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Phylogenomics of caspase-activated DNA fragmentation factor

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Abstract

The degradation of nuclear DNA by DNA fragmentation factor (DFF) is a key step in apoptosis of mammalian cells. Using comparative genomics, we have here determined the evolutionary history of the genes encoding the two DFF subunits, DFFA (also known as ICAD) and DFFB (CAD). Orthologs of DFFA and DFFB were identified in *Nematostella vectensis*, a representative of the primitive metazoan clade cnidarians, and in various vertebrates and insects, but not in representatives of urochordates, echinoderms, and nematodes. The domains mediating the interaction of DFFA and DFFB, a caspase cleavage site in DFFA, and the amino acid residues critical for endonuclease activity of DFFB were conserved in *Nematostella*. These findings suggest that DFF has been a part of the primordial apoptosis system of the eumetazoan common ancestor and that the ancient cell death machinery has degenerated in several evolutionary lineages, including the one leading to the prototypical apoptosis model, *Caenorhabditis elegans*.

Keywords: Apoptosis; DNA fragmentation factor; CAD; ICAD; DNase; Caspase; Evolution; *Nematostella vectensis*; *Caenorhabditis elegans*

Apoptosis is a cell death program that removes surplus cells and damaged cells in metazoan animals. The molecular machinery of apoptosis has been characterized in model organisms such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the mouse *Mus musculus*. The most simple apoptosis regulatory system is found in *C. elegans*. The core molecular machinery of nematode apoptosis consists of the protease ced-3, the ced-3 activator ced-4, the ced-4 inhibitor ced-9, and the ced-9 inhibitor egl-1. While apoptotic protein breakdown is mediated by ced-3, nuclear DNA is degraded by the lysosomal endonuclease nuc-1 [1].

The cell death machinery of the fly and of mammals is more complex and includes a multiplicity of ced-3 homologs, constituting the so-called caspase family of proteases, as well as a series of direct and indirect regulators of caspases. Apoptotic DNA breakdown in fly, mouse and human cells involves the activity of a nuc-1 homolog, DNaseII, which however is preceded by the activity of the DNA fragmentation factor (DFF) [2]. DFF consists of two subunits, named DFFA/DFF45/ICAD and DFFB/DFF40/CAD, which interact via their amino-terminal CIDE-N domain [3]. DFFA functions as a folding chaperone for DFFB and abolishing the expression of DFFA abrogates the protein expression of DFFB [4–6]. Binding of DFFA inhibits DFFB, the enzymatically active component of the complex, and thereby keeps DFF in an inactive state in non-apoptotic cells. The activation of DFF is regulated differently in mammals and in the fly. In mammalian cells DFFA is cleaved by caspase-3 at two sites, which leads to the release of active DFFB [7,8]. In the fly DFFA is cleaved by a caspase at a single site [6] and DFFB is also proteolytically processed by a caspase [9].

The simple apoptosis process of nematodes is often regarded as the primordial system which has evolved to more complexity in "higher" organisms [10]. This is a valid assumption within the classical model of animal phylogeny according to which nematodes branched from the evolutionary tree before the split of the lineages leading to arthropods and vertebrates, i.e. animals with a true coelom (Coelomata). However, recent molecular systematics

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studies have led to a revision of this model and provided evidence that nematodes and arthropods form a clade of molting animals (Ecdysozoa) and are more closely related to each other than to humans [11,12]. Therefore, commonalities between the human and the fly apoptosis system, such as the presence of DFF, are likely to have been present in the last common ancestor of human, fly and nematode. It is uncertain whether the primordial apoptosis system of bilaterians (comprising human, fly, and nematode) included caspase cleavage of both DFFA and DFFB (like in the fly), cleavage of DFFA only (like in human) or cleavage of neither DFFA nor DFFB.

To clarify the evolution of the apoptotic DNA degradation system, we performed a phylogenomic analysis [13] using genome sequences from species of multiple metazoan lineages including the sea anemone *Nematostella vectensis*, a cnidarian. Since the evolutionary lineage of cnidarians has branched from the animal tree before the appearance of bilaterians, the analysis of this genome is highly informative about the components of the apoptotic DNA breakdown processes in the last common ancestor of nematodes, flies and mammals. Our results reveal the evolutionary history of DFF and suggest that DFF is an ancient caspase-activated apoptosis factor that has been lost in *C. elegans*.

Materials and methods

Nucleotide sequences of DFFA and DFFB orthologs were retrieved from the databases of annotated genes, expressed sequence tags (ESTs) and genome sequences in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and from a series of additional databases [14–16] listed in Table 1. The amino acid sequences of human DFFA (GenBank Accession No. NP_004392) and DFFB (NP_004393) were used as query sequences in the respective BLAST searches. The analysis of the genomes of *N. vectensis*, *C. elegans*, *Strongylocentrotus purpuratus*, *Ciona intestinalis*, and *Ciona savignyi* also included a search for proteins containing a CIDE-N domain (pfam02017), which is present in both DFFA and DFFB. Proteins with a predicted CIDE-N domain were analyzed for the presence of additional protein regions with amino acid sequence similarity to DFFA or DFFB.

Amino acid sequences were aligned with the ClustalW algorithm at the website of the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/). Protein domain structure predictions were made with the InterProScan algorithm at http://www.ebi.ac.uk/InterProScan/ [17].

Results

Identification and structural features of DNA fragmentation factor genes in N. vectensis

Genes encoding orthologs of DFFA and DFFB were found in the cnidarian *N. vectensis*, in arthropods and vertebrates (zebrafish, frog, chicken, and human). By contrast, the assembled genome sequences of the sea squirts (tunicates) *C. intestinalis* and *C. savignyi*, the sea urchin *S. purpuratus*, and the nematodes *C. elegans*, *C. briggsae*, and *Pristionchus pacificus* did not contain genes of significant sequence similarity to DFFA nor DFFB.

The *N. vectensis* DFFA gene encoded a protein of 210 amino acids in length and 25% amino acid sequence identity to the N-terminal part of human DFFA (amino acids 1–215) (Fig. 1). Expression of the gene was confirmed by the identification of two DFFA ESTs (gi82871047, gi82867372) from *N. vectensis* embryos. The *N. vectensis* DFFA gene consisted of 2 exons and an intron, the position of which corresponded to the site of the second intron of human DFFA (Fig. 1). The conserved position and phase (position within the triplet) of this intron suggested that it was derived from the DFFA gene in the last common ancestor of the sea anemone and humans. The amino acid sequence encoded by exon 1 of *N. vectensis* DFFA was similar to that deduced from exons 1 and 2 of human DFFA and was predicted by the InterProScan domain search algorithm to fold into a CIDE-N structure. The amino acid sequence encoded by exon 2 of *N. vectensis* DFFA was most similar to domain I2 of mammalian DFFA [18] (encoded by exons 3 and 4 of the human DFFA gene) (Fig. 1A), and weakly similar to the domain I3, also known as DFF-C domain [18,19] (encoded by exons 5 and

Table 1
Species and databases used for comparative genomics analyses

Clade	Species	Database
Cnidaria	Sea anemone (*Nematostella vectensis*)	http://evodevo.bu.edu/stellabase/
Nematoda	Nematode (*Caenorhabditis elegans*)	http://www.ensembl.org; http://www.wormbase.org/
	Nematode (*Caenorhabditis briggsae*)	http://www.wormbase.org/
	Nematode (*Pristionchus pacificus*)	http://www.pristionchus.org/
Arthropoda	Floor beetle (*Tribolium castaneum*)	http://www.bioinformatics.ksu.edu/BeetleBase/
	Honey bee (*Apis mellifera*)	http://racerx00.tamu.edu/bee_resources.html
	Yellow fever mosquito (*Aedes aegypti*)	http://www.ensembl.org; http://www.vectorbase.org/
	African malaria mosquito (*Anopheles gambiae*)	http://www.ensembl.org; http://www.vectorbase.org/
	Fruit fly (*Drosophila melanogaster*)	http://www.ensembl.org; http://flybase.bio.indiana.edu/
Echinodermata	Sea urchin (*Strongylocentrotus purpuratus*)	http://annotation.hgsc.bcm.tmc.edu/Urchin/cgi-bin/pubLogin.cgi
Urochordata	Sea squirt (*Ciona intestinalis*)	http://www.ensembl.org; http://genome.jgi-psf.org
	Sea squirt (*Ciona savignyi*)	http://www.ensembl.org; http://www.broad.mit.edu/annotation/ciona/
Vertebrata	Zebrafish (*Danio rerio*)	http://www.ensembl.org
	Clawed frog (*Xenopus tropicalis*)	http://www.ensembl.org
	Chicken (*Gallus gallus*)	http://www.ensembl.org
	Human (*Homo sapiens*)	http://www.ensembl.org

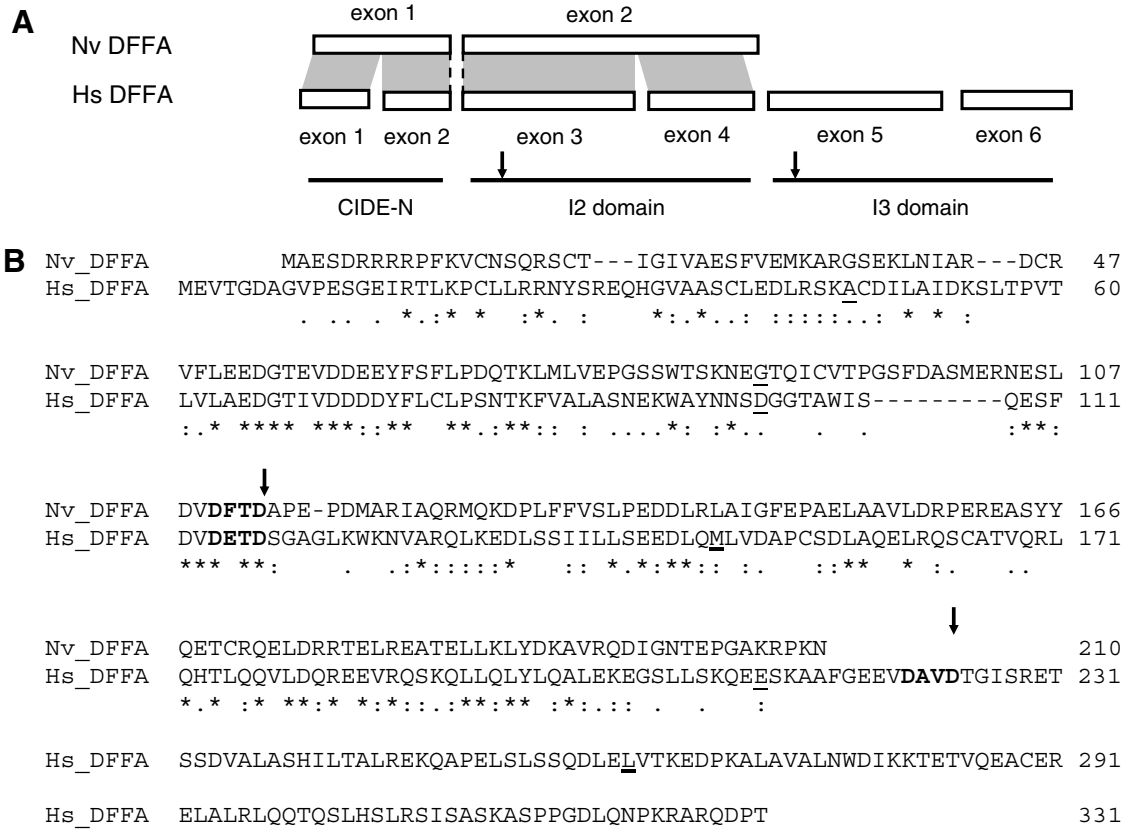

Fig. 1. Gene structure and amino acid sequence alignment of *Nematostella vectensis* and human DFFA. (A) Schematic depiction of the exon structure of *N. vectensis* (Nv) and human (Hs) DFFA. Bars represent protein-coding exons. Regions of sequence similarity between *N. vectensis* and human DFFA are linked by areas of grey shading. Vertical discontinuous lines indicate the position of conserved exon borders. At the bottom of the panel the protein domain organization and the position of caspase cleavage sites (arrows) of mammalian DFFA are shown. (B) Amino acid sequence alignment of *N. vectensis* and human DFFA. Caspase cleavage motifs are shown in bold. The position of caspase cleavage sites is indicated by arrows. Amino acid residues corresponding to exon borders are underlined by a thick line (phase 0), a thin line (phase 1) or a double line (phase 2). Stars, periods, and colons below the alignment indicate identical, similar and highly similar amino acids, respectively.

6 of the human DFFA gene) (not shown). *N. vectensis* DFFA contained a caspase cleavage motif at a site corresponding to the caspase processing site of human DFFA domain I2 (Fig. 1B, bold letters).

The *N. vectensis* DFFB protein showed 40% amino acid sequence identity to human DFFB and was encoded by a gene consisting of 4 exons (Fig. 2). The position and the phase of the introns following exons 1 and 3 of *N. vectensis* DFFB were homologous to those of the introns following exons 2 and 6 of human DFFB, respectively, suggesting an evolutionary ancient origin of these introns. The amino acid sequence encoded by exon 1 of *N. vectensis* DFFB was similar to that encoded by exons 1 and 2 of human DFFB and was predicted by the InterProScan domain search algorithm to fold into a CIDE-N structure. In addition, a WGR domain, a structure proposed to be involved in nucleic acid binding, was predicted by the SMART algorithm [20] for the central region of the *N. vectensis* DFFB protein. Five amino acid residues (D259, H260, H307, K309, H312 of human DFFB) (Fig. 2B) critical for catalytic activity and four residues (C226, C235, H239, C306) involved in the binding of a zinc ion [18] were conserved in *N. vectensis* DFFB.

Comparative analysis of the caspase processing sites in DFFA and DFFB

DFF components from diverse species were analysed for the conservation of the caspase cleavage sites previously defined in the mouse [7] and in *Drosophila* [9]. Amino acid sequence alignment showed that the first of the two DFFA caspase motifs was well conserved among all orthologs (Fig. 3A). The second caspase cleavage site of mammalian DFFA was conserved among vertebrates whereas it was absent in the sea anemone, the floor beetle *T. castaneum* and the honey bee *A. mellifera*, which encoded DFFA proteins lacking an I3 domain. Fruit fly and mosquito DFFAs contained a potential second caspase cleavage motif (Fig. 3A) which was followed by a C-terminal protein domain of minimal sequence similarity to the mammalian I3 domain [6] (data not shown).

The caspase cleavage site previously described in *Drosophila* DFFB (dCAD) was localized to a protein region that lacked a homolog in both human and sea anemone DFFB (Fig. 3B). This region was also absent in DFFB of the floor beetle whereas a homologous region was present in the DFFB genes of two mosquito species and the honey

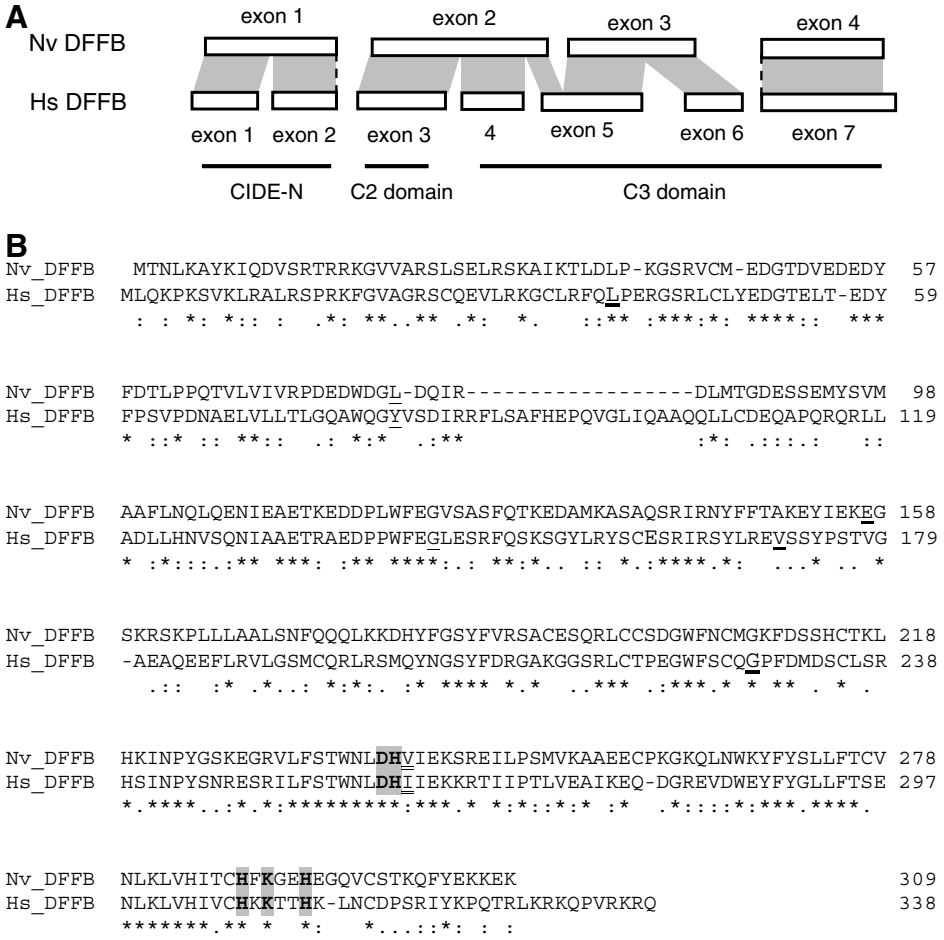

Fig. 2. Gene structure and amino acid sequence alignment of *Nematostella vectensis* and human DFFB. (A) Schematic depiction of the exon structure of *N. vectensis* (Nv) and human (Hs) DFFB. Bars represent protein-coding exons. Regions of sequence similarity between *N. vectensis* and human DFFB are linked by areas of grey shading. Vertical discontinuous lines indicate the position of conserved exon borders. At the bottom of the panel the domain organization of mammalian DFFB is shown. (B) Amino acid sequence alignment of *N. vectensis* and human DFFB. Amino acid residues involved in endonuclease catalytic activity are represented by bold letters and grey shading. Amino acid residues corresponding to exon borders are underlined by a thick line (phase 0), a thin line (phase 1) or a double line (phase 2). Stars, periods, and colons below the alignment indicate identical, similar and highly similar amino acids, respectively.

bee. In the latter case, the nucleotide sequence encoding this region had characteristics of an intron and was predicted to be spliced out from the transcript in the honey bee genome database. In all insect DFFB proteins containing a *D. melanogaster*-like insert, a putative caspase cleavage site was conserved (Fig. 3B).

DFFA and DFFB genes have been lost in several evolutionary lineages

The presence or absence of DFFA and DFFB was mapped onto a phylogenetic tree of animals [11] (Fig. 4). The species distribution pattern of both genes suggested that the DFF genes were inactivated at least in three different evolutionary lineages. The same number of gene loss events was inferred from DFF gene distribution when an alternative phylogenetic tree representing the Coelomata hypothesis was used (Supplementary material 2). In all cases of DFF gene loss both DFFA and DFFB were lost.

Discussion

Our data demonstrate that DFF has originated very early in the evolution of metazoan animals. Cnidarians diverged from the bilaterian lineage more than 600 million years ago [21], and representatives of both lineages have retained the DFF genes up to the present. We show that several features of DFFA and DFFB have been well conserved during evolution whereas others have diverged in different branches of the evolutionary tree.

The conservation of the caspase cleavage site in the I2 domain of DFFA indicates that caspase-mediated activation of nuclear DNA breakdown is an evolutionarily ancient process. Indeed various caspase genes are present

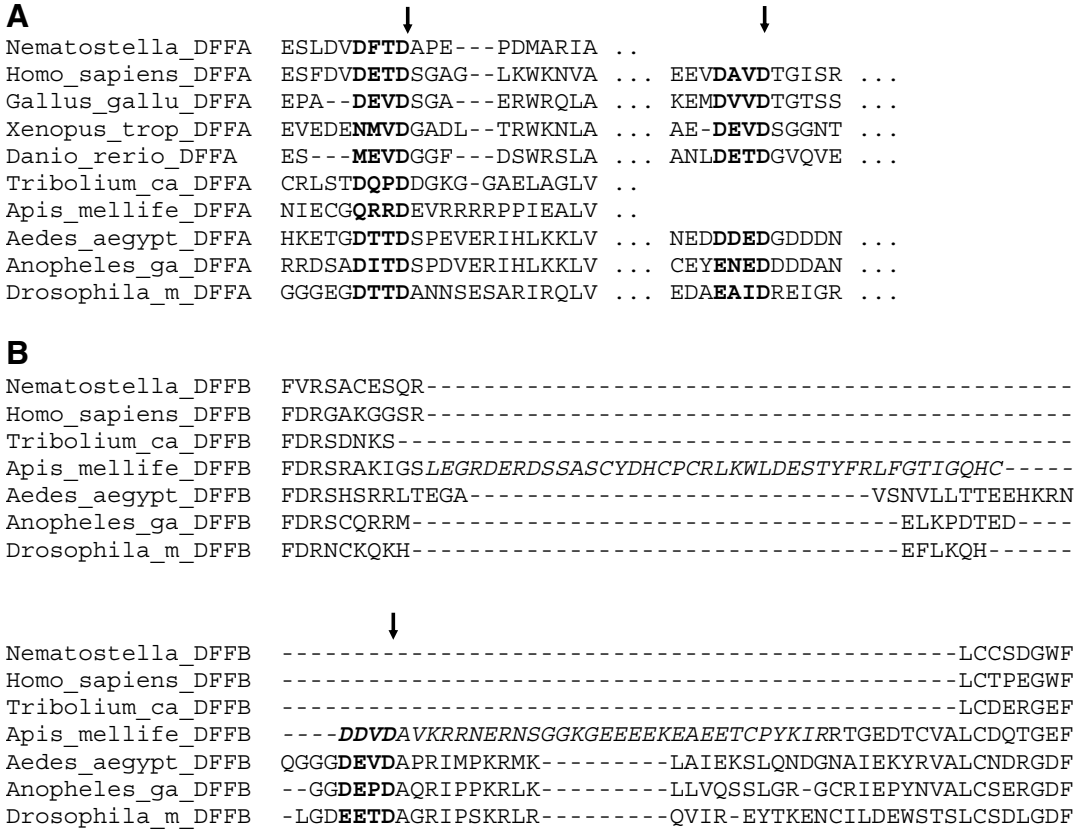

Fig. 3. Conservation of caspase cleavage sites in DFFA and DFFB. Partial amino acid sequence of DFFA (A) and DFFB (B) proteins from diverse species were aligned. Only the protein regions corresponding to the caspase cleavage sites of human DFFA (A) and to the caspase cleavage site of *Drosophila melanogaster* DFFB (B) are shown. The DFFA sequences in the region of the second caspase cleavage were aligned according to [6]. Caspase cleavage motifs are represented by bold letters. The position of caspase cleavage sites is indicated by arrows. In the *Apis mellifera* DFFB sequence italics represent amino acid residues encoded by a gene region with intron characteristics. The amino acid sequences were derived from the following database entries: *Homo sapiens* DFFA (GenBank gi4758148), DFFB (gi4758150), *Gallus gallus* DFFA (gi50759301), *Xenopus tropicalis* DFFA (gi60416006), *Danio rerio* DFFA (gi68402368), *Tribolium castaneum* DFFA (translation of EST gi75724543), DFFB (gi91088411), *Apis mellifera* DFFA (gi110772194; Beebase prelease protein gnl|Amel|GB11102), DFFB (Beebase prelease protein gnl|Amel|GB16455), *Aedes aegypti* DFFA (gi108868709), DFFB (gi108879353), *Anopheles gambiae* DFFA (gi118785332, vectorbase EST BM617507.1), DFFB (gi118790646), *Drosophila melanogaster* DFFA (gi24652882), DFFB (gi19921238).

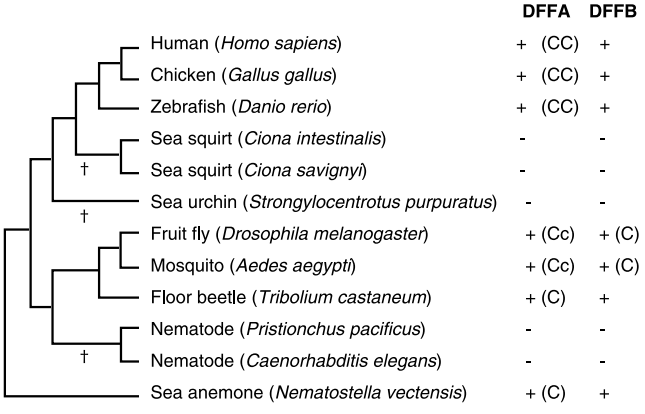

Fig. 4. Evolutionary history of DFFA and DFFB genes. The presence (+) or absence (−) of a DFFA and DFFB ortholog was mapped onto the phylogenetic tree of animals according to the Ecdysozoa hypothesis (simplified from [11]). "C" and "CC" indicate the conservation of one and two caspase cleavage sites, respectively. "Cc" indicates the fact that the second DFFA caspase cleavage site has not been confirmed experimentally in insects [6]. Each loss of DFF genes is indicated by a cross next to the respective branch of the evolutionary tree.

in the genomes of the established model species and of *N. vectensis* [22] (our unpublished data). High levels of conservation were also found in the CIDE-N domains of DFFA and DFFB and at sites involved in DFFB catalytic activity. The fact that no species could be identified in which only one DFF component was present confirms the concept that these proteins depend on each other to function properly [4].

The C-terminal region of DFFA was highly variable among different species. The I3 domain was conserved among vertebrate DFFA orthologs, whereas the C-terminus of mosquito and fly DFFA showed little, if any, significant sequence similarity to I3, and a domain equivalent to I3 was completely absent in DFFA orthologs from other insects and the sea anemone. Since there is significant sequence similarity between vertebrate I2 and I3 domains, and both I2 and I3 contain a caspase cleavage site and are encoded by two exons that are separated by an intron of conserved phase, it is likely that I3 has arisen by duplication of the gene region encoding I2. In a previous study

the C-terminus of *Drosophila* DFFA has been shown to have little sequence similarity to the I3 domain of mammalian DFFA and proteolysis at the second potential caspase cleavage site of *Drosophila* DFFA (Fig. 3) could not be detected [6]. Therefore, it is possible that the gene regions encoding the C-terminal domains of vertebrate and fly DFFAs have not been derived from a common ancestral gene but have evolved independently and may even serve different functions.

The second significant difference among DFF proteins relates to the role of DFFB as direct target of processing by caspases. The caspase cleavage motif of *D. melanogaster* DFFB [9] is conserved among various species of the order *Drosophila* (data not shown), mosquitos and the honey bee. The absence of this site in the sea anemone and in humans suggests that DFFB has not been cleaved by a caspase in the ancestral apoptosis system of eumetazoans (cnidarians + bilaterians) and that this proteolytic interaction represents an evolutionary innovation specific to some arthropods.

In addition to showing the ancient origin of DFF, this study provides evidence that DFF genes were lost during the evolution of some species. This indicates the existence of alternative apoptotic DNA degradation pathways. This assumption is also supported by previous studies which have shown that DFF is not expressed in all human tissues which undergo apoptosis efficiently [23] and that absence of DFFB does not lead to developmental abnormalities in the mouse [24]. However, other studies have indicated that DFF, together with DNase II, prevents the inadvertant activation of innate immunity against chromosomal DNA within apoptotic cells [6,24]. Whether possible differences in anti-DNA immune responses in different evolutionary lineages may have contributed to the observed pattern of conservation or loss of DFF, remains to be evaluated.

The results of this study have implications on the interpretation of molecular mechanisms of apoptosis as defined in various model organims, most notably *C. elegans*. Like two other nematode species, *C. briggsae* and *P. pacificus*, this prototypical apoptosis model has lost DFF. We conclude that the mechanism of apoptotic DNA breakdown in *C. elegans* differs significantly from that of the eumetazoan common ancestor and that the apoptosis program of *C. elegans* represents a lineage-specific degeneration of the primordial apoptosis system.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.122.

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