Novel α7-like nicotinic acetylcholine receptor subunits in the nematode *Caenorhabditis elegans*

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Abstract

We have used reverse-transcription-polymerase chain reaction (RT-PCR) and DNA sequencing techniques to confirm the transcription of seven (six α and one non-α) novel candidate nicotinic acetylcholine receptor (nAChR) subunit-encoding genes identified in the genome sequence of the nematode *Caenorhabditis elegans*. Compared to vertebrate nAChR subunits, they most closely resemble the homomer-forming, neuronal α7 subunit. Comparison of the predicted amino acid sequences of the new nAChR subunits with those described previously in *C. elegans* reveals five subunits (four α and one non-α) which resemble the DEG-3-like group of subunits. To date, this highly divergent nAChR subunit group is unique to *C. elegans*. ACR-22 is the first non-α member of the DEG-3-like group of subunits to be identified. Two new members of the related ACR-16-like nAChR group of subunits have also been shown to be transcribed, making the ACR-16-like subunit group the largest in *C. elegans*. Residues in the α subunit second transmembrane region (M2) which contribute to the channel lining show variations with implications for channel function. For example, in ACR-22, the highly conserved 0′ lysine of M2 is replaced by histidine. Restrained molecular dynamics simulations have been used to generate molecular models of homo-pentameric M2 helix bundles for the novel subunits, enabling identification and display of pore-lining and protein interface residues. The number and diversity of genes encoding *C. elegans* nAChR subunits with similarities to the homomer-forming vertebrate α7 subunits and the identification of related non-α subunits, only found in *C. elegans* to date, suggest that at least some of these subunits may contribute to heteromers in vivo.

Keywords: Nicotinic acetylcholine receptors; *Caenorhabditis elegans*; ion channel; molecular modeling; α7 subunit

Nicotinic acetylcholine receptors (nAChRs) are pentameric membrane proteins which mediate fast synaptic transmission at neuromuscular junctions and in the nervous system of many organisms (Changeux and Edelstein 1998). The vertebrate nAChR gene family is diverse, with separate sets of genes encoding the currently known 5 muscle and 12 neuronal subunit isoforms. Subunits of nAChRs fall into two main categories: α subunits are defined by adjacent cysteines which contribute to the ACh binding site; non-α subunits lack this motif (Changeux and Edelstein 1998). Most vertebrate nAChR isoforms are expressed in the nervous system, and among these the homomer-forming α7–9 subunits are the most divergent.

Caenorhabditis elegans provided the first viable mutants resistant to the cholinergic anthelmintic compound, levami-

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sole (Lewis et al. 1980). Subsequent molecular and genetic analyses revealed that several of the levamisole resistance mutations were in genes encoding structural components of heteromeric nAChRs (Fleming et al. 1997). Other *C. elegans* nAChR subunits have been identified by cross-species hybridization [*acr-2* (Squire et al. 1995), *acr-16* (previously referred to as Ce21) (Ballivet et al. 1996), and *acr-3* (Baylis et al. 1997)] and genetic screens [*deg-3* (Treinin and Chalfie 1995) and *des-2* (Treinin et al. 1998)]. Also, 8 novel nAChR α subunits were identified by Mongan et al. (1998) from the genome sequence of *C. elegans* (The *C. elegans* Sequencing Consortium, 1998), making a total of 13 α subunits, by far the largest number of nAChR α subunits reported for any organism.

ACR-16, a nematode homolog of α7 (Ballivet et al. 1996), like vertebrate α7 subunits (Couturier et al. 1990), can form functional homomeric receptors when expressed in *Xenopus laevis* oocytes. The amino acid sequence of the *C. elegans* homomer-forming subunit ACR-16 is 47% identical to chicken α7, and aspects of the pharmacology of these recombinant nematode and vertebrate receptors have been compared (Ballivet et al. 1996; Raymond et al. 2000). Four additional α subunits and two non-α subunits most similar to ACR-16 have been identified in *C. elegans* (Mongan et al. 1998). Thus, although ACR-16 is capable of forming a homomeric nAChR when expressed in *Xenopus* oocytes (Ballivet et al. 1996), heteromeric receptors containing other ACR-16-like subunits cannot be ruled out in vivo.

A second group of *C. elegans* nAChR subunits, distinct from the ACR-16 group, with most similarity to the α7–9 vertebrate neuronal nAChR subunits (Mongan et al. 1998), has also been found in *C. elegans*. The first member identified was DEG-3 (Treinin and Chalfie 1995). Two additional DEG-3-like subunits have been identified, ACR-5 (Mongan et al. 1998) and DES-2 (Treinin et al. 1998). The DEG-3-like subunits (Treinin and Chalfie 1995; Mongan et al. 1998) are unique so far to *C. elegans*. It was shown that DEG-3 and DES-2 can form functional heteromeric nAChRs when coexpressed in *Xenopus* oocytes (Treinin et al. 1998). However, physiological and genetic studies of these receptor subunit-encoding genes suggest that other subunits participate in DEG-3-containing channels in vivo (Treinin et al. 1998).

Here we report the transcription of five novel DEG-3-like nAChR subunits. This includes the first report of a non-α nAChR subunit relative of DEG-3. The transcription of two additional ACR-16-like nAChR subunits is also demonstrated, making ACR-16-like subunits the largest nAChR subunit group identified to date in any organism. Initial clues to aspects of functional diversity in this large nAChR gene family have been sought using molecular modeling of the second transmembrane domain (M2), a region known to influence channel selectivity and gating (Wilson et al. 2000). Of particular interest is the finding that the residues which are oriented away from the channel lumen and therefore interact directly with other transmembrane domains show much greater variability than those residues exposed to the pore.

Results

RT-PCR demonstration of nAChR subunit-gene transcription

The *C. elegans* genome sequence databases were screened with a range of nAChR protein sequences using the BLAST algorithm. Genes encoding nAChR α subunits were identified among these sequences by the presence of the vicinal cysteine motif corresponding to residues 192 and 193 of the *Torpedo* α subunit (Noda et al. 1982). RT-PCR was used to confirm the transcription of six novel nAChR α subunits and one putative non-α subunit (Fig. 1). The partial cDNA products were gel-purified and sequenced to confirm their identity. All fall into either the ACR-16 or DEG-3-like nAChR subunit groups (Fig. 2, Table 1). ACR-22 is most similar to the DEG-3-like α subunits but lacks the vicinal cysteine motif found in α subunits and must therefore be designated a non-α subunit. Together with the 19 subunits previously reported (Treinin and Chalfie 1995; Ballivet et al. 1996; Fleming et al. 1997; Mongan et al. 1998), this is by some margin the largest known family of nAChR subunits (20 α and 7 non-α subunits).

Extensive DEG-3-like and ACR-16-like groups of C. elegans *nAChR subunits*

We identified genes encoding four novel α subunits (ACR-17, ACR-18, ACR-20, ACR-23) and one putative non-α subunit (ACR-22) most similar to DEG-3 (Fig. 2). Unlike the *des-2* and *deg-3* α subunit genes, which are adjacent on chromosome V and under the control of the same promoter (Treinin et al. 1998), *acr-17*, *acr-18*, and *acr-23* are widely

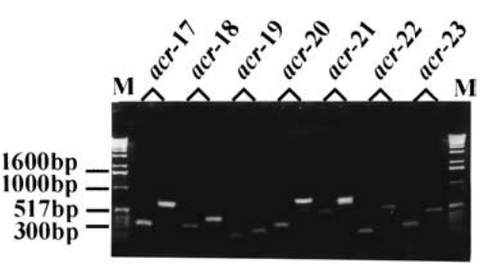

Fig. 1. RT-PCR confirms transcription of novel nAChR subunits. PCR products are shown after electrophoresis on a 1.3% agarose-TBE gel. The gel image shows pairs of adjacent cDNA and genomic PCR products corresponding to the following nAChR subunits: *acr17* (F53E10.2); *acr-18* (F28F8.1); *acr<-19* (C31H5.3); *acr-20* (R06A4); *acr-21* (F27B3); *acr-22* (F48E3.7), and *acr-23* (F59B1.9).

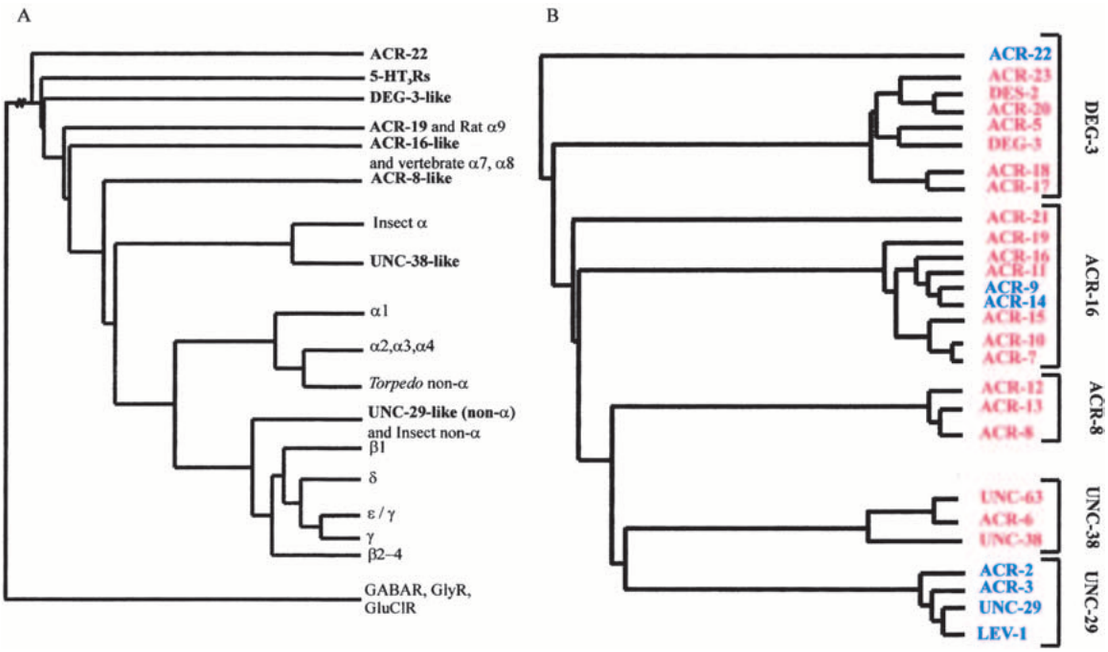

Fig. 2. (*A*) An alignment of all nAChR subunit sequences available in public databases and a selection of anionic selective GABAR, GlyR, and GluClR was generated using default parameters of ClustalW (Thompson et al. 1994). The anionic channels were selected as the out-group to root the tree. The global tree was constructed using the Protdist (Dayhoff PAM algorithm) and Neighbor programs of the PHYLIP package. For reasons of clarity, related groups of vertebrate, insect, and *C. elegans* subunits groups are illustrated by single branches in *A*. The insect branch includes the two available *Myzus persicae* sequences (X81887, X81888) in addition to the *Manduca sexta* (Y09795), *Locusta migratoria* (AJ000390, AJ000391, AJ000393), *Heliothis virescens* (AJ000399) and *Drosophila melanogaster* ALS (X07194), SBD (X55676) and Dα3 (Y15594) subunits. UNC-38-like homologs have been isolated from the parasitic nematodes *Ascaris suum* (AJ011382), *Trichostrongylus columbriformis* (U56903), *Haemonchus contortus* (U72490), and a non-α subunit, which is more similar to UNC-38 than other non-α subunits, from *Onchocerca volvulus* (L20465). (*B*) This tree shows the *C. elegans* groups expanded to reveal all *C. elegans* nAChR subunits. Five distinct groups of *C. elegans* subunits can be recognized: four groups of mainly α subunits; DEG-3-like, ACR-16-like, UNC-38-like, and ACR-8-like nAChR α subunits, in addition to the UNC-29-like group containing only non-α subunits. The alignment and global tree used to generate this Fig. are available on request from the authors.

distributed on the same chromosome, while the genes encoding ACR-20 and ACR-22 are located on chromosomes II and X, respectively.

Two additional α subunit members of the ACR-16-like subunits of *C. elegans* (ACR-19 and ACR-21) were identified. This is currently the largest nAChR subunit group in *C. elegans* (nine members total) and includes two non-α subunits, ACR-9 and ACR-14. The genes encoding ACR-19 and ACR-21 are located on chromosomes I and III, respectively. ACR-21 is the most diverged member of this group, and its predicted amino acid sequence derived from analysis of the genome sequence is most similar (55%) to the homomer-forming vertebrate α9 subunit, which is itself the most distant relative of vertebrate α7 nAChRs (Elgoyhen et al. 1994).

Molecular modeling of the channel lining M2 regions

Molecular modeling of homo-pentameric M2 helix bundles derived from the six novel nAChR α subunits revealed that the physicochemical properties of the pore-lining residues were highly conserved between *C. elegans* and all other nAChR subunits, with the exception of the previously described ACR-8-like subunits (Mongan et al. 1998). The protein interface residues of the DEG-3-like subunits were also investigated (Fig. 3). There is greater variability in residues predicted to interact with the transmembrane protein environment of these subunits than in residues exposed to the channel lumen. Although understanding of the role of transmembrane residues is increasing (Bouzat and Barrantes 1997), little is known of the physical interactions between the M2 helices and the surrounding transmembrane domains.

Furthermore, variability in M4 residues, which are oriented away from the pore but which interact with the lipid environment, has recently been shown to strongly influence channel function (Bouzat et al. 2000; Tamamizu et al. 2000). This shows that transmembrane helices not exposed to the channel lumen can also play a role in channel gating. The unexpected variability of residues at the protein inter-

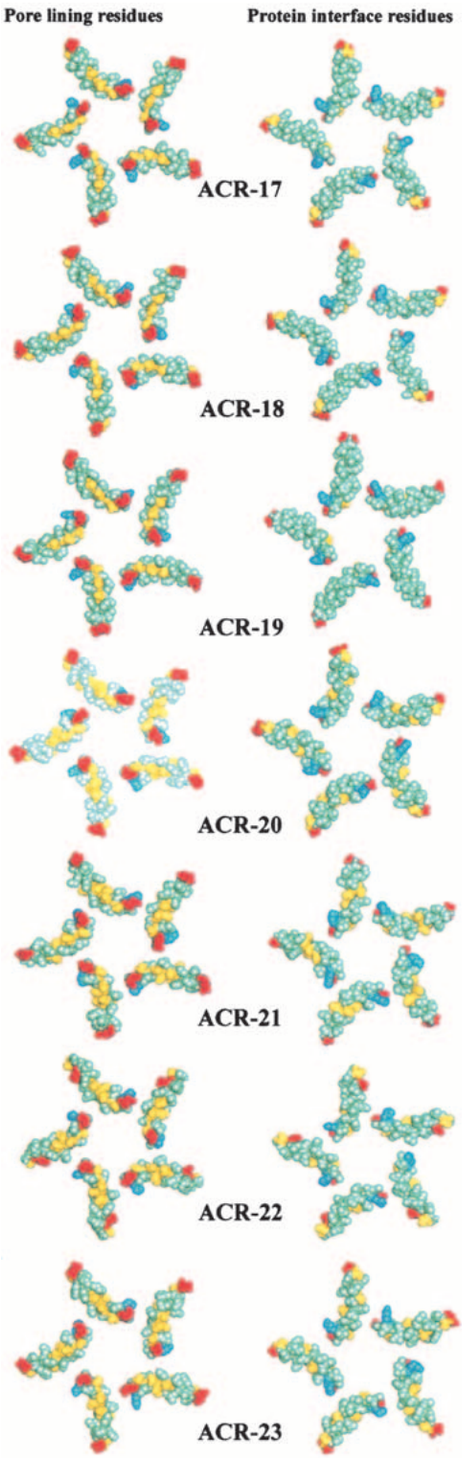

Fig. 3. Pore-lining and protein interface residues of the M2 α helix were generated using PORELINING (G.R. Smith and M.S.P. Sansom, unpubl.). The M2 helices are rotated into a plane to form a disk, one surface of which corresponds to the pore-lining surface of the M2 helices (left-hand diagrams) and the other surface of which (right-hand diagrams) corresponds to the protein interface residues. The color code for residues is: gray, apolar; yellow, polar and neutral; red, acidic; blue, basic.

face of the M2 domains of the *C. elegans* nAChR subunits may give rise to subtle differences in the allosteric transitions of each helix relative to neighboring transmembrane domains, resulting in distinct channel characteristics.

When pore models were compared in terms of their pore radius profiles (Fig. 4), in each case the narrowest region was close to the 2′ residue and had a van der Waals radius of approximately 6Å, corresponding to an effective radius of about 3Å when pore solvation is taken into account (Sankararamakrishnan et al. 1996). The pore diameters of DEG-3 and ACR-19 are smaller relative to the other novel *C. elegans* nAChR subunits due to the presence of the asparagine residue at the 2′ position in place of the more common small and/or polar residues (T, S, or G; Fig. 4). The 2′ residue is believed to form the narrow region of the pore, and substitution of this residue increases or decreases the channel conductance depending on the volume of the side chain of the substituted residue (Villarroel et al. 1991; Villarroel and Sakmann 1992).

Discussion

This study has identified seven novel candidate nAChR subunit-encoding genes (ACRs-17 to -23) in the nematode *C. elegans*. They possess features common to nAChR subunits: the di-cysteine loop (Fig. 5); aromatic residues in loops A, B, C, and D which are important for ligand binding (Fig. 5; for review, see Corringer et al. 2000); a glutamate residue at the −1′ position of M2 (Fig. 4) which has been shown to be involved in cation selectivity and calcium permeability; and four putative transmembrane regions (Figs. 4, 6). Overall, conservation is stronger in the M2 region with the seven novel subunits described in this study, showing 25%–60% identity with the M2 region of the ACR-16 subunit. For the other three transmembrane regions, identities of 21%–53% (M1), 6%–50% (M3), and 11%–32% (M4) were observed. Six of the novel subunits (ACRs-17 to 21 and ACR-23) possess two adjacent cysteine residues in loop C, marking them as α subunits, whereas ACR-22 lacks the vicinal cysteine motif and is therefore designated a non-α subunit. The combined results from this and other laboratories (Treinin and Chalfie 1995; Ballivet et al. 1996; Fleming et al.1997; Mongan et al. 1998) demonstrate an extensive and diverse gene family of nAChR subunits (20 α and 7 non-α subunits), the largest currently known for any organism.

Compared to vertebrate nAChR sequences, the seven subunits showed greatest homology with homomer-forming subunits, most notably α7. Several nAChR subunits can form functional homomeric receptors when expressed in *Xenopus* oocytes. These include vertebrate α7 (Couturier et al. 1990), α8 (Schoepfer et al. 1990; Gerzanich et al. 1994), α9 (Elgoyhen et al. 1994), *Schistocerca migratoria* αL1 (Marshall et al. 1990), and *C. elegans* ACR-16 (= Ce21)

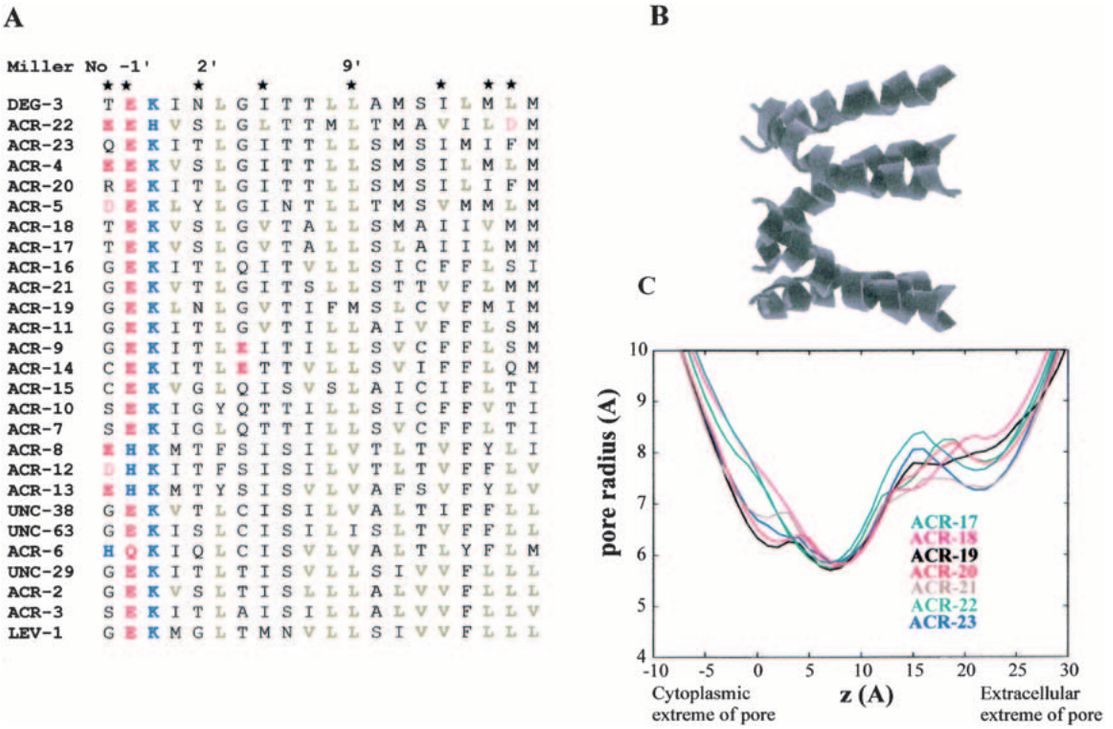

Fig. 4. (*A*) An alignment of the predicted amino acid sequences of all *C. elegans* nAChR subunits was calculated using ClustalW. Only the sequence of M2, the second transmembrane region, is illustrated. Amino acid residues are numbered according to Miller (1989) and are colored according to side chain properties: acidic (red), basic (blue), and aliphatic (green). (*B*) Molecular models of M2 homo-pentameric α-helix bundles were generated by simulated annealing/molecular dynamics using restraints derived from structural and mutagenesis data (Sankararamakrishnan et al. 1996; Adcock et al. 1998). The upper diagram shows an M2 helix bundle, with the peptide backbone shown as helical ribbons. The N-termini of the helices (corresponding to the intracellular mouth of the pore) are shown on the left-hand side of the diagram. (*C*) Pore radius profiles determined for the M2 α-helix bundle models. The HOLE algorithm (Smart et al. 1996) was used to determine pore radius profiles.

(Ballivet et al. 1996). The chicken α7 (Couturier et al. 1990) and α8 subunits form pharmacologically distinct homomeric nAChRs when expressed in *Xenopus* oocytes (Gerzanich et al. 1994). However, these recombinant nAChRs differ pharmacologically from native chicken nAChRs containing the α7 subunit (Anand et al. 1993).

There is some evidence that α7 homomers can form functional receptors in vivo (Drisdel and Green 2000). However, other studies have indicated that the α7 subunit may be part of more complex receptors. For instance, the functional properties of α7-containing nAChRs were shown to be dependent on cell type (Cuevas and Berg 1998). In contrast to rat hippocampal α7-nAChRs (Séguéla et al. 1993), the α7-containing nAChRs present on rat intracardiac ganglia desensitize slowly and recover rapidly from ^{125}I-α-bungarotoxin (α-bgt) blockade (Cuevas and Berg 1998). It was speculated that such modification of receptor functional properties could arise from the combination of α7 with as yet unknown nAChR subunits to form heteromeric receptors or from cell-specific or location-specific regulatory interactions. The ability of α7 to coassemble with the β3 subunit when expressed in *Xenopus* oocytes demonstrates the possibility of the α7 subunit combining with other nAChR subunits to form functional heteromeric receptors (Palma et al. 1999). Indeed, α-bgt binding studies showed that nAChRs composed of both α7 and α8 subunits constitute 17% of ^{125}I-α-bgt-binding protein (Keyser et al. 1993), indicating that at least some native chicken nAChRs containing α7 are heteromers in vivo. Moreover, physiological studies of embryonic chick sympathetic neurons indicated that the α7 subunit contributed to at least three subtypes of native nAChRs, leading to the proposal that the α7 subunit was forming heteromeric channels with other nAChR subunits (Yu and Role 1998). Thus, the subunit makeup of native α7-containing receptors as well as their functional diversity remains to be fully elucidated.

The *C. elegans* ACR-16-like group is now the largest collection of nAChR subunits resembling the neuronal α7-like homomer-forming subunits of vertebrates found in any organism to date. Our present finding of non-α subunit members of this subunit group (see also Mongan et al. 1998) is therefore of interest. This suggests that although ACR-16

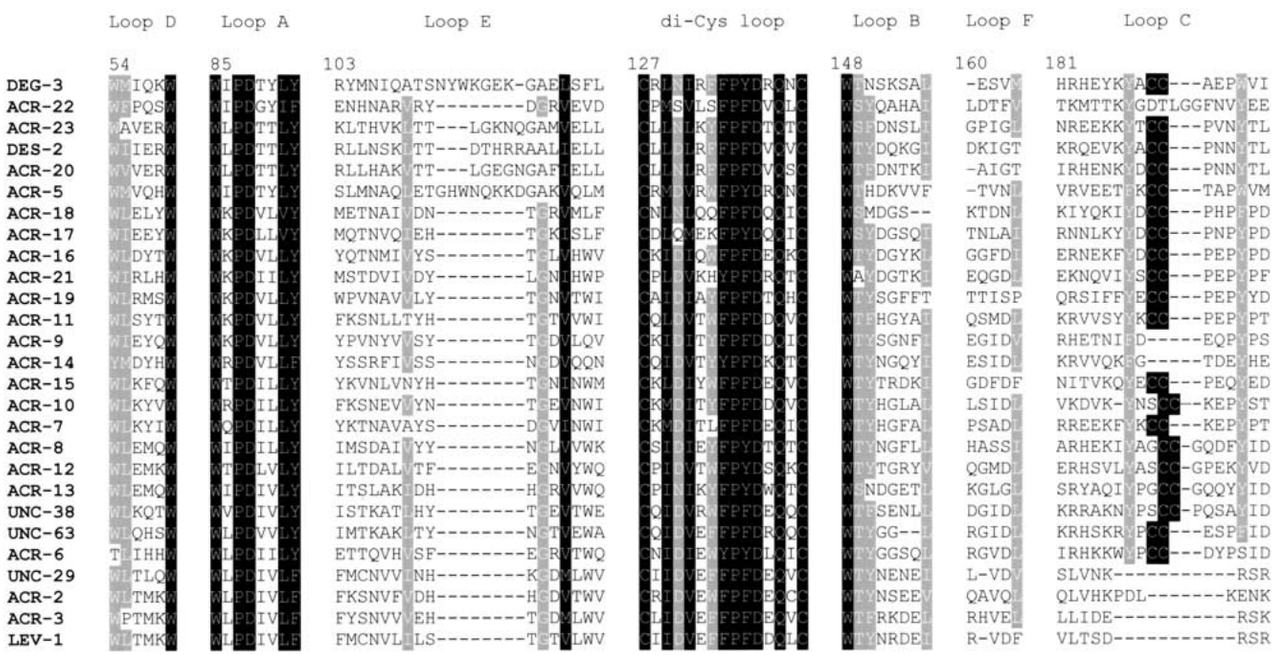

Fig. 5. An alignment of the predicted amino acid sequences of the N-terminal (extracellular) domains of all *C. elegans* nAChR subunits was calculated using Clustal W and presented by the GENEDOC program (available from its authors at http://www.cris.com/~ketchup/genedoc.shtml). The conserved di-cysteine loop and the loops contributing to the ACh binding site are shown. Residues are numbered according to the chicken α7 nAChR subunit.

is capable of forming a homomeric nAChR when expressed in *Xenopus* oocytes (Ballivet et al. 1996), there is the possibility that heteromeric receptors containing other ACR-16-like subunits exist in vivo, as is the case for DEG-3 and DES-2 (Yassin et al. 2001).

To date, the ACR-16 homomer and the DEG-3/DES-2 heteromer are the only subunits of the ACR-16 and DEG-3 groups to have been functionally expressed (Tables 1, 2). Other *C. elegans* nAChR subunits have been expressed as heteromeric channels in *Xenopus* oocytes (Table 2). These subunits are members of the UNC-38 and UNC-29 groups (Fig. 2B), which share close homology with vertebrate muscle nAChR subunits (Mongan et al. 1998). It will be of interest to express more ACR-16-like α subunits and see whether they, like ACR-16 and the vertebrate α7 subunits, are able to form functional homomeric channels. In addition, coexpression of ACR-16-like non-α subunits with other members of this group may help determine the influence of α7-like non-α subunits on generating functional diversity. It is worth noting that in ACR-22, the highly conserved 0′ lysine of M2 is replaced by histidine (Fig. 4). Functional studies with this subunit may contribute to our understanding of the effect of variations within the channel lining region on receptor activity. Sequence comparisons revealed that the newly discovered ACR-21, the most divergent ACR-16-like α subunit, is most similar to the α9 subunit. If functional nAChRs containing ACR-21 can be generated, it will be of interest to see if they share the atypical "mixed" pharmacology exhibited by vertebrate α9 homomeric nAChRs (Elgoyhen et al. 1994; Rothlin et al. 1999).

To identify pore-lining and protein interface residues in the novel *C. elegans* α subunits, molecular models of homopentameric M2 helix bundles were generated. It is interesting to note that residues oriented away from the channel lumen show much greater variability than those directly exposed to the pore. Most studies have focused on the contribution of the channel lining residues to the functional characteristics of the receptor (for review, see Hucho et al. 1996). However, the finding of such variability in residues which can interact directly with the transmembrane "superstructure" composed of M1, M3, and M4 may reflect a role for these residues in allosteric transitions required for channel gating.

This study adds to our current understanding of nAChR subunit diversity (Le Novère and Changeux 1995; Tsunoyama and Gojobori 1998) and has contributed to the characterization of the largest known family of nAChR subunit genes. Analysis of the predicted amino acid sequence of the subunits has highlighted diversity in residues known to contribute to the channel lining region. The observed molecular diversity among *C. elegans* nAChR α subunits suggests greater functional diversity in the actions of ACh than previously envisaged.

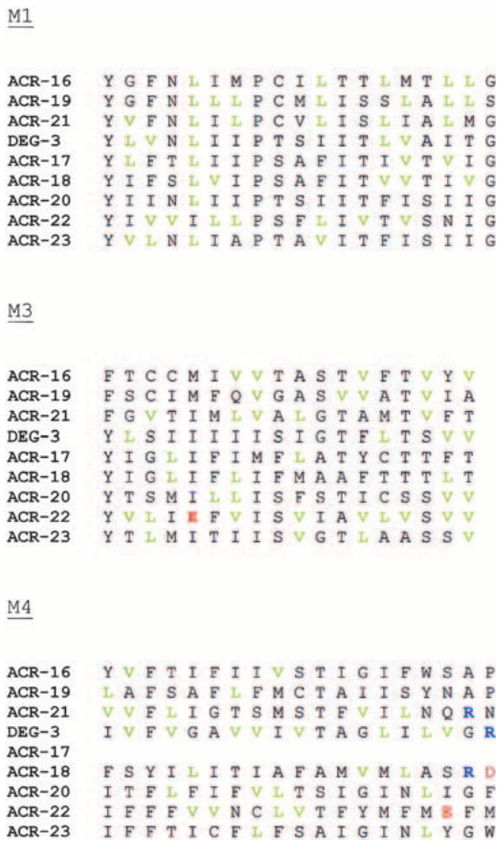

Fig. 6. An alignment of the predicted amino acid sequences of the M1, M3, and M4 regions of the novel seven *C. elegans* nAChR subunits was calculated using Clustal W. The corresponding sequences of ACR-16 and DEG-3 are included for comparison. Amino acid residues are colored according to side chain properties: acidic (red), basic (blue), and aliphatic (green). At present there is no M4 sequence for the ACR-17 subunit. Since we have shown that this subunit is transcribed, the predicted protein sequence for ACR-17 in the *C. elegans* databases is likely to be incomplete.

It has been shown that a mutation in the M2 region of the DEG-3 subunit leads to neuronal degeneration in *C. elegans*, probably due to increased receptor activity leading to Ca^{2+}-mediated toxicity (Treinin and Chalfie 1995). Interestingly, mice homozygous for a similar mutation in the α7 subunit die within 24 hours of birth and show cell death in neuronal cells, perhaps also due to increased intracellular Ca^{2+} levels (Orr-Urtreger et al. 2000). In addition, receptors with the DEG-3 and DES-2 subunits have been localized to nonsynaptic regions where they are thought to perform a chemosensory role (Yassin et al. 2001). The α7 subunit is also expressed in non-neuronal cells such as cells of the thymus (Navaneetham et al. 1997) and also in bronchial epithelial cells (Wang et al. 2001). It will be of interest to investigate further the functions of these non-neuronal α7-containing nAChRs. Thus, studies of nAChRs from *C. elegans* may contribute to our understanding of the function of similar receptors in higher organisms.

A preliminary search of the larger *Drosophila* genome sequence revealed a smaller nAChR subunit gene family with seven α subunits identified to date and three candidate non-αs (Littleton and Ganetzky 2000; M. Grauso, pers. comm.). Nevertheless, it may be that functional diversity is maintained in *Drosophila* through alternative processing of nAChR gene transcripts, as is well established in the case of *Drosophila* GABA receptors (Hosie et al. 1997). In the future it will be of interest to determine whether genomes of higher eukaryotes reveal a similarly complex nAChR gene family.

Materials and methods

Identification of novel nAChR α subunits in databases

The complete genome sequence of *C. elegans* is available at URL: http://www.sanger.ac.uk and http://genome.wustl.edu/gsc. The *C. elegans* databases were searched with the following representative nAChR α subunits: *Drosophila melanogaster* ALS (X07194); *Torpedo californica* muscle α subunit (J00963); chicken α7 (X68586); DEG-3 (U19746); ACR-16 (X83887); UNC-38 (X98600) and ACR-8 (Z50029) using BLAST (Altschul et al. 1990). Putative nAChR α subunits were identified by the presence of the adjacent cysteine residues in addition to the cysteine-loop which defines members of this superfamily of receptors.

Reverse transcription and polymerase chain reaction (RT-PCR)

Genomic DNA was prepared as described in detail elsewhere (Mongan et al. 1998). Total RNA was isolated from mixed-stage cultures of *C. elegans* using TRIzol (Life Technologies). Messenger RNA was purified using an Oligotex Direct mRNA isolation kit (QIAGEN) and first-strand cDNA synthesized using Superscript II (Life Technologies). The cDNA was diluted to 100 μL with sterile water, phenol-extracted and ethanol-precipitated. cDNA pellets were resuspended in 100 μL of sterile ultrapure water and stored at −20°C. Subunit-specific oligonucleotide primers were designed to flank the sequence predicted to encode the vicinal Cys motif and M2, and also span an intron/exon boundary. In this way cDNA and genomic PCR products differ in size. The sequences of primers used are as follows:

acr-17: 5′CAACCACTACATCCCAAATATGGA3′
5′CCAATGTAATATTGCCCGAGAAGAG3′
acr-18: 5′GACAACAACGCAGTGACTCCC3′
5′GTTTCGTAACATTTGCATCGTGGAG3′
acr-19: 5′CGATATTTTTCTATGAATGCTGCCC3′
5′CACTTGTTTGAGGCATTGCTTCAGC3′
acr-20: 5′CGAAAGCGAAGCAATCGGGACCAC3′
5′GTCCGATGAGTGGAATGAAAGATG3′
acr-21: 5′GGAAGTGCTTCGGCTGTCGGAGGC3′
5′CAGACGCCCAGTACATGAAATCTG3′
acr-22: 5′GAATGGGACATTGTACGTTTAATGC3′
5′CCATGGTAAGCATTGTAGTGAGAC3′
acr-23: 5′GTGGAACACATGTAGACGTCGATG3′
5′CACGTGCTGTCCGGTCAACTACAC3′

Subunit-specific primers were employed in PCR reactions using either cDNA or genomic DNA in a 50 μL total reaction volume

Table 1. *Percent similarity and identity between the predicted protein sequences of novel nAChR subunits and key* C. elegans *subunits*

	% Identity of derived protein sequences												
	DEG-3	ACR-16	ACR-8	UNC-38	ACR-17	ACR-18	ACR-19	ACR-20	ACR-21	ACR-22	ACR-23	UNC-63	UNC-29
DEG-3	~	28	22	24	30	25	25	43	33	27	27	25	23
ACR-16	37	~	31	37	31	28	40	34	45	23	22	37	44
ACR-8	32	41	~	38	24	24	30	28	30	23	40	41	33
UNC-38	34	48	51	~	26	21	32	30	39	23	22	52	39
ACR-17	41	41	36	38	~	47	27	32	36	26	23	27	24
ACR-18	35	37	34	29	59	~	25	30	35	25	24	26	28
ACR-19	35	49	40	41	39	32	~	27	41	23	20	34	28
ACR-20	56	43	40	42	42	38	41	~	34	28	49	29	29
ACR-21	46	56	43	51	47	47	54	46	~	28	26	40	33
ACR-22	40	35	38	35	40	34	33	44	40	~	19	25	25
ACR-23	37	35	40	34	30	31	29	57	37	31	~	25	20
UNC-63	36	47	53	63	39	36	42	43	53	35	34	~	41
UNC-29	34	34	47	50	34	31	40	41	46	35	29	52	~
	% Similarity of derived protein sequences												

The GAP analysis program (Wisconsin Package, version 9.1, Genetics Computer Group (GCG), Madison, WI) was used to determine the percentage similarity and identity between the predicted protein sequences of novel nAChR subunits and key *C. elegans* subunits, DEG-3, ACR-16, ACR-8, UNC-38, and UNC-29. Default parameters were used (gap creation penalty of 12 and gap extension penalty of 4).

composed of 1 × PCR buffer supplemented to 2.5 mM $MgCl_2$ (Promega), 0.2 μM each primer, and 0.2 mM dNTP mix (Pharmacia). Either 1 μL first-strand cDNA or 0.1 μg genomic DNA was added as template. Negative control PCRs were carried out without the addition of template DNA for all amplifications. The following conditions were used for 40 cycles in a Perkin Elmer (480 model) thermocycler: 94°C for 5 min, prior to addition of 2.5 units of *Taq* polymerase (Promega); 94°C for 30 sec, 55°C for 30 sec, 72° for 90 sec. All PCR products were analyzed by electrophoresis in a 1.3% agarose-TBE gel. RT-PCR products were gel purified using a QIAquick gel extraction kit (QIAGEN) and sequenced by the dye termination method using an ABI automated sequencer. The most abundant amplification products were gel purified, as low levels of nonspecific amplification products may interfere with direct sequencing of PCR products. The RT-PCR products were sequenced and intron-exon boundaries confirmed by comparison with the genomic sequence.

Molecular modeling and pore radius calculations

The restrained molecular dynamics (MD) methods employed have been described in detail (Sansom et al. 1995; Sankararamakrishnan et al. 1996; Adcock et al. 1998). Briefly, MD simulations with experimentally derived restraints taken from 9Å resolution cryo-electron microscopy images (Unwin 1995) and from pore-lining side chains identified by site-directed mutagenesis (Hucho et al. 1996) were used to generate models of homopentameric M2 helix bundles for the *C. elegans* nAChR sequences. MD simulations were performed using Xplor V3.1 (Brünger 1992) with the

Table 2. *Expression of* C. elegans *nAChRs*

Receptor	Heterologous expression	Expression in *C. elegans*	Reference
DEG-3/DES-2	Cation channel preferentially activated by choline	Sensory neurons including nonsynaptic regions	Yassin et al. 2001
ACR-5	—	DB/VB motor neurons	Esmaeili et al. 2002
ACR-16	Homomeric cation channel, levamisole is antagonist	—	Ballivet et al. 1996 Raymond et al. 2000
UNC-38/UNC-29/LEV-1	Cation channel, levamisole is agonist	UNC-29: body and head muscles, central neurophil	Fleming et al. 1997
UNC-63/UNC-29/LEV-1	Cation channel, levamisole is agonist	UNC-63: body muscles, motor and head ganglia neurons LEV-1: body muscles, motor neurons	E. Culetto and D.B. Sattelle, pers. comm.
UNC-38/ACR-2	Cation channel, levamisole is agonist	—	Squire et al. 1995
UNC-38/ACR-3	Cation channel, levamisole is agonist	—	Baylis et al. 1997

The α subunits are underlined.

CHARMM PARAM19 (Brooks et al. 1983) parameter set. Display and examination of models were carried out using Quanta98 (Molecular Simulations), and diagrams of structures drawn using Molscript (Kraulis 1991). Simulations were carried out on Silicon Graphics workstations. Pore-lining residues in the generated models were identified using diagrams produced by rotating the constituent helices onto a plane, forming a disc of helices with one surface corresponding to their pore-lining faces and the other surface revealing the M2 residues which interact directly with the other transmembrane domains. In these diagrams, the innermost ring of side chains corresponds to the intracellular end and the outermost ring corresponds to the extracellular end of the pore.

The "HOLE" algorithm (Smart et al. 1996) was employed to estimate the pore dimensions of the nAChR models generated by SA/MD. HOLE determines progressively the radius of the largest sphere that can be accommodated without overlap with the van der Waals radii of residues exposed to the channel lumen. The net result is a series of dimensions determined by measuring the radius of a flexible sphere "squeezing" through the ion channel.

Computational sequence analysis

The Genefinder program was developed (P. Green and L. Hillier, unpublished software) to identify potential protein coding genes within genome data. The program uses statistical criteria based on genomic features characteristic of protein encoding sequences including splice consensus sites, translation start and stop sequences, G-C characteristics, and codon biases (Wilson 1999). The computer-generated gene predictions are then inspected manually, and EST and protein homologies are used to improve the final annotation, presented in ACeDB. Predicted proteins are then compared with existing protein databases and matches recorded in the public *C.elegans* databases. This method of gene prediction has proven to have greater than 70% accuracy, although errors do occur in the identification of translational start sites and intron-exon boundaries. Genefinder has identified 19,099 predicted genes on the *C. elegans* genome (The *C. elegans* Genome Sequencing Consortium, 1998).

Novel nAChR subunits were identified using BLAST analysis of the *C. elegans* genomic and protein databases, and alignments were constructed using ClustalW (Thompson et al. 1994). The relationship between protein sequences may be inferred by detailed analysis of multiple alignments. Although our analyses were based on predicted protein sequences, these novel nAChR subunits were identified using BLAST homology searches, which found the features highly conserved in known nAChR subunits. Hence only small regions of highly divergent sequence may not have been identified by our searches, which should not alter the overall result of our analyses.

Phylogenetic studies have investigated the molecular evolution of ligand-gated ion channels (LGICs) (Ortells and Lunt 1995), the nAChR subunit gene family (Le Novère and Changeux 1995; Tsunoyama and Gojobori 1998), avermectin-sensitive GluClR (Vassilatis et al. 1997), and inhibitory ligand-gated ion channels (Xue 1998). The Phylip package [PHYLIP (Phylogeny Inference Package) version 3.5c, distributed by the author, J. Felsenstein, Department of Genetics, University of Washington, Seattle, USA] can be used to construct trees representing the phylogenetic relationship between sequences. However, in the present study the programs were employed to determine the sequence relationship of novel nAChR subunits relative to a functionally distinct out-group containing ionotropic receptors that form anion channels (GluClRs, GABAR, and GlyRs). As in our previous study (Mongan et al. 1998), the Protdist program of the Phylip package was used to compute a distance measurement for the protein sequences, the Dayhoff PAM matrix. The neighbor-joining (NJ) method was then used to produce an unrooted tree by examining the distance matrix calculated by Protdist. Although the tree generated by the NJ method was unrooted, the inclusion of a functionally distinct but structurally related group of sequences in the dataset (termed the out-group) allowed the tree to be rooted and the final relationship determined.

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