Xenopus laevis RIC-3 enhances the functional expression of the C. elegans homomeric nicotinic receptor, ACR-16, in Xenopus oocytes

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Abstract
RIC-3 enhances the functional expression of certain nicotinic acetylcholine receptors (nAChRs) in vertebrates and invertebrates and increases the availability of functional receptors in cultured cells and Xenopus laevis oocytes. Maximal activity of RIC-3 may be cell-type dependent, so neither mammalian nor invertebrate proteins is optimal in amphibian oocytes. We cloned the Xenopus ric-3 cDNA and tested the frog protein in oocyte expression studies. Xenopus laevis RIC-3 shares 52% amino acid identity with human RIC-3 and only 17% with that of Caenorhabditis elegans. We used the C. elegans nicotinic receptor, ACR-16, to compare the ability of RIC-3 from three species to enhance receptor expression. In the absence of RIC-3, the proportion of oocytes expressing detectable nAChRs was greatly reduced. Varying the ratio of acr-16 to X. laevis ric-3 cRNAs injected into oocytes had little impact on the total cell current. When X. laevis, human or C. elegans ric-3 cRNAs were co-injected with acr-16 cRNA (1 : 1 ratio), 100 µM acetylcholine induced larger currents in oocytes expressing X. laevis RIC-3 compared with its orthologues. This provides further evidence for a species-specific component of RIC-3 activity, and suggests that X. laevis RIC-3 is useful for enhancing the expression of invertebrate nAChRs in X. laevis oocytes.

Keywords: chaperone, nicotinic acetylcholine receptor, whole-cell voltage clamp, Xenopus oocyte.

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suppress functional expression of nAChRs (Halevi et al. 2003; Castelan et al. 2008), 5-HT₃Rs (Halevi et al. 2003; Castillo et al. 2005; Cheng et al. 2007) and α₇ nAChR/5-HT₃R chimaeras (Castillo et al. 2005; Gee et al. 2007).

RIC-3 is an integral membrane protein with two membrane-spanning domains, TM1 and TM2, the second of which is well conserved between species and has been identified as both necessary and sufficient for receptor modulation (Cohen Ben-Ami et al. 2005; Biala et al. 2009). Studies in Xenopus oocytes with the homologous ACR-16 receptor from C. elegans suggest a conserved TM2 domain is crucial for enhancing expression levels (Cohen Ben-Ami et al. 2009). TM1 may be a cleavable signal sequence in human RIC-3 (Cheng et al. 2007). On the C-terminal end, the TM domains are followed by one or two coiled-coil domains, which are suggested to be responsible for some of the subunit specificity (Halevi et al. 2003; Biala et al. 2009). Alternatively spliced transcripts, some of which do not encode the coiled-coil regions, have been suggested as a means by which the protein may exert even more control over subtypes of nAChRs (Halevi et al. 2003). A recent model suggests that the coiled-coil domains of mammalian RIC-3 do not bind α₇ nAChR directly, but may be part of a homotypic assembly method, bringing multiple subunits, each bound to RIC-3 via the transmembrane region, together (Wang et al. 2009).

The idea that the function of RIC-3 may vary in different cellular environments was first raised by Cheng et al. (2007) in discussing how the effect of RIC-3 on receptor expression can be opposite in mammalian cells versus amphibian oocytes. Lansdell et al. (2008) took this further and demonstrated that Drosophila RIC-3 enhanced nAChR expression to a greater extent in a Drosophila cell line than in a human one, and that human RIC-3 was more effective at enhancing nAChR expression in human cells compared with Drosophila cells. Studies using Xenopus oocytes have so far only utilized C. elegans, D. melanogaster or human RIC-3. Therefore, we have cloned the Xenopus laevis ric-3 cDNA to investigate whether this could be a useful tool for expression studies and screening of nAChRs in oocytes, particularly in respect of invertebrate nAChRs, as in vitro expression of these nAChRs is challenging (Millar and Lansdell 2010).

Invertebrate nAChRs are of medical and economical importance as targets of many important drugs that act against nematode and insect parasites, vectors and pests (Lees et al. 2012). Nematodes express a large number of different nAChR subunits (Mongan et al. 1998), including those making up the well-characterized levamisole-sensitive (L-type) and nicotine-sensitive (N-type) receptors (Lees et al. 2012) as well as those more recently exploited as novel targets for drugs such as monepantel (Kaminsky et al. 2002; Boulin et al. 2008, 2011). We are using the homologous nAChR encoded by the Caenorhabditis elegans acr-16 gene (Raymond et al. 2000) to explore the development of improved systems for the expression of nematode nAChR.

Co-expression of C. elegans ACR-16 with RIC-3 produces robust currents (Biala et al. 2009; Cohen Ben-Ami et al. 2009; Sattelle et al. 2009), and offers a suitable experimental system for comparing RIC-3 from three different species (X. laevis, C. elegans and human). Evidence that RIC-3 itself can be regulated by other proteins in vivo (Shteingauz et al. 2009) has raised the question as to whether an excess of RIC-3 may be deleterious to nAChR expression. In this study, we use differing ratios of the X. laevis ric-3 and C. elegans nAChR subunit acr-16 mRNA to address this issue, and compare the results obtained with the nematode receptor to those from oocytes expressing the human α₇ nAChR (Peng et al. 1994).

Materials and methods

Cloning of X. laevis ric-3 cDNA

One adult X. laevis was terminally anaesthetised using a solution of 0.2% (w/v) benzocaine in tap water and the heart excised. Within 15 min, the brain and spinal cord were removed using an outlined dissection technique (Rowell, 1953), then placed in a 1.5 mL microcentrifuge tube and snap frozen in liquid nitrogen, then stored at −80°C. After thawing, the tissue was placed, along with 2 mL of Trizol® reagent (Invitrogen, Carlsbad, CA, USA), in a 10 mL borosilicate glass homogenizer tube (Jencons, Leighton Buzzard, UK) pre-cleaned with RNase-Away™ (Invitrogen) and the tissue mechanically pulsed using the accompanying pestle. A phenol:chloroform (1:1) extraction was used to isolate the RNA, which was then reversed transcribed into cDNA and used as the template for PCR amplification. Primer sequences for PCR were based on the X. tropicalis ric-3 sequence (NCBI accession number BC118843); the forward sequence was ATGGCTCTGTCCGCTGTCCA and the reverse ATGTAGCAATCAGTACACAATGC. The resulting 1117bp product was cloned into the pGEM®-T Easy (Promega, Madison, WI, USA) and the insert sequenced.

Bioinformatics

Sequences were translated using an online Expasy tool (http://expasy.org/tools/dna.html). Translated sequences were interrogated for transmembrane regions and coiled-coil domains using two servers: TMPred (www.ch.embnet.org/software/TMPRED_form.html) and COIL (www.ch.embnet.org/software/COLLS_form.htm). Signal peptide sequences were predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). ClustalW2 was used for alignments (www.ebi.ac.uk/Tools/msa/clustalw2/).

Expression in Xenopus oocytes

RNA was prepared from plasmid DNA linearized using NcoI (X. laevis ric-3), NotI (C. elegans and human ric-3), Apal (acr-16) or XbaI (human α₇) and synthesized by the T7 (pCDNA3.1) or SP6 (pGEM®-T Easy) mMessage mMachine kits (Ambion, Austin, TX, USA). The C. elegans ric-3 cDNA used was that described in Sattelle et al. (2009); the human ric-3 cDNA encoded variant 1, 912
isoform a. All the cDNA clones were sequenced prior to use to confirm that they encoded a functional protein. The human Stage V and stage VI oocytes were selected from X. laevis ovaries (NASCO, Fort Atkinson, WI, USA) treated with 2.5 mg/mL collagenase for 30 min, washed, then manually defolliculated using fine forceps. The oocytes were kept in chilled Standard Oocyte Saline (SOS) (pH 7.5, 100 mM NaCl, 1.0 mM MgCl₂, 5.0 mM HEPES, 2.0 mM KCl, 1.8 mM CaCl₂) before being injected with a Nanoject 3-00-203-X (Drummond, Broomall, PA, USA). In experiments where the ratio of ric-3 cRNA:acr-16 cRNA was varied, oocytes were injected with 50 ng of acr-16 mRNA with either 200 ng (1 : 4), 50 ng (1 : 1), 12.5 ng (4 : 1) or no (1 : 0) X. laevis ric-3 cRNA. In experiments comparing RIC-3 from the three different species (human, C. elegans and X. laevis), 50 nL of 1 µg/µL cRNA was injected, pre-mixed at a 1 : 1 ratio. After injection, oocytes were incubated 48–72 h at 18°C in a modified SOS solution containing 2.5 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin.

Oocytes were transferred to an oocyte recording chamber and impaled with two mounted glass microelectrodes (3 M KCl and resistance between 0.5 and 5 MΩ in SOS). The membrane potential was clamped with a GeneClamp 500 Amplifier (Molecular Devices, Sunnyvale, CA, USA) at ~100 mV and a resting current below 1 µA. SOS solution was applied at a rate of 3 mL/min, a Minipuls peristaltic pump was used to control the membrane potential (Gilson, Middleton, WI, USA) at +ACH or nicotine were applied; we were not able to measure those currents accurately so they have been discarded in all the subsequent data analyses. However, in a minority of oocytes, ACR-16 expression in the absence of RIC-3, ACR-16 produced very small or undetectable currents in the majority of oocytes, when 100 µM ACh or nicotine were applied; we were not able to measure those currents accurately so they have been discarded in all the subsequent data analyses. However, in a minority of oocytes, ACR-16 expression in the absence of any RIC-3 resulted in substantial ACh-induced currents (Fig. 3).

We tested the effects of varying the relative expression of the X. laevis RIC-3, compared with the ACR-16 receptor, by injecting the two cRNAs at a ratio of 1 : 4, 1 : 1 and 4 : 1 ric-3:acr-16. In each case, the same amount of receptor cRNA was injected into the oocytes. The proportion of oocytes that produced measurable currents in response to application of 100 µM ACh was increased for all ric-3:acr-16 cRNA ratios compared with those cells injected with acr-16 cRNA alone (Table 2). We produced and compared concentration-response curves for various ratios of ric-3:acr-16 cRNAs injected into oocytes. All the EC₅₀ values for acetylcholine were similar (Table 2), regardless of the ratio of acr-16:ric-3 cRNA that was injected into the oocytes and the confidence intervals for the 1 : 1 ratio overlapped with that shown in Fig. 2. In addition, the peak response to application of 100 µM ACh was the same, regardless of the ratio of ric-3 to acr-16 cRNAs tested (Fig. 3). The injection of equal amounts of ric-3 and acr-16 cRNAs was therefore used in subsequent experiments.

A comparison of the effects of RIC-3 from X. laevis, C. elegans and human on the functional expression of ACR-16 and human α7 nAChR

We injected oocytes with acr-16 cRNA together with either X. laevis, human or C. elegans ric-3 cRNA. In each case, equal amounts of the ric-3 and acr-16 cRNAs were injected. Co-injection of the X. laevis ric-3 cRNA with acr-16 resulted in an 1117 bp product that was cloned and sequenced. A BLASTP search using this sequence, translated into protein, showed that it was very similar to RIC-3 from several species and had the highest identity to the full length predicted polypeptide from X. tropicalis. Virtual translation of the cDNA sequence and analysis of the resultant polypeptide sequence revealed that two membrane-spanning regions were predicted by TMPred; the first of these is likely to be a signal peptide (Fig. 1). The predicted polypeptide sequence also contained two predicted coiled-coil domains (Fig. 1) and as such, can be considered equivalent to full length C. elegans RIC-3, rather than the truncated form reported by Halevi et al. (2003). The sequence was deposited in the NCBI sequence database under Accession Number ACF74450.1. Alignment of the translated Xenopus RIC-3 sequence with those from mammals and invertebrates showed 52% identity to the human protein, but only 17% with that from C. elegans (Table 1), despite human RIC-3 lacking one of the coiled-coil domains. The second membrane-spanning regions are especially well conserved, with the X. laevis and human RIC-3 sharing over 90% sequence identity in this region.

The effect of X. laevis ric-3 on the expression of ACR-16 in X. laevis oocytes

Expression of the ACR-16 nAChR was robust when co-expressed with the X. laevis RIC-3, with an EC₅₀ for ACh of 24 µM, (95% CI = 19–30 µM), and a Hill Slope of 2.18 (Fig. 2), consistent with previously published data (Raymond et al. 2000). The EC₅₀ for nicotine was 22 µM (95% CI = 15–33 µM), and a Hill Slope of 1.91. When expressed in the absence of RIC-3, ACR-16 produced very small or undetectable currents in the majority of oocytes, when 100 µM ACh or nicotine were applied; we were not able to measure those currents accurately so they have been discarded in all the subsequent data analyses. However, in a minority of oocytes, ACR-16 expression in the absence of any RIC-3 resulted in substantial ACh-induced currents (Fig. 3)
in significantly larger peak currents upon application of 100 μM ACh (Fig 4) than did co-injection of the human or C. elegans ric-3. The mean current responses to 100 μM ACh were 854 nA for X. laevis RIC-3, 160 nA for C. elegans RIC-3, 247 nA for human RIC-3 and 187 nA for ACR-16 alone. The responses from the oocytes injected with X. laevis ric-3 cRNA were significantly higher than those injected with C. elegans or human ric-3 cRNA, or with none. There were no significant differences in peak amplitude to 100 μM ACh between the C. elegans, human and acr-16 alone conditions, though the proportion of oocytes producing

**Fig. 1** The predicted amino acid sequence and domain structure of X. laevis RIC-3. (a) Alignment of translated sequences for X. laevis, C. elegans and human RIC-3 showing first and second transmembrane (TM) regions (bold) predicted by TMPred; and the first (bold, highlighted light grey) and second (bold, highlighted dark grey) coiled-coil (CC) domains predicted by COILS. (b) Schematic representation of the domains found in RIC-3 from the three species.

**Table 1** Amino acid identity between the predicted domains (Fig 1B) of X. laevis RIC-3 and the human and C. elegans orthologues

| Domain       | C. elegans | Human |
|--------------|------------|-------|
| TM1          | 36% (TM2)  | 91%   |
| CC1          | 37%        | 68%   |
| CC2          | 22%        | N/A   |
| Full-length  | 17%        | 52%   |

The TM2 domain of the C. elegans RIC-3 is likely to be functionally equivalent to the TM1 domain of the human and X. laevis proteins.
detectable currents was significantly lower in the absence of any RIC-3, which confirms that the human and *C. elegans* proteins were being functionally expressed.

As co-expression of the invertebrate ACR-16 receptor with the *X. laevis* RIC-3 led to larger amplitude ACh-induced currents than was achieved with the human or nematode orthologues, we compared the ability of all the three RIC-3 proteins to stimulate expression of the human α7 nAChR. Oocytes were injected with α7 cRNA with or without one of the RIC-3 cRNAs. Interestingly, mean currents detected in response to application of 1 mM ACh were very similar, irrespective of the presence or absence of exogenous RIC-3 (Fig. 5), and a high proportion (> 90%) of oocytes injected with the α7 cRNA expressed currents regardless of whether they were also injected with ric-3 cRNA or not. No statistically significant differences were observed in the EC50 values for ACh from any of these experimental conditions (No RIC-3 = 56 ± 17 μM; human RIC-3 = 38 ± 7 μM; + *C. elegans* RIC-3 = 65 ± 19 μM; + *X. laevis* RIC-3 = 47 ± 20 μM).

**Discussion**

RIC-3 from nematodes, insects and mammals enhances the *in vitro* expression of certain ligand-gated cation channels, including vertebrate α7 nAChRs (*Halevi et al. 2002, 2003; Lansdell et al. 2005*) and the nematode ACR-16 receptor (*Biala et al. 2009; Sattelle et al. 2009*), and RIC-3 is required for the expression of nematode levamisole-sensitive receptors (*Boulin et al. 2008, 2011*). Many of those studies are carried out in the *Xenopus* oocyte system using the mammalian or nematode RIC-3 proteins; however, there is evidence that the activity of RIC-3 is host-cell dependent, with the insect protein being more effective in insect cell cultures and the mammalian protein working better in mammalian cell lines (*Lansdell et al. 2008*). We therefore hypothesized that, for maximum expression in the *Xenopus* oocyte, co-expression with the frog RIC-3 would be optimal. As a first step in

![Fig. 2](image-url)  
**Fig. 2** Concentration-response curves for ACR-16 co-expressed with *X. laevis* RIC-3. The mean peak current amplitude [normalized to 100 μM acetylcholine (ACh)] was recorded from voltage clamped oocytes 48 h post-injection with 50 ng acr-16/Xenopus ric-3 cRNA at a 1 : 1 ratio (n = 8, n = 4). ACh (closed circles) EC50 = 24 μM (95% CI = 19–30 μM), Hill Slope = 2.177. Nicotine (open circles) EC50 = 22 μM (95% CI = 15–33 μM), Hill Slope = 1.906.

![Fig. 3](image-url)  
**Fig. 3** The effect of varying the amount of *X. laevis* RIC-3 on the expression of ACR-16 in *Xenopus* oocytes. (a) The percentage of oocytes expressing detectable currents (>10 nA) in response to 100 μM acetylcholine (ACh) was determined on the experimental weeks in which oocytes from all conditions were screened. The mean percentage expression with the injection of different ratios of acr-16:*X. laevis-ric-3* cRNA was as follows; 49 % for 1 : 4, 32 % for 1 : 1, 23 % for 4 : 1 and 7 % for 1 : 0. A One-Way ANOVA with a Tukey’s Multiple Comparison Test revealed that the 1 : 4 and 1 : 1 ratio conditions gave significantly more frequent detectable expression than in the 1 : 0 ratio condition (** and * respectively). The 1 : 4 ratio condition was also significantly different to the 4 : 1 condition (*). (b) Mean peak amplitude to 100 μM ACh on ACR-16 expressed in oocytes injected with different ratios of *X. laevis* ric-3 cRNA to subunit cRNA showing SEM (n = 5, total n for oocytes equal or above 7). Each symbol represents the mean peak amplitude response from a single oocyte on application of 100 μM ACh. The mean results for each condition were as follows; acr-16:*X. laevis* ric-3 1 : 4 = 662.5 nA, 1 : 1 = 850.7 nA, 1 : 4 = 690 nA, 1 : 0 = 434.4 nA. A one-way ANOVA found that there was no significant difference between the conditions.
testing this hypothesis, we cloned a full length cDNA encoding RIC-3 from *Xenopus laevis*, and found the protein to possess high sequence similarity to human RIC-3 but with two predicted coiled-coil domains, as in the *C. elegans* protein (Halevi et al. 2003). *X. laevis* RIC-3 is also predicted to contain two membrane-spanning domains, the first of which is likely to represent a signal peptide.

To test the ability of the *Xenopus* RIC-3 to enhance invertebrate receptor expression in the oocyte system, we used the *C. elegans* ACR-16 nAChR as a model. ACR-16 forms a homomeric nAChR whose expression in the oocyte system is improved by co-expression with RIC-3 (Biala et al. 2009). The pharmacology of the ACR-16 nAChR was similar to that described previously (Ballivet et al. 1996; Sattelle et al. 2009). One apparent difference between the data reported here and that published previously is in the size of the responses produced by ACR-16 expression in the absence of RIC-3; these have been previously reported to be very small (Biala et al. 2009). The reason for this apparent discrepancy is that we were not able to reliably quantify the size of the currents in the majority of the oocytes expressing ACR-16 alone, and they have been excluded from the data shown. A proportion (16%) of the oocytes injected with acr-16 cRNA, but no ric-3, produced robust currents and the peak amplitudes of these are presented; this proportion was much lower than in the groups injected with acr-16 plus any of the ric-3 cRNAs. These differences were statistically significant (Fig. 4) and demonstrate that the human and *C. elegans* RIC-3 proteins were functional in these experiments. We do not know what allows ACR-16 to be expressed in the absence of the additional RIC-3 chaperone in these few oocytes; it is possible that a small proportion of the oocytes might express low amounts of endogenous chaperones, RIC-3 and others, that permit this expression. Similar ACR-16 currents have been observed by other groups in the absence of RIC-3 (Ballivet et al. 1996; Raymond et al. 2000).

We found no noticeable difference in amplitude of currents or the dose-response curves in oocytes co-injected with
different ratios of *X. laevis* ric-3 cRNA alongside the *acr-16* cRNA, and no effects from injecting up to four times more ric-3 than *acr-16* cRNA. We therefore used a 1 : 1 ratio of ric-3 : *acr-16* cRNAs, when we compared the ability of RIC-3 from *X. laevis*, human and *C. elegans* to enhance the expression of ACR-16. Our data indicate that *Xenopus* RIC-3 co-expressed with ACR-16 produced higher current amplitudes in response to 100 μM ACh than human or *C. elegans* RIC-3. We observed no significant difference between the ability of *C. elegans* and human RIC-3 to enhance the expression of ACR-16, suggesting that the species specificity of the chaperone, at least for some receptors, is dependent more on the host cell than on the receptor being expressed (Landsell et al. 2008).

If the *X. laevis* RIC-3 is more effective than the human or *C. elegans* protein in chaperoning the expression of a nematode nAChR, it raises the question of whether this would also apply to mammalian nAChR. As expression of the human homomeric α7 nAChR resulted in robust responses in a high proportion of oocytes, as previously reported (Peng et al. 1994), regardless of the addition of exogenous RIC-3, we were unable to determine if these findings could be generalized to vertebrate receptors.

Overall, our data suggest that the *X. laevis* RIC-3 might be a useful chaperone for oocyte expression of invertebrate ligand-gated cation channels and, for certain applications, may have advantages over the use of either invertebrate or mammalian forms of the protein.

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