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The *C.elegans ric-3* gene is required for maturation of nicotinic acetylcholine receptors

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Mutations in *ric-3* (resistant to inhibitors of cholinesterase) suppress the neuronal degenerations caused by a gain of function mutation in the *Caenorhabditis elegans* DEG-3 acetylcholine receptor. RIC-3 is a novel protein with two transmembrane domains and extensive coiled-coil domains. It is expressed in both muscles and neurons, and the protein is concentrated within the cell bodies. We demonstrate that RIC-3 is required for the function of at least four nicotinic acetylcholine receptors. However, GABA and glutamate receptors expressed in the same cells are unaffected. In *ric-3* mutants, the DEG-3 receptor accumulates in the cell body instead of in the cell processes. Moreover, co-expression of *ric-3* in *Xenopus laevis* oocytes enhances the activity of the *C.elegans* DEG-3/DES-2 and of the rat α-7 acetylcholine receptors. Together, these data suggest that RIC-3 is specifically required for the maturation of acetylcholine receptors.

Keywords: biogenesis/*Caenorhabditis elegans*/ion channel/nicotinic acetylcholine receptor

Introduction

Ionotropic acetylcholine receptors are members of the ligand-gated ion channel superfamily. These acetylcholine receptors are pentamers, usually heteromers, composed of specific α and non-α subunits. These subunits have a large N-terminal extracellular domain, four transmembrane domains, and a large intracellular loop between transmembrane domains III and IV (Numa *et al*., 1983). The receptor subunits fold and assemble into pentamers in the endoplasmic reticulum (ER), and are then exported through the Golgi apparatus to the plasma membrane. A strict requirement for folding and pentamer assembly prior to exit from the ER has been suggested (Smith *et al*., 1987; Gu *et al*., 1991; Kreienkamp *et al*., 1995; Keller *et al*., 2001). This maturation process is time consuming, requiring 2 h (Merlie and Lindstrom, 1983), and depends upon poorly understood cellular machinery. The low surface expression of several acetylcholine receptors when expressed in heterologous cells indicates that cell-specific assembly and trafficking proteins may be required (Cooper and Millar, 1997; Dineley and Patrick, 2000; Sweileh *et al*., 2000). However, except for the broad-specificity ER chaperones, BiP, calnexin and 14-3-3η, little is known about proteins that mediate folding, assembly or targeting of acetylcholine receptors (Blount and Merlie, 1991; Chang *et al*., 1997; Jeanclos *et al*., 2001).

To identify genes needed for acetylcholine receptor activity in *Caenorhabditis elegans*, we screened for suppressors of a dominant mutation in the acetylcholine receptor subunit DEG-3 (Treinin and Chalfie, 1995; Treinin *et al*., 1998). The DEG-3(u662) mutation is a substitution of an amino acid in the pore-forming transmembrane domain (TMDII) of this subunit. The mutant subunit assembles with DES-2 to form a non-desensitizing channel that ultimately causes degeneration and necrotic death of neurons that express this gene (Treinin and Chalfie, 1995; Treinin *et al*., 1998). This screen led to the identification of mutations in *des-2*, a subunit of the DEG-3 receptor, and also in *ric-3* (resistant to inhibitors of cholinesterase). *ric-3* mutations were also identified in screens for mutants defective for cholinergic transmission at the neuromuscular junction (Nguyen *et al*., 1995; Miller *et al*., 1996). These data suggested that *ric-3* activity is required for the function of both receptors: the neuronal acetylcholine receptor and the muscle acetylcholine receptor. Here we demonstrate that RIC-3 is needed for the activity of multiple acetylcholine gated channels and is not needed for the activity of other ligand gated channels. Our characterization of *ric-3* function suggests that in the absence of this gene, acetylcholine receptors are not transported to the cell surface and that heterologous expression of this protein greatly improves surface expression of these receptors. Thus, RIC-3 is the first identified component of the biosynthetic machinery specifically required for the functional maturation of acetylcholine receptors.

Results

Screen for suppressors of deg-3-induced cell death

deg-3(u662) is a missense mutation in the pore-forming domain of the acetylcholine receptor subunit DEG-3. This mutation causes degeneration of the neurons expressing this subunit, and these cell deaths lead to uncoordinated movement and mechanosensory defects in mutant worms (Treinin and Chalfie, 1995; Treinin *et al*., 1998). We screened for mutations that suppressed the behavioral defects caused by the *deg-3(u662)* mutation. Presumably, suppressor mutations would identify genes required for DEG-3 receptor function. We identified 51 mutations that suppressed the behavioral defects as well as neuronal

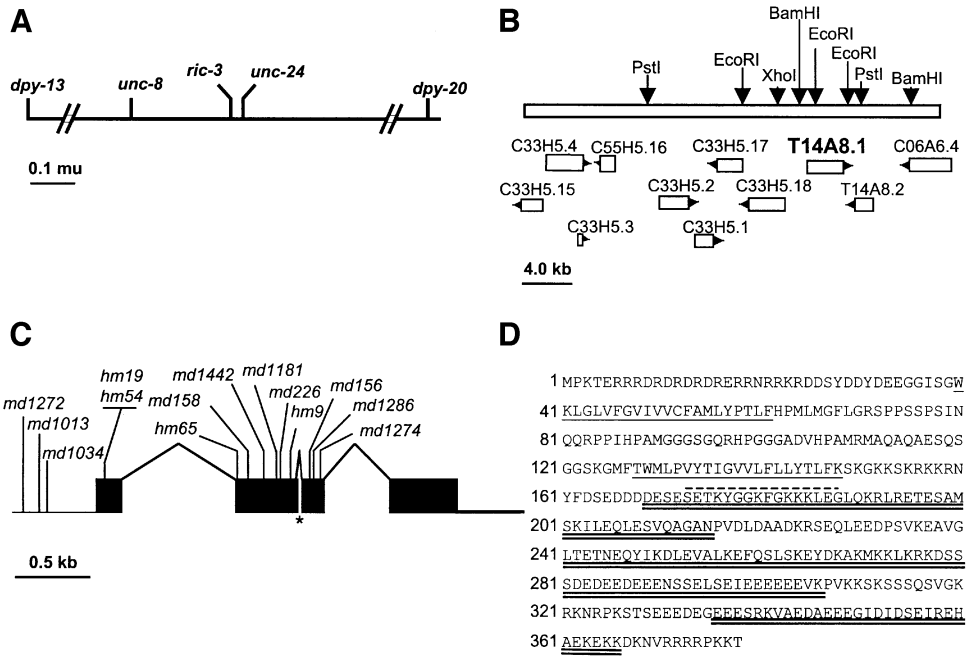

Fig. 1. Cloning and molecular analysis of *ric-3*. (**A**) Genetic map of chromosome IV showing the position of *ric-3* relative to nearby genes. (**B**) Physical map of cosmid T14A8, showing positions of predicted genes (T14A8.1, which encodes *ric-3*, is in bold) and of restriction sites used in this study. (**C**) Structure of the *ric-3* gene. Exon structure was determined from the analysis of four cDNAs. Blocks, exons; bold line, non-translated regions; thin line, 5′ upstream non-transcribed region. The alternatively spliced exon is marked with an asterisk. Sites of mutation in *ric-3* are indicated: *md1013*, *md1034*, *md1181*, *md1272*, *md1274*, *md1286* and *md1442* are Tc1 insertions, *hm9*, *hm19*, *hm54* and *hm65* are nonsense mutations, and *md146*, *md156*, *md158* and *md226* are deletions (12.5 kb, 7 bp, 5 bp and 12 bp, respectively). (**D**) Sequence of the RIC-3 protein derived from cDNAs (yk704a8 and yk722a6). Single underlines indicate transmembrane regions, double underlines indicate coiled-coil regions, and the dashed line indicates amino acids that are absent in the alternatively spliced cDNA variants (yk719f3 and yk266d12), in which the last G in this segment is converted to an R.

degeneration. These mutations fall into three groups: mutations in *deg-3* itself (36 mutations), mutations in *des-2* (a second subunit of the DEG-3 receptor; 11 mutations; Treinin *et al*., 1998) and mutations in a third gene that we originally named *des-5* (four mutations: *hm9*, *hm19*, *hm54* and *hm65*).

des-5 mutations are strong suppressors of *deg-3(u662)*-induced cell degeneration. Cell swelling, the visible hallmark of *deg-3(u662)*-induced degenerations, is seen in 96% of *deg-3(u662)* mutants 2 h after hatching. By contrast, only 4% of *des-5(hm65)deg-3(u662)* and 14% of *des-5(hm19)deg-3(u662)* animals exhibit degenerating cells (50 animals each). In wild-type adults there are ~11 cells (10.6 ± 1.9, $n = 28$ animals) which can be observed expressing a *deg-3lacZ* construct (*hmIs1*). In *deg-3(u662)*, only three cells survive (3.36 ± 1.3, $n = 50$). However, seven cells survive in *des-5(hm9)deg-3(u662)* double mutants (7.1 ± 2.6, $n = 50$). Although *des-5* mutations suppress the uncoordinated phenotype caused by the *deg-3(u662)* mutation, the double mutants do not exhibit wild-type movement, and *des-5* animals are uncoordinated in the absence of the *deg-3(u662)* mutation. The *des-5* animals coil when moving backwards. Moreover, they are slightly small and transparent, which is a phenotype of starved animals, a starved appearance that is probably caused by reduced pharyngeal pumping (see below). They also display a temperature-sensitive growth phenotype: animals will either arrest growth or display reduced fertility upon transfer to 25°C.

We mapped *des-5* to an interval between *unc-8* and *unc-24* on chromosome IV (Figure 1A). *ric-3* is also a gene that falls in this region, and *des-5*-associated phenotypes are similar to phenotypes seen in *ric-3* mutants (Nguyen *et al*., 1995; Miller *et al*., 1996). A complementation test demonstrated that *des-5(hm9)* is allelic to *ric-3(md158)*. In addition, *ric-3(md158)*, like the *des-5* alleles, suppresses *deg-3(u662)*-induced degeneration [only 4% of *ric-3(md158)deg-3(u662)* newly hatched larvae have degenerating cells; 50 animals]. We concluded that *des-5* and *ric-3* are alleles of the same gene, and the gene will be described henceforth by its previously published name of *ric-3*.

Cloning of ric-3

ric-3 maps to a narrow region between *unc-8* and *unc-24* on chromosome IV (Figure 1A). This region is covered by 26 cosmids. These cosmids were injected into *ric-3 (md1181)* animals and assayed for their ability to rescue *ric-3*-associated phenotypes. The cosmid T14A8 rescued *ric-3*. In addition, we probed this interval using Southern blot analysis. Probing with a 9.7 kb *Bam*HI fragment from the T14A8 cosmid revealed DNA polymorphisms in five *ric-3* mutants isolated from the mutator strain TR638 (Miller *et al*., 1996; *md1181*, *md1274*, *md1286*, *md1013* and *md1272*; Figure 1B and C). Insertions consistent with the size of the *C.elegans* transposon Tc1 were identified in two adjacent *Eco*RI fragments of the single gene T14A8.1. *ric-3(md1181hm57)* is a wild-type revertant of *ric-3-(md1181)*. The insertion observed in *md1181* was absent in the revertant, indicating that this putative transposon is the relevant mutation in this strain. A 6.5 kb *Xho*I–*Pst*I subclone (Figure 1B) containing only this gene was

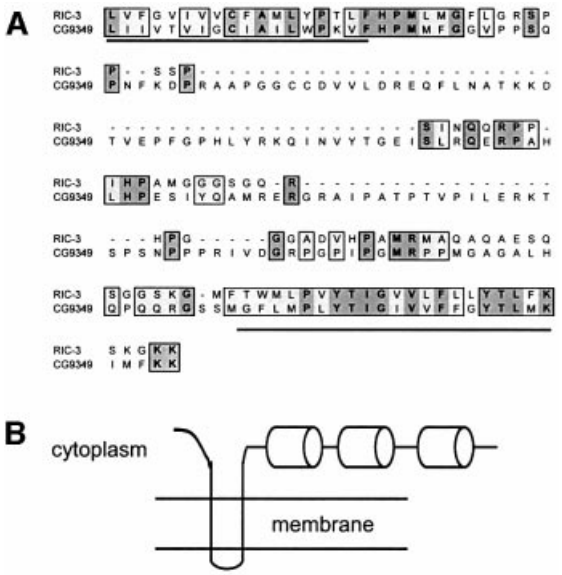

Fig. 2. A RIC-3 homolog. (**A**) Homology with *Drosophila* CG9349 protein in the region spanning the transmembrane domains. Underlines indicate putative transmembrane domains. (**B**) Structure and topology predictions for RIC-3 and CG9349. Barrels indicate positions of coiled-coil regions. CG9349 has only one predicted coiled-coil domain.

sufficient for rescue of *ric-3*-associated phenotypes in transgenic animals (three of three transgenic lines), demonstrating that T14A8.1 is the coding region for *ric-3*.

Four cDNA clones (gift of Y.Kohara) from this locus were obtained and sequenced. Two of these cDNAs were SL1 spliced and thus indicate that the entire open reading frame (ORF) has been identified (Krause and Hirsch, 1987). Two of these four cDNAs lacked a small second intron and thus indicate that the *ric-3* transcript is alternatively spliced. These cDNAs encode proteins of 363 or 378 amino acids (Figure 1D). The first ATG found in these ORFs is preceded by CAAA, a close match to the *C.elegans* translation initiation consensus (Blumenthal and Steward, 1997). Three Tc1 insertions are located within a 0.5 kb fragment in the promoter of this gene; these Tc1 insertions are likely to affect transcription of *ric-3*. All other *ric-3* mutations, including Tc1 insertions, deletions and point mutations, affect the first half of these ORFs. All point mutations and deletions except *md226* lead to severe truncation of the protein (Figure 1C and D). *md158* is a 5 bp deletion and *hm9* is a nonsense mutation, and are used as null mutations in these studies.

The RIC-3 protein

Sequence analysis of *ric-3* shows that it encodes a novel protein (Altschul *et al*., 1990) showing no homology to characterized proteins in the databases. RIC-3 is a highly charged protein, comprised of 8% aspartic acid, 13% glutamic acid, 10% lysine and 8% arginine (Figure 1D). Structure predictions suggest that RIC-3 lacks a signal peptide but contains two transmembrane domains followed by three coiled-coil regions (Berger *et al*., 1995; Sonnhammer *et al*., 1998). According to these predictions, RIC-3 is located on membranes with its N-terminal domain and C-terminal coiled-coil domains in the cytoplasm (Figure 2B; Hartmann *et al*., 1989). Coiled-coil motifs have previously been implicated in protein–protein interactions where they directly associate with other coiled-coil domain-containing proteins (Stefancsik *et al*., 1998).

Although RIC-3 is not homologous to previously studied proteins, it is weakly homologous to a protein, CG9349, identified by the *Drosophila* genome project (The Drosophila Sequencing Consortium, 2000). This protein is 35% identical to RIC-3 in the two transmembrane domains and in a small region between these domains (Figure 2A). In addition, both genes contain coiled-coil regions C-terminal to the transmembrane domains. Structure predictions also suggest that, like RIC-3, the *Drosophila* protein CG9349 has no signal peptide and has its coiled-coil domain in the cytoplasm (Hartmann *et al*., 1989).

To determine in which cells the *ric-3* gene is expressed, we inserted the coding region for green fluorescent protein (GFP) after the first ATG and in-frame with the rest of the *ric-3* gene in the rescuing genomic fragment. Expression of the GFP-tagged protein in *ric-3(md1181)* fully rescued all *ric-3*-associated phenotypes. GFP was found in the pharyngeal muscles, the body wall muscles and in many neurons (Figure 3). Using the number and position of RIC-3-expressing cell bodies as our criteria, we could account for all neurons in the body (apart from the major head and tail ganglia). As the density of cell bodies in these ganglia precludes accurate counting, we specifically focused on one group of sensory neurons, the amphid and phasmid neurons, most of which stain with the lipophilic dye DiD. All DiD-staining neurons also expressed *ric-3gfp* (data not shown). Thus, RIC-3 is expressed in most if not all *C.elegans* neurons, including all motor neurons of the ventral cord, many sensory neurons (touch receptor and chemosensory neurons) and interneurons.

In addition to the identification of cells expressing RIC-3, we also looked at the intracellular localization of this functional GFP fusion. In neurons, fluorescence was absent in the nucleus and was strong in the soma, and in the body muscles fluorescence was often seen in cytoplasmic membranous structures (Figure 3B). Because RIC-3 was found in the cell soma and is a membrane protein, it is possible that the protein is localized to the ER. Localization to the ER would suggest a role for RIC-3 in the assembly of acetylcholine receptors. However, weak GFP fluorescence is also seen in neuronal processes including the ventral cord and nerve ring; thus, other roles such as in transport cannot be excluded.

ric-3 is specifically required for cholinergic transmission

ric-3 mutants are resistant to the acetylcholinesterase inhibitor aldicarb (Nguyen *et al*., 1995), which indicates a defect in cholinergic transmission at neuromuscular junctions of the body wall muscle. This neurotransmission defect appears to be postsynaptic, since *ric-3* animals are also resistant to the anthelmintic levamisole (Miller *et al*., 1996). Levamisole is an agonist of the body wall muscle acetylcholine receptor (Lewis *et al*., 1980). Given that RIC-3 is also required for the neuronal acetylcholine receptor DEG-3/DES-2, these data suggested that RIC-3 may play a general role in acetylcholine receptor function.

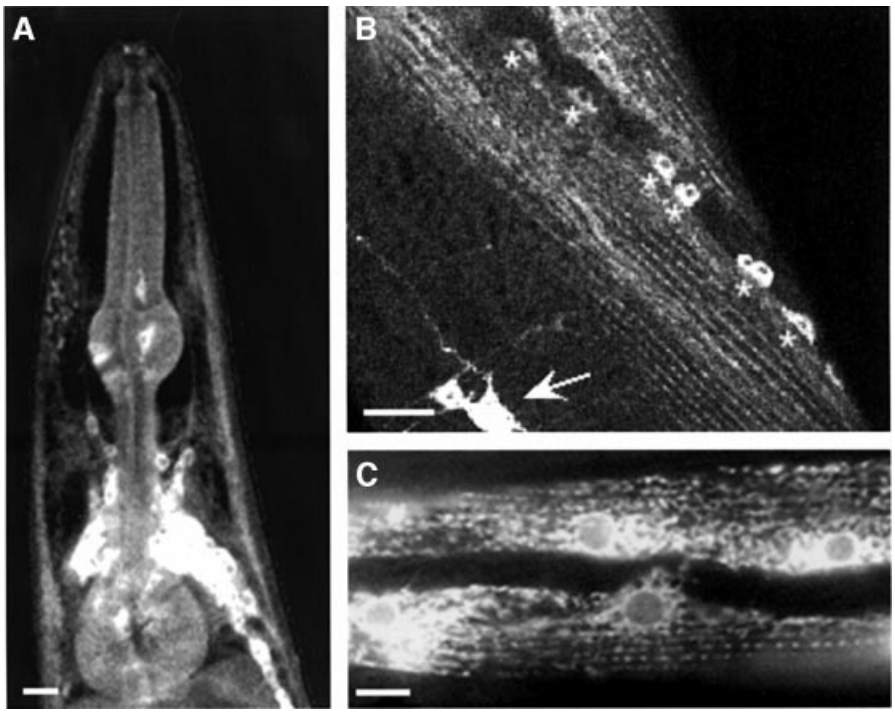

Fig. 3. Localization of RIC-3. RIC-3 localization was visualized using a RIC-3::GFP functional fusion. (**A**) Head region, showing the pharyngeal muscles (the double-lobed organ in the middle) and head ganglia neurons seen between the two lobes of the pharynx. Confocal section, scale bar: 10 μM. (**B**) Body muscles and neurons. The row of neurons in the middle marked by asterisks are motor neurons, on both sides of which are seen the body muscles. In the left corner, marked by an arrow, are the posterior–lateral ganglion neurons showing strong fluorescence in cell bodies and weak fluorescence of processes. Confocal section, scale bar: 50 μM. (**C**) RIC-3 localization in the body muscles as visualized using a *myo-3ric-3gfp* fusion. Scale bar: 20 μM.

There are four well characterized acetylcholine receptors in *C.elegans*: the neuronal receptor DEG-3/DES-2, the pharyngeal muscle receptor, the body muscle levamisole-sensitive receptor and the body muscle nicotine-sensitive receptor. *In vivo* electrophysiological recordings of the last three receptors are currently feasible. To assay directly the effects of *ric-3* mutations on the activity of acetylcholine receptors, we examined acetylcholine currents from these receptors in *ric-3* mutants.

Pharyngeal contraction is regulated by two neurotransmitters: acetylcholine initiates muscle contraction and glutamate terminates it. The effects of these transmitters can be assayed using extracellular recordings called electropharyngeograms (EPGs; Raizen and Avery, 1994). In accordance with the starved appearance of *ric-3* mutants, pharyngeal pumping is greatly reduced in *ric-3(hm9)* adults (45 ± 5 pumps/min) compared with the wild type (>200 pumps/min; 20 adult animals each). Interestingly, the reduced pumping rates are observed in adults but not in larvae. Contraction of the pharyngeal muscle is initiated by the cholinergic MC neuron (Raizen *et al*., 1995). Synaptic currents from MC can be observed as a small inward current in the electropharyngeogram of the wild type (Figure 4). In *ric-3(hm9)* mutants, normal MC spikes are not seen; however, 70% of pumps are still preceded by a small depolarizing peak (40 pumps), possibly sub-threshold MC spikes (Figure 4). Thus, it is likely that the activity of the pharyngeal muscle acetylcholine receptor is also affected by *ric-3* mutations. Pharyngeal contractions are terminated by inhibitory glutamate transmission from the M3 motor neuron. In contrast to cholinergic transmission, these glutamate-mediated outward currents are unaffected in *ric-3* mutants (Figure 4). Thus, in the pharyngeal muscle, RIC-3 is specifically required for cholinergic transmission and does not cause a general defect in synaptic transmission.

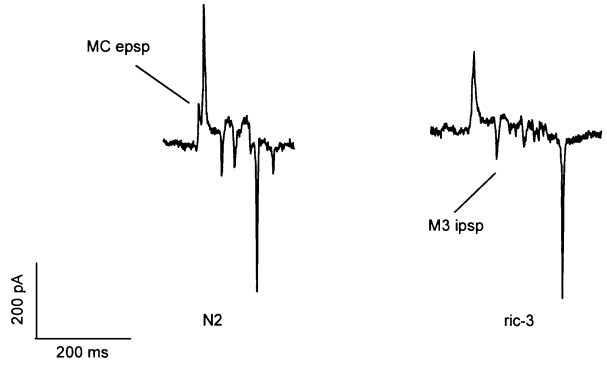

Fig. 4. Defective cholinergic transmission in the *C.elegans* pharyngeal muscle. Representative traces for the electrical activity of the pharynx of wild-type (N2) and *ric-3(hm9)* mutant animals. MC EPSP is the excitatory cholinergic activity, seen as a small spike preceding muscle depolarization (large upward spike). M3 IPSP is the inhibitory glutamatergic activity, seen as a series of small spikes preceding muscle repolarization (large downward spike). In the *ric-3(hm9)* mutant, the small depolarizatory current seen in the beginning of the trace may represent I phase activity, i.e. a sub-threshold MC spike.

The body wall muscle expresses two distinct acetylcholine receptors and one GABA receptor (Richmond and Jorgensen, 1999). Using whole-cell, voltage-clamp

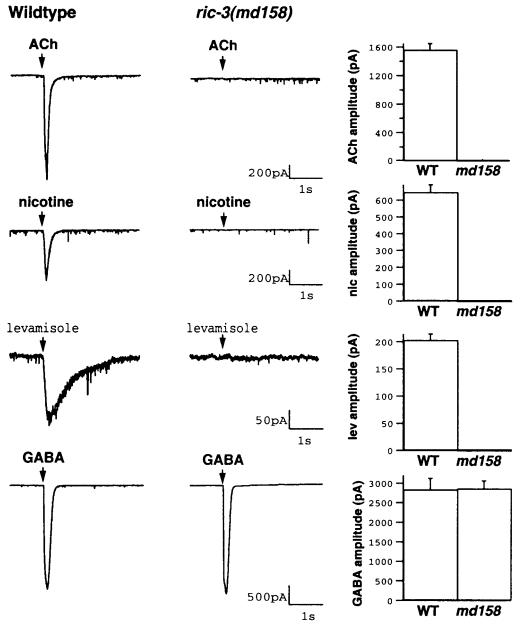

Fig. 5. Defective response to acetylcholine receptor agonists in the body muscles. Electrical responses of wild-type and mutant *ric-3(md158)* body muscles to puffs of ACh, nicotine, levamisole or GABA (100 μM each). On the left are representative traces of the responses; arrows indicate the time of agonist application. On the right is shown the average of the responses.

recordings of individual body wall muscles, it is possible to distinguish the two acetylcholine receptors because one is sensitive to levamisole while the other is sensitive to nicotine (Richmond and Jorgensen, 1999). Application of acetylcholine in wild-type animals activates both receptors. In *ric-3(md158)* animals, the response to acetylcholine was greatly diminished compared with the wild type (12 ± 2 pA, $n = 6$ and 1560 ± 255 pA, $n = 8$, respectively), suggesting that both receptors are inactive in *ric-3* mutants (Figure 5). Responses to nicotine were absent in *ric-3(md158)* animals but were robust in the wild type (0.1 ± 0.1 pA, $n = 9$ and 646 ± 124 pA, $n = 8$, respectively). Response to levamisole was also eliminated in *ric-3(md158)* compared with the wild type (0.3 ± 0.3 pA, $n = 6$ and 203 ± 30 pA, $n = 8$, respectively). However, responses were observed at very high agonist concentrations; 1 mM acetylcholine was able to elicit very low amplitude responses (97.1 ± 11 pA, $n = 4$), suggesting that in *ric-3* mutants small numbers of functional acetylcholine receptors are still present on the cell surface. In contrast, application of GABA elicits the same response in both *ric-3* (2860 ± 636 pA, $n = 10$) and wild-type animals (2840 ± 714 pA, $n = 6$; Figure 5). Thus, *ric-3* is specifically required for cholinergic transmission in both pharyngeal muscles and body muscles.

ric-3 mutants do not respond to acetylcholine receptor agonists, evidence for a defect in receptor biogenesis or activity. To determine whether RIC-3 is required within the same cells that express these receptors, we examined whether expression of RIC-3 within the body muscles is sufficient for normal activity of the body muscle levamisole receptor. Indeed, expression of RIC-3 from the *myo-3* promoter (Fire and Waterson, 1989) is sufficient for rescue of the levamisole insensitivity of *ric-3(md1181)* mutants; transgenic *ric-3(md1181)* animals expressing RIC-3 in the body muscle alone arrest movement on 1 mM levamisole (82%), unlike the original *ric-3(md1181)* animals, which show no arrest of movement (0%, 50 animals each). In order to rule out the possibility that RIC-3, like agrin, an extracellular matrix protein needed for clustering of nAChRs, can be supplied by both muscles and neurons (Reist *et al.*, 1992), we examined whether expression of RIC-3 in neurons could also rescue the insensitivity of *ric-3(md1181)* to levamisole. For this purpose we fused the *ric-3* ORF to the *aex-3* promoter, a general neuronal promoter (Iwasaki *et al.*, 1997). This construct, unlike the *myo-3::ric-3* construct, did not rescue levamisole insensitivity in two lines of *ric-3(md1181)* transgenic animals (0% arrested animals, 50 animals each). However, this analysis and our RIC-3 localization studies (above) could not rule out the possibility that RIC-3 functions in the synapses for trafficking or function of the levamisole-sensitive receptor. Thus we examined whether rescue of this receptor's activity depends on synaptic localization of RIC-3. For this purpose we looked at the localization of a *myo-3::ric-3gfp* fusion that rescues the levamisole insensitivity of *ric-3(md1181)* in two lines examined (68 and 82% arrested animals, $n = 50$ each). This fusion is seen in muscles only, and is localized to membranous structures within these cells (Figure 3C). No GFP staining was detected in the ventral cord or dorsal cord, sites of *C.elegans* neuromuscular junctions (50 animals). Neither could we detect synaptic RIC-3 in confocal thin sections (1 μm sections, $n = 10$). Thus, it is likely that the effect of RIC-3 on the levamisole-sensitive receptor is carried out within the muscle, and does not require co-localization in the synapse.

RIC-3 is needed for receptor assembly or trafficking

ric-3 mutations could affect acetylcholine receptors by altering expression, localization or function. To determine whether these mutations affect the expression of an acetylcholine receptor subunit, we examined DEG-3 immunostaining in *ric-3* mutants. In *ric-3* mutants, strong DEG-3 staining is seen in the cell bodies, demonstrating that RIC-3 is not required for DEG-3 expression. However, there was a change in distribution of DEG-3 immunoreactivity in *ric-3* mutants. For example, in wild-type animals, both cell bodies and axons of the PVD neurons stain with similar intensities. However, in *ric-3* animals, DEG-3 immunoreactivity in axons is always much weaker than immunoreactivity of cell bodies (50 animals; Figure 6). These data suggest that the absence of cholinergic transmission is not caused by a functional defect in properly transported receptors, but is caused by a defect in receptor folding, assembly or trafficking.

To directly test a role for RIC-3 in acetylcholine receptor maturation we examined RIC-3 function in *Xenopus* oocytes. We previously demonstrated that the DEG-3/DES-2 receptor can be expressed in oocytes (Treinin *et al.*, 1998). Choline is the preferred ligand of the DEG-3/DES-2 receptor (Yassin *et al.*, 2001). Reliable but small currents (83 ± 8 μA, $n = 38$ oocytes; 11 experiments) can be detected when non-saturating concentrations of cRNA for both DES-2 and DEG-3 are

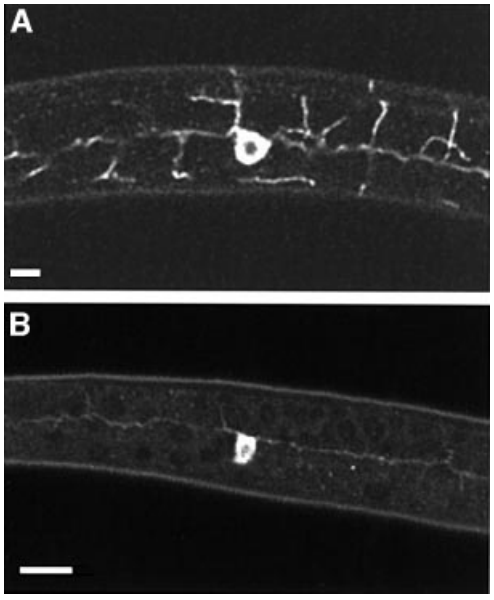

Fig. 6. DEG-3 accumulates in cell bodies of *ric-3* mutants. Immunohistochemical analysis using DEG-3 antibodies on young adults. Shown is the PVD neuron and processes in (**A**) wild type and (**B**) *ric-3(md158)*. Note the size difference between wild type and *ric-3,* both of which are young adults. Confocal section, scale bar: 20 μM.

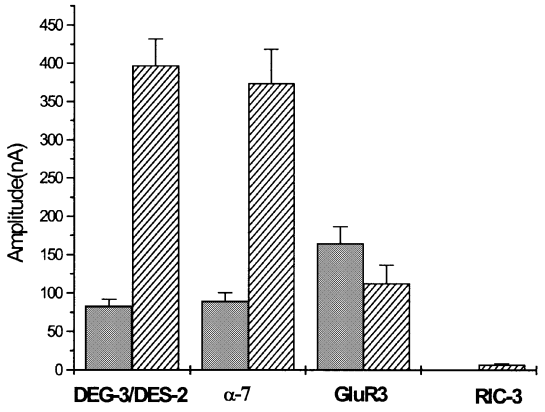

Fig. 7. RIC-3 co-expression enhances acetylcholine receptor activity in *Xenopus* oocytes. Amplitude of choline- (3.2 mM) or glutamate- (1 mM) dependent currents measured in oocytes together with (striped) or without (filled) co-expression of RIC-3. The DEG-3/DES-2 experiments represent $n = 38$ (oocytes), $N = 6$ (frogs) each. α-7, $n = 23$, $N = 4$; GluR3, $n = 7$, $N = 2$; and RIC-3 alone, $n = 10$, $N = 3$. The effects of co-expressing RIC-3 on DEG-3/DES-2- and α-7-dependent current amplitudes are significant using a paired *t*-test at >99%.

co-injected (Figure 7). However, when RIC-3 cRNA is injected with DES-2 and DEG-3 cRNAs, peak currents are increased nearly 5-fold (397 ± 36 μA, $n = 38$ oocytes; 11 experiments). This enhancement is seen on the second and third day following injection, and disappears on the fourth day. Expression of RIC-3 alone does not produce detectable choline-activated currents, nor was there any increase in the amplitude of glutamate-activated currents when GluR3 was co-expressed with RIC-3 (Figure 7). Thus, RIC-3 maintains specificity for acetylcholine receptors in *Xenopus* oocytes. Although RIC-3 is not absolutely necessary for the maturation of acetylcholine receptors in *Xenopus* oocytes, it does confer increased efficiency on the maturation of acetylcholine receptors.

To examine whether RIC-3 could enhance the maturation of non-*C.elegans* acetylcholine receptors, we co-expressed RIC-3 with the rat α-7 homomeric receptor (Seguela *et al*., 1993). Although the sequence of rat α-7 is only 30% identical to those of DEG-3 or DES-2 (Treinin *et al*., 1998), co-expression of RIC-3 with rat α-7 leads to a 4.2 ± 0.36-fold increase in peak current amplitude (Figure 7; $n = 23$ oocytes; six experiments). Thus, it appears that the ability of RIC-3 to increase acetylcholine receptor activity is mediated by conserved domains within these subunits.

Discussion

Mutations in *ric-3* were isolated because they were able to suppress necrotic cell death caused by the deregulated activity of a mutant acetylcholine receptor subunit DEG-3. We cloned *ric-3* and found that it encodes a novel protein expressed in muscles and neurons (Figures 1 and 3). Detailed examination of *ric-3* mutants shows a reduction in cholinergic transmission mediated by four different ionotropic acetylcholine receptors: the DEG-3/DES-2 receptor, the pharyngeal muscle receptor, and the levamisole- and nicotine-sensitive body muscle receptors (Figures 4 and 5). While *ric-3* mutations reduce synaptic transmission in a large number of cells, the gene appears to be specific for acetylcholine-sensitive ligand-gated ion channels. Neither GABA transmission mediated by the UNC-49 receptor (Richmond and Jorgensen, 1999) nor glutamate transmission mediated by the pharyngeal receptor AVR-15 (Dent *et al*., 1997) is affected in *ric-3* mutants (Figures 4 and 5). This specificity of the effects of RIC-3 appears to conflict with its wide expression. However, the *C.elegans* genome codes for an unusually large number of nAChRs (~40 predicted nAChR subunits; Bargmann, 1998; Mongan *et al*., 1998). Thus, the wide expression of RIC-3 suggests that it is needed for the activity of many of these nAChRs. For example, B motor neurons express RIC-3 and also ACR-5, a nAChR α subunit (Winnier *et al*., 1999); thus, it is possible that RIC-3 is also needed for the activity of ACR-5.

Co-expression with RIC-3 is required for acetylcholine receptor activity in *C.elegans* body muscles and is sufficient for enhanced acetylcholine receptor activity in *Xenopus* oocytes. RIC-3 appears to be a protein required for acetylcholine receptor biogenesis. First, the RIC-3 protein appears to be localized to a membranous organelle in the cell body, consistent with localization to the ER (Figure 3). Secondly, the DEG-3 receptor subunit accumulates in neuronal cell bodies in *ric-3* mutants (Figure 6). It is known that acetylcholine receptors are assembled in the ER and that improperly folded or assembled receptors accumulate in the ER (Smith *et al*., 1987). Thus, RIC-3 may function during folding, assembly or transport from the ER into the Golgi apparatus. Consistent with this role, co-expression of the *C.elegans* DEG-3/DES-2 or the rat α-7 acetylcholine receptors with RIC-3 was sufficient for enhanced acetylcholine receptor activity, likely to be a result of increased surface expression of these receptors.

RIC-3 is the first example of a protein specifically required for the maturation of acetylcholine receptors.

Biogenesis of membrane proteins is a complex process requiring 2–3 h (reviewed in Green, 1999; Keller and Taylor, 1999). Specifically for acetylcholine receptors, this process includes folding, generation of the appropriate membrane topology, assembly with specific subunits to form a pentamer, glycosylation, disulfide bond formation and, finally, targeting to specific sites on the membrane. The only proteins previously implicated in acetylcholine receptor biogenesis were BiP, calnexin and 14-3-3η (Blount and Merlie, 1991; Chang *et al*., 1997; Jeanclos *et al*., 2001). However, these are proteins required for the folding and maturation of many proteins. Our work on *ric-3* provides evidence for an acetylcholine receptor-specific processing pathway. However, it seems unlikely that this processing pathway is unique to *C.elegans*. Evidence for specific requirements for acetylcholine receptor biogenesis in mammals is provided by studies showing low cell-surface expression of acetylcholine receptors following heterologous expression. Specifically, cell-surface expression of the α-7 acetylcholine receptor was shown to be cell type dependent, suggesting that a specific biogenesis machinery is required for α-7 cell-surface expression (Cooper and Millar, 1997; Sweileh *et al*., 2000). Proteins similar to RIC-3 may mediate the interaction between acetylcholine receptors and a general protein-processing machinery, thus conferring on this machinery acetylcholine receptor specificity. A requirement for a RIC-3-like protein in vertebrate receptors is consistent with enhanced α-7 activity observed with co-expression of RIC-3 in *Xenopus* oocytes.

RIC-3 is one of a limited number of molecules that have a protein-specific role in the biogenesis of membrane proteins (reviewed in Herrmann *et al*., 1999). The reasons for such specificity are unknown. One possibility is that specificity is only required in special cases. For example, *Drosophila* rhodopsin molecules depend on interactions with the photoreceptor specific chaperone NinaA for their surface expression (Schneuwly *et al*., 1989; Colley *et al*., 1995). It has been suggested that the requirement for NinaA is related to the unusually large numbers (1×10^8 functional receptors per cell) of rhodopsin molecules required in *Drosophila* photoreceptors, numbers that cannot be processed by the generalized processing machinery (Baker *et al*., 1994). The formation of a functional heteromeric pentamer having the proper order and stoichiometry, as is needed for the formation of acetylcholine receptors, may also be such a special case. However, an interesting alternative is that the ability to recognize and separately process different receptors provides an additional mechanism for regulating one class of receptors while leaving other membrane proteins unaffected. The inefficient maturation of acetylcholine receptors (only 30% of newly synthesized subunits assemble to a functional receptor; Merlie and Lindstrom, 1983) opens the way for regulating acetylcholine receptor activity through changes in the efficiency of receptor maturation. It is interesting in this regard to note that RIC-3 is expressed widely and affects at least four different acetylcholine receptors. Thus, RIC-3 may mediate global signals that regulate cholinergic transmission. An example of such a control mechanism, where a global signal regulates surface expression, is provided by the effect of insulin on the $GABA_A$ receptor (Wan *et al*., 1997).

Materials and methods

Strain maintenance and genetics

The wild type was N2 Bristol and all strains were grown as previously described (Wood *et al*., 1988). Screens for suppressors of *deg-3(u662)* were described previously (Treinin and Chalfie, 1995). In short, >30 000 EMS mutagenized haploid genomes were screened for suppression of the Unc and Mec phenotypes associated with *deg-3(u662)*.

Complementation tests were conducted by crossing *ric-3(md158)*/+ males with *dpy-13(e184)des-5(hm9)* hermaphrodites. Half of the non-Dpy progeny of this cross were coilers and resistant to levamisole. Mapping was carried out as follows: among 256 Dpy non-Ric progeny of *dpy-20(e1282)ric-3(md146)/unc-24(e138)*, 251 had progeny with the *unc-24(e138)* phenotype; among 74 Dpy non-Ric progeny of *dpy-13(e184)ric-3(md146)/unc-8(e49)*, 69 had progeny with the *unc-8(e49)* phenotype; and among 74 Dpy non-Ric progeny of *dpy-13(e184)ric-3(md146)/unc-24(e138)*, all 74 had progeny with the *unc-24(e138)* phenotype. This analysis shows that *ric-3* is within the 0.26 m.u. interval between *unc-8* and *unc-24*, approximately at position 3.52 on the genetic map.

Pharmacology

Levamisole resistance was examined on NGM plates (Wood *et al*., 1983) containing 1 mM levamisole (Sigma). Sensitive L4-adult animals contract, arrest movement within minutes, and eventually die. Aldicarb resistance was examined on NGM plates with 1 mM aldicarb (Chemservice). Two L4 animals were placed on each plate and then examined for the generation of fertile progeny 7 and 14 days later.

Electrophysiology of C.elegans muscles

Extracellular recordings from the pharynx were made as in Raizen and Avery (1994). For the pumping rate data, L4s were transferred to fresh agar plates 1 day prior to the pumping rate assay and kept at 20°C. Immediately before the assay, animals were transferred to room temperature in order to count pumps. Electrophysiology of the body muscles was as in Richmond and Jorgensen (1999).

Antibody, lacZ and DiD staining

DEG-3 localization was visualized as described in Yassin *et al*. (2001). Strains carrying the following alleles of *ric-3* were examined: *md146*, *md158*, *hm9*, *md226* and *md1181*. We saw the same defect in all strains. Survival of DEG-3 expressing neurons and effects of RIC-3 on DEG-3 transcription were assayed using a *deg-3*-lacZ construct (Treinin *et al*., 1998). DiD C18 (Molecular Probes D-7757) was dissolved in DMF at 10 mg/ml. *ric-3gfp* transgenics were incubated for 1 h at room temperature with rocking in 1:1000 DiD diluted in M9 buffer, followed by 2 h destaining on seeded NGM plates. Visualization was performed on 2% agar pads + 4% paraformaldehyde.

Identification of RIC-3 expressing neurons

RIC-3::GFP stained all 57 motor neurons in the ventral cord in five of 10 animals examined, all six cells in the left posterior lateral ganglion in five of eight animals examined, all four cells of the right lateral ganglion in two of seven animals examined, and all six other cells between the vulva and head in nine of 10 animals examined. In the tail ganglia, we never counted the full complement (33 cells), rather we counted 26 ± 2 cells ($n = 10$). Among the cells in the tail ganglia, we identified PVC L, DVA, DVB and DVC by position, and PHA L/R and PHB L/R using DiD staining. In the head ganglia, no attempt was made to count cells. However, the amphid cells ASH L/R, ASJ L/R, ASK L/R and ADL L/R were identified using DiD staining.

Molecular biology

General molecular biology methods followed the protocols in Sambrook *et al*. (1989). Southern blots were probed with random primed cosmids or DNA fragments. Specifically, using a 9.7 kb *Bam*HI fragment from cosmid T14A8, we could visualize 1.6 kb insertions in DNA from the *ric-3* mutant strains. Specifically, in *md1013* and *md1272*, the 4.5 kb *Eco*RI fragment was shifted, and in *md1181*, *md1274* and *md1286*, the adjacent 2.2 kb *Eco*RI fragment shifted. Both of these bands were absent in *md146*. For rescue experiments, cosmids or subclones were co-injected

with the *rol-6* DNA (pRF4) (Mello and Fire, 1995) and roller progeny of stable transgenic lines were examined for pumping and levamisole resistance. The smallest rescuing subclone is a 6.5 kb *Xho*I–*Pst*I fragment from cosmid T14A8 ligated into pBluescript SK$^-$ (Stratagene).

To tag RIC-3 with GFP, an *Eco*RV site was inserted immediately downstream of the first ATG of RIC-3 using PCR-mediated mutagenesis. An *Eco*RV fragment containing GFP from the plasmid pPD102.33 (Fire *et al*., 1990) was ligated to this *Eco*RV site. This reporter construct was injected at 5 ng/μl and supplemented to 50 ng/μl with pBluescript. Integration of the extrachromosomal array was obtained using UV irradiation (Mitani, 1995).

ric-3 alleles were sequenced directly from PCR-amplified DNA. Sites of Tc1 insertions were estimated from the size of the PCR product generated by Tc1-specific primers combined with RIC-3-specific primers. The structure of the RIC-3 gene was obtained from the sequences of four cDNAs, yk266d12, yk704a8, yk719f3 and yk722a6, which were kindly provided by Y.Kohara. Three of these, yk704a8, yk719f3 and yk722a6, were obtained from libraries enriched for full-length cDNAs (Suzuki *et al*., 1997). yk266d12, yk704a8 and yk722a6 all terminate 12 bp upstream of the first ATG in the RIC-3 ORF. In both yk704a8 and yk722a6, this site is trans-spliced to an SL-1 leader sequence (Krause and Hirsch, 1987); only yk719f3 terminates downstream of this site with its 5′ end 77 bp downstream, and may represent a truncated cDNA. The second intron encoding 15 amino acids is not spliced out of yk704a8 and yk722a6.

Expression from the *myo-3* promoter was achieved by inserting a 2.5 kb *Sal*I–*Asc*I fragment containing the *myo-3* promoter (Fire and Waterson, 1989) in place of a 2.5 kb *Xho*I–*Asc*I fragment containing the RIC-3 promoter. For this purpose, an *Asc*I site was inserted immediately upstream of the RIC-3 ATG using PCR-mediated mutagenesis. A *Sal*I GFP fragment from pPD103.87 was inserted into an internal *Sal*I site in the same construct to generate the *myo-3ric-3gfp* construct. In this construct, GFP is fused to the full-length RIC-3 downstream of the first coiled-coil domain. The *aex-3ric-3* construct was generated by inserting an *Apa*I–*Pst*I *aex-3* promoter fragment from pMH50 into appropriate sites generated using linkers upstream of the RIC-3 ATG. These sites were inserted into the *myo3ric-3* construct in place of the *myo-3* promoter.

Sequence analysis

Structure prediction analysis was carried out using programs on http://www.expasy.ch and http://psort.nibb.ac.jp. The Flybase accession number for CG9349 is FBgn0034575. Alignment was generated using Clustal_W analysis (McVector).

Heterologous expression and electrophysiology

An *Eco*RI fragment containing RIC-3 ORF was cut out of the yk266d12 cDNA clone and cloned into a pCDNA3 variant that includes 5′ and 3′ UTRs from the *Xenopus* β-globin gene (Invitrogene). This clone contains 12 bp of the *ric-3* 5′ untranslated sequences and no 3′ untranslated sequences. *In vitro* transcribed cRNAs were injected at a final concentration of 0.1–0.5 ng. In each experiment, final concentrations of DEG-3, DES-2, α-7 or GluR3 were kept constant between injections with or without RIC-3. Injections and recordings were carried out as described in Treinin *et al*. (1998). Choline, which is the preferred agonist of the DEG-3/DES-2 channel (Yassin *et al*., 2001), was used at saturating concentrations of 3.2 mM and glutamate was used at 1 mM. For the glutamate receptor experiments we used a mutant GluR3(L507Y), which enables better visualization of glutamate-induced currents in *Xenopus* oocytes, as the L507Y point mutation eliminates AMPA receptor desensitization without significantly affecting other parameters (Stern-Bach *et al*., 1998). In each experiment, 3–5 oocytes with RIC-3 and 3–5 oocytes without RIC-3 were examined. Comparisons of current amplitude were made for experiments where the average current amplitude for oocytes not injected with RIC-3 was between 50 and 150 nA, reducing the variability inherent to the oocyte expression system. Errors are standard errors of means, and significance was examined using the paired *t*-test.

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