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Nematode acetylcholinesterases are encoded by multiple genes and perform non-overlapping functions

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Abstract

Nematodes are unusual in that diverse molecular forms of acetylcholinesterase are the product of distinct genes. This is best characterised in the free living organism *Caenorhabditis elegans*, in which 3 genes are known to give rise to distinct enzymes, with a fourth likely to be non-functional. ACE-1 is an amphiphilic tetramer associated with a hydrophobic non-catalytic subunit, analogous to vertebrate T enzymes, whereas ACE-2 and ACE-3 are glycosylphosphatidylinositol-linked amphiphilic dimers. The different *ace* genes show distinct anatomical patterns of expression in muscles, sensory neurons and motor neurons, with only a few examples of coordinated expression. Clear homologues of *ace-1* and *ace-2* have now been isolated from a variety of parasitic nematodes, and the predicted proteins have very similar C-terminal amino acid sequences, implying an analogous means of anchorage to membranes. In addition to these membrane-bound enzymes, many parasitic nematodes which colonise mucosal surfaces secrete acetylcholinesterases to the external (host) environment. These hydrophilic enzymes are separately encoded in the genome, so that some parasites may thus have a total complement of six *ace* genes. The secretory enzymes have been characterised from the intestinal nematode *Nippostrongylus brasiliensis* and the lungworm *Dictyocaulus viviparus*. These show a number of common features, including a truncated C-terminus and an insertion at the molecular surface, when compared to other nematode acetylcholinesterases. Although the function of these enzymes has not been determined, they most likely alter host physiological responses to promote survival of the parasite.
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Acetylcholinesterases (AChEs) are encoded by multiple genes in nematodes. Genetic analysis in *Caenorhabditis elegans* originally defined three AChE genes (*ace-1*, *ace-2* and *ace-3*), which code for three classes of enzyme. ACE-A is encoded by *ace-1* on chromosome X, ACE-B by *ace-2* on chromosome I, and ACE-C by *ace-3* on chromosome II [1–4]. Homozygous mutants in *ace-1* or *ace-2* have only slight alterations in locomotion, whereas *ace-3* mutants have no visible phenotype. Double mutants ($ace\text{-}1^{-}/ace\text{-}2^{-}$) are severely uncoordinated and the triple mutation ($ace\text{-}1^{-}/ace\text{-}2^{-}/ace\text{-}3^{-}$) is lethal [1,2,4]. Subsequent cloning and analysis of the AChE-encoding genes have revealed the structure, tissue distribution and deduced functions of these enzymes. Three

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major classes of AChE have also been characterised in the plant-parasitic nematodes *Heterodera glycines* and *Meloidogyne* [5,6]. These data, and the recent isolation of homologues of the *C. elegans* enzymes in other species suggest that the function and pattern of expression of AChEs in *C. elegans* may represent a paradigm for nematodes in general.

1. ACE-1

C. elegans ACE-1 takes the form of an amphiphilic tetramer associated with a hydrophobic non-catalytic subunit, analogous to vertebrate T AChEs [7], and a similar association has been found for ACE-1 of the root-knot nematode *Meloidogyne* [6]. Genes which are clear homologues of *C. elegans ace-1* have now been cloned from *Meloidogyne* [8] and the bovine lungworm *Dictyocaulus viviparus* (Lazari *et al*. unpublished data). The derived C-terminal sequences of each of these enzymes show features of a tryptophan amphiphilic tetramerization (WAT) domain previously characterized in vertebrate AChE T subunits. The nematode sequences contain eight conserved aromatic residues, six of which align with those in *Torpedo* ACE_T, including three tryptophan residues crucial for tetramerisation [9]. All contain a cysteine residue at position-3 or -4 from the C-terminus, again analogous to vertebrate T peptides. In the latter, assembly into an amphiphilic tetramer involves association of the WAT domain with a proline rich attachment domain (PRAD) on a non-catalytic structural subunit. It is therefore highly likely that a similar organization occurs with the nematode AChE ACE-1 enzymes, although the associated non-catalytic domain remains to be defined in these species [7].

The spatial distribution of *ace-1* expression has been determined in *C. elegans* using microinjection of green fluorescent protein (GFP) reporter constructs. GFP expression was observed in all body wall muscle cells, in the pharyngeal muscle cells pm5, and in three pairs of sensory neurons in head ganglia [10]. The pharyngeal muscle cells are essential for feeding. Pharyngeal pumping has been shown to be regulated via cholinergic innervation through neuron M5 [11], and is defective in *ace-1*$^{-}$/*ace-2*$^{-}$ double mutants [12]. Interestingly, *ace-1* is expressed in the majority of stages of the root-knot nematode *Meloidogyne*, but is undetectable in adult female worms, which are sedentary in plant root tissue. Pharyngeal pumping continues essentially unaffected in *C. elegans ace-1* mutants [1], and therefore expression of *ace-1* may not be necessary for survival of adult female *Meloidogyne*.

2. ACE-2

C. elegans ACE-2 is a glycosylphosphatidylinositol (GPI)-linked amphiphilic dimer [7]. The C-terminus is hydrophobic, similar to vertebrate H enzymes, with a consensus site for peptide cleavage and GPI addition [13]. It also contains a cysteine residue just upstream of the predicted cleavage site, which by analogy with mammalian enzymes is likely to contribute to an intramolecular disulphide bond.

A cDNA encoding a clear homologue of *C. elegans ace-2* has been cloned from *D. viviparus* [14]. The predicted protein shows 65% identity in amino acid sequence, with conserved residues in the catalytic triad, W84 in the choline binding site, and six cysteines known to contribute to intra-molecular disulphide bonds in *Torpedo* AChE. Like *C. elegans* ACE-2, Dv-ACE-2 also has a predicted consensus site for GPI addition at the C-terminus, and a cysteine residue which most likely contributes to an intermolecular disulphide bond [7].

Injection of GFP reporter constructs showed that *ace-2*:GFP has an almost exclusive neuronal pattern of expression which is established early in development. It is particularly prominent in cephalic sensory neurons, in pm5 cells, and motor neurons in the anal ganglion and hypodermal cells [15].

3. ACE-3

Two genes (*ace-3* and *ace-4*) are present in tandem on chromosome II, with just 356 nucleotides separating the stop codon of *ace-4* and the putative initiation codon of *ace-3*. ACE-3 is assembled into a GPI-linked amphiphilic dimer, although it lacks the C-terminal cysteine immediately proximal to the predicted cleavage site [7]. It is unclear whether ACE-4 is produced as a functional protein. ACE-1 and ACE-2 have been estimated to account for 95% of the total enzyme activity in worms, and ACE-3 for the remainder [1–4]. Northern blot analysis revealed that levels of *ace-4* mRNA are almost undetectable [15]. The same analysis indicated a high abundance of *ace-3* mRNA, suggesting that the enzyme may have a low catalytic efficiency [7]. Expression of *ace-3* was examined by fusing GFP to a 4 kb 5′ region of the *ace-3*/*ace-4* operon, and was observed in pharyngeal muscle cells, and neurons in the cephalic region, anal ganglion, and associated with the medial canals [15].

ACE-3 is unusual in that it has a very low K_m for ACh and is insensitive to inhibition by eserine [3]. There is an analogy here with ACE-C of *Meloidogyne*, which is relatively resistant to organophosphates and carbamates [6].

This may be significant, because if *Meloidogyne* ACE-C is also expressed primarily in pharyngeal muscles, then sedentary adult worms which have established a feeding site may be able to feed and persist in the presence of nematicides [6].

ACE-1, ACE-2 and ACE-3 show only about 35% identity in amino acid sequence, although ACE-3 and ACE-4 are more similar, indicative of a recent duplication event [7]. Gene expression is largely non-overlapping in terms of tissue distribution, indicative of non-redundant functions [15]. Although *ace-4* is transcribed along with *ace-3*, the extremely low levels of mature processed *ace-4* mRNA, the failure to detect associated enzymatic activity in vivo, and the substitution of glutamic acid 199 by glutamine adjacent to the active site serine residue suggest that ACE-4 is non functional.

4. Secreted acetylcholinesterases of parasitic nematodes

Many parasitic nematodes which colonise mucosal surfaces synthesize AChEs in specialised secretory glands and release these enzymes to their external environment, usually the host gastrointestinal tract [16]. The enzymes are invariably hydrophilic, although both monomeric and dimeric forms have been described [17–19]. We have characterised secreted AChEs from *Nippostrongylus brasiliensis*, the adult stage of which inhabits the jejunum of rats, and *D. viviparus*, which is slightly unusual in that it colonises the trachea and main stem bronchi of bovine lungs. *N. brasiliensis* secretes three variants of AChE, sACE-A, -B and -C, each of which is encoded by a separate gene [20–22]. In contrast, *D. viviparus* secretes 5 electrophoretic variants [23]. It is unclear whether these are all products of separate genes, although we have isolated and characterised cDNAs for two discrete secreted enzymes, designated Dv-sACE-1 and -2 [24]. All of these enzymes show absolute specificity for acetylcholine (ACh), in contrast to nematode neuromuscular enzymes, which also hydrolyse butyrylcholine (BuCh) to a variable degree [25,26]. This broader substrate specificity appears to be characteristic of neuromuscular AChEs from other invertebrates [27]. The ability of invertebrate AChEs to hydrolyse larger choline esters has been linked to substitution of F288 in the acyl pocket by smaller non-aromatic residues [25]. All three *N. brasiliensis* secreted AChEs have F288 substituted by methionine, yet still show minimal activity against BuCh. The nematode enzymes contain an insertion of two residues in the acyl binding pocket, leading to a predicted clash of BuCh with W331, and an alleviation of the steric tightness by mutation of M288 to glycine [22]. This is consistent with experiments which showed that the single mutants W331F or M288G by themselves did not enhance BuCh hydrolysis, whereas the triple mutant W331F/M288G/W290F did [21].

At the phylogenetic level, the neuromuscular enzymes Dv-ACE-2 and Dv-ACE-1 segregate separately with *C. elegans* ACE-2 and ACE-1, respectively. In contrast, the secreted enzymes from both *Nippostrongylus* and *Dictyocaulus* are most closely related to *C. elegans* ACE-2, but form independent clusters, suggesting that they evolved independently after separation of the species. This relationship is illustrated in Fig. 1.

The nematode secreted AChEs we have defined show some similarity to hydrophilic enzymes found in the venom of Elapid snakes [28,29]. They share the characteristic of a truncated C-terminus, which lacks any means of membrane anchorage. Another unusual feature of the nematode secreted AChEs is an insertion at the molecular surface, 32 residues long in the *D. viviparus* enzymes, and 17 amino acids in *N. brasiliensis* [22,24]. These insertions are present in all nematode secreted enzymes identified thus far, but their location and sequences are species-specific. In *D. viviparus*, the insertion is positioned between residues 55 and 61, and in *Nippostrongylus*, between residues 258 and 259 (*Torpedo* numbering). It is unclear whether these insertions play any defined functional role, but they are notably absent in neuromuscular AChEs, and it is therefore possible that they may be involved in tissue targeting, given that they are secreted into the host environment. The insertions from both species contain cysteine residues (two in *N. brasiliensis* and four in *D. viviparus*), and in the former species these are involved in formation of a fourth intramolecular disulphide bond (unpublished data). Mapping of splice sites in the *Dictyocaulus* AChE genes indicates that the 32 amino insertion is encoded by a dedicated exon, suggestive of a specific function [14].

The possible function of secreted AChEs in nematode parasites has long been debated [30,31]. Possible roles include regulation of local muscle contraction which might otherwise dislodge parasites from their niche [32]. Alternatively, they might function to inhibit cholinergic stimulation of fluid and mucus secretion by enterocytes, and exocytosis by Paneth cells [33–35]. Whatever their function, immune stress appears to positively regulate AChE secretion, suggesting that the enzymes aid parasites in maintaining their position until they can reproduce [36–38]. We have utilised RNA interference to suppress synthesis of secreted AChEs in adult *N. brasiliensis*. Soaking adult worms in double stranded RNA corresponding to the 5′ region of the coding sequence of

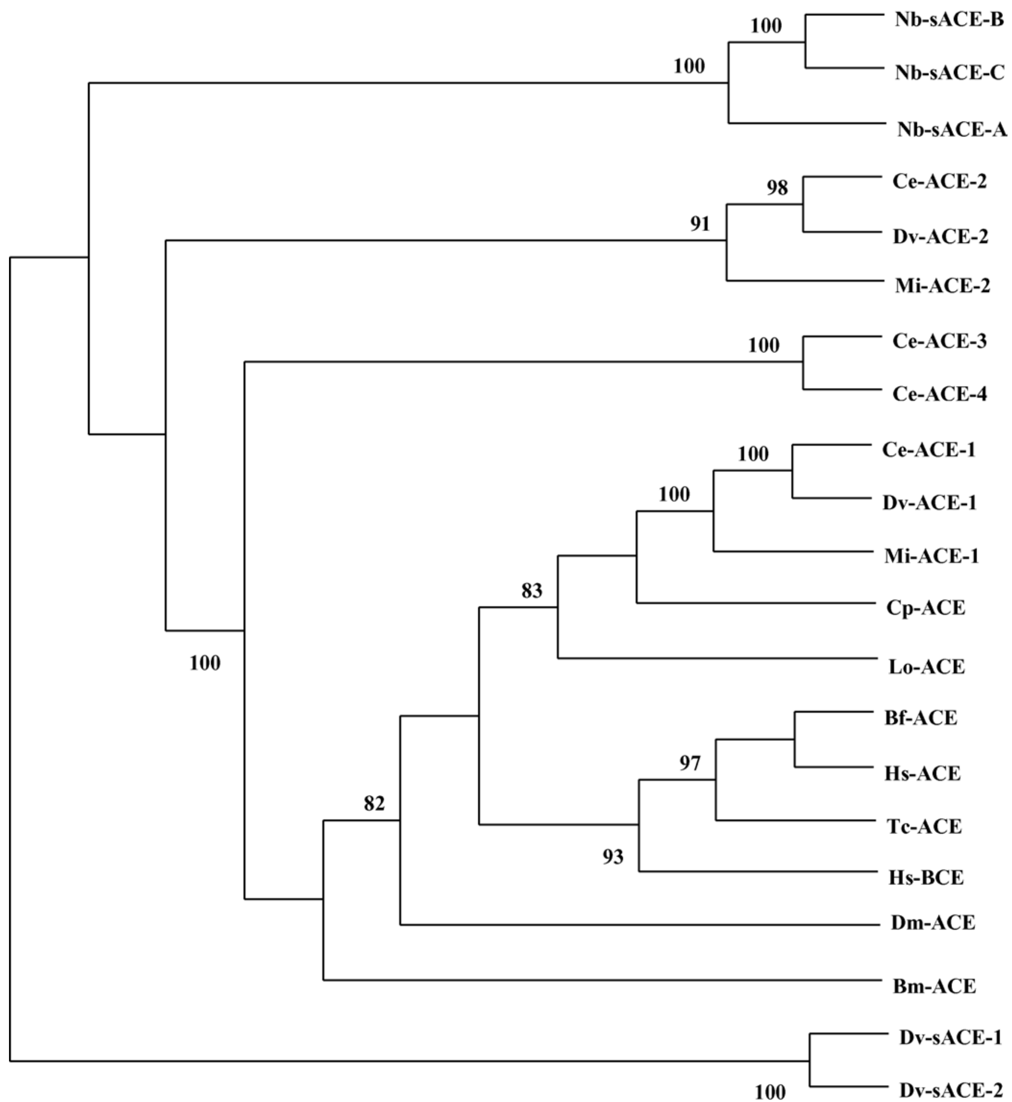

Fig. 1. Molecular phylogeny of nematode AChEs. Protein sequences representing AChEs from diverse organisms were aligned in CLUSTALW [41] and were manually edited to remove ambiguities. Phylogenetic analysis was performed using Phylo_Win [42], using the maximum parsimony setting and performing 500 bootstrapped replicates. Bootstrap values with scores above 70 are represented on the figure, which is adapted from Lazari et al. [14]. Abbreviations and GenBank accession numbers are as follows: Nb-sACE-A: *N. brasiliensis* secretory AChE A, Nb-sACE-B: *N. brasiliensis* secretory ACE B, Nb-sACE-C: *N. brasiliensis* secretory ACE C, Dv-sACE-1: *D. viviparus* secretory ACE-1, Dv-sACE-2: *D. viviparus* secretory ACE-2, Dv-ACE-1: *D. viviparus* ACE-1, Dv-ACE-2: *D. viviparus* ACE2, Ce-ACE-1: *C. elegans* ACE 1, Ce-ACE-2: *C. elegans* ACE-2, Ce-ACE-3: *C. elegans* ACE-3, Ce-ACE-4: *C. elegans* ACE-4, Mi-ACE-1: *Meloidogyne incognita* ACE-1, Mi-ACE-2: *Meloidogyne incognita* ACE-2, Cp-ACE: *Culex pipiens* AChE, Lo-ACE: *Loligo opalescens* ACE, Bf-ACE: *Bungarus fasciatus* ACE, Hs-ACE: *Homo sapiens* ACE, Tc-ACE: *Torpedo californica* ACE, Hs-BCE: *Homo sapiens* BuCE, Dm-ACE: *Drosophila melanogaster* ACE, Bm-ACE: *Boophilus microplus* ACE-1.

sACE-B resulted in over 90% suppression of synthesis of all three secreted enzymes with no apparent effect on parasite motility [39]. When transplanted into the jejunum of rats, these parasites appeared to be compromised in their ability to maintain their position, however these data need to be confirmed.

An alternative role for these enzymes is protection against inhibitors (e.g. chaconines and solanines) which, if ingested in the host diet, could lead to worm expulsion by acting on neuromuscular AChEs. An analogy here lies in *Drosophila melanogaster* which produce soluble AChE secreted into the haemolymph, resulting in resistance of flies to organophosphates [40]. Whatever the role of the nematode secreted AChEs, they clearly perform non-overlapping functions with those expressed in the neuromuscular systems of these organisms, and

future work should shed light on their interaction with physiological processes of the mammalian host.

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