The EGF-like domain of chick acetylcholine receptor-inducing activity (ARIA) contains its full biological activity

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Abstract **Acetylcholine receptor-inducing activity (ARIA) is a glycoprotein initially purified from chick brain based on its ability to increase the synthesis of acetylcholine receptor (AChR) on cultured myotubes. cDNA encoding ARIA contains different domains and the functions of each domain in ARIA activity are not known. We used molecular genetic methods to construct a chimeric fusion protein, designated $ARIA_{S136-K205}$-Fc, that contained the leader sequence, the EGF-like domain of chick ARIA (S_{136} to K_{205}) and the Fc region of human immunoglobulin. The $ARIA_{S136-K205}$-Fc cDNA was transfected into HEK 293 cells and stable cell lines secreting soluble $ARIA_{S136-K205}$-Fc were obtained. The secreted $ARIA_{S136-K205}$-Fc has a molecular mass of ~60 kDa and can be purified by protein G chromatography. The purified $ARIA_{S136-K205}$-Fc retained its full biological activity of chick ARIA that included: (i) induction of tyrosine phosphorylation of erbB 3 receptor in C2C12 myotubes; and (ii) ~12-fold stimulation of AChR α-subunit mRNA synthesis when applied onto cultured chick myotubes. This Fc-tagged ARIA could be rapidly purified and provides a very useful ligand for identifying its true receptor(s) on muscle cell surface.**

Key words: Acetylcholine receptor; Extracellular matrix; Neuromuscular junction; Synapse formation

1. Introduction

During the development of neuromuscular junctions, there occurs the formation of postsynaptic specializations directed by the motor neurons approaching the muscle fibers [1]. These postsynaptic specializations include the aggregation of acetylcholine receptors (AChRs), acetylcholinesterase (AChE) and other synaptic proteins [2]. The increase in postsynaptic AChR density, up to approx. 10 000 receptors/μm^2, is primarily due to an increase in local AChR synthesis, and to the aggregation of AChRs already present in the membrane at the time of nerve-muscle contact [1,3].

In the local synthesis of post-synaptic molecules, RNAs encoding AChR subunits, AChE and other synapse specific proteins are highly concentrated in the synaptic regions [4–7]. The up-regulation of AChR synthesis at the neuromuscular junctions is induced by motor neurons. The motor nerve provides two distinct mechanisms to achieve this striking localization of AChRs: (i) axons release factors, like calcitonin gene-related peptide [8], ascorbic acid [9] and *a*cetylcholine *r*eceptor *i*nducing *a*ctivity (ARIA) [10], that stimulate the synaptic expression of AChR; and (ii) nerve-evoked electrical activity represses the synthesis of AChR in the extrasynaptic regions [11,12].

ARIA was first identified from chick brain and is the best candidate for the nerve-derived signal for such postsynaptic gene regulation. ARIA could mimic the effects of motor axons on the muscle target that include: inducing the synthesis of AChR in aneural myotubes [10], and increasing the number of voltage-gated sodium channels [13] and the expression of the ε-subunit of AChR characteristic of the adult AChR [14–16]. In addition, ARIA was shown to stimulate the tyrosine phosphorylation of both erbB2 and erbB3, and the activation of mitogen-activated protein kinase indicating that ARIA's signaling pathway in muscle might be mediated by erbB2 and/or erbB3 receptors [14–16]. Recent studies, however, indicate that the true receptor for ARIA could just heterodimerize and then phosphorylate either one of its homologous EGF-like receptors, such as erbB2 and/or erbB3 [17]. Based on these observations, Loeb and Fischbach [18] proposed that ARIA is released from the developing motor nerve terminals, activates its receptor on the postsynaptic muscle membrane and thus induces postsynaptic gene expression at the neuromuscular junctions.

cDNA encoding ARIA was first isolated from a λgt 10 chick brain library. Sequencing analysis has revealed that ARIA is a member of a growing family of regulatory factors, named the neuregulin family, which are derived from the same gene by alternative RNA splicing [15,19]. Several members of this family have been identified: *neu* differentiation factor from rat (NDF) [20], heregulin from human [21] and glial growth factor from bovine (GGF) [19]. The cloned ARIA cDNA encoded for a considerably larger transmembrane precursor denoted pro-ARIA with a predicted protein of 602 amino acids. From the N-terminus to the C-terminus, pro-ARIA has immunoglobulin (Ig)-like, epidermal growth factor (EGF)-like, hydrophobic and intracellular domains [22]. Mature ARIA, which is 42 kDa in size, is believed to be a product after proteolytic cleavage of pro-ARIA at the dibasic amino acid residues (K_{205} and R_{206}) adjacent to the hydrophobic domain [22,23]. The N-terminus containing Ig-like domain is able to bind to the extracellular matrix through charged interactions at the synaptic clefts [18]. The EGF-like domain can be released by unknown proteases and acts on the postsynaptic muscle membrane. The EGF-like domain derived from other members of neuregulin has been shown to induce the tyrosine phosphorylation of erbB receptors and the induction of AChR expression [15,16,18]. In order to support the

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Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; ARIA, acetylcholine receptor-inducing activity; EGF, epidermal growth factor; GGF, glial growth factor; Ig, immunoglobulin; NDF, neu differentiation factor; PCR, polymerase chain reaction; TTX, tetrodotoxin.

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'ARIA release' hypothesis and to create a ligand for receptor identification, we constructed a protein fragment of ARIA containing only the EGF-like domain tagged with Fc region of human Ig, namely $ARIA_{S136-K205}$-Fc. The Fc-tagged ARIA fusion protein was functionally expressed in mammalian cells and the fusion protein could be purified by protein G column. The purified $ARIA_{S136-K205}$-Fc shows full biological activity of ARIA in the induction of erbB3 receptor tyrosine phosphorylation and an increase in the expression of mRNA encoding muscle AChR α-subunit.

2. Materials and methods

2.1. cDNA construction and transfection

The leader sequence from hemagglutinin of the avian influenza virus FPV was purified from the expression vector of pM4-3, and this leader sequence was inserted into a mammalian expression vector pcDNA I (Invitrogen, San Diego, USA) as described in [24]. Downstream of the leader sequence was a cloning site *Eco*RI of the pcDNA I, followed by a ~0.6 kb cDNA fragment encoding the 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 [22]. The standard reaction mixture contained chick pro-ARIA cDNA, 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 reaction mixture was subjected to PCR under the following conditions: 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C for 30 cycles with 10 min of extension at 72°C at the last cycle. The PCR product was digested with *Eco*RI and purified on silica gel (Geneclean II kit, BIO 101, USA); the DNA fragment was subcloned into the *Eco*RI site of the modified pcDNA I vector as shown in Fig. 1. The $ARIA_{S136-K205}$ DNA fragment was confirmed to be in-frame with the leader sequence at the 5′ end and human Ig Fc at the 3′ end by DNA sequence analysis with a Sequenase 2.0 kit (USB, OH, USA). The modified pcDNA I containing the $ARIA_{S136-K205}$-Fc cDNA fragment was transfected into the human embryonic kidney fibroblast cell line, HEK 293, by the calcium phosphate method as described [25]. The stably transfected cells were selected by co-transfecting a G418 resistance gene followed by the use of antibiotic G418 (Gibco Laboratories, NY, USA) and flow cytometry for single cell cloning.

2.2. Purification of $ARIA_{S136-K205}$-Fc fusion protein

Protein G agarose (Boehringer Mannhein, Germany) was in a 1:1 volume, pre-washed twice with 0.1 M glycine pH 3.0 and then with phosphate-buffered saline (PBS; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 8.0). About 0.5 ml of protein G agarose beads were mixed with 50 ml conditioned medium from $ARIA_{S136-K205}$-Fc cDNA transfected HEK 293 cells, buffered with 10 mM Tris and 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was rotated at 4°C for 2 h, and then the beads was centrifuged down. The beads were transferred to an Eppendoff tube and washed 3 times with PBS pH 8.0. The $ARIA_{S136-K205}$-Fc fusion protein was eluted using 0.1 M glycine pH 3.0 and then neutralized with 1/10 volume of 1 M Tris-HCl pH 8.0. The purified protein was stored at −80°C until further use.

2.3. Muscle cell cultures

For primary chick myotube cultures, hind-limb muscles dissected from 11-day-old New Hampshire chick embryos were dissociated according to the method of Fischbach [26] with minor modifications [27]. Muscle cells were routinely cultured in MEM supplemented with 10% heat-inactivated horse serum, 2% chick embryo extract, 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. C2C12 myoblasts were maintained in DMEM supplemented with 10% heat inactivated horse serum, 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. C2C12 myoblasts were induced to fuse at confluent stages by serum starvation to 2% finally. The AChR-inducing activity assay was performed on 4-day-old chick muscle cultures, and treatment of ARIA or tetrodotoxin (TTX) was carried out overnight.

2.4. Immunochemical analysis

In phosphorylation studies, fused C2C12 myotubes were treated with purified recombinant $ARIA_{S136-K205}$-Fc for 30 min. Treated cells were lysed in RIPA buffer (PBS pH 7.4, 1% NP-40, 0.5% dexocoxylate, 0.1% SDS, 1 mM PMSF, 1 mM orthovanadate, 1 mM aprotinin). The erbB3 receptor was immunoprecipitated with a rabbit antibody against erbB3 (C17; Santa Cruz Bitotech. CA, USA) at 1:1000 dilution. Immunoprecipated proteins were collected on protein G agarose beads, and fractionated on 7.5% SDS-PAGE. Electrophoresed proteins were transferred onto nitrocellulose membrane (MSI, MA, USA) as described in [25]. The membrane was blocked with 2.5% dry milk containing 20 mM Tris HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, for 1 h at 37°C, followed by incubation with horseradish peroxidase-conjugated anti-tyrosine phosphorylation antibodies RC 20 (Transduction Lab., USA) diluted 1:1000. Immunoreactivity was detected on an ECL Western Blot System (Amersham, UK) following the instructions of the supplier.

For histochemical staining, the $ARIA_{S136-K205}$-Fc cDNA transfected HEK 293 cells were washed with PBS and fixed for 10 min at room temperature with PBS, pH 7.4, containing 2% paraformaldehyde, 5% sucrose. Cells were permeabilized for 3 min with 0.1% Triton X-100 in PBS followed by washing 3 times in PBS. Cells were then incubated with peroxidase-conjugated protein A diluted 1:5000 in PBS with 10% fetal calf serum for 3 h at room temperature. The cells were washed 3 times with PBS, and the peroxidase activity was developed in diaminobenzidine for 30 min with a Zymed liquid DAB-black substrate kit. The cells were washed with PBS and mounted with glycerol. Labeled cells were visualized under a Zeiss Axiophot microscope.

2.5. Northern blot analysis of AChR α-subunit

Total RNA was collected from treated chick myotubes using the guanidium thiocyanate method [28]. Total RNA was electrophoresed in an 1% formaldehyde-agarose gel, transferred overnight onto a nylon membrane (MSI) and hybridized with a cDNA probe of ~1.2 kb chick AChR α-subunit [5]. Probes were labeled by the random oligonucleotide priming method using [α-^{32}P]dCTP (Amersham) with a commercially available labeling kit (Pharmacia, Sweden). Hybridization was performed at 42°C overnight in 50% formamide, 6×SSC,

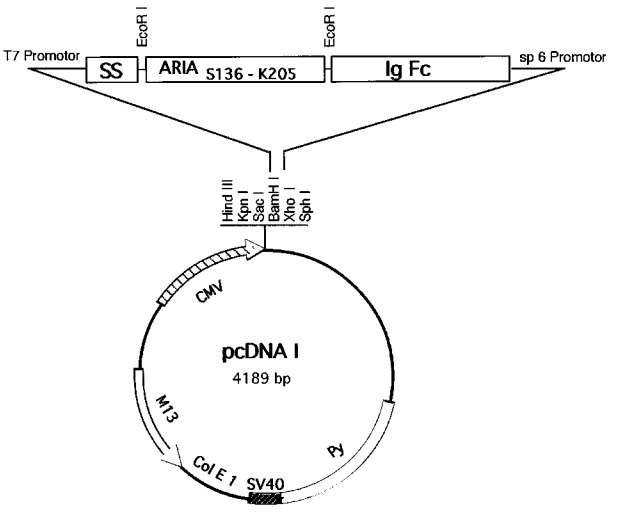

Fig. 1. cDNA encodes $ARIA_{S136-K205}$-Fc. The signal sequence (S S) from hemagglutinin of the avian influenza virus FPV was inserted into a mammalian expression vector pcDNA I with a downstream *Eco*RI site followed by a ~0.6 kb cDNA fragment encoding Fc region of human immunoglobulin G1 (Ig Fc). The EGF-like domain of ARIA was created by PCR with a pair of primers flanking S_{136} to K_{205}, with an artificial *Eco*RI site at both ends, and subcloned between the signal sequence and Ig Fc. The integrity of the construct was confirmed by DNA sequencing, and the DNA was used for transfection analysis.

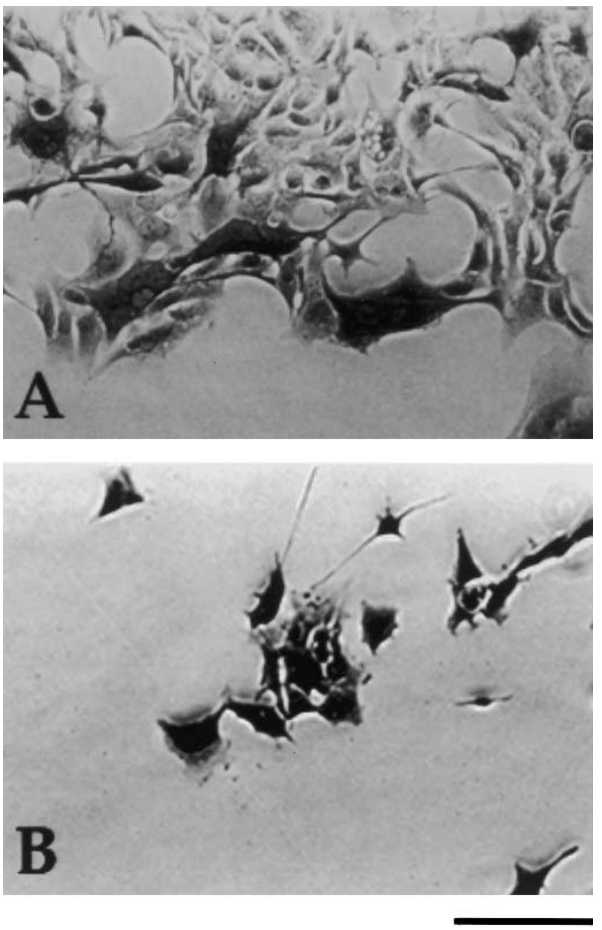

Fig. 2. HEK 293 cells transfected with $ARIA_{S136-K205}$-Fc cDNA expressed human Ig proteins. Transfected 293 cells were washed, fixed and permeabilised with 0.1% Triton X-100 in PBS followed by washing in PBS. Cells were then incubated with peroxidase-conjugated protein A for 3 h at room temperature. The peroxidase activity was developed in diaminobenzidine with a Zymed liquid DAB-black substrate kit. The labeled cells were visualized under Zeiss Axiophot microscopy. (A) Transient transfection of $ARIA_{S136-K205}$-Fc cDNA for 24 h had about 30% of cells showed peroxidase staining. (B) Stable transfected cells were selected by antibiotic and single cell cloning, and all the cells showed peroxidase staining. Scale bar, 80 μm.

5×Denhart's solution, 0.1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. After hybridization, filters were washed with decreasing concentrations of SSC to a final concentration of 0.1×SSC with 0.1% SDS at 55°C, and exposed to X-ray film with intensifying screens at −80°C.

3. Results

The cDNA encoding $ARIA_{S136-K205}$-Fc fusion protein was constructed and subcloned into pcDNA I with an artificial leader sequence as in Fig. 1. The $ARIA_{S136-K205}$-Fc cDNA in pcDNA I was purified and transfected into HEK 293 cells by the calcium phosphate method. After 2 days of transfection, the cells were fixed and stained with peroxidase-conjugated protein A. About 30% of cells showed positive staining of peroxidase-conjugated protein A (Fig. 2A). Stable transfectants were selected by antibiotic and flow cytometry. The selected HEK 293 cells, as determined using peroxidase-conjugated protein A, show the expression of $ARIA_{S136-K205}$-Fc fusion protein (Fig. 2B). A clonal cell line was obtained. Because of the artificial signal sequence, $ARIA_{S136-K205}$-Fc from cultured stably transfected HEK 293 cells was secreted into the conditioned medium. When the conditioned medium was loaded onto SDS gels for immunoblotting analysis, a predominant protein band at ~60 kDa was recognized by anti-human Ig Fc antibody (Fig. 3, lane 1). The $ARIA_{S136-K205}$-Fc could be purified by protein G affinity chromatography. Conditioned medium of stably transfected HEK 293 cells was collected and recirculated onto a protein G column, the bound protein being eluted with low pH buffer. The purified $ARIA_{S136-K205}$-Fc protein demonstrated a protein band at ~60 kDa on a Coomassie stained SDS gel (Fig. 3, lane 2). The molecular mass of $ARIA_{S136-K205}$-Fc protein remained unchanged before and after protein G purification, indicating the integrity of the recombinant protein (compare Fig. 3, lanes 1,2). The identity of the purified protein was further confirmed by its recognition of anti-human Ig Fc antibody, and with anti-ARIA polyclonal antibody (Fig. 3, lanes 3,4). In general, over 20 μg of fusion protein from 100 ml of conditioned medium could be purified by protein G chromatography and this only required 4 h. The predicted size of $ARIA_{S136-K205}$-Fc protein from its cDNA is ~40 kDa; the larger size of the recombinant $ARIA_{S136-K205}$-Fc protein observed could be the result of heavy glycosylation in the fusion protein, and the likelihood is that it is at the human Ig Fc region, since the size of human Ig Fc alone is almost the same as that of $ARIA_{S136-K205}$-Fc (Fig. 3, lane 5). When similar expression analysis was performed on a full-length ARIA molecule (from K_{44} to K_{205}) that contained an Ig-like domain,

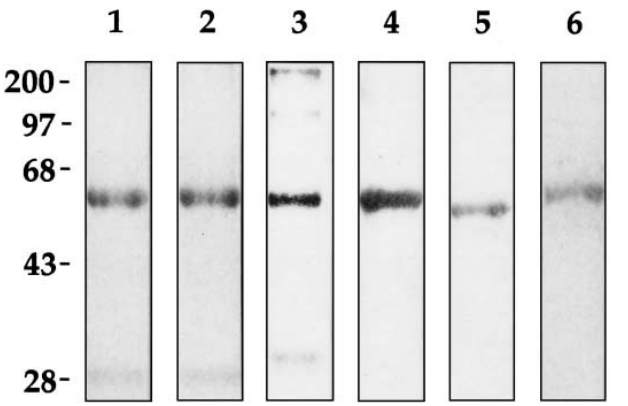

Fig. 3. Expression and purification of $ARIA_{S136-K205}$-Fc from transfected HEK 293 cells. Samples were loaded onto a 10% polyacrylamide gel, and some of them were transferred onto a nitrocellulose paper for the recognition by antibodies. Lanes: 1, conditioned medium from $ARIA_{S136-K205}$-Fc cDNA stable transfected HEK 293 cells and probed with anti-human Ig antibody conjugated with peroxidase in a Western blot analysis; 2, about 3 μg of protein G purified $ARIA_{S136-K205}$-Fc was analyzed by SDS gel electrophoresis and stained with Coomassie blue; 3, about 0.1 μg of protein G purified $ARIA_{S136-K205}$-Fc was recognized by anti-human Ig antibody; 4, about 0.1 μg of purified $ARIA_{S136-K205}$-Fc was recognized by anti-ARIA antibody on a western blot analysis. The production of anti-ARIA antibody is described elsewhere; 5, about 0.1 μg of purified human Ig Fc fragment from Fc cDNA transfected cells and recognized by peroxidase-conjugated anti-human Ig antibody; 6, conditioned medium of HEK 293 cells transfected with full-length ARIA cDNA containing Ig-like domain, EGF-like domain and tagged with human Ig Fc. The protein band was recognized by peroxidase-conjugated anti-human Ig antibody. Molecular markers are in kDa.

an EGF-like domain and was tagged with human Ig Fc, a similar size of ~60 kDa fusion protein was obtained (Fig. 3, lane 6). This indicates that a potential cleavage site exists between the Ig-like and EGF-like domains of ARIA, and the release of active EGF-like fragment could be a prerequisite for its functions.

The biological activity of $ARIA_{S136-K205}$-Fc protein was confirmed by its induction of tyrosine phosphorylation and stimulation of the synthesis of AChR α-subunit in cultured myotubes. Application of $ARIA_{S136-K205}$-Fc induced the phosphorylation of erbB 3 receptor in C2C12 myotube cultures (Fig. 4), that was similar to the activity of full-length recombinant ARIA previously described [6,15,23]. Both ARIA and $ARIA_{S136-K205}$-Fc induced tyrosine phosphorylation of a protein with a molecular mass of ~185 kDa. The identity of the phosphorylated protein was confirmed by its recognition with anti-erbB 3 receptor antibody indicating the specific induction of erbB 3 phosphorylation by ARIA in C2C12 myotubes (Fig. 4). The purified $ARIA_{S136-K205}$-Fc protein was able to stimulate the synthesis of mRNA encoding the AChR α-subunit of cultured chick myotubes. A transcript size of ~3.2 kb was detected by using an AChR α-subunit cDNA probe (Fig. 5). The expression of AChR α-subunit mRNA in cultured chick myotubes was increased to ~12-fold after treatment of $ARIA_{S136-K205}$-Fc for 1 day, while TTX was used as a positive control and showed ~50-fold induction in AChR α-subunit mRNA expression.

4. Discussion

Our studies support the release hypothesis of ARIA that the EGF-like domain from S_{136} to K_{205} contains the full biological activity of chick ARIA in the induction of erbB3 receptor phosphorylation and the increase of AChR α-subunit mRNA expression. Although we did not compare the potency of $ARIA_{S136-K205}$-Fc activity with full-length ARIA in our activity assay, the preliminary results from our transfection studies showed that their activity in AChR induction is very comparable (Yang et al., unpublished results). In addition, when we expressed the full-length ARIA containing the Ig-like domain, EGF-like domain and tagged with human Ig Fc, a protein size of ~60 kDa similar to that of $ARIA_{S136-K205}$-Fc was obtained. This indicates that a protease cleavage site between the Ig-like and EGF-like domains of chick ARIA exists even in transfected fibroblasts; the release of the EGF-like fragment seems to be a prerequisite step for the AChR-inducing activity of ARIA. In our laboratory, we are creating different mutants at the region between the Ig-like and EGF-like domains so as to determine the exact cleavage site for the release of the active EGF-like fragment of ARIA.

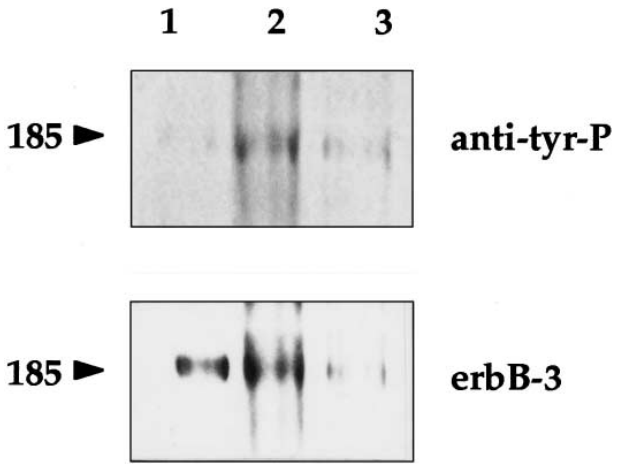

Fig. 4. $ARIA_{S136-K205}$-Fc induced tyrosine phosphorylation of erbB 3 on C2C12 myotubes. The C2C12 myotubes were treated with purified $ARIA_{S136-K205}$-Fc for 30 min. Treated cells were lysed in RIPA buffer. The erbB3 receptor was immunoprecipitated with 1:1000 of a rabbit antibody (C17) against erbB3. The immunoprecipitated proteins were collected on protein G agarose beads and fractionated by 7.5% SDS-PAGE. Electrophoresed proteins were transferred onto nitrocellulose membrane, and detected by peroxidase-conjugated anti-tyrosine phosphorylation antibodies RC 20 (upper panel). The same filter was washed and probed with anti-erbB 3 receptor antibody as to confirm the identity of the recognized band (lower panel). Lanes: 1, control untreated myotubes; 2, recombinant full-length ARIA; 3, recombinant $ARIA_{S136-K205}$-Fc. Arrowheads indicate the molecular size of recognized proteins.

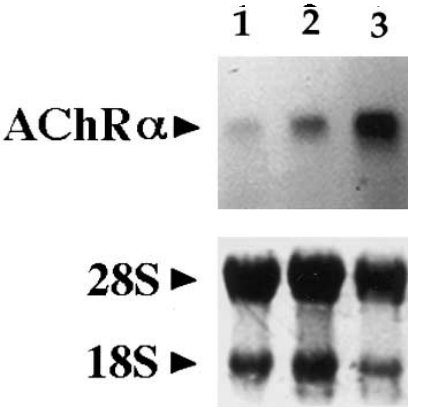

Fig. 5. $ARIA_{S136-K205}$-Fc induced the synthesis of mRNA encoding AChR α-subunit. 4-day-old chick myotubes were treated overnight with ~3 μg of purified $ARIA_{S136-K205}$-Fc (lane 2) or 1 μM TTX (lane 3). The untreated myotube is the control (lane 1). Total RNA was isolated from the cells and 10 μg of RNA were subjected to 1% formaldehyde-agarose gel. The membrane was probed with AChR α-subunit cDNA (~1.2 kb), and a transcript of ~3.2 kb was detected. $ARIA_{S136-K205}$-Fc and TTX increased the AChR α-subunit mRNA ~12- and ~50-fold, respectively. Two independent experiments showed similar mRNA induction. Lower panel shows the ribosomal RNA staining with 18S and 28S as markers.

The exact mechanism for the release of the bioactive EGF-like fragment from the ARIA molecule at the neuromuscular junctions is not known. According to the hypothesis proposed by Loeb and Fischbach [18], the motor neurons express pro-ARIA in a transmembrane form, which is released into the synaptic cleft in the form of ARIA by protease cleavage at K_{205} and R_{206}. The released ARIA binds to glycosaminoglycans at the synaptic cleft by charge interaction on the Ig-like domain. By further action of protease at the neuromuscular junctions, the biologically active EGF-like domain of ARIA is released, and activates a receptor on the postsynaptic muscle membrane in causing the up-regulation of AChR and other synaptic gene expression. Several lines of evidence support such a hypothesis: (i) an activity found to increase the local transcript of AChR subunits is associated with the basal lamina of the neuromuscular junctions after axotomy [29,30]; (ii) the ARIA immunoreactivity is revealed to be localized at the synaptic basal lamina [15]; (iii) both glycosaminoglycan and proteases are commonly found in the synaptic cleft; and (iv) the Ig-like and EGF-like domains show activity in glycosami-

noglycan binding and AChR induction, respectively ([18,31]; this study). Thus, the specific regulation of protease in the synaptic cleft could be a determining step in releasing the functional fragment of ARIA for AChR induction, and thus regulates the formation of neuromuscular junctions during development and nerve regeneration. However, one should bear in mind that the direct proof of this hypothesis has not been established, and that the regulatory pathway for these protease actions within the synaptic cleft is not known.

While the cloned chick ARIA does not have the spacer domain between the Ig-like and EGF-like domains, the spacer domain has been identified in other neuregulin homologs [19–21]. The functional role of the spacer domain in other members of the neuregulin family is not known, but it has been proposed that this domain is the site for carbohydrate attachment and may be responsible for glycosaminoglycan binding. Recently, various isoforms of ARIA containing a spacer domain were also identified in chick muscle (Ng et al., manuscript submitted). Whether the existence of a spacer domain at ARIA sequence will affect the activity and the cleavage of EGF-like fragment is not clear. However, it is possible that such a heavy glycosylated spacer domain could regulate the susceptibility of ARIA to protease actions at the synaptic cleft.

We report here a procedure for the rapid purification of recombinant $ARIA_{S136-K205}$-Fc which provides an excellent ligand for ARIA receptor identification. Although erbB kinases have been demonstrated to be phosphorylated by the application of ARIA in cultured muscle cells, direct evidence for the erbB kinases to be the true receptor for ARIA is lacking. The true receptor for ARIA could be a homolog of erbB kinase, and the activation of ARIA receptor requires heterodimerization with erbB kinase(s) in the postsynaptic muscle membranes [17]. $ARIA_{S136-K205}$-Fc possesses several advantages in receptor binding and in receptor cross-linkage analysis. The fusion protein could be purified with relative ease and is stable for weeks at cold temperature. Detection of the ligand could be accomplished using protein A that is highly specific and commercially available in many conjugated forms. The utilization of the EGF-like fragment for our binding assay would obviate the problem of interference by molecules that interact with other parts of ARIA, such as the Ig-like domain. Moreover, the specificity of the cross-linked protein could include an excess amount of untagged $ARIA_{S136-K205}$ as the displacement ligand. Using this ligand, researchers at our laboratory are searching for different binding components of ARIA in many chick tissues.

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