with multiple LRRs. However, a distinguishing feature of Nac/Defcap's primary sequence is that Nac/Defcap contains a pyrin-like motif (PLM) and a proline-rich sequence (PR) at its amino-terminus. Nac/Defcap is widely expressed in adult tissues but is present at high levels only in blood leukocytes, spleen, heart, and thymus. The authors demonstrated that the Nac/Defcap splicing gives rise to two splice-variants of the protein, long and short, which retain the potential of interacting with Casp2 and, to a lesser extent, Casp9. The CARD of Nac/Defcap interacts selectively with the CARD domain of Apaf1 and this interaction plays a role in Cyt-c-

A

Gene	Protein Domains	MW	Functions	Expression	Refs
Apaf1	CARD NBD WD-40	137 KDa	Activation of Casp9, physical component of the apoptosome	Broad expression	2
FLASH (?)	CED-4-like or NBD (?) DRD	220 KDa	Activation of Casp8	Broad expression	9
Nod1/CARD4	CARD NBD LRRs	106 KDa	Activation of NF-κB through RICK	Broad expression	11, 12
Nod2	CARD CARD NBD LRRs	115 KDa	Activation of NF-κB through RICK and the IKK complex	Monocytes	3
Nac/Defcap	PLM PR NBD LRRs CARD	164 KDa	Cytochrome c-inducible interaction with Apaf1 and Casp9; interaction with Casp2 and Casp9;	Broad expression. Stronger in peripheral blood leukocytes, spleen, heart, and thymus	4, 5

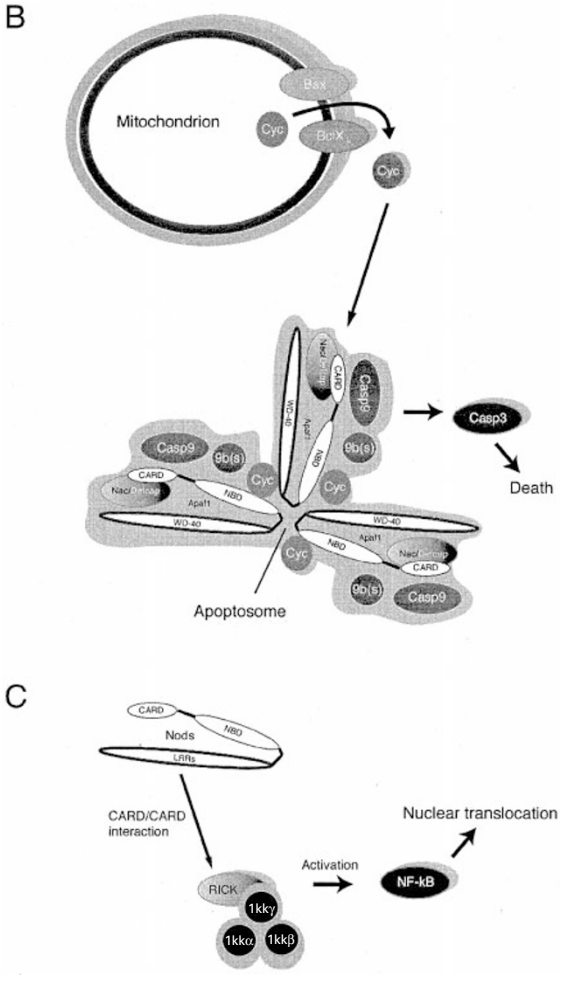

Figure 1 The Apaf1 family and its pathways. (**A**) The *Apaf-1*-like gene family in mammals. CARD, caspase recruitment domain; LRRs, multiple leucine-rich repeats; MW, molecular weight; NBD, nucleotide binding domain; PLM, pyrin-like motif; PR, proline-rich sequence. The domain structures of Apaf1 (xL isoform), FLASH, Nod1/CARD4, Nod2 and Nac/Defcap (L isoform) are compared. FLASH similarity to Apaf1 has been argued.[10] The domains reciprocal proportions are not preserved. (**B**) The balance between Bax, Bcl-X_L and other Bcl-2-like proteins may regulate the efflux of Cytochrome *c* (Cyc) from mitochondria. Cyc activates the apoptosome, whose stoichiometry is not known (as an example, three molecules of each components are shown). Active Apaf1 can induce Casp9 self-processing through a CARD/CARD interaction. The direct interaction of Casp9 is inhibited by a Casp9 splicing isoform, 9b(s), which plays a dominant-negative role by inhibiting Apaf1 CARD binding. Also, Nac/Defcap binding to Apaf1 can increase its potential to activate Casp9. Cytosolic-Cyc increase, in a sort of feedback loop, can enhance Nac/Defcap affinity for Apaf1 binding. The active form of Casp9 can thus activate Casp3, which rapidly leads to cell death. (**C**) Other LRRs-containing Apaf1-like molecules, the Nod proteins, can act on nuclear function, through RICK/IKK complex-mediated activation of NF-κB

mediated activation of caspases in cytosolic extracts and in cells.[4] Furthermore, the association of Nac/Defcap with Apaf1 is Cyt-c-inducible, and the resulting apoptosome is a mega-complex, which contains both Nac/Defcap and Apaf1. Nac/Defcap association to the apoptosome correlates with enhanced recruitment and proteolytic processing of pro-Casp9. Nac/Defcap, in our opinion, represents the first member of a novel Apaf1-like subfamily. Its functions are obviously more closely related to Apaf1 functions (see Figure 1B). Several papers have recently shown that Bcl-2 has Apaf1-independent death-protective functions and that Bcl-X_L and Bcl-2 do not directly interact with Apaf1.[16–18] We are presently trying to unravel the functional correlation of Apaf1 and Bcl-X_L in inner ear and brain development (Francesco Cecconi, Marjo Salminen and Peter Gruss, unpublished results). It remains to be elucidated if Nac/Defcap is a target of Bcl-2 or Bcl-X_L regulation.

Last but not the least, a series of interesting findings in hematopoietic cells point to the existence of a further member of the *Apaf1*-like family (Vanessa Marsden, Jerry Adams and Andreas Strasser, personal communication). Hematopoietic cells from *Apaf-1*$^{-/-}$ and *Casp9*$^{-/-}$ mice undergo normal apoptosis when exposed to developmental programmed death stimuli *in vivo*, when starved of cytokines *in vitro* or exposed to experimentally applied cytotoxic stimuli (glucocorticoids, radiation, phorbol esters etc.). On the basis of this model we may speculate that a specific (ced-4 related) adapter for some known or novel caspases exists in hematopoietic cells. In other words, a novel member of the Apaf1-like family.

There are no experimental evidences about the roles of these Apaf1-like proteins *in vivo* and knockout studies have not yet been performed. Besides the basic research goals, analyzing their activity in the cell death networks will be crucial for the future of apoptosome research in neurodegeneration and cancer.

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