MCN

Molecular and Cellular Neuroscience **9,** 132–143 (1997)
Article No. CN970613

Chick Muscle Expresses Various ARIA Isoforms: Regulation during Development, Denervation, and Regeneration

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Acetylcholine receptor inducing activity (ARIA) is a glycoprotein released from the motor neuron to stimulate the synthesis of acetylcholine receptors (AChRs) on the postsynaptic muscle fiber. Transcripts encoding ARIA were detected not only in brain but also in muscle, and immunohistochemical staining showed that muscle-derived ARIA was restricted to the neuromuscular junctions. RT-PCR analysis revealed three biological active isoforms of ARIA in chick muscle, namely ARIA$_{\beta 1}$, ARIA$_{\alpha 2}$, and ARIA$_{\beta 2}$ that were classified based on their variation in the carboxyl-terminus of the EGF-like domain. The expression of these ARIA isoforms in muscle changed during development, denervation, and nerve regeneration. ARIA$_{\beta 1}$, ARIA$_{\alpha 2}$, and ARIA$_{\beta 2}$ were expressed in embryonic and young chick muscles, while ARIA$_{\beta 1}$ was the major isoform expressed in adult chicken. The embryonic-like expression of ARIA$_{\alpha 2}$ and ARIA$_{\beta 2}$ was induced after nerve injury in adult chicken. However, the prominent expression of ARIA$_{\beta 1}$ in adult-like profile was restored after nerve regeneration. A splicing variation in the region between Ig-like and EGF-like domains of ARIA was also revealed; a zero-amino acid insertion (ARIA$_{SP0}$), a 17-amino acid insertion (ARIA$_{SP17}$), or a 34-amino acid insertion (ARIA$_{SP34}$) were identified. Unlike ARIA$_{SP0}$, the expression of ARIA$_{SP17}$ and ARIA$_{SP34}$ was found in muscle and sciatic nerve only. The expression of ARIA$_{SP0}$, ARIA$_{SP17}$, and ARIA$_{SP34}$ in chick muscle remained unchanged during development and after nerve injury. Moreover, the specific expression of these ARIA isoforms in cultured myotubes was not affected by drug treatments or by coculturing with neurons. Our findings provide strong evidence that muscle ARIA may play an important role in the formation of neuromuscular junctions.

INTRODUCTION

During the formation of neuromuscular junctions, the motor neuron innervates the target muscle and induces the postsynaptic specializations that include the aggregation of nicotinic acetylcholine receptors (AChRs) and the increase of AChR expression on the postsynaptic membrane (Salpeter, 1987; Hall and Sanes, 1993). Agrin, a synapse-organizing protein, is implicated to be important in directing the aggregation of AChRs and other postsynaptic molecules at the neuromuscular junctions (McMahan, 1990), while acetylcholine receptor inducing activity (ARIA) is a nerve-derived factor that stimulates the synthesis of AChR on the surface of myotubes (Falls *et al.*, 1990, 1993). It has been demonstrated that the application of ARIA increases the rate of AChR synthesis in cultured avian and mammalian muscles; the increase is accompanied by a large and selective increase in the expression of AChR subunit mRNAs (Harris *et al.*, 1988, 1989; Martinou *et al.*, 1991; Chu *et al.*, 1995; Pun and Tsim, 1995). The adult expression of AChR ϵ-subunit mRNA was also induced by the application of ARIA in cultured myotubes (Chu *et al.*, 1995; Si *et al.*, 1996; Tansey *et al.*, 1996). In addition, ARIA increases the number of voltage-gated sodium ion channels in cultured chick muscles (Corfas and Fischbach, 1993). Thus, it is believed that ARIA plays a pivotal role in the development and maintenance of postsynaptic specializations at the neuromuscular junctions.

cDNA encoding chick ARIA was cloned (Falls *et al.*, 1993). Sequence analysis shows that ARIA belongs to a neuregulin family and has diverse functions in neuron development (Meyer and Birchmeler, 1995; Jo *et al.*, 1995). Members of neuregulin include rat *neu* differentiation factor (NDF; Wen *et al.*, 1994), human heregulin (HRG; Holmes *et al.*, 1992), and bovine glial growth factor (GGF; Marchionni *et al.*, 1993). ARIA is synthesized as a transmembrane precursor molecule, called pro-ARIA, that has Ig-like domain, EGF-like domain, hydrophobic domain, and intracellular domain (Falls *et*

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al., 1993). Like other members of the neuregulin family, ARIA exists in many isoforms that are derived by alternative RNA splicing. The most common splicing site is at the carboxyl-terminus of the EGF-like domain that determines two major classes of isoforms termed α and β. Within each class, there are subclasses that are classified according to the region downstream of the intracellular domain (Holmes *et al.*, 1992; Wen *et al.*, 1994).

Two potential proteolytic cleavage sites have been proposed in pro-ARIA: (i) between the EGF-like and the hydrophobic domains at K_{205} and R_{206} for the release of soluble ARIA; and (ii) between the Ig-like and the EGF-like domains for the release of functional EGF-like fragment from the soluble ARIA. According to the ARIA release hypothesis, proposed by Loeb and Fischbach (1995), pro-ARIA is synthesized by the motor neuron as a transmembrane molecule and released as a soluble molecule at the proteolytic cleavage site at K_{205} and R_{206} dibasic residues. The soluble ARIA is bound to the negatively charged glycosaminoglycan in the synaptic cleft by electrostatic interactions with the Ig-like domain of ARIA, and it is believed that the glycosaminoglycan is the storage site of ARIA at neuromuscular junctions. Further cleavage at the region between the Ig-like and the EGF-like domains releases the functional EGF-like fragment from soluble ARIA. The active EGF-like fragment diffuses across the synaptic cleft and acts on the appropriate receptor on postsynaptic membrane to trigger its biological actions.

While ARIA is expressed predominantly in nervous tissues (Corfas *et al.*, 1995), ARIA expression is also detected at the neuromuscular junctions. Immunohistochemical analysis on mouse muscle fibers revealed the expression of ARIA at synaptic sites. ARIA immunoreactivity was also extended around the circumference of muscle fibers (Chu *et al.*, 1995; Jo *et al.*, 1995; Moscoso *et al.*, 1995; Sandrock *et al.*, 1995). In comparison with the synaptic staining, the level of extrasynaptic staining reduced during development from the first postnatal week to the adult stage (Moscoso *et al.*, 1995). Besides, ARIA mRNA was expressed in chick muscles (Pun and Tsim, 1995), rat muscles, and cultured sol 8 mouse muscle cells (Moscoso *et al.*, 1995). These results suggest that muscle expresses its own ARIA. Whether they contribute to the AChR-inducing activity during the formation of neuromuscular junctions is, however, unknown. In order to determine the possible roles of muscle ARIA in the formation of neuromuscular junctions, we identified different isoforms of ARIA transcript from chick muscles and determined its expression profile during development. Embryonic and adult animals exhibit a very different expression profile of ARIA isoforms, and the profile of expression was regulated during development, denervation, and regeneration after nerve injury.

RESULTS

Isoforms of Muscle ARIA

By using primers (S-2 and AS-2) flanking the carboxyl-terminus of EGF-like domain of chick ARIA (Fig. 1), RT-PCR was performed on various tissues, including brain, pectoral muscle, sciatic nerve, and optic lobe from E18 chick embryos. All tissues gave a major band of 150 bp, and two additional PCR products with sizes of 144 and 125 bp were exclusively detected in muscle and sciatic nerve (Fig. 2A). The identified PCR products were confirmed by DNA sequencing, and they represented the partial sequence at the carboxyl-terminus of the EGF-like domain of chick ARIA β1, α2, and β2 based on their sequence comparison with the mammalian counterparts (Fig. 2B). The chick ARIA isoforms share over 80% amino acid homology to rat NDF (Fig. 2C). Other isoforms of chick ARIA were not identified after screening the PCR products.

To determine the splicing variants at the region between the Ig-like and the EGF-like domains, S-1 and AS-1 primers (see Fig. 1) were used for RT-PCR analysis (Fig. 3A). Splicing variants of ARIA were identified in muscle and sciatic nerve, and the splicing region corresponded to the known spacer domain of neuregulin. These ARIA variants are: a zero-amino acid insertion, a 17-amino acid insertion, and a 34-amino acid insertion, namely $ARIA_{SP0}$, $ARIA_{SP17}$, and $ARIA_{SP34}$, respectively (Fig. 3B). $ARIA_{SP0}$ was detected in nerve tissue, muscle, and sciatic nerve, while the expression of $ARIA_{SP17}$ and $ARIA_{SP34}$ was found only in sciatic nerve, muscle, and cultured myotubes (Fig. 3A). Thus, the source of $ARIA_{SP17}$ and $ARIA_{SP34}$ at the neuromuscular junction was from muscle and Schwann cells instead of motor neurons. Although the length and the site of spacer domain insertion in chick ARIA are very similar to other members of neuregulin, the amino acid sequence within the spacer domain shows only 30% amino acid homology to its mammalian counterparts (Fig. 3C). The two splicing sites, the carboxyl-terminus of EGF-like domain and the spacer domain, were interchangeable. RT-PCR analysis, by using S-1 and AS-2 primers flanking both of the splicing sites (see Fig. 1), showed that the cDNAs encoding nine different combinations of ARIA isoforms were identified. However, the exact amount of each ARIA isoform within muscle was not determined.

The RT-PCR analysis was performed on RNA isolated

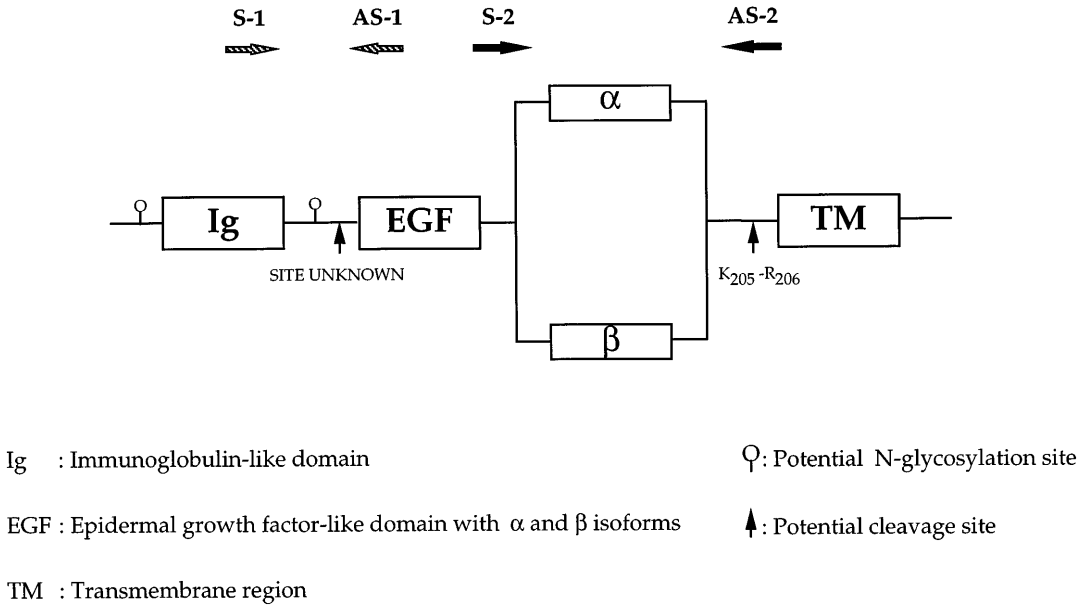

FIG. 1. A schematic diagram shows the full-length pro-ARIA. The predicted pro-ARIA consists of 602 amino acids. From N-terminal to C-terminal, it has immunoglobulin-like domain (Ig), epidermal growth factor-like domain (EGF) with two major splicing variants, α and β, and transmembrane region (TM). Soluble form of ARIA is released by the cleavage at the dibasic residues K_{205}-R_{206} at the C-terminus of the EGF-like domain. The active EGF-like domain is released by proteolytic activity at a region between the Ig-like and the EGF-like domains (termed "spacer" domain) from the soluble ARIA. Primers used for RT-PCR analysis were indicated by arrows.

from muscle masses; the detected ARIA expression could be derived from other cell types instead of muscle cells. Anti-ARIA antibodies, both anti-α isoform and anti-α, β isoforms specific antibodies, were used to stain a P14 chick muscle section. The antibody staining was colocalized with rhodamine-conjugated α-bungarotoxin, indicating the restricted localization of ARIA at the neuromuscular junctions (Fig. 4). The staining of anti-ARIA α isoform-specific antibody at the junctions indicated that the muscle and the Schwann cell could contribute part of the synaptic ARIA, because the motor neuron did not express the α isoform.

Functional Activity of Muscle ARIA

To determine the biological activities of identified muscle ARIA isoforms (β1, α2, and β2), cDNAs encoding the EGF-like domain of these isoforms were tagged with human immunoglobulin Fc cDNA and subcloned into mammalian expression vector. The chimeric constructs were transfected into human embryonic kidney fibroblast (HEK) 293 cells and the ARIA-Fc fusion proteins were purified by protein-G column (Yang *et al.*, 1997). Application of the purified protein onto cultured myotubes induced (i) the expression of AChR α-subunit by ~3-fold (Fig. 5A), and (ii) the tyrosine phosphorylation of erbB 3 receptor (Fig. 5B). Although we did not quantify the precise potencies of different ARIA isoforms, they all exhibited the AChR-inducing activity in cultured myotubes. The functional expression of different variants within the spacer domain were not tested, because they do not contain the functional region of ARIA.

Developmental Expression of Muscle ARIA

Studies on the expression of muscle ARIA isoforms during development revealed that $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ were detected from E7 to P14 chicks, indicating an embryonic-like expression profile. $ARIA_{\beta 1}$ was the major form of ARIA being detected in adult muscle representing an adult-like expression profile (Fig. 6A). When we analyzed the relative amount of each ARIA isoform expression, the levels of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ remained relatively unchanged from E7 to P14 chicks. The expression of $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ was reduced to less than 10% of the total ARIA expression in adult chicken muscles, while the $ARIA_{\beta 1}$ became the prominent species in adult chicken muscles (Fig. 6A). Expression of ARIA mRNA in spinal cord at the corresponding time points was used as a control, and $ARIA_{\beta 1}$ was the major isoform being detected throughout the development. For quantifying the muscle ARIA mRNA

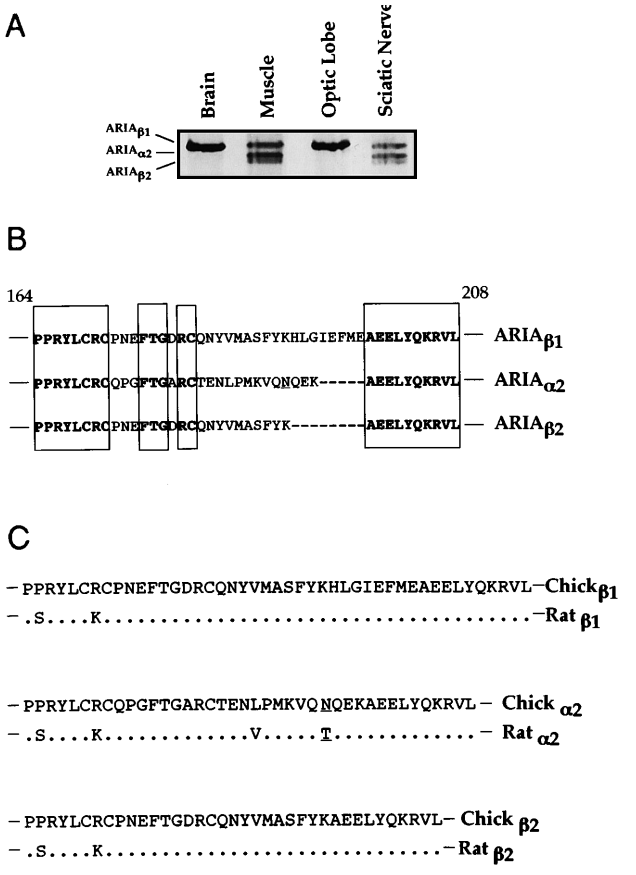

FIG. 2. $ARIA_{\beta1}$, $ARIA_{\alpha2}$ and $ARIA_{\beta2}$ are expressed in chick muscle. (A) Total RNAs extracted from different tissues were reverse transcribed with random hexamer to obtain the first strand cDNAs. PCR was done with S-2 and AS-2 primers (see Fig. 1). The PCR products were analyzed by a 12% acrylamide gel and stained by ethidium bromide. (B) $ARIA_{\beta1}$, $ARIA_{\alpha2}$, and $ARIA_{\beta2}$ cDNAs revealed by RT-PCR were sequenced. Homologous regions among the three isoforms are boxed, and the amino acid position is numbered according to Falls *et al.* (1993). (C) Sequence comparison of the three isoforms with rat NDF counterparts at the same region. Unmatched amino acids are indicated. The underlined amino acid in the α2 form determine a and b subclasses.

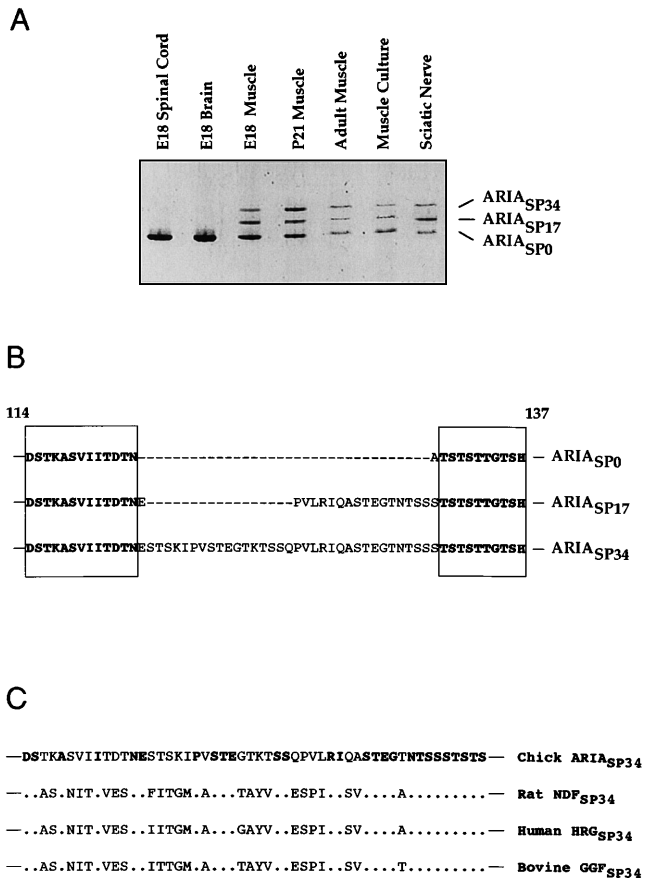

FIG. 3. $ARIA_{SP0}$, $ARIA_{SP17}$, and $ARIA_{SP34}$ are expressed in chick muscle. (A) Reverse transcription was used to prepare first strand cDNAs from total RNAs. The cDNAs were amplified by PCR with S-1 and AS-1 primers (see Fig. 1). Ages of chicken are indicated from Embryonic Day 18 (E18) to 3-month-old chicken (Adult). $ARIA_{SP17}$ and $ARIA_{SP34}$ are specifically expressed in muscle and sciatic nerve. (B) Sequence alignment of $ARIA_{SP0}$, $ARIA_{SP17}$, and $ARIA_{SP34}$ cDNAs. Homologous regions among the three isoforms are boxed. Amino acid position is numbered according to Falls *et al.* (1993). (C) Sequence comparison of the 34 amino acid insertion with rat, human, and bovine neuregulins. Unmatched amino acids are indicated.

expression, RNase protection assay was performed to detect the ARIA mRNA from E7 to adult chicken; a peak expression of ARIA was revealed during the early stages of development. However, the expression of ARIA in muscle was barely detectable in adult chicken indicating the decrease of ARIA expression during synapse maturation (Fig. 6B).

Expression of Muscle ARIA during Denervation and Nerve Regeneration

In P18-denervated gastrocnemius muscle, the embryonic-like expression profile of ARIA ($ARIA_{\beta1}$, $ARIA_{\alpha2}$, and $ARIA_{\beta2}$) was detected; this embryonic-like expression profile remained after 30 days of postdenervation (Fig. 7 Denervation). In contrast, the adult-like expression profile (primarily $ARIA_{\beta1}$) was detected in the sham-operated control (Fig. 7 Control). In the nerve-crushed muscle, the adult-like expression profile of muscle ARIA was restored after 30 days of nerve regeneration (Fig. 7 Regeneration). Denervation increased the level of ARIA mRNA expression; Northern blot analysis of the denervated muscles showed that the mRNA expression of ARIA was up-regulated by ~5-fold after denervation, in particular, in the first 2 days postsurgery (Fig. 8). The effectiveness of the muscle

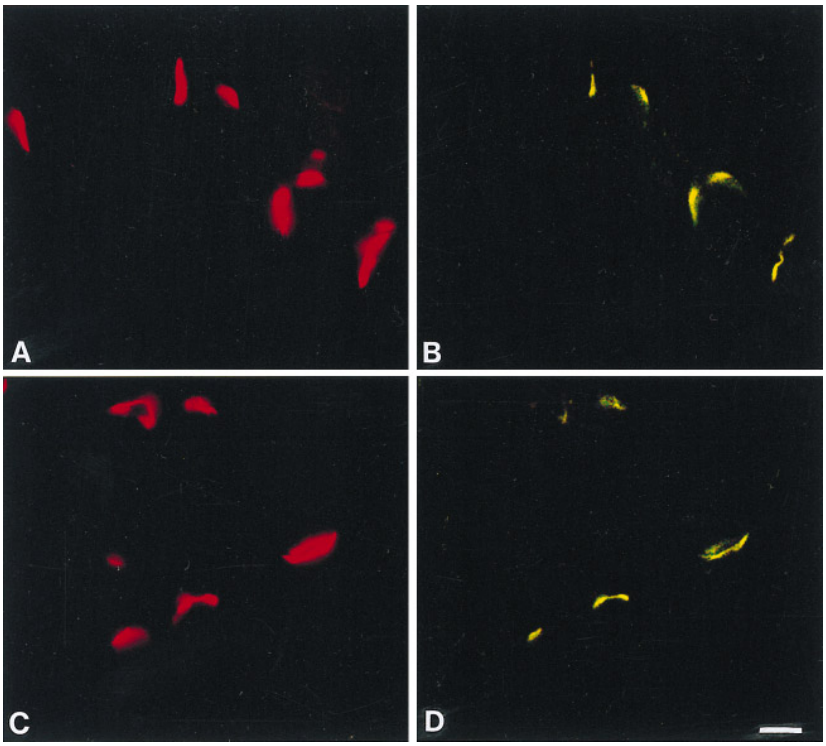

FIG. 4. Colocalization of ARIA immunoreactivity with AChR aggregate at the neuromuscular junctions. Sixteen-micrometer section of 2-week-old chick pectoral muscle was used. The muscle section was double stained with rhodamine-conjugated α-bungarotoxin and anti-ARIA antibodies followed by fluorescein isothiocyanate-conjugated secondary antibody. (A) Rhodamine-conjugated α-bungarotoxin staining; (B) Same view as (A) but stained with anti-ARIA antibody specific for both α and β isoforms. (C) Rhodamine-conjugated α-bungarotoxin staining; (D) Same view as (C) but stained with ARIA α isoform-specific antibody. All the rhodamine and fluorescein staining are colocalized. Bar, 15 μm.

denervation and nerve regeneration was confirmed by the regulation of AChR α-subunit mRNA as previously reported (Ip *et al.*, 1996; Tsim *et al.*, 1997).

An induction of $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ expression was revealed in the contralateral muscle of denervated chicken; the effect was similar to the ipsilateral denervated muscle (Fig. 9). The sham-operated chicken showed no change in its ARIA isoform expression (Fig. 9). In the analysis of ARIA isoforms at the spacer domain, there was no significant change in the expression of ARIA isoforms ($ARIA_{SP0}$, $ARIA_{SP17}$, and $ARIA_{SP34}$) in response to denervation (Fig. 10). Thus, denervation could affect the RNA splicing of muscle ARIA in the region close to the carboxyl-terminus, but not the expression of the spacer domain with different amino acid insertions.

ARIA in Cultured Chick Myotube Cultures

Because the expression of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ isoforms in muscle was regulated during development and denervation, we then analyzed the effects of paralyzing agents on the profile of ARIA isoform expression in cultured myotubes. All the agents examined, including tetrodotoxin (TTX), veratridine, a depolarizing concentration of KCl, and ascorbic acid, showed little or no effect on the expression of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ isoforms in cultured myotubes (Fig. 11A). The expression of AChR α-subunit mRNA was altered by these drug treatments, indicating the effectiveness of such treatments (data not shown). A similar observation was made in myotubes cocultured with a neuroblastoma X glioma hybrid cell line NG108-15; the expression of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ isoforms in myotubes remained unchanged in the presence of neurons (Fig. 11B). Thus, the switch of ARIA isoform expression in developing muscles might not be a result of direct nerve contact or polarization and depolarization of the postsynaptic muscle membrane.

DISCUSSION

Our data provide the first demonstration that chick muscle expresses different isoforms of ARIA and their

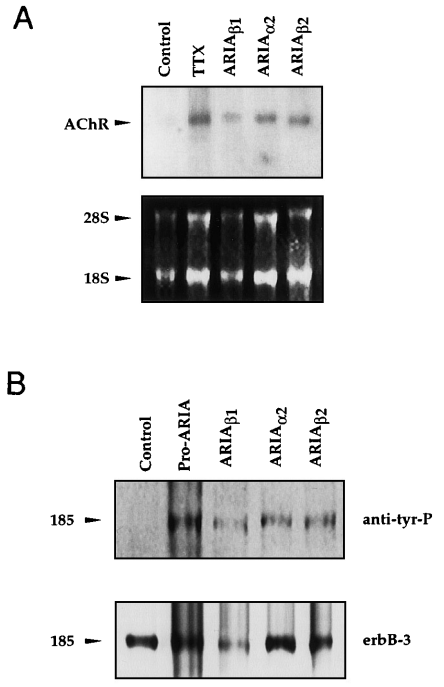

FIG. 5. Full biological activity of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$. The EGF-like domain of different muscle ARIA isoforms (β1, α2, and β2) were tagged with human immunoglobulin Fc fragment. The chimeric fusion protein was purified by protein-G column and ~1 μg of the purified protein was applied onto cultured myotubes for activity assay. (A) The expression of AChR α-subunit mRNA in cultured chick myotubes was increased by recombinant ARIA-Fc proteins. About 20 μg of total RNA was loaded onto an 1% formaldehyde-agarose gel and transferred overnight onto a nylon membrane and hybridized with a cDNA probe of ~1.2 kb chick AChR α-subunit. Myotube cultured alone and 1 μ*M* tetrodotoxin-treated (TTX) myotubes served as negative and positive controls, respectively. Isoforms of muscle ARIA (β1, α2, and β2) show the AChR-inducing activity. Similar results were obtained in several experiments. Lower panel shows ribosomal RNA at 28S and 18S. (B) The erbB 3 receptor was tyrosine phosphorylated by the recombinant ARIA-Fc proteins. The ARIA-treated C2C12 myotubes were lysed in PBS, pH 7.4, 1% NP-40, 0.5% dexocoxylate, 0.1% SDS, 1 m*M* PMSF, 1 m*M* orthovanadate, 1 m*M* aprotinin, and immunoprecipitated by anti-erbB 3 antibody, followed by protein-G beads. The proteins were separated by a 7.5% acrylamide gel and blotted. Upper panel shows the recognition of anti-phosphotyrosine antibody. Lower panel shows the same blot probed with anti-erbB 3 antibody, indicating the phosphorylated protein is, indeed, erbB 3 receptor. Recombinant pro-ARIA as described in Pun and Tsim (1995) is a positive control. Molecular marker of kDa is shown.

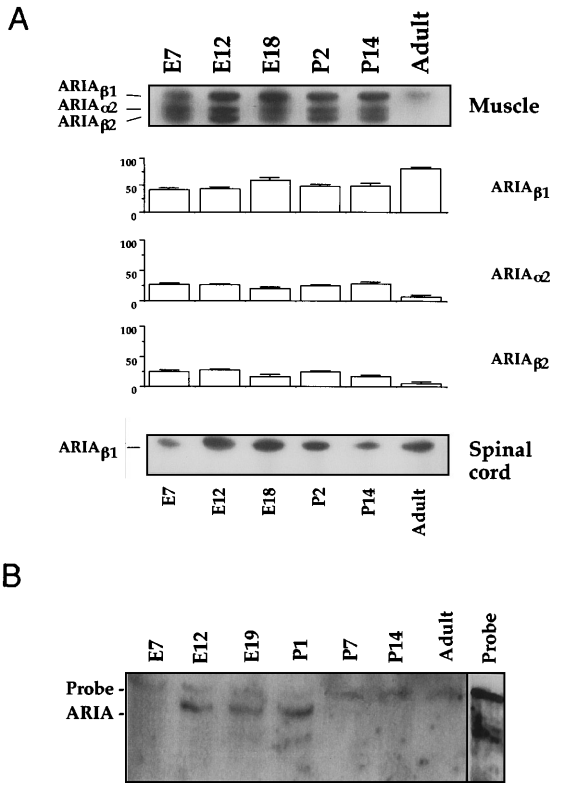

FIG. 6. Differential expression of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ in developing chick pectoral muscle. (A) RT-PCR was performed on muscle RNAs by using S-2 and AS-2 primers flanking the C-terminus of the EGF-like domain as described in the legend to Fig. 2. Ages of chicken are indicated from Embryonic Day 7 (E7) to 3-month-old (Adult). Histogram shows the relative abundance of each ARIA isoform in different ages of chick muscle. Densitometer was used to determine the intensity of each ARIA isoform transcript, values (Mean ± SEM, $n = 3$) are expressed in percentage of total intensity in each muscle sample. Spinal cords at the corresponding time points were used as controls. (B) RNase protection assay of developing pectoral muscles, using the cRNA probe from V_{27} to E_{154} according to the sequence of pro-ARIA. Ages of chicken are from Embryonic Day 7 (E7) to 3-month-old (Adult). The peak of expression is at the early stage of development.

expression profile is regulated during development, denervation, and regeneration after nerve injury. Two splicing variation sites within pro-ARIA are found: (i) the region at the carboxyl-terminal end to give $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ isoforms; (ii) the region between the Ig-like and the EGF-like domains to form a spacer domain with zero, 17, or 34 amino acids insertions, namely $ARIA_{SP0}$, $ARIA_{SP17}$, and $ARIA_{SP34}$. In embryonic or young chicks, muscles express $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ isoforms of ARIA. In adult chicken muscle, the expression of $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ decreases to a minimal while $ARIA_{\beta 1}$ remains the major ARIA isoform being expressed. This differential expression of ARIA isoforms in muscle is altered by denervation, and is restored to normal expression profile by nerve regeneration. In contrast, the splicing variants at the spacer domain are unchanged during development and denervation.

The expression of different ARIA isoforms is likely to

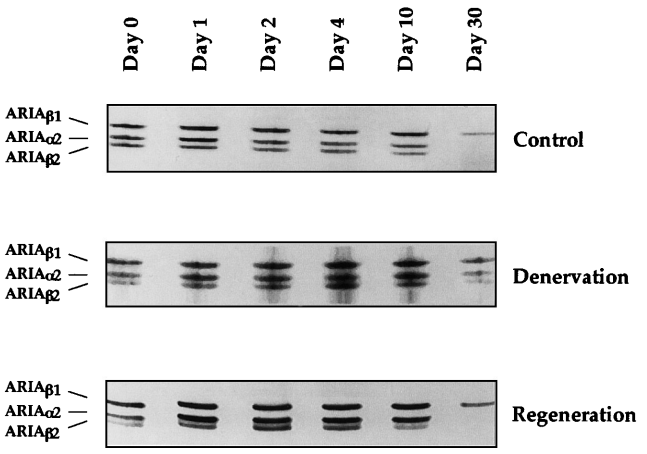

FIG. 7. Transcripts encoding $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ were regulated following denervation and nerve regeneration. Denervation: An ~0.5-cm-long portion of the left sciatic nerve was removed from the upper thigh of P18 chicks. Regeneration: Nerve crush was performed on P18 chicks with a prechilled fine forceps on the left sciatic nerve in the upper thigh. Denervation and regeneration were confirmed by the analysis of AChR expression in the muscle (see Ip *et al.*, 1995). Control: sham-operated chicken. Gastrocnemius muscles were collected for total RNA extraction. RT-PCR was conducted as in Fig. 2. Day of postoperation is shown.

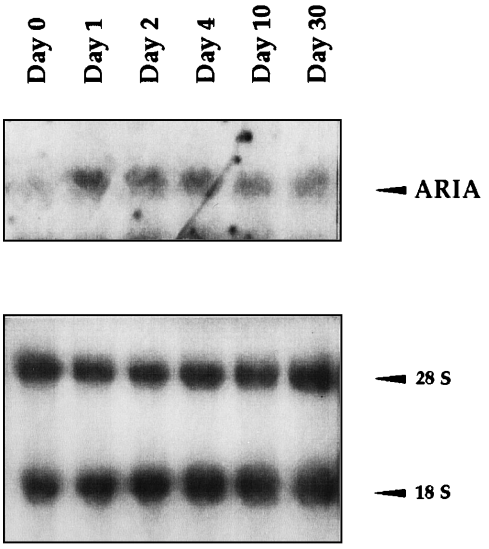

FIG. 8. Denervation induces the expression of ARIA mRNA in muscle. Denervation of P18 chick gastrocnemius muscle was described in the legend to Fig. 7. Cloned pro-ARIA cDNA was used as a probe for Northern blot analysis. A transcript of ~7.5 kb was detected as indicated. Ethidium bromide stained RNA gel showing the 28S and 18S ribosomal RNA illustrates the equal loading of total RNAs. Day of postoperation is shown.

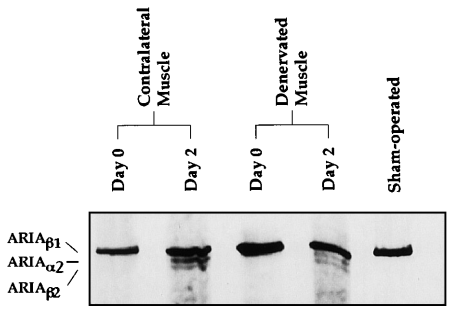

FIG. 9. Expression of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ in the contralateral side of denervated chicken are changed. Denervation was performed by removing an ~0.5-cm-long portion of the left sciatic nerve from the upper thigh of 3-month-old chickens. Gastrocnemius muscles of the operated (denervated) and the unoperated sides (contralateral) were collected for total RNA extraction. RT-PCR was conducted as described in the legend to Fig. 2. Two days after sham-operated chicken was used as control; both ipsilateral and contralateral muscles expressed only β1 isoform. Day of postoperation is shown.

be derived from muscle cell instead of other cell types within the muscle masses. Several lines of evidence supported the hypothesis: (i) ARIA was restricted at the neuromuscular junctions and the extrajunctional ARIA was not detected in chick muscle, and similar ARIA staining was reported from other species (Chu *et al.*, 1995; Jo *et al.*, 1995; Moscoso *et al.*, 1995; Sandrock *et al.*, 1995); (ii) the neuromuscular junction contains three different cell types, neuron, muscle, and Schwann cell. The synaptic expression of ARIA α isoform, revealed by anti-ARIA α isoform-specific antibody, could be derived from muscle or Schwann cell since the motor neuron expressed only the β isoform; (iii) the pattern of ARIA immunostaining at the neuromuscular junctions changed during development and after denervation (Jo *et al.*, 1995; Moscoso *et al.*, 1995); and (iv) the cultured myotubes expressed ARIA and showed similar profile of

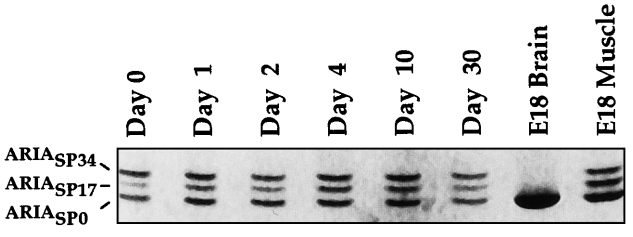

FIG. 10. Expression of $ARIA_{SP0}$, $ARIA_{SP17}$, and $ARIA_{SP34}$ are unaffected after denervation. Gastrocnemius muscles of P18 chicks were denervated as in Fig. 7. RT-PCR was conducted as described in the legend to Fig. 3 by using S-1 and AS-1 primers flanking the Ig-like and the EGF-like domains. E18 chick muscle and brain served as controls. Day of postoperation is shown.

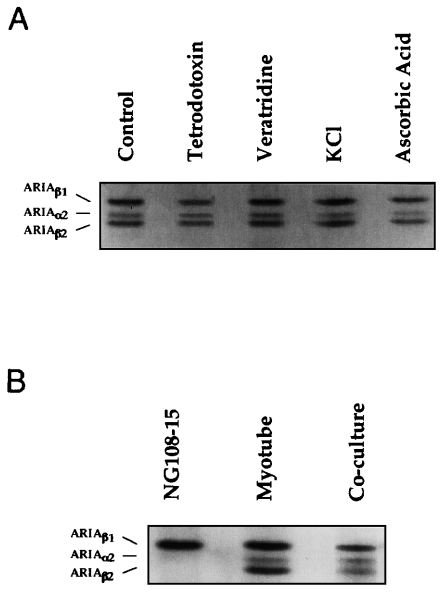

FIG. 11. Expression of $ARIA_{\beta 1}$, $ARIA_{\alpha 2}$, and $ARIA_{\beta 2}$ in cultured myotubes under the drug treatments and cocultured with NG108-15 cells. (A) Three-day-old chick myotubes were treated with TTX (1 μ*M*), veratridine (20 μ*M*), KCl (15 m*M*), or ascorbic acid (30 μg/ml) for 2 days. (B) In neuron-muscle cocultures, NG108-15 cells ($\sim 2 \times 10^5$) were plated onto 3-day-old primary chick muscle culture in a 100-mm culture plate for 2 days. Total RNA was isolated and RT-PCR was performed as described in the legend to Fig. 2 with S-2 and AS-2 primers flanking the carboxyl-terminus of the EGF-like domain. ARIA isoform expression was not significantly affected in all cases as compared to the untreated control myotubes. In NG108-15 cells cultured alone, $ARIA_{\beta 1}$ was detected and served as background for the cocultures.

ARIA isoform as in embryonic chick muscle (Moscoso *et al.*, 1995). Although ARIA is expressed in muscle, it is not known whether the postsynaptic muscle fibers release their ARIA to the synaptic cleft per se. Furthermore, whether muscle ARIA could play a role in the formation of postsynaptic specializations remains to be explained.

Several lines of evidence suggest that the muscle-derived ARIA could have similar functions as the neuron-derived ARIA. First, the cloned cDNA encoding muscle ARIA shows no sequence difference to the cDNA isolated from neurons (Fall *et al.*, 1993 and this study). Second, the EGF-like domain of different ARIA isoforms in various species is the active fragment that induces the phosphorylation of receptor tyrosine kinase and has the AChR-inducing activity when applied onto cultured myotubes (Tzahar *et al.*, 1994; Loeb and Fischbach, 1995; Jo *et al.*, 1995; Si *et al.*, 1996; Yang *et al.*, 1997). Indeed, we showed that the EGF-like domain of chick muscle ARIA could induce the up regulation of AChR α-subunit and the activation of erbB receptors. Thus, the postsynaptic muscle cell has the potential to synthesize and secrete active ARIA at the synaptic cleft. However, the direct proof of muscle ARIA having the AChR-inducing activity *in situ* remains to be established. It is highly possible that the muscle-derived ARIA is biologically inactive due to the posttranslation modification within the muscle. Alternatively, other factor(s) from muscle could inhibit the muscle ARIA to elicit autocrine effects. Another line of evidence to support the hypothesis that muscle ARIA could play a role in the formation of neuromuscular junctions is the alteration of ARIA isoform expression during development, denervation, and nerve regeneration. The specific expression of $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ in developing muscle and in denervated muscle provide a hint that these two ARIA isoforms may be involved in the early events of synaptogenesis. Indeed, the primary source of $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ is from muscle or Schwann cell while $ARIA_{\beta 1}$ is derived from muscle, Schwann cell, and spinal cord. Moreover, the reexpression of $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ in the contralateral side of denervated muscle in adult chicken might be a result of denervation-induced nerve sprouting in the contralateral side as previously reported (Rotshenker and Tal, 1985; Fadic *et al.*, 1988). Although the expression pattern of $ARIA_{\alpha 2}$ and $ARIA_{\beta 2}$ in muscle is related to the time of synapse formation, the specific functions of these two ARIA isoforms at the synaptic cleft have not been reported. Denervation of the sciatic nerve increases the ARIA mRNA expression in the gastrocnemius muscle to about ~5-fold, indicating that the motor nerve, not only affecting the ARIA isoform expression profile, is able to down regulate the expression of ARIA mRNA in muscle. This regulation pattern is in parallel with other postsynaptic molecules in response to nerve denervation (Salpeter, 1987; Hall and Sanes, 1993).

Motor neurons can exert influence on the postsynaptic muscle fiber either via the release of trophic factors or through the activity evoked by synaptic transmission. Several lines of evidence show that muscle activity could regulate the postsynaptic gene expression of AChR, AChE, and voltage-dependent sodium channels (Hall and Sanes, 1993; Vallette and Mosssoulié, 1991; Yang *et al.*, 1991). However, other neuron-derived chemical factors, such as ascorbic acid and calcitonin gene-related peptide (CGRP) could also affect the postsynaptic gene activity. Ascorbic acid has been demonstrated to increase the expression of AChR α-subunit mRNA in rat-derived cloned L5 muscle cells (Hororvity *et al.*, 1989), and CGRP has been shown to increase the number of AChR and AChE in cultured chick myotubes (Fontaine *et al.*, 1987; Choi *et al.*, 1996). To determine the

possible nerve-derived factor(s) that could regulate the expression of ARIA on the muscle cells, we studied the effect of paralyzing agents (TTX and veratridine), a depolarizing concentration of KCl, and ascorbic acid on the expression of ARIA mRNA in cultured chick myotubes. Results showed that the above agents caused little or no effect on the expression profile of muscle ARIA isoforms. Previous studies have shown that the neuroblastoma cell NG108-15 is able to form functional synapses when cocultured with myotubes (Christian *et al.*, 1978). However, the expression profile of muscle ARIA isoforms remains unchanged in the neuron–muscle cocultures. Thus, the activity of muscle membrane and the physical contact of neuroblastoma cells have no effect in the alteration of muscle ARIA isoform expression profile, while other factor(s) may possibly control such ARIA isoform alteration.

The spacer domain of ARIA is a region spanning the Ig-like and the EGF-like domains, where there are sites for N- and O-linked glycosylations (Wen *et al.*, 1994; Meyer and Birchmeler, 1995). Unlike the previously reported neuronal form of ARIA that has 34 amino acids missing in the spacer domain (Falls *et al.*, 1993), we have identified three possible insertions with 0, 17, or 34 amino acids at that region. According to the ARIA release hypothesis (Loeb and Fischbach, 1995), the active EGF-like fragment is released from the stored ARIA by local protease activity at the synaptic cleft. The different spacer domain insertions may control the susceptibility of ARIA to protease action in releasing the active EGF-like fragment at the synaptic cleft. Besides, it has been proposed that one possible role of the high concentration of N- and O-linked glycosylation sites at the spacer domain is to keep the adjacent functional domains, Ig-like and EGF-like domains, in proper exposure and being accessible for molecular interactions (Peles and Yarden, 1993). The different spacer domain insertions may allow different conformations of muscle-derived ARIA to proceed with their functions. Site-directed mutagenesis studies at the spacer domain of various isoforms of ARIA are ongoing in our laboratory to determine the exact functions of ARIA's spacer domain during the formation of neuromuscular junctions.

EXPERIMENTAL METHODS

Animal Surgery

Chick tissues including brain, optic lobe, spinal cord, sciatic nerve, and pectoral muscle of New Hampshire chicks at different ages were collected, frozen in liquid nitrogen, and stored in −80°C for total RNA extraction. P18 chicks or adult chicken (P90) were anesthetized by isoflurane, and ~0.5-cm portion of the left sciatic nerve was removed using asptic surgical techniques (Ip *et al.*, 1995; Tsim *et al.*, 1997). Gastrocnemius muscle of the operated ipsilateralside and the unoperated contralateral side were collected at various time points after the surgery, frozen in liquid nitrogen, and stored in −80°C. Nerve crush was performed with a prechilled fine forceps on the left sciatic nerve of upper thigh of P18 chicks, and the gastrocnemius muscles were collected as described above.

Cell Culture

Primary chick myotube cultures were prepared from the hind-limb muscles dissected from E11 chick embryos. The muscles were then dissociated according to modified protocol previously described (Fischbach *et al.*, 1972; Wallace, 1989). Muscle cells were cultured in MEM supplement with 10% heat inactivated horse serum, 2% (v/v) chick embryo extract, 1 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Drug treatment was performed in normal medium on 4-day-old chick myotube culture as follows: tetrodrotoxin (TTX; 1 μ*M*), veratridine (20 μ*M*), KCl (15 m*M*), and ascorbic acid (30 μg/ml). The cultures were incubated for 2 days, pelleted, and then stored in −80°C for total RNA extraction. In the cocultures, about 2×10^5 NG108-15 cells were plated onto the 3-day-old chick myotube culture in a 100-mm tissue culture plate for 2 days, and then the cells were collected as above. The C2C12 myoblasts were cultured and induced to fuse by reducing the serum to 1.5% (Si *et al.*, 1996). All tissue culture chemicals were from GIBCO-BRL (MD).

Immunocytochemical Staining

The pectoral muscle of chicken was dissected, embedded in a tissue freezing medium, and frozen in an isopentane/liquid nitrogen bath. Sixteen-micrometer sections were cut on a cryostat and thaw-mounted on gelatin-coated slides and then allowed to air-dry overnight. Tissue sections were then fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS; 80 m*M* Na_2HPO_4, 20 m*M* NaH_2PO_4, 100 m*M* NaCl, pH 7.6) for 15 min followed by three 5-min washes in PBS, and a blocking step in 2% normal goat serum in PBS containing 0.05% Triton X-100 for 30 min. Anti-ARIA α isoform specific antibody was purchased from Transduction Lab. (Lexington, KY) and anti-ARIA α and β isoforms-specific antibody was raised in our laboratory by using

the recombinant EGF-like domain of ARIA as antigen (Yang *et al.,* 1997; Ng *et al.,* unpublished results). They were used at 1:2000 to 1:3000 dilution in PBS with 2% normal goat serum and 0.05% Triton X-100, were added onto each slide and then incubated at 4°C for 18 h. After three 10-min washes in PBS, the sections were incubated with fluorescent-conjugated secondary antiserum (1 in 200 dilution) in PBS, 2% normal goat serum, and 0.05% Triton X-100 for 2 h at room temperature. Sections were washed in PBS for 3× 10 min. For double staining, rhodamine-conjugated α-bungarotoxin (10^{-8} *M*; Molecular Probes, Eugene, OR) was incubated together with the secondary antibody. Sections were dehydrated and mounted with glycerol-based mounting media and examined with a Zeiss Axiophot microscope equipped with fluorescent and rhodamine optics.

RNA Isolation and RT-PCR Analysis

Total RNA was prepared from the collected tissues by using LiCl method (Chirgwin *et al.,* 1979). For the cultured myotubes, total RNA was isolated by using Micro RNA Isolation kit (Stratagene, CA). RNA concentration and purity were determined by ultraviolet absorbance at 260 nm. In RT-PCR analysis, 5 μg of total RNAs was reverse transcribed by Moloney Murine Leukemia Virus reverse transcriptase (GIBCO-BRL) by random oligonucleotide priming in a 20-μl reaction. One-fifth of the reverse transcription product was used as a template in PCR analysis with primers described below. PCR was carried out for 30 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min in a 25-μl volume containing 0.8 m*M* dNTPs, 1× PCR buffer, and 0.625 U of *Taq* Polymerase (GIBCO-BRL). The PCR products were analyzed in a 12% polyacrylamide gel (Sambrook *et al.,* 1989). For subcloning of the PCR products, amplified DNAs were directly cloned into pCR II vector (Invitrogen, CA). The identity of the cloned PCR products was confirmed by DNA sequencing using T7 Sequencing kit (Pharmacia Biotech, Sweden). The PCR primers were designed according to chick pro-ARIA cDNA sequence (Falls *et al.,* 1993). A set of primers flanking the Ig-like domains, S-1: 5′-TGCAGAGTGAGCAGCAAACT-3′ (sense; from 342–361) and AS-1: 5′-ACTCTCCCCCATTTACACAG-3′ (antisense; from 492–473), and a set of primers flanking the EGF-like domain S-2: 5′-GACCTCCCAAACCCTCCACG-3′ (sense; from 507–526) and AS-2: 5′-AGCACCCGTTTCTGGTACAGT-3′ (antisense; from 652–632) were used.

Northern Blot and RNA Protection Assay

RNA samples were fractionated on an 1% formaldehyde gel. Ethidium bromide was used to assess the equivalency of loading among different samples. After the electrophoresis, samples were transferred to a charged nylon membrane (Hybond-N, Amersham, UK) and were UV cross-linked. Blots were hybridized using a partial pro-ARIA cDNA fragment (from 24 to 953 bp; Pun and Tsim, 1995). Probes were labeled with [α-^{32}P]dCTP (Amersham) by an Oligolabeling kit (Pharmacia Biotech). Hybridization was performed at 42°C overnight in 40% deionized formamide, 5× Denhart's solution, 0.5% SDS, 5× SSC, 10% dextran sulfate, and 0.1 mg / ml denatured salmon sperm DNA. After hybridization, the filters were washed twice with 2× SSC with 0.1% SDS at room temperature for 30 min each, and then twice with 0.1× SSC with 0.1% SDS at 55°C for 30 min each. The washed filters were exposed to X-ray film with double intensifying screens at −80°C for 3 weeks.

Antisense cRNA probe corresponding to V_{27} to E_{154} of Pro-ARIA (Falls *et al.,* 1993) was synthesized by using a SP6 / T7 Transcription kit with DIG RNA Labeling Mix (Boehringer Mannheim, Germany). RNase protection analysis was performed with an RNase Protection kit (Boehringer Mannheim). Fifty micrograms of total RNA extracted from muscle were hybridized for 5 h at 45°C in 80% formamide, 400 m*M* NaCl, 1 m*M* EDTA, 40 m*M* Pipes, pH 6.4, with 10 ng of DIG-labeled antisense cRNA probe. After digestion with RNase T1, samples were separated by electrophoresis with 8% denaturing polyacrylamide gel (Sambrook *et al.,* 1989). After the electrophoresis, the samples were transferred to a charged nylon membrane (Hybond-N, Amersham). The blot was developed using an anti-DIG antibody conjugated with alkaline phosphatase and the chemiluminiscent substrate according to the protocols provided in the DIG High Prime DNA Labeling and Detection Starter kit II (Boehringer Mannheim).

Functional Expression of Muscle ARIA

The construction of cDNA encoding ARIA EGF-like domain tagged with human immunoglobulin Fc was described elsewhere (Yang *et al.,* 1997). In brief, an artificial leader sequence was inserted into a mammalian expression vector pcDNA I (Invitrogen, San Diego). The downstream of the leader sequence was a *Eco*RI cloning site and then followed by a ~0.6-kb cDNA fragment encoding Fc region of human immunoglobulin G1. The EGF-like domain of ARIA was constructed by PCR with a pair of primers flanking S_{136} to K_{205}, with an artificial *Eco*RI site at both ends, according to the published sequence of pro-ARIA. The PCR was carried out for 30 cycles standard reaction mixture containing chick muscle ARIA cDNAs, 2.5 units of *Vent* DNA

polymerase, and 0.3 μg of forward primer, 5′ CGG AAT TCC AGT CAT CTC ACA AAA 3′ (sense, 435–449), and backward primer, 5′ CTG AAT TCT TTC TGG TAC AGT TCC 3′ (anti-sense, 628–643). The DNA fragment was subcloned into *Eco*RI site of the modified pcDNA I vector. The modified pcDNA I containing Fc tagged ARIA cDNA was transfected into HEK 293 cells by calcium phosphate precipitation (Sambrook *et al.*, 1989). The Fc-tagged ARIA EGF-like fragment was purified by protein-G column (Yang *et al.*, 1997).

In the AChR α-subunit induction assay, the chick myotubes were treated with 1 μg Fc-tagged ARIA protein overnight, and total RNA was collected from the treated chick myotubes. Total RNA was electrophoresed in an 1% formaldehyde-agarose gel and transferred overnight onto a nylon membrane and hybridized with a cDNA probe of ~1.2 kb chick AChR α-subunit (Tsim *et al.*, 1992; Pun and Tsim, 1995). In the phosphorylation studies, fused C2C12 myotubes were treated with 1 μg Fc-tagged ARIA protein for 30 min. The treated cells were lysed and immunoprecipitated with a rabbit antibody against erbB 3 (C17; Santa Cruz Bitotech, CA) at 1:1000 dilution. The immunoprecipitated proteins were collected on protein G agarose beads, fractionated by 7.5% SDS polyacrylamide gel for immunoblotting. The membrane was stained with peroxidase-conjugated anti-tyrosine phosphorylation antibodies RC 20 (Transduction Lab) diluted 1 in 1000. Immunoreactivity was detected by ECL Western Blot System (Amersham, UK) following the instructions from the supplier.

ACKNOWLEDGMENTS

We are grateful to Tina Dong, Lisa Yung, H. Y. Choi, and Matthew Au from our laboratory, to William Chau from the University Animal Care Facility for their expert technical assistance. The research was supported by grants from Research Grants Council of Hong Kong and Biotechnology Research Institute at The Hong Kong University of Science and Technology.

REFERENCES

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18:** 5294–5299.

Choi, R. C. Y., Leung, P. W. Y., Dong, T. T. X., Wan, D. C. C., and Tsim, K. W. K. (1996). Calcitonin gene-related peptide increases the expression of acetylcholinesterase in cultured chick myotubes. *Neurosci. Lett.* **217:** 165–168.

Christian, C. N., Daniels, M. P., Sugiyama, H., Vogel, Z., Jacques, L., and Nelson, P. G. (1978). A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells. *Proc. Natl. Acad. Sci. USA* **75:** 4011–4015.

Chu, G. C., Mosocoso, L. M., Sliwkowski, M. X., and Merlie, J. P. (1995). Regulation of the acetylcholine receptor ϵ-subunit gene by recombinant ARIA: An in vitro model for transynaptic gene regulation. *Neuron* **14:** 329–339.

Corfas, G., and Fischbach, G. D. (1993). The number of Na+ channels in cultured chick muscle is increased by ARIA, an acetylcholine receptor-inducing activity. *J. Neurosci.* **13:** 2118–2125.

Corfas, G., Rosen, K. M., Aratake, H., Krauss, R., and Fischbach, G. D. (1995). Differential expression of ARIA isoforms in the rat brain. *Neuron* **14:** 103–115.

Fadic, R., Soza, M. A., and Inestrosa, N. C. (1988). Changes in contralateral synaptic acetylcholinesterase following motor nerve section in rats. *Neurosci. Lett.* **90:** 229–233.

Falls, D. L., Harris, D. A., Johnson, F. A., Morgan, M. M., Corfas, G., and Fischbach, G. D. (1990). Mr 42,000 ARIA: A protein that may regulate the accumulation of acetylcholine receptors at the developing chick neuromuscular junctions. *Cold Spring Harbor Symp. Quant. Biol.* **55:** 397–406.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the Neu ligand family. *Cell* **72:** 801–815.

Fischbach, G. D. (1972). Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* **28:** 407–429.

Fontaine, B., Klarsfeld, A., and Changeux, J. P. (1987). Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptor α-subunit mRNA levels by distinct intracellular pathways. *J. Cell Biol.* **105:** 1337–1342.

Hall, Z. W., and Sanes, J. R. (1993). Synaptic structure and development: The neuromuscular junction. *Cell/Neuron* **72** (suppl.): 99–121.

Harris, D. A., Falls, D. L., Dill-Devor, R. M., and Fischbach, G. D. (1988). Acetylcholine receptor-inducing factor from chicken brain increases the level of mRNA encoding the receptor α-subunit. *Proc. Natl. Acad. Sci. USA* **85:** 1893–1897.

Harris, D. A., Falls, D. L., and Fischbach, G. D. (1989). Differential activation of myotube nuclei following exposure to an acetylcholine receptor-inducing factor. *Nature* **337:** 173–176.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. (1992). Identification of heregulin, a specific activator of p185 erbB2. *Science* **256:** 1205–1210.

Horovitz, O., Knaack, D., Podleski, T. R., and Salpeter, M. M. (1989). Acetylcholine receptor α-subunit mRNA is increased by ascorbic acid in cloned L5 muscle cells: Northern blot analysis and in situ hybridization. *J. Cell Biol.* **108:** 1823–1832.

Ip, F. C. F., Fu, A. K. Y., Tsim, K. W. K., and Ip, N. Y. (1995). Cloning of the α-component of the chick ciliary neurotrophic factor receptor: Developmental expression and down-regulation in denervated skeletal muscle. *J. Neurochem.* **65:** 2393–2400.

Jo, S. A., Zhu, X., Marchionni, M. A., and Burden, S. J. (1995). Neuregulins are concentrated at nerve-muscle synapses and activate ACh-receptor gene expression. *Nature* **373:** 158–161.

Loeb, J. A., and Fischbach, G. D. (1995). ARIA can be released from extracellular martix through cleavage of a heparin-binding domain. *J. Cell Biol.* **130:** 127–135.

Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Denehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBury, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. (1993). Glial growth factors are alternatively spliced erbB-2 ligands expressed in the nervous system. *Nature* **362:** 312–318.

Martinou, J. C., Falls, D. L., Fischbach, G. D., and Merlie, J. P. (1991). Acetylcholine receptor-inducing activity stimulates expression of the ϵ-subunit of the muscle acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **88:** 7669–7673.

McMahan, U. J. (1990). The agrin hypothesis. *Cold Spring Harbor Symp. Quant. Biol.* **55:** 407–418.

Meyer, D., and Birchmeler, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378:** 386–390.

Moscoso, L. M., Chu, G. C., Gautam, M., Noakes, P. G., Merlie, J. P., and Sanes, J. R. (1995). Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA / heregulin, and its putative receptors, erbB2 and erbB3, in developing mammalian muscle. *Dev. Biol.* **172:** 158–169.

Peles, E., and Yarden, Y. (1993). Neu and its ligands: from an oncogene to neural factors. *BioEssays* **15:** 815–824.

Pun, S., and Tsim, K. W. K. (1995). Truncated form of pro-acetylcholine receptor-inducing activity (ARIA) induces AChR α-subunit but not AChE transcripts in cultured chick myotubes. *Neurosci. Lett.* **198:** 107–110.

Rotshenker, J., and Tal, M. (1985). The transneuronal induction of sprouting and synapse formation in intact mouse muscles. *J. Physiol. (London)* **360:** 387–396.

Salpeter, M. M. (1987). Developmental and neural control of the neuromuscular junction and of the junctional acetylcholine receptor. In *The Vertebrate Neuromuscular Junction* (M. M. Salpeter, Ed.), pp. 55–115. A. R. Liss, New York.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandrock, Jr., A. W., Goodearl, A. D. J., Yin, Q. W., Chang, D., and Fischbach, G. D. (1995). ARIA is concentrated in nerve terminals at neuromuscular junctions and at other synapses. *J. Neurosci.* **15:** 6124–6136.

Si, J., Luo, Z., and Mei, L. (1996). Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase. *J. Biol. Chem.* **271:** 19752–19759.

Tansey, M. G., Chu, G. C., and Merlie, J. P. (1996). ARIA / HRG regulates AChR ϵ subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and Ras / MAPK pathway. *J. Cell Biol.* **134:** 465–476.

Tsim, K. W. K., Greenberg, I., Rimer, M., Randall, W. R., and Salpeter, M. M. (1992). Transcripts for AChR and AChE show distribution differences in cultured chick muscle cells. *J. Cell Biol.* **118:** 1201–1212.

Tsim, K. W. K., Choi, R. C. Y., Dong, T. T. X., and Wan, D. C. C. (1997). A globular, not asymmetric, form of AChE is expressed in chick motor neurons: Down-regulation towards maturity and after denervation. *J. Neurochem.* **68:** 479–487.

Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994). ErbB-3 and erbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor / heregulin isoforms. *J. Biol. Chem.* **269:** 25226–25233.

Vallette, F. M., and Massoulié, J. (1991). Regulation of the expression of acetylcholinesterase by muscular activity in avian primary cultures. *J. Neurochem.* **56:** 1518–1525.

Wallace, B. G. (1989). Argin-induced specializations contain cytoplasmic, membrane, and extracellular matrix-associated components of the postsynaptic apparatus. *J. Neurosci.* **9:** 1294–1302.

Wen, D., Suggs, S. V., Karunagaran, D., Liu, N., Cupples, R. L., Luo, Y., Janssen, A. M., Ben-Baruch, N., Trollinger, D. B., Jacobsen, V. L., Meng, S. Y., Lu, H. S., Hu, S., Chang, D., Yang, W., Yanigahara, D., Koski, R. A., and Yarden, Y. (1994). Structural and functional aspects of the multiplicity of Neu differential factors. *Mol. Cell. Biol.* **14:** 1909–1919.

Yang, J. F., Ng, Y. P., Pun, S., Ip, N. Y., and Tsim, K. W. K. (1997). The EGF-like domain of chick acetylcholine receptor-inducing activity (ARIA) contains its full biological activity. *FEBS Lett.* **403:** 163–167.

Yang, J. S. J., Sladky, J. T., Kallen, R. G., and Barchi, R. L. (1991). TTX-sensitive and TTX-insensitive sodium channel mRNA transcripts are independently regulated in adult skeletal muscle after denervation. *Neuron* **7:** 421–427.

Received January 17, 1997
Revised March 14, 1997
Accepted April 9, 1997