

Alternative Splicing of *gar-1*, a *Caenorhabditis elegans* G-Protein-Linked Acetylcholine Receptor Gene

Yang-Seo Park,^{*,1} Yong-Seok Lee,^{†,1} Nam Jeong Cho,^{*,2} and Bong-Kiun Kaang^{†,2}

^{*}School of Life Sciences, Chungbuk National University, Cheongju, Korea; and [†]Molecular Neurobiology Laboratory, Institute of Molecular Biology and Genetics, Department of Biology, Seoul National University, Seoul, Korea

Received December 20, 1999

We have recently identified a gene, designated *gar-1*, coding for a novel form of G-protein-linked acetylcholine (ACh) receptor in *Caenorhabditis elegans*. Although this receptor is most closely related to muscarinic ACh receptors (mAChRs), electrophysiological analyses have shown that ligand binding specificity of the receptor is distinct from that of mAChRs. Here we report that three receptor isoforms are generated by alternative splicing of the *gar-1* transcript. These receptor isoforms differ only in the third intracellular loop that is considered to be important for G protein coupling. The three splice variants, when expressed in *Xenopus* oocyte, displayed similar pharmacological profiles and signaling activities. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that the three *gar-1* mRNAs are present at all developmental stages examined. The results in this study provide evidence that alternative splicing is involved in promoting molecular diversity of G-protein-linked ACh receptors. © 2000 Academic Press

Key Words: *gar-1*; G-protein-linked acetylcholine receptor; alternative splicing; *Caenorhabditis elegans*.

Acetylcholine (ACh), a major neurotransmitter in the nervous system, exerts its effects through two types of receptors, nicotinic ACh receptor and muscarinic ACh receptor (mAChR). Nicotinic ACh receptors are cation channels, while mAChRs are G-protein-linked receptors that have seven transmembrane domains. To date, five mAChR subtypes (m1–m5) have been identified in mammals (1, 2). The m1, m3, and m5 subtypes are known to preferentially activate phospholipase C, while the m2 and m4 subtypes preferentially

Abbreviations used: ACh, acetylcholine; *C. elegans*, *Caenorhabditis elegans*; GIRK, G-protein-gated inwardly rectifying K⁺ channel; i3 loop, third intracellular loop; mAChR, muscarinic acetylcholine receptor; RT-PCR, reverse transcriptase-polymerase chain reaction.

¹ These authors contributed equally to this work.

² To whom correspondence may be addressed. Fax: 82-431-267-2306. E-mail: namjcho@chungbuk.ac.kr (N.J.C.) or Fax: 82-2-874-1206. E-mail: kaang@snu.ac.kr (B.-K.K.).

inhibit adenylyl cyclase (3, 4). These receptor subtypes share a high degree of amino acid sequence similarity, except for the third intracellular loop (i3 loop). This highly variable i3 loop is believed to determine specificity for G protein coupling (5).

Recently, we reported the isolation of the *gar-1* gene that codes for a novel form of G-protein-linked ACh receptor in the nematode *Caenorhabditis elegans* (*C. elegans*) (6). This GAR-1 receptor is most similar to but pharmacologically different from mAChRs. Electrophysiological studies have shown that muscarinic antagonists, such as scopolamine and pirenzepine, did not effectively inhibit the ACh-induced activation of the GAR-1 receptor and a muscarinic agonist, oxotremorine, failed to activate the receptor (6). In the present study we show that alternative splicing of the *gar-1* gene produces three functional receptor isoforms that differ only in the i3 loop region.

MATERIALS AND METHODS

Isolation of three splice variants of gar-1. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the RNA PCR kit (Takara) following the procedures recommended by the manufacturer. PCR primers used were described (6). PCR products were ligated into pBluescript (Stratagene) and the nucleotide sequences of the clones were determined using the Cy5 AutoRead sequencing kit and ALFexpress DNA sequencer (Pharmacia).

Electrophysiology of receptor isoforms expressed in Xenopus oocytes. Prepreparation and injection of cRNAs encoding GAR-1a, GAR-1b, GAR-1c, pig m2, and G-protein-gated inwardly rectifying K⁺ channel (GIRK1) into oocytes of *Xenopus laevis* were carried out as described in Lee *et al.* (6). The details of perfusion, drug application, and voltage-clamping experiments were also described in Lee *et al.* (6).

Preparation of stage-specific C. elegans RNAs and RT-PCR. Stage-specific *C. elegans* RNAs were prepared as described previously (7). Each stage-specific RNA (1 μg) was reverse transcribed in a total volume of 10 μl using the 1st strand cDNA synthesis kit (Boehringer Mannheim) according to the manufacturer's instructions. For PCR, 5 μl RT reaction mix was added to a 20-μl solution containing 1.875 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 μM forward primer (MF-i3), 0.25 μM reverse primer (MR-i3), and 0.625 U *Taq* polymerase (Takara). Conditions for PCR were as follows: an initial denaturation at 94°C for 2 min, 30 cycles of amplification (30 s at 94°C, 30 s at 55°C, and

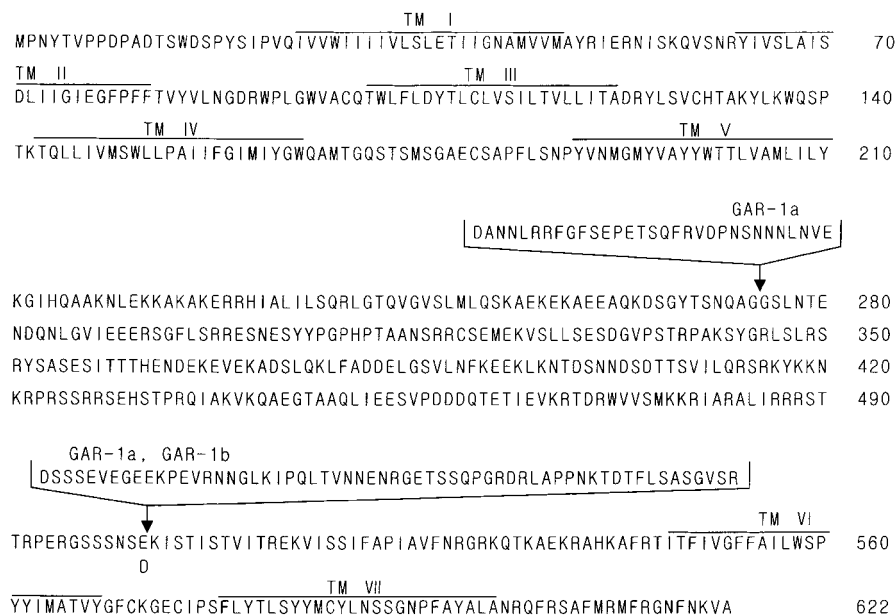


FIG. 1. The deduced amino acid sequences of the three *C. elegans* G-protein-linked ACh receptor (GAR-1) isoforms. The amino acid sequence of GAR-1c is shown and numbered. The extra residues present in GAR-1a and GAR-1b are indicated. Glutamate (E), the 502nd residue in GAR-1c, is converted to aspartate (D) in GAR-1a and GAR-1b as a result of alternative splicing (see Fig. 2 and text). Seven transmembrane domains (TM I-TM VII) are indicated. The nucleotide sequences of *gar-1a*, *gar-1b*, and *gar-1c* have been submitted to GenBank under Accession Nos. AF117300, AF075245, and AF117301, respectively.

2 min at 72°C), and a final extension at 72°C for 10 min. PCR primers used were: MF-i3, 5'-CGTGAATCAAGGGTATTTCATCAAGCA-3'; MR-i3, 5'-CGGGAATTCGTCTTCTGAATGCTTTATGAGC-3' (*EcoRI* site created is underlined).

RESULTS

We have previously isolated and characterized a cDNA encoding GAR-1, a *C. elegans* G-protein-linked ACh receptor (6). The *gar-1* gene was shown to contain 16 introns in the coding region, suggesting a possibility of alternative splicing. To test this possibility, we performed RT-PCR experiments using primers designed to span various parts of the coding region. When we used a primer pair flanking the i3 loop, we observed three PCR products that hybridized to the *gar-1* gene. This suggested to us that *gar-1* encodes at least three alternatively spliced transcripts.

To determine the molecular details of the alternative splicing in the *gar-1* gene, we isolated RT-PCR products and analyzed them by restriction enzyme mapping and DNA sequencing. Three distinct cDNAs were obtained and named, from the longest, *gar-1a*, *gar-1b*, and *gar-1c*. GAR-1a, GAR-1b, and GAR-1c are predicted to have 713, 682, and 622 amino acids, respectively. GAR-1b corresponds to the receptor that we described previously (6). The three GAR-1 isoforms differ only in the i3 loop region. GAR-1a contains an insertion of 31 amino acids in the i3 loop compared with GAR-1b (Fig. 1). GAR-1c is identical to GAR-1b except for a deletion of 60 amino acids and

a substitution of one amino acid in the i3 loop, Glu⁵⁰² for Asp (Fig. 1).

Comparison of the cDNAs with the corresponding genomic sequence revealed an additional exon (exon 9) that was not identified in the previous study (6) (Fig. 2). Furthermore, it showed that the three *gar-1* transcripts result from alternative splicing of three exons (exons 9, 15, and 16) (Fig. 2). The 93-bp exon 9 is present only in *gar-1a*, while the 171-bp exon 15 is present in *gar-1a* and *gar-1b*. Exon 16 is present in all three *gar-1* cDNAs, but the size of the exon in *gar-1c* (129 bp) is 9 bp shorter than the other two cDNAs (138 bp). This is due to alternative use of two splice acceptor sites at the 5' end of exon 16 (Fig. 2). In *gar-1a* and *gar-1b*, exon 15 is spliced to the first splice acceptor site in exon 16, whereas in *gar-1c*, exon 14 is spliced to the second splice acceptor site in exon 16, thus skipping exon 15 and omitting the first 9 bp of exon 16. This results in the conversion of the last amino acid of exon 14 from aspartate in *gar-1a* and *gar-1b* to glutamate in *gar-1c*.

Since the i3 loop is known to be critical for G protein coupling, the alternative splicing in this region might affect the coupling property of the receptor. To examine this possibility, we compared the coupling efficacy of the three GAR-1 isoforms by expressing in *Xenopus* oocytes each receptor isoform together with GIRK1 channel. As shown in Fig. 3, the GIRK1-mediated current was activated by ACh in the high K⁺ solution irrespective of the receptor isoforms (209.9 ± 39.8 nA,

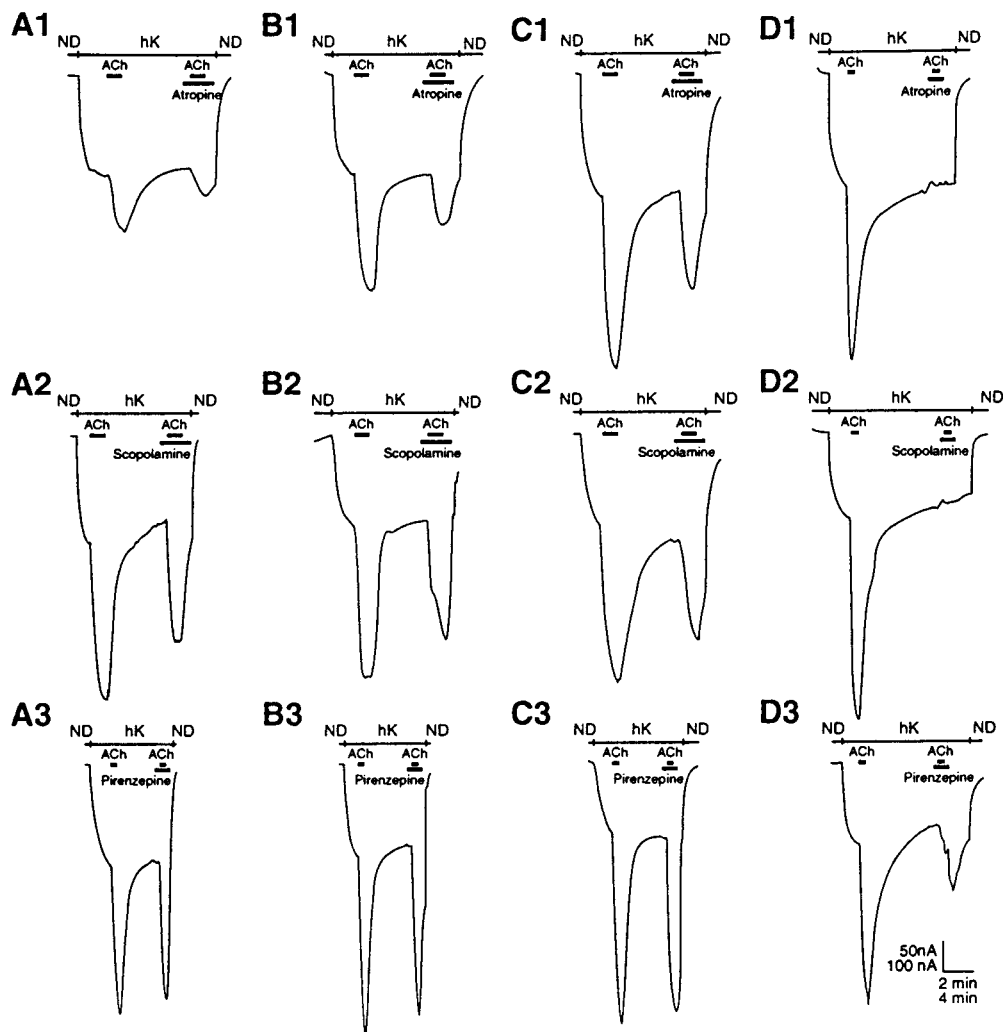


FIG. 3. Functional expression of GAR-1 isoforms in *Xenopus* oocytes and their antagonist specificity. Oocytes were injected with cRNA (2.5 ng each) for GAR-1a (**A1**, **A2**, **A3**), GAR-1b (**B1**, **B2**, **B3**), GAR-1c (**C1**, **C2**, **C3**), or pig m2 mAChR (**D1**, **D2**, **D3**) together with GIRK1 cRNA (2.5 ng). All the current traces were recorded at a holding potential of -70 mV in two-electrode voltage-clamp. The presence of drugs in the bathing solution is indicated by bars. Perfusion of ND96 (ND) and high K^+ (hK) solutions and drug applications were done as described in Lee *et al.* (6). To evoke the GIRK1-mediated inward current, $1 \mu\text{M}$ acetylcholine (ACh) was applied. **A1**, **B1**, **C1**, **D1**, atropine ($1 \mu\text{M}$); **A2**, **B2**, **C2**, **D2**, scopolamine ($1 \mu\text{M}$); **A3**, **B3**, **C3**, **D3**, pirenzepine ($1 \mu\text{M}$). The scale bar indicates 100 nA and 4 min for **A3**, **B3**, and **C3** and 50 nA and 2 min for all the other traces.

be alternatively spliced. In fact, no intron has been found in the coding region of mammalian G-protein-linked ACh receptor (that is, mAChR) genes. Although *Drosophila* mAChR gene has been reported to contain three introns in the coding region (12), the existence of splice variants has not been known. Collectively, our data indicate that alternative splicing is a mechanism to increase molecular diversity of G-protein-linked ACh receptors in *C. elegans*.

Electrophysiological experiments using the *Xenopus* oocyte expression system showed that the three GAR-1 isoforms are coupled to the activation of GIRK1 channel. The receptor isoforms appeared functionally similar with respect to ligand specificity (Fig. 3) and dose-response curve (Fig. 4). The physiological significance of alternative splicing of the *gar-1* gene is not clear yet.

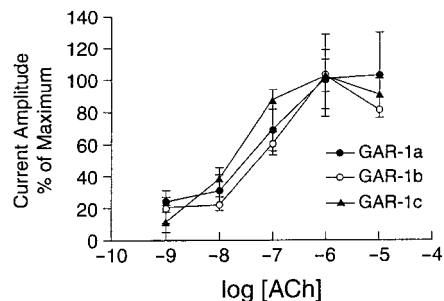


FIG. 4. Dose-response curves for three GAR-1 isoforms. The amplitude of the peak current was measured and normalized to the maximum value obtained for each isoform. Each data point in the curves consists of recordings from 3 to 6 oocytes.

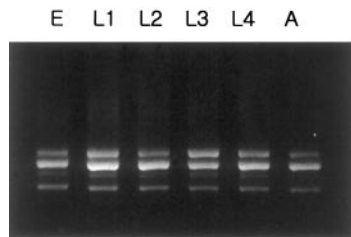


FIG. 5. RT-PCR of the three *gar-1* mRNAs at various developmental stages. RT-PCR was performed, with a primer pair flanking the i3 loop (MF-i3/MR-i3), using stage-specific RNA as a template (see Materials and Methods). The PCR products were analyzed on a 1% agarose gel. The expected sizes of the PCR products for *gar-1a*, *gar-1b*, and *gar-1c* are 1281, 1188, and 1008 bp, respectively (from top to bottom). E, embryo; L1, first larval stage; L2, second larval stage; L3, third larval stage; L4, fourth larval stage; and A, young adult.

It is possible that the three receptor isoforms may interact with distinct G proteins since the three isoforms differ only in the i3 loop region that is thought to be critical for determining G protein specificity. Actually this has been seen in the case of D2 dopamine receptor, another well-known G-protein-linked neurotransmitter receptor. Two alternatively spliced D2 receptor isoforms, that are identical except for an insertion of 29 amino acids in the i3 loop (13, 14), were reported to have differential specificity to G proteins (15). It is also possible that sequestration of the GAR-1 isoforms may be different as the i3 loop has been implicated in receptor sequestration (16). In fact, the extent of sequestration between the two D2 receptor isoforms was reported to be different upon agonist treatment (17).

In *C. elegans* two additional genes, designated *gar-2* and *gar-3*, code for G-protein-linked ACh receptors. GAR-3, when expressed in Chinese hamster ovary cells, has been shown to stimulate phosphatidylinositol metabolism and exhibit virtually identical pharmacological profile to mammalian mAChRs (7). GAR-2, which is encoded by a gene located on cosmid F47D12, activates GIRK1 channel in response to ACh and displays pharmacological properties distinct from those of mAChRs (manuscript in preparation). Our preliminary data indicate the existence of three *gar-2* and two *gar-3* splice variants, suggesting that alternative splicing serves as a general mechanism for diversifying G-protein-linked ACh receptors in the nematode.

ACKNOWLEDGMENTS

We thank Ueon Suk Kim for her help with DNA sequencing. This study was supported by Grant 1998-019-D00157 to N.J.C and B.-K.K. from the Ministry of Education, Republic of Korea, and partly by Korea Ministry of Science and Technology under the Brain Science Research Program to B.-K.K.

REFERENCES

- Bonner, T. I., Buckley, N. J., Young, A. C., and Brann, M. R. (1987) *Science* **237**, 527–532.
- Bonner, T. I., Young, A., Brann, M. R., and Buckley, N. J. (1988) *Neuron* **1**, 403–410.
- Peralta, E. G., Ashkenazi, A., Winslow, J. W., Ramachandran, J., and Capon, D. J. (1988) *Nature* **334**, 434–437.
- Ehlert, F. J., and Yamamura, H. I. (1995) in *Molecular Mechanisms of Muscarinic Acetylcholine Receptor Function* (Wess, J., Ed.), pp. 47–69, R. G. Landes, Austin.
- Wess, J., Liu, J., Bluml, K., Yun, J., Schoneberg, T., and Blin, N. (1995) in *Molecular Mechanisms of Muscarinic Acetylcholine Receptor Function* (Wess, J., Ed.), pp. 1–17, R. G. Landes, Austin.
- Lee, Y.-S., Park, Y.-S., Chang, D.-J., Hwang, J. M., Min, C. K., Kaang, B.-K., and Cho, N. J. (1999) *J. Neurochem.* **72**, 58–65.
- Hwang, J. M., Chang, D.-J., Kim, U. S., Lee, Y.-S., Park, Y.-S., Kaang, B.-K., and Cho, N. J. (1999) *Receptors Channels*, in press.
- Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) *Nature* **365**, 166–170.
- Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., and Journot, L. (1993) *Nature* **365**, 170–175.
- Moore, E. E., Kuestner, R. E., Stroop, S. D., Grant, F. J., Matthewes, S. L., Brady, C. L., Sexton, P. M., and Findlay, D. M. (1995) *Mol. Endocrinol.* **9**, 959–968.
- Shyu, J.-F., Inoue, D., Baron, R., and Horne, W. C. (1996) *J. Biol. Chem.* **271**, 31127–31134.
- Shapiro, R. A., Wakimoto, B. T., Subers, E. M., and Nathanson, N. M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9039–9043.
- Giros, G., Sokoloff, P., Martres, M.-P., Riou, J.-C., Emorine, L. J., and Schwartz, J.-C. (1989) *Nature* **342**, 926–929.
- Monsma, F. J., Jr., McVittie, L. D., Gerfen, C. R., Mahan, L. C., and Sibley, D. R. (1989) *Nature* **342**, 926–929.
- Montmayeur, J.-P., Guiramand, J., and Borrelli, E. (1993) *Mol. Endocrinol.* **7**, 161–170.
- Shockley, M. S., Lameh, J., and Sadee, W. (1995) in *Molecular Mechanisms of Muscarinic Acetylcholine Receptor Function* (Wess, J., Ed.), pp. 183–195, R. G. Landes, Austin.
- Itokawa, M., Toru, M., Ito, K., Tsuga, H., Kameyama, K., Haga, T., Arinami, T., and Hamaguchi, H. (1996) *Mol. Pharmacol.* **49**, 560–566.