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Review

Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death

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Abstract

Although much emphasis has been laid on the role of caspase in cell death, recent data indicate that, in many instances, mammalian cell death is caspase-independent. Thus, in many examples of mammalian cell death the 'decision' between death and life is upstream or independent of caspase activation. Similarly, it is unclear whether PCD of plants and fungi involves the activation of caspase-like enzymes, and no caspase-like gene has thus far been cloned in these phyla. Apoptosis inducing factor (AIF) is a new mammalian, caspase-independent death effector which, upon apoptosis induction, translocates from its normal localization, the mitochondrial intermembrane space, to the nucleus. Once in the nucleus, AIF causes chromatin condensation and large scale DNA fragmentation to fragments of ~50 kbp. The AIF cDNA from mouse and man codes for a protein which possesses three domains (i) an amino-terminal presequence which is removed upon import into the intermembrane space of mitochondria; (ii) a spacer sequence of approximately 27 amino acids; and (iii) a carboxyterminal 484 amino acid oxidoreductase domain with strong homology to oxidoreductases from other vertebrates (*X. laevis*), non-vertebrate animals (*C.elegans*, *D. melanogaster*), plants, fungi, eubacteria, and archaebacteria. Functionally important amino acids involved in the interaction with the prosthetic groups flavin adenine nucleotide and nicotinamide adenine nucleotide are strongly conserved between AIF and bacterial oxidoreductase. Several eukaryotes possess a similar domain organisation in their AIF homologs, making them candidates to be mitochondrial oxidoreductases as well as caspase-independent death effectors. The phylogenetic implications of these findings are discussed.

Keywords: mitochondria; cytochrome *c*

Abbreviations: AIF, apoptosis inducing factor; ANT, adenine nucleotide translocator; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine nucleotide

Phylogeny of apoptosis in the three metazoan kingdoms

Programmed cell death (PCD) is known to play a major role in the development and/or stress responses of all three metazoan kingdoms (Plantae, Animalia, and Fungi). Two opposite scenarios may account for the evolution of PCD. As a first possibility, PCD would have evolved during the metozoan radiation in each major evolutionary brench independently. In this case, shared characteristics of PCD across phyla such as partial chromatin fragmentation and condensation, increased generation of reactive oxygen species, or loss of plasma membrane asymmetry[1] would be understood as *post mortem* manifestations of entropic processes. As a second possibility, PCD would have evolved in a primitive, unicellular eukaryotic ancestor, before the separation of the three metozoan kingdoms. We prefer this latter hypothesis, because some unicellular eukaryotes (*Trypanosoma cruzi*, *Trypanasoma brucei rhodesiense*, *Leishmania amazonensis*, *Tetrahymena thermophyla*, *Euglena gracilis*, *Schizosaccharomyces pombe*) can undergo cell death with some features of PCD/apoptosis such as chromatin condensation and large scale DNA fragmentation.[2,3] Moreover some domains of apoptosis-regulatory proteins (e.g. the 'apoptotic ATPase' domain of CED4/Apaf-1) first identified in animals can be found in proteins from plants, eubacteria (*Actinomycetes* and *Bacillus subtilis*.) and the archaeon *Pyococcus horikoshi*.[4] We and others have speculated that the primitive mechanisms of apoptosis could have been established as a by-product of the host-endosymobiont micro-ecosystem generated by incorporation of the primitive mitochondrion into the proto-eukaryotic host cell.[5–8] If PCD has arisen in the primitive eukaryote, then some basic features of cell death such as a regulated permeabilization of mitochondrial membranes should be the same in all metozoan branches of the evolutionary tree. In contrast, the mechanisms connecting the basic apoptotic 'program' to signals elicited by development or stress, could have evolved independently in different phyla (and perhaps within different classes).

Our present knowledge on death-regulatory mechanisms and death effectors in plants, fungi, and unicellular

eukaryotes is scarce, and no convincing evidence for universal PCD regulators/effectors is available. Some key effectors of cell death are known to modulate PCD in cross-philum experiments. Thus, human Bax or *Caenorhabditis elegans* CED4 are known to kill *S. pombe* cells upon transfection-enforced overexpression.[9,10] In contrast, the cytoprotective role of Bcl-2 in *Saccharomyces cerevisiae* is a matter of debate,[11] and transgenic Bcl-$2X_L$ does not inhibit PCD in plants.[12] Importantly, mammalian PCD (apoptosis) is mostly coupled to the activation of caspases, which are indispensable for the acquisition of several hallmarks of advanced apoptosis (oligonucleosomal DNA fragmentation, formation of nuclear bodies, marked shrinkage).[13] However, there is limiting evidence for the existence of caspases in plants,[14] and end-stage differentiation-associated death of *Dictiostelium discoideum* cells has been demonstrated to occur in the presence of caspase inhibitors.[15] Moreover, oligonucleosomal DNA fragmentation, one of the caspase-dependent hallmarks of mammalian apoptotic cell death,[16] is not a central feature of plant or fungus cell death. Thus, whatever is the natural history of cell death, caspase activation is not a central feature of cell death across the metazoan kingdoms.

The CED3/4/9 'apoptosome': equally important in worms and mammals?

Our current view of the phylogeny of apoptosis has been profoundly influenced by pioneering work performed on the nematode *C. elegans*.[17] Three *C. elegans* death (CED) genes (*CED3*, *CED4*, *CED9*) have a major role in developmental cell death control, and current biochemical studies suggest that the death-inhibitory protein CED9 (a homolog of mammalian Bcl-2) interacts with the death-inducing proteins CED4 (a homolog of mammalian Apaf-1) and CED3 (a homolog of mammalian caspase-9), thereby preventing CED4 from activating the caspase CED3.[18] This molecular complex has been baptized as 'apoptosome'.[19] The basic platform of the apoptosome is also found in mammals, where it contains additional molecules such as cytochrome *c*, which interacts with the mammalian CED4 homolog Apaf-1.[20,21] Cytochrome *c* does not interact with CED4.[22] This difference in apoptosome structure between worms and mammals is important, because it implies that accidental, stress-induced permeabilization of the outer mitochondrial membrane, which would induce cytochrome *c* release, will facilitate activation of the apoptosome in mammals, but not in *C. elegans*. This may explain, at least in part, why *C. elegans* cells do not undergo

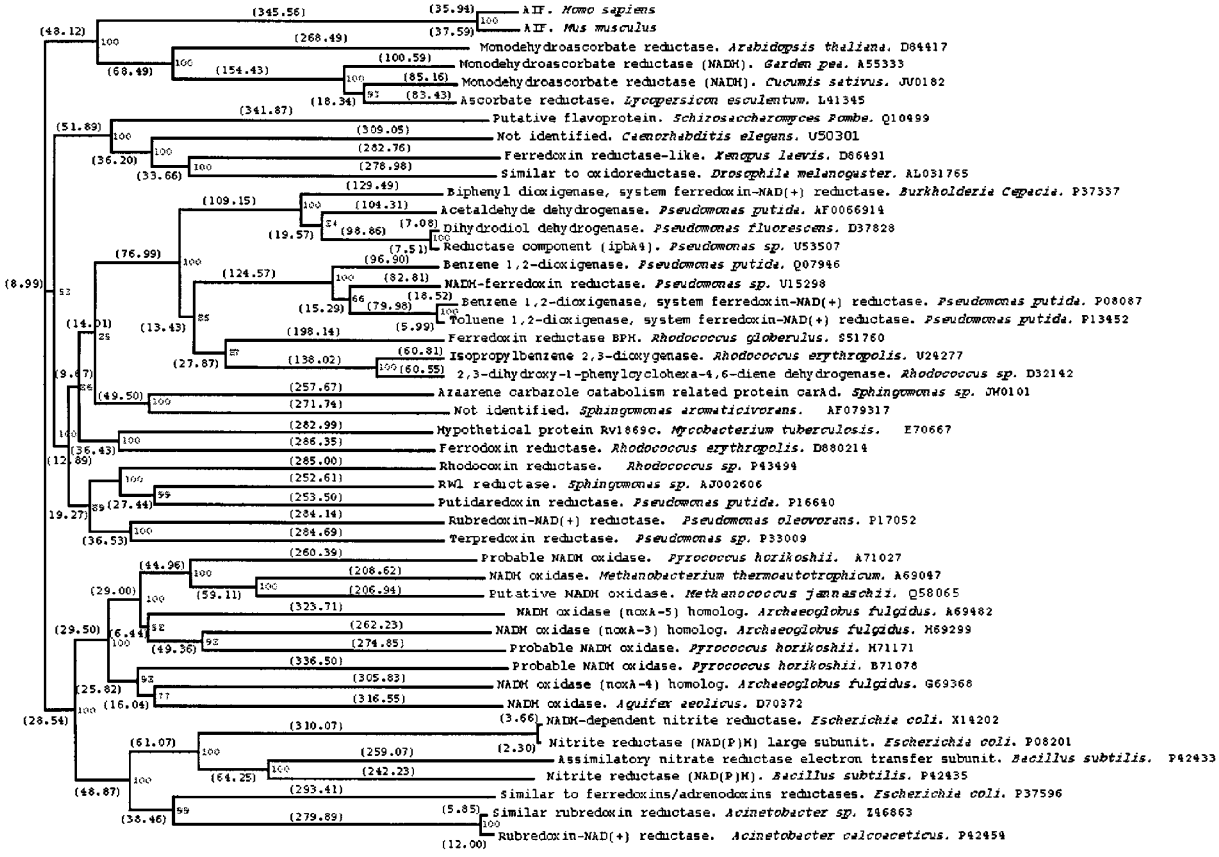

Figure 1 Phylogenetic organization of AIF homologs. Branch lengths are indicated in parentheses. Bootstrap resampling values from 100 replicates are presented at nodes. The GenBank accession codes of each AIF homolog are enumerated. The general topology of the tree remained unaltered, irrespective of the method chosen for its calculation (maximum parsimony or maximum likelihood). Moreover, high bootstrap resampling values support the non-randomness of internal branching order

apoptosis in response to environmental stress such as nutrient starvation or γ-irradiation. Indeed, stress-induced cell death is unwarranted in *C. elegans* because such cell death would compromise the survival of the entire worm.

Gain-of-function mutations of CED9 or loss-of-function mutations of CED4 or CED3 have a major effect on the death of the 131 cells which are normally dismissed during the development of *C. elegans*. In contrast, the knock-out of Apaf-1, caspase-9, or Bcl-2 (or Bcl-XL) entails a surprisingly weak phenotype in the mouse.[23–27] Although these alterations cause death during the embryonic or early postnatal stage and cause major perturbations in the central nervous system,[23–27] most organs of the mouse develop near-to-normally, implying that cell death has not been deranged during development of, for instance, the cardiovascular system (vessel lumina), limb buds (interdigital spaces), or the lymphoid system.

Two possible explanations can be forwarded to explain the apparent discrepancy between genetic data obtained in the worm and in mammals. First, this difference may be attributed to the increased genetic redundancy of mammals leading to the compensation of defective death control by homologous genes of the same gene family. Second, it may be speculated that mammals possess additional, apoptosome-independent pathways involved in developmental cell death. Arguments in favor of this hypothesis, in particular the existence of caspase-independent death pathways, will be enumerated below. As a possibility, *C. elegans* may have lost most of the developmental cell death pathways during evolution, with the exception of the (presumably stress-independent) apoptosome pathway. Alternatively, it may be argued that mammals have developed several apoptosome-independent pathways by adding additional death control modules (e.g. CD95/FADD/caspase-8) to a primitive, apoptosome-based death program.

Caspase-independent death in mammals

In contrast to previous belief, inhibition of caspases does not prevent cell death in most mammalian models of apoptosis induction. Thus, when cell death is induced by Bax,[28–30] Bak,[31] c-Myc,[31] ligation of glucocorticoid receptors,[32,33] tumor necrosis factor,[34] interferon-γ,[35] crosslinking of CD2,[36] staurosporin,[36,37] ganglioside GD3,[38] DNA damage,[32,39] or infection with HIV-1,[40] cells normally die from full-blown apoptosis and manifest caspase activation. However, pan-caspase inhibitors, do not prevent cytolysis, nor do they prevent mitochondrial membrane permeabilization, although they usually abolish oligonucleosomal DNA fragmentation. Even Fas/Apo-1/CD95-induced cell death, a paradigm of receptor-mediated primary caspase activation,[41] is only retarded but not prevented by caspase inhibition, at least in L929 cells.[42] When caspase activation is inhibited indirectly, for instance, by culturing cells in conditions in which both glycolytic and respiratory ATP generation are prevented, cells also die without oligonucleosomal DNA fragmentation and without cellular shrinkage,[43–45] yet manifest nuclear condensation and DNA cleavage into large 50–150 kbp fragments undistinguishable from that seen in the early stage of apoptosis.[46]

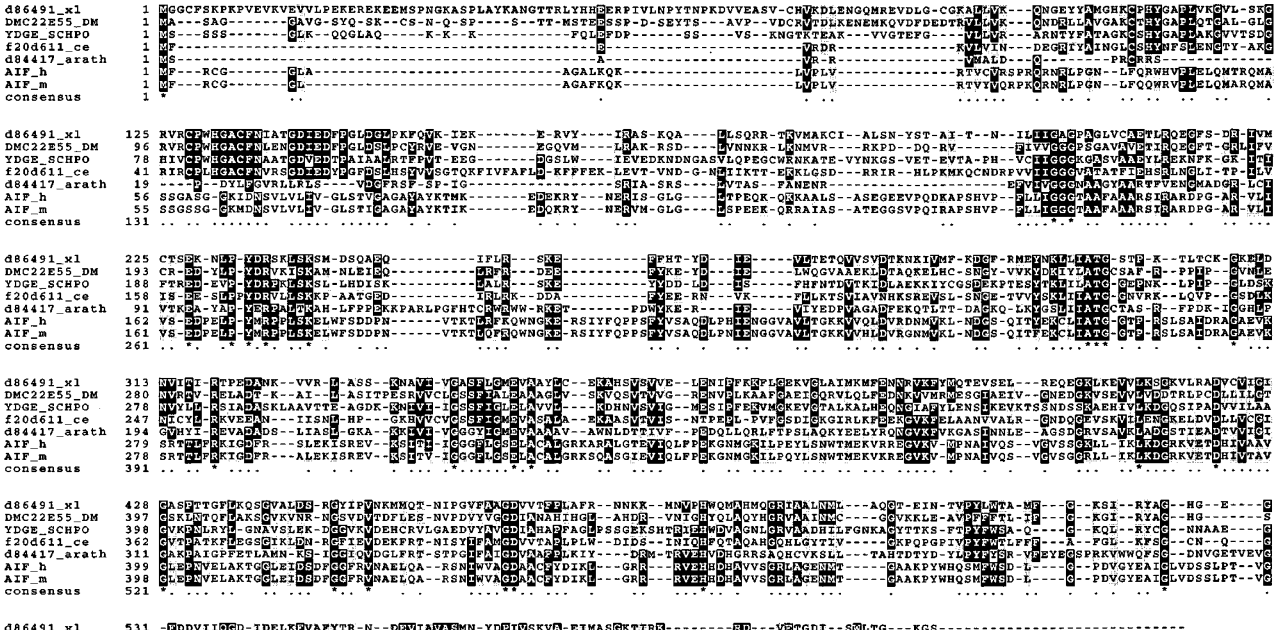

Figure 2 Alignment of amino acid sequences AIF (human and murine) against other AIF-like proteins from various sources: *Xenopus laevis* (D86491), *Drosophila melanogaster* (DMC22E5_5), *Schizosaccharomyes pombe* (Q10499), *Caenorhabditis elegans* (accession number F20d6.11), and *Arabidopsis thaliana* (D86491). Residues identical to at least 50% of the aligned sequences are shaded in black and less conserved residues in gray

A similar pattern of death with mitochondrial alterations and phosphatidylserine exposure on the cell surface, yet absent caspase activation and advanced DNA breakdown, is found when cell death is induced by cross-linking CD4,[47] CD45,[48] CD99[49] or CXRC4,[47] overexpression of PMN,[35] adenovirus E4orf4,[50] or FADD.[51] Again, in these models of cell death induction, caspase inhibition has no cytoprotective effect whatsoever. Thus, specific interventions on cells, including cross-linking of surface receptors may cause a type of cell death that does not involve the activation of caspases.

Altogether, these observations underline the probable role of caspase-independent death mechanisms in the mammalian system. Caspases are required for the complete manifestation of apoptotic morphology, yet are dispensable for cell death to occur in many systems. In the presence of caspase inhibitors, only some features of apoptosis such as initial chromatin condensation is found. Of note, the morphology of cells dying in the presence of caspase inhibitors resembles that of unicellular eukaryotes (which lack caspases) induced to undergo PCD-like death.

Apoptosis inducing factor (AIF) – an evolutionary conserved, caspase-independent death effector

During apoptosis, soluble mitochondrial intermembrane proteins are released through the outer mitochondrial membrane.[52–57] The mitochondrial intermembrane protein fraction contains an activity which suffices to force isolated HeLa nuclei to adopt an apoptotic morphology and to lose at least part of their DNA content.[52,54] We have baptized this activity 'apoptosis inducing factor' (AIF). Based on a cytofluorometric assay allowing measurement of the frequency of subdiploid nuclei exposed to mitochondrial proteins,[58] we have purified a protein which maintains its bioactivity in the presence of the caspase inhibitor Z-VAD.fmk.[56] This protein was found to be an ubiquitous FAD-binding flavoprotein.[57] Cloning of the full-length cDNAs corresponding to mouse AIF (612 amino acids) and human AIF (613 aa)[57] revealed that AIF is strongly conserved between the two mammalian species (92% aa identity in the whole protein) and bears a highly significant homology with oxidoreductases from all eukaryotic and prokaryotic kingdoms in its C-terminal portion (aa 128–612 for mAIF; 95% aa identity between mouse and human) (Figures 1 and 2).

We have investigated the evolutionary origins of AIF using database searches and phylogram calculations. AIF possesses significant homology with NADH ferredoxin reductases from both eubacteria and archaebacteria. Among eukaryotes, strongest homology is seen with several plant ascorbate oxidoreductases, in particular with dehydroascorbate reductase from *Arabidopsis thaliana*, monodehydroascorbate reductase from *Cucumis sativus* (cucumber), and the ascorbate free radical reductase from *Lycopersicon esculentum* (tomato). Several among these plant genes are induced by stress such as heat, cold, superoxide anion, wounding, or fungal pathogens.[59–64] Further phylogenetic analysis reveals that AIF also has a highly significant homology with four putative oxidoreductases from vertebrate (*Xenopus laevis*) and invertebrate (*C. elegans*, *Drosophila melanogaster*) animals, as well as *S. pombe*. In contrast no homolog has been found in *S. cerevisiae* whose entire genome is sequenced (Figures 1 and 2). The N-terminal portion of AIF has no homology to oxidoreductases. It bears a mitochondrial localization sequence (aa 1–101 for mAIF; 84% aa identity between mouse and human), as well as a 'spacer' region (aa 102–127 for mAIF; 60% aa identity between mouse and human). The mitochondrial presequence is removed after import of AIF into the intermembrane space.[57] It appears that the animal and *S. pombe* AIF homologs have a similar overall architecture, with a mitochondrial presequence (Figure 2), suggesting that they may have the same subcellular distribution and perhaps the same function as mammalian AIF. Similarly, the monodehydroascorbate dehydrogenase reductase from *A. thaliana* bears, in addition to its C-terminal oxidoreductase domain, an N-terminal mitochondrial presequence with a canonical arginine (R) residue at position 10 (relative to N-terminal residue of the mature protein) at residue 46 of the precursor sequence (Figure 2). Future studies will have to determine whether these proteins have an apoptogenic function. Intriguingly, it has been reported that dehydroascorbate reductase activity redistributes from mitochondria to the cytosol in the dark-induced senescence of *Pisum sativum* (pea) leaves.[65] Drought also increases the dehydroascorbate reductase activity in the cytosol (but not in chloroplasts) of *Sorghum bicolor* and *Helianthus annuus* (sunflower).[66] These data suggest that the putative plant AIF homolog undergoes a stress-induced subcellular redistribution, as this has been shown for mammalian AIF.

Subcellular fractionation, immunofluorescence analysis, and immunoelectron microscopy have established that AIF is normally confined to mitochondria, yet subject to mitochondrio-nuclear translocation upon induction of apoptosis by diverse agents such as ceramide, staurosporin, or glucocorticoids.[57] Thus, in contrast to cytochrome *c* (which stays cytosolic), AIF moves to the nucleus, concomitant to the initial phase of chromatin condensation. This nuclear relocalization of AIF is compatible with the presence of several putative nuclear localization signals within the oxidoreductase-like domain of AIF.[57] Importantly, the mitochondrio-nuclear translocation of AIF is caspase-independent (unpublished observation). When added to purified nuclei from HeLa cells, recombinant AIF protein induces DNA loss, peripheral chromatin condensation, and digestion of chromatin into $\sim$50 kbp fragments but no oligonucleosomal fragmentation,[57] probably by activating a sessile nuclear DNAse. In addition to its nuclear effects, recombinant AIF acts on mitochondria. In the presence of a thermolabile cytosolic co-factor, AIF causes purified mitochondria to dissipate their $\Delta\Psi_m$ and to release cytochrome *c* and caspase-9. Microinjection of recombinant AIF into the cytoplasm of live cells

induces several hallmarks of apoptosis: nuclear chromatin condensation and DNA loss, dissipation of the $\Delta\Psi_m$, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane.[57] None of these AIF effects, either on isolated organelles or on intact cells, is prevented by the broad spectrum caspase inhibitor Z-VAD.fmk, indicating that they are caspase-independent.[57] Thus AIF is a logical candidate for a caspase-independent cell death effector causing some features of nuclear apoptosis. That this is the case is suggested by experiments in which an anti-AIF antiserum microinjected into the cytoplasm of life cells prevents early chromatin condensation induced by some apoptosis-inducing agents such as staurosporin.[57]

A recombinant protein corresponding to the mAIF precursor does not bind FAD, whereas a shorter protein lacking the mitochondrial targeting sequence and part of the 'spacer' region (Δ1-120) does bind FAD.[57] Similarly, mature AIF purified from mitochondria (Δ1-101) is a flavoprotein. These data suggest that the FAD prosthetic group is attached to the AIF protein within the mitochondrion, after removal of the targeting sequence, as this has been described for other mitochondrial flavoproteins.[67,68] Most if not all amino acids supposed to interact with the prosthetic groups FAD and NAD are strongly conserved between AIF and two reductases whose three-dimensional structure has been elucidated, namely dihydrolipoamide dehydrogenase from *Pseudomonas putida* and human glutathion reductase (Figure 3). The core consensus of the typical motif GXGXXG/A of the Rossman fold[69] is found at two distinct regions of the sequence (aa 138-143 and 307-312 in human AIF): the more N-terminal motif seems to be involved in the NAD(P)H binding, whereas the more C-terminal one probably binds FAD. In contrast, AIF does not belong to the subfamily of disulfide reductases because it lacks two cysteines essential to form the redox-active disulfide bond in the catalytic site (Figure 3a). Thus, AIF does not belong to the superfamily of flavoprotein disulfide oxidoreductases (which includes glutathione reductase, dihydrolipoamide reductase, mercuric reductase, alkylhydroperoxide reductase and thioredoxin reductase). However, the strong conservation of NAD/FAD binding motifs strongly suggests that AIF possesses an oxidoreductase activity, in addition to its apoptogenic

a

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beda_psep   4 HVAIIGNGVA GFTTAQALRA EGYEGRISLI GEEQHLPYDR ----PSLSKA VLDGSF----
rodo_rhs    1 SIVIIGSGQA GFEAAVSLRS HGFSGTITLV GDEPGVPYQR ----PPLSKA YLHSDP----
AIFh_Δ132 133 PFLLIGGGTA AFAAARSIRA RDPGARVLIV SEDPELPYMR ----PPLSKE LWFSDDPNVT
gshr_hum   22 DYLVIGGGSG GLASAR--RA AELGARAAVV -ESHKLGGTC VNVGCVPKKV MWNTAVHSEF
dld1_psep   8 TLLIIGGGPG GYVAA--IRA GQLGIPTVLV -EGQALGGTC LNIGCIPSKA LIHVAEQFHQ
interactions      nff  f                        ff    fff    fn   f

beda_psep  94 -------DGS TISADAIVIA TGSRARMLSL ---PGSQLPG VVTL-RTYGD VQLLRDSWTP
rodo_rhs   92 -------DAT AIEYDHLILA TGARNRLLPV ---PGANLPG VHYL-RTAGE AESLTSSMAS
AIFh_Δ132 247 -------DGS QITYEKCLIA TGGTPRSLSA IDRAGAEVKS RTTLFRKIGD FRSLEKISRE
gshr_hum  136 PKPTIEVSGK KYTAPHILIA TGGMPSTPHE SQIPGASLGI TSDGFFQLEE LP--------
dld1_psep 125 --KQVEVDGQ RIQCEHLLLA TGSSSVELPM --LPLGGPVI SSTEALAPKA LP--------
interactions                      f ff

beda_psep 143 NTRLLIVGGG LIGCEVATTA RKLGLSVTIL EAGDELL--- -VRVLGRRIG AWLRGLLTEQ
rodo_rhs  141 CSSLVVIGAG FIGLEVAAAA RKKGLDVTVV EAMDRPM--- -ARALSSVMS GYFSTAHTEH
AIFh_Δ132 300 VKSITIIGGG FLGSELACAL GRKARALGTE VIQLFPEKGN MGKILPEYLS NWTMEKVKRE
gshr_hum  198 -GRSVIVGAG YIAVEMAGIL SALGSKTSLM IRHDKVLRSF DSMISTNCTE ELENAGVEVL
dld1_psep 173 -QHLVVVGGG YIGLELGIAY RKLGAQVSVV EARERILPTY DSELTAPVAE SLKKLGIALH
interactions        nn   Cn                    nn

beda_psep 199 GVQVELKTGV SGFSG-EGQL EKMVN---DG RSFI-ADNAL ICVGADPADQ LARQAGLECD
rodo_rhs  197 GVHMRLSTGV KTINAADGRA AGTTN---SG DVIH-ADAVV VGIGVVPNIE LAALTGLPV-
AIFh_Δ132 360 GVKVMPNAIV QSV-GVSSGK LLKLK---DG RKVE-TDHIV AAVGLEPNVE LAKTGGLEID
gshr_hum  247 KFSQVKEVKK TLSGLEVSMV TAVPGRLPVM TMIPDVDCLL WAIGRVPNTK DLSLNKLGIQ
dld1_psep 232 LGHSVEGYEN GCLLANDGKG G-------QL RLE--ADRVL VAVGRRPRTK GFNLECLDLK
interactions      n                                    nnnn  f

beda_psep 255 -----RGVVVD HRGATSAKGI FAVGDVATWP L-HSGGKRSL ETVMNAQRQA TAVAKAILGK
rodo_rhs  253 ---DNGIVVD EYLRTPDENI SAIGDCAAYP IPGKAGLVRL ESVQNAVDQA RCLAAQLTGT
AIFh_Δ132 416 SD-FGGFRVN AELQARS-NI WVAGDAACFY DIKLG-RRRV EHHDHAVVSG RLAGENMTGA
gshr_hum  307 TDDKGHIIVD EFQNTNVKGI YAVGDVCGKA LLTPVAIAAG RKLAHRLFEY KEDSKLDYNN
dld1_psep 273 MNGA-AIAID ERCQTSMKNV WAIGDVAGEP MLAHRAMAQG EMVAEIIA-G KA-RRFEPAA
interactions                          f   n  Cfff
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function. These two activities can be separated because the entire AIF protein precursor (aa 1–612), which does not bind FAD (the prosthetic group indispensable for the putative electron donor/acceptor function), becomes apoptogenic when refolded *in vitro*.[57] Thus, in analogy to cytochrome *c*, AIF appears to be a bifunctional protein with two independent functions, an electron acceptor/donor (oxidoreductase) function and an apoptogenic function.

Based on the above data, AIF and its homologs appear to be candidate death effectors acting in different phyla, including in fungi and in plants. This possibility is currently under active investigation in our laboratory.

Table 1 Amino acid sequence similarity and identity in different apoptosis regulatory proteins

Human	Caspase-9	Apaf-1	Bcl-2	Cyt *c*	ANT1	AIF
M. musculus	73 (65)[a]	93 (85)	95 (90)	96 (91)	99 (95)	98 (92)
D. melanogaster	47 (22)	–	–	85 (66)	90 (71)	55 (26)
X. laevis	46 (23)	–	65 (35)	–	–	54 (27)
C. elegans	55 (24)	81 (16)[b]	52 (25)[c]	83 (54)	85 (65)	56 (25)
S. pombe	–	–	–	47 (38)	73 (47)	57 (29)

[a]Values indicate the percentage of identical+similar amino acids in interspecies comparisons. Values in parenthesis indicate the percentage of identical amino acids. Dashes indicate that the corresponding gene has not been cloned. [b]Note that human Apaf-1 (1194 aa) possesses N-terminal WD domains missing in *C. elegans* CED-4 (549 aa), indicating a difference in the overall domain organization. [c]Note that Bcl-2 (239 aa) lacks a functionally important N-terminal domain present in CED-3 (280 aa)

Figure 3 Conservation of amino acids involved in the binding of FAD or NAD in AIF. (**a**) Conservation of dehydrogenase motifs (boxed) in human AIF and other recognized oxidoreductase components (bedapsep, benzene 1,2-dioxygenase system ferredoxin reductase component from *Pseudomona putida*; GenBank accession number Q07946; rodo_rhs, rhodocoxin reductase from *Rhodococcus*; P43494) and two reductases whose 3D structure has been elucidated (gshr_h: human glutathion reductase; P00390; dld1_psep, dihydrolipoamide dehydrogenase from *P. putida*; P09063). Residues that interact with FAD or NAD (in dld1_psep) are marked as 'f' or 'n', respectively. Residues that interact with both NAD and FAD bear the annotation 'C'. Boxed cysteines (absent in AIF) are typical for the pyridine nucleotide-disulphide oxidoreductase family. (**b**) The probable conservation of the NAD/FAD-binding residues in AIF is based on the 3D structure of dihydrolipoamide dehydrogenase from pseudomona putida (dld1_psep; Brookhaven Protein Data Bank access code 1LVL). The colored residues represent the most conserved motifs as in (**a**) (same color code). Space-filling symbols indicate the position of NAD (orange) and FAD (red)

Concluding remarks

Our present knowledge of PCD phylogeny is incomplete. Thus, it remains elusive whether basic mechanisms of mammalian cell death such as the loss of mitochondrial membrane barrier function are also found in fungi and plants.[11] Similarly, the exact nature of effector molecules causing irreversible degradation of essential cellular structures are unknown. On teleological grounds, it can be speculated that the core of cell death control would involve structures which are essential both for death and life.[5,70] Only based on this condition, the 'social control'[71] of cell death would be maintained throughout phylogeny and ontogeny. If cell death control is exerted by proteins which fulfill an essential metabolic function, then somatic mutations cannot lead to the acquisition of total PCD resistance. Indeed, tumor cells only manifest a partial resistance to PCD induction, and thus far no example of complete PCD resistance, even among dedifferentiated tumors, is reported.

Proteins with dual vital/lethal functions include cytochrome *c* (essential for respiration and caspase activation),[72] and ANT (an inner mitochondrial membrana ADP/ATP antiporter which can become a lethal pore).[73] In accord with their bifunctional nature, such proteins are more conserved between different species than are proteins from the 'apoptosome' (Table 1). Conventional strategies for the identification of PCD-regulatory genes are based on the systematic mutation/deletion of genes and the search of apoptotic-resistant phenotypes. Although this approach has defined important PCD-regulatory genes in *C. elegans*, *D. melanogaster*, and *A. thaliana*, it cannot lead to the identification of such bifunctional genes. As a result, more subtle, biochemical approaches may be required for the identification of proteins (and possibly non-protein structures?) participating in the central mechanisms of cell death control.

Methods of sequence analyses

Gapped-Blast searches[74] were done for AIF homologs on the Internet Server of the National Center for Biotechnology Information. Other specific databases were consulted using FASTA: *C. elegans* protein database (from The Sanger Center web page, http://www.sanger.ac.uk/Projects/C_elegans/) and Rickettsia prowazekii (from Rickettsia prowazekii Sequencing Project web page, http://evolution.bmc.uu.se/~siv/gnomics/Rickettsia.html). The protein sequences found were aligned through ClustalW (version 1.7) with the maximum degree of freedom and using the Henikoff BLOSUM30 scoring matrix to assess the pairwise similarity. These alignments were further manually refined using the program MPSA (Multiple Protein Sequence Analysis, version 0.66b) and MEME (Multiple Expectation Maximum for Motif Elicitation) algorithm.[75] Unrooted phylogenetic trees, were constructed using 'protdist' (Dayhoff PAM 001 matrix method) to compute a distance matrix from protein sequences. The scores obtained from alignments were assembled into the matrix distance. Then the method of Fitch and Margoliash[76] was applied to obtain the best branching and to confirm the tree topology. Bootstrap resampling analysis[77] from 100 replicates was used to evaluate the support for internal branches.

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