ARIA Can Be Released from Extracellular Matrix through Cleavage of a Heparin-binding Domain

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Abstract. ARIA, or acetylcholine receptor-inducing activity, is a polypeptide that stimulates the synthesis of acetylcholine receptors in skeletal muscle. Here we demonstrate that the ability of ARIA to induce phosphorylation of its receptor in muscle is blocked by highly charged glycosaminoglycans. ARIA constructs lacking the NH_2-terminal portion, containing an immunoglobulin-like domain, are fully active and are not inhibited by glycosaminoglycans. Limited proteolysis of ARIA with subtilisin blocks the glycosaminoglycan interaction by degrading this NH_2-terminal portion, but preserves the active, EGF-like domain. We also show that ARIA can be released from freshly dissociated cells from embryonic chick spinal cord and cerebellum by either heparin, high salt or limited proteolysis with subtilisin, suggesting that ARIA is bound to the extracellular matrix through charged interactions. We present a model of how ARIA may be stored in extracellular matrix at developing synapses and how its release may be mediated by local proteolysis.

SYNAPSE formation is a complex process bringing together elements from the presynaptic neuron and postsynaptic cell to form a highly specialized node for communication. The molecular architecture at the synapse may be regulated by polypeptides which serve as first messengers in a cascade that activates crucial programs for differentiation (see Hall and Sanes, 1993 for recent review). Acetylcholine receptor-inducing activity (ARIA)[1] is a factor produced by spinal motor neurons that promotes the synthesis of acetylcholine receptors in embryonic myotubes (Jessell et al., 1979; Usdin and Fischbach, 1986; Falls et al., 1993). Other effects of ARIA on muscle that mimic effects of motor neuron axons include increasing the number of voltage-gated sodium channels (Corfas and Fischbach, 1993*b*) and increasing the expression of the ε acetylcholine receptor subunit characteristic of the adult acetylcholine receptor (Martinou et al., 1991). We postulate that ARIA is released from developing motor nerve terminals and activates its receptor on the postsynaptic membrane. Supporting this, ARIA is expressed in embryonic spinal motor neurons at the time of nerve–muscle synapse formation (Corfas et al., 1995). Also consistent with this hypothesis is that ARIA binds to and causes the phosphorylation of its receptor (p185) on tyrosine residues in primary chick myotubes and the L6 muscle-derived cell line (Corfas et al., 1993*a*).

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1. *Abbreviations used in this paper*: ARIA, acetylcholine receptor-inducing activity; GGF, glial growth factor; NDF, Neu differentiation factor; hNDF, human NDF.

Sequence analysis of ARIA from cloned cDNA suggests that the purified 42-kD polypeptide is first expressed as a considerably larger transmembrane precursor called pro-ARIA (Falls et al., 1993). Sequence analysis has also revealed that ARIA is one isoform of a growing family of regulatory factors all of which are probably derived from a common gene by alternative mRNA splicing (Marchionni et al., 1993). Other members of this family include Neu differentiation factor (NDF), heregulin, and glial growth factor (for review see Peles and Yarden, 1993). Each of these factors was independently purified on the basis of a unique biological activity. NDF was purified from the medium of ras-transformed rat fibroblasts based on its ability to cause tyrosine phosphorylation of the Neu oncogene (Wen et al., 1992). It also induces differentiation of mammary tumor cells to milk-producing cells. The heregulins are homologous human isoforms derived from a human breast tumor cell line (Holmes et al., 1992). Glial growth factor (GGF) was purified from bovine pituitary on the basis of its mitogenic activity in Schwann cells (Goodearl et al., 1993; Marchionni et al., 1993). Each of these factors contains an EGF-like domain, which has been shown to be sufficient for both receptor binding and phosphorylation of p185 (Holmes et al., 1992; Wen et al., 1994), and an immunoglobulin-like domain, of unclear function. Recent experiments have suggested that the Neu proto-oncogene may not in fact be the true receptor for ARIA and its isoforms, but becomes phosphorylated on tyrosine only following binding to and hetereodimerization with either one of its homologous EGF-like receptors, erbB-3 or erbB-4 (for review see Carraway and Cantley, 1994).

One feature of chicken brain ARIA that enabled its purification was its ability to bind to heparin columns (Falls

et al., 1993). This suggested to us that binding to glycosaminoglycans in the extracellular matrix may be important in regulating ARIA's actions. There are a growing number of examples of polypeptide growth and differentiation factors that bind to extracellular matrix proteoglycans. These include acidic and basic fibroblast growth factors, transforming growth factor-β, granulocyte-macrophage colony-stimulating factor, interleukin-3, interferon γ, the netrins, and neurotrophin-6 (Ruoslahti and Yamaguchi, 1991; Gotz et al., 1994; Serafini et al., 1994). Interactions with extracellular matrix are thought to provide a readily accessible store of factors, to protect them from degradation, and in some instances, enhance their biological activities. Release of bound factors from extracellular matrix can be achieved by proteolysis of the core proteoglycan or by local release of heparin (Hardingham and Fosang, 1992).

An attractive hypothesis is that ARIA may be concentrated at synapses through binding to heparin-like glycosaminoglycans in the extracellular matrix. Along this line, in vivo studies have suggested that after axonal transection, neuromuscular basal lamina retains an activity which promotes the synthesis of acetylcholine receptor subunits (Goldman et al., 1991; Jo and Burden, 1992). Furthermore, ARIA (neuregulin) immunoreactivity has been localized to the neuromuscular synapse and remains associated with basal lamina in the absence of nerve and muscle (Jo et al., 1995).

In this paper we have localized the heparin-binding portion of ARIA to its NH_2 terminus containing an immunoglobulin-like domain. We find that both limited proteolysis with subtilisin or heparin treatment can release ARIA from the extracellular matrix of chick embryonic spinal cord and cerebellum. We present evidence that this release can be achieved through proteolytic inactivation of the heparin-binding domain resulting in a partially degraded form of ARIA which is soluble and active. Unlike other growth factors whose activity is potentiated by heparin, we find that heparin blocks ARIA-induced receptor phosphorylation.

Materials and Methods

ARIA Forms

Recombinant ARIA was purified from the media of transfected COS-7 cells. Cells were transfected as described previously (Fall et al., 1993) with a construct of the λ12 clone to which an NH_2-terminal FLAG epitope was added and inserted into the Bam H1 site of the vector pcDNA I/AMP (Invitrogen, San Diego, CA). This was accomplished by first introducing a 48-nucleotide oligonucleotide encoding the flag epitope into the BstX1 site. The resultant NH_2-terminal predicted amino acid sequence for this construct is MGDYKDDDDKGPVCWNSGVLRGM, where the last methionine residue corresponds to the first amino acid of the λ12 insert. OptiMEM I media (GIBCO BRL, Gaithersburg, MD) was harvested 3 d after transfection and applied to a 0.5-ml column of anti-FLAG (M2) antibody-coupled agarose (International Biotechnologies, Inc., New Haven, CT) and eluted with 0.1 M glycine (2.5). The fractions were rapidly neutralized with 0.1 vol of 1 M Tris-HCl (8.0). Fractions with phosphorylation activity were pooled and applied to an Altex C4 reverse phase column in 0.1% trifluoroacetic acid. It was eluted on a 0-60% gradient of acetonitrile and came off as a single peak. This material was stored at 4°C and dried down before use. Constructs of the human forms of ARIA were generously provided by AMGEN (Thousand Oaks, CA). The isolated EGF-like domain corresponds to amino acids 177–246 of the human β1 form expressed in *Escherichia coli*. The Ig-EGF form corresponds to amino acids 14–246 of the human β1 form expressed also in *E. coli*.

Glycosaminoglycan Inhibition of ARIA-induced Receptor Phosphorylation

Various preparations of recombinant ARIA were diluted into 150 μl of L6 media (DME containing 10% heat-inactivated fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin, all purchased from GIBCO BRL). Stock solutions of glycosaminoglycans were added to give the final indicated concentrations and incubated at room temperature with various forms of ARIA for 5 min before addition to L6 muscle cells. p185 phosphorylation on tyrosine residues was performed as described previously (Corfas et al., 1993*a*). Essentially, samples were applied to cells plated 6-10 d previously at a density of 50,000 cells per well of a 48-well tissue culture dish. After 30–45 min of treatment, the media was discarded, the cells solubilized in 2× SDS sample buffer, and boiled for 5 min. Phosphotyrosine on p185 was detected by Western blot analysis using a monoclonal antibody directed against phosphotyrosine (mAb 4G10 generously provided by B. Druker and T. Roberts, Dana Farber Cancer Center, Boston, MA) after resolution on overrun 5% reducing SDS–polyacrylamide gels. The filters were then probed with a goat anti–mouse IgG coupled to peroxidase (Boehringer Mannheim Corp., Indianpolis, IN) and exposed to film after treatment with chemiluminescence reagents (Dupont-NEN, Boston, MA). Heparin (porcine intestinal mucosa, ~13,000 M_r), heparan sulfate, dextran sulfate, dermatan sulfate, keratan sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Chondroitin sulfate (chondroitin sulfate A) was from Boehringer Mannheim.

HPLC Gel Filtration of Proteolytic Fragments

An equal amount of COS-expressed ARIA was treated without and with 50 μg/ml of subtilisin at room temperature for 20 min. The reaction was stopped with the addition of PMSF at a final concentration of 1 μg/ml. 100 μl of each was loaded in series onto the same Altex TSK 3000 (0.75 × 30 mm) gel filtration HPLC column preequilibrated with 30% acetonitrile, 0.1% trifluoroacetic acid, flowing at 0.4 ml/min. 0.4-ml fractions were collected and 0.2 ml of each were then lyophilized, reconstituted in L6 media, and assayed for p185 phosphorylation activity. Densitometry of films using a computing densitometer (model 300B; Molecular Dynamics, Sunnyvale, CA) was used to quantify the amount of activity in each fraction. The total activity was normalized to 100% and plotted as percent of total. Activity from chick cerebellum was obtained by treating 10 million freshly isolated cerebellum cells, isolated as described below, with 10 μg/ml of subtilisin (Boehringer Mannheim) in PBS, 1 mM $CaCl_2$ for 20 min at room temperature. The cells were spun down at 14,000 rpm in an Eppendorf centrifuge and the 100-μl supernatant was removed, made 1 mg/ml PMSF, and immediately applied to the same HPLC gel filtration column run exactly as above. Fractions were assayed and quantified as above. Using identical running conditions the following size standards were used for column calibration: carbonic anhydrase (29,000 D), cytochrome C (12,400 D), and aprotinin (6,500 D) (Sigma). The elution positions of standards were measured at OD_{280} and confirmed by SDS–polyacrylamide gel electrophoresis followed by staining with coomassie brilliant blue.

Nonenzymatic Preparation of Cells from Chick Embryonic Cerebellum and Spinal Cord

Whole cerebellum or spinal cord were removed from E17 chick embryos and placed on ice in Hank's balanced salt solution containing 10 mM Hepes, pH 7.4 (HBSS/7.4; GIBCO BRL). The meninges were carefully removed and the tissue transferred to a tube containing the above buffer with 1% bovine serum albumin (RIA grade; Sigma) and 500 U/ml of DNAseI (Sigma) at room temperature. The tissue was gently triturated 12 times through a blue p1000 pipette tip. The tissue still remaining was removed by 1 min centrifugation at 500 rpm in an IEC HNSII clinical centrifuge. The supernatant was transferred to a new tube containing 10 ml of cold HBSS/7.4 and spun for 7 min at 1,200 rpm in the same centrifuge. Cells were resuspended in cold PBS, pH 7.4 (GIBCO BRL) and counted. This method gives a high yield of mostly single cells with a viability of ~50–60% by trypan blue exclusion.

Subtilisin- and Heparin-induced Release of Cell Surface ARIA

Five million cells isolated as described above were used for each data point. Aliquots of cells were spun at 5,000 rpm for 5 min at 4°C in an Eppendorf centrifuge and resuspended in 45 μl of PBS, pH 7.4, sometimes

containing 1 mM $CaCl_2$ for digestions with subtilisin. 5 μl containing a 10-fold final concentration of either freshly prepared subtilisin or heparin was added, mixed by vortexing, and incubated for either 20 min at room temperature (subtilisin) or at 4°C (heparin). The cells were mixed intermittently. After 20 min the cells were spun at 14,000 rpm in the same centrifuge at 4°C for 5 min. For analysis of ARIA activity in the supernatants, the entire supernatant was added to 100 μl of L6 media, mixed and added to L6 cells for assay of receptor phosphorylation as described above. To measure acetylcholine receptor insertion rate, 100 μl of this released material was diluted ninefold with chick muscle culture media and added to primary chick myotube cultures as described previously (Falls, 1993). For some experiments the cells were first treated with heparin, centrifuged at 5,000 rpm in the Eppendorf centrifuge, and then resuspended in 50 μl of PBS, pH 7.4 containing 1 mM $CaCl_2$ and subtilisin to examine the amount of activity remaining after heparin treatment. A polyclonal antisera made against amino acids 14–241 of an *E. coli*-expressed human NDF α2 form generously provided by AMGEN (no. 1915) was used to block activity released from cerebellum cells.

Western blots of released activity by heparin, subtilisin, or 1 M NaCl were all performed by resuspending a pellet of five million cells with 25 μl of the indicated solution incubating as above for 20 min at room temperature. The cells were centrifuged and the supernatants were added to 20 μl of 2× sample buffer, boiled for 5 min and then analyzed on 10% reducing SDS–polyacrylamide gels by Western blot using an affinity purified antisera generated against a synthetic peptide consisting of the first 27 amino acids of chicken ARIA (HM21). The blots were developed using a goat anti–mouse IgG linked to peroxidase and chemiluminescence as described above. Densitometry of the Western blots was performed as described above.

Results

ARIA Contains a Heparin-binding Domain Which When Bound to Heparin Blocks Receptor Phosphorylation

One key feature that enabled the purification of ARIA from chicken brain was that ARIA could bind tightly to a heparin column, requiring 0.45–0.65 M NaCl to elute the activity (Falls et al., 1993). Since heparan sulfate is present in the synaptic basal lamina of neuromuscular junctions, we were interested in characterizing the role of heparin on ARIA's actions (Sanes et al., 1986). Heparin was added to recombinant chicken ARIA before the addition of both to cultured L6 muscle cells. Receptor interaction was assessed by measuring the extent of p185 tyrosine phosphorylation using Western blot analysis. At 500 μg/ml, heparin blocks nearly all of the p185 phosphorylation (Fig. 1). The half maximal blocking concentration of heparin is ~5 μg/ml or 40 nM. When the same concentrations of chondroitin sulfate are added to ARIA, there is no inhibition of phosphorylation. Thus, heparin, in addition to binding to ARIA, blocks its ability to induce phosphorylation of its receptor.

To determine which portion of ARIA interacts with heparin, various constructs were examined for their ability to induce receptor phosphorylation in the presence of glycosaminoglycans. Fig. 2 summarizes the predicted domain structure of proARIA along with three constructs used for these studies. Both chicken ARIA and human NDF (hNDF) constructs were used, since no differences in biological activity have thus far been detected between these forms. Two of the constructs, ARIA and hNDF-$\beta1_{14\text{-}246}$ contain a β1 EGF-like domain connected by a variable length serine/threonine-rich spacer to an immunoglobulin-like domain. The third construct was hNDF-$\beta1_{177\text{-}246}$, which has only a β1 EGF-like domain.

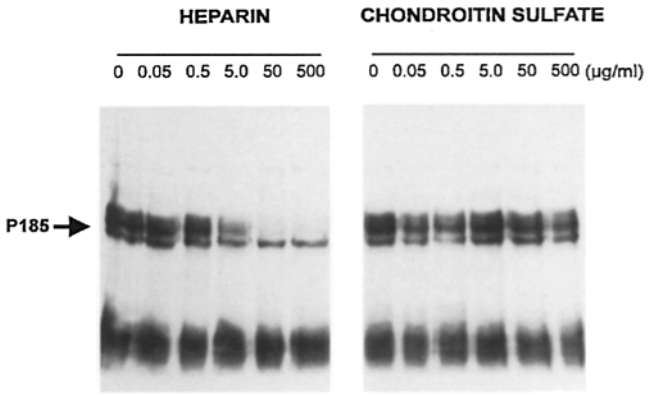

Figure 1. Heparin blocks ARIA-induced receptor phosphorylation in cultured L6 muscle cells. Recombinant ARIA was incubated in L6 media with the indicated final concentrations of heparin or chondroitin sulfate and added to confluent L6 cell cultures for 30 min. The cells were solubilized in SDS sample buffer and p185 phosphorylation activity (*arrows*) was measured by Western blot analysis using anti-phosphotyrosine antibodies.

hNDF-$\beta1_{14\text{-}246}$-induced receptor phosphorylation is blocked by heparin at a half-maximal concentration which is similar to that observed with ARIA (Fig. 3 *a*). In contrast, heparin does not block the phosphorylation activity of hNDF-$\beta1_{177\text{-}246}$ that consists of only the EGF-like domain. No decrease is observed even at the highest heparin concentrations. Consistent with this, hNDF-$\beta1_{177\text{-}246}$ does not bind to heparin-linked agarose as do all constructs containing the immunoglobulin-like domain (data not shown). These results suggest that heparin binds to the NH_2-terminal portion of ARIA, containing the immunoglobulin-like domain, and prevents the association of the EGF-like domain with its receptor. To explore the specificity of this glycosaminoglycan interaction, various glycosaminoglycans, differing in charge density, were then added to hNDF-$\beta1_{14\text{-}246}$. While heparin, heparan sulfate, dermatan sulfate, and dextran sulfate block p185 phosphorylation induced by hNDF-$\beta1_{14\text{-}246}$, keratan sulfate and chondroitin sulfate, which are less negatively charged, have no effect (Fig. 3 *b*).

Limited Proteolysis of ARIA Restores Activity in the Presence of Heparin

The presence of a heparin-binding domain specifies a means by which ARIA could bind to negatively charged molecules such as glycosaminoglycans on extracellular matrix proteoglycans. Once bound, however, ARIA is unable to induce receptor phosphorylation. Since the EGF-like domain alone is sufficient for biological activity, one way to avoid this inhibition by glycosaminoglycans is to remove or inactivate the heparin-binding domain while preserving the function of the EGF-like domain. We found that we could accomplish this by using limited proteolysis with the nonspecific protease, subtilisin. Fig. 4 shows that while heparin completely blocks p185 phosphorylation induced by hNDF-$\beta1_{14\text{-}246}$, after limited proteolysis with subtilisin, heparin no longer blocks phosphorylation of p185. This result suggests that the heparin-binding activity can in fact be dissociated from the receptor phosphorylation ac-

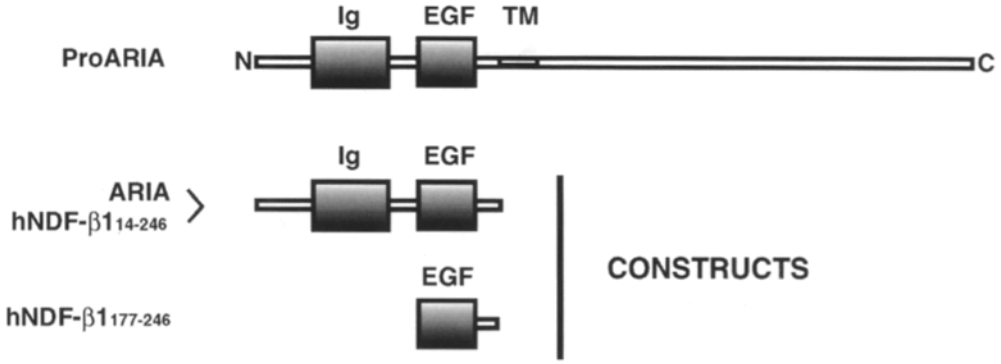

Figure 2. ARIA domain structure and constructs. ProARIA has an NH_2-terminal immunoglobulin-like domain (*Ig*), an EGF-like domain (*EGF*), a putative transmembrane sequence (*TM*), and a large COOH-terminal domain. Cleavage occurs somewhere between the EGF-like domain and the transmembrane domain to produce a soluble polypeptide containing both the immunoglobulin and EGF-like domains. Constructs used in these studies include chicken ARIA and human hNDF-β_{14-246}, each containing both the immunoglobulin-like domain and the EGF-like domain and hNDF-$\beta_{177-246}$ that consists of an isolated EGF-like domain.

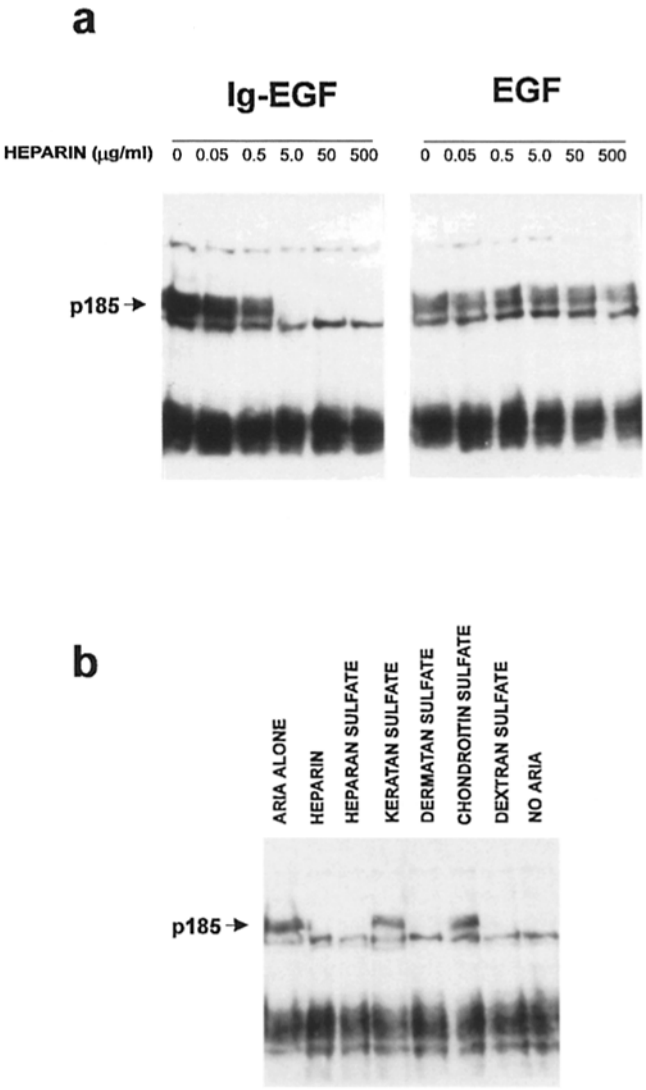

Figure 3. The NH_2 terminus of ARIA is a selective glycosaminoglycan binding domain. (*a*) hNDF-β_{14-246} (*Ig-EGF*) or hNDF-$\beta_{177-246}$ (*EGF*) at 100 pM each were mixed with increasing concentrations of heparin and applied to L6 cells to measure p185 phosphorylation as in Fig. 2. (*b*) 100 pM hNDF-β_{14-246} Ig-EGF, was mixed with 500 μg/ml of the indicated glycosaminoglycans or dextran sulfate and analyzed for p185 phosphorylation activity in L6 cells.

tivity by proteolysis. Subtilisin treatment also reduces the total amount of activity perhaps due to partial degradation of the EGF-like domain.

The size of active fragments produced by limited proteolysis of recombinant ARIA with subtilisin was examined by HPLC gel filtration chromatography. Before treatment with subtilisin, ARIA has an apparent molecular mass of 25 kD (Fig. 5). This same glycoprotein migrates with an apparent molecular mass of 42 kD on reducing SDS–polyacrylamide gels. After brief treatment with subtilisin and application to the same column, the receptor phosphorylating activity now elutes as two lower molecular mass peaks of 11.5 and 5.3 kD. The 5.3-kD activity is consistent in size with an isolated EGF-like domain. The 11.5-kD activity likely contains the EGF-like domain, since it is active, with varying portions of the degraded NH_2 terminus.

Thus limited proteolysis effectively inactivates the heparin-binding activity by removing part or all of the NH_2 terminus containing the immunoglobulin-like domain while preserving the function of the EGF-like domain, which is relatively resistant to proteolysis. This relative susceptibility to proteolysis may be an important mechanism employed for releasing ARIA from the extracellular matrix in vivo.

ARIA Can Be Released from Embryonic Chick Spinal Cord and Cerebellum

In light of the above findings, we asked whether ARIA

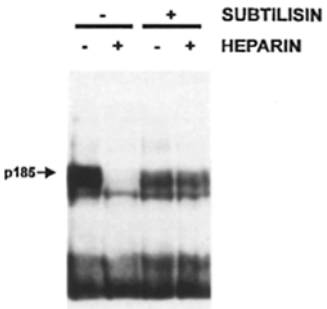

Figure 4. Heparin no longer blocks receptor phosphorylation after limited proteolysis of ARIA. hNDF-β_{14-246}, containing both the immunoglobulin and EGF-like domains (100 pM final concentration) was digested with 0.1 μg/ml subtilisin in PBS, 1 mM $CaCl_2$ for 20 min at room temperature. The reaction was stopped by the addition of L6 media. Both digested and nondigested hNDF-β_{14-246} were mixed with or without 200 μg/ml of heparin before addition to the L6 cells to assess p185 receptor phosphorylation.

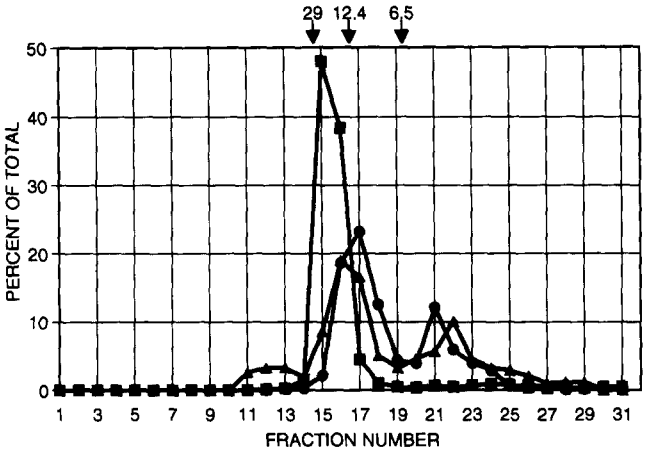

Figure 5. HPLC gel filtration analysis of ARIA after limited proteolysis with subtilisin reveals two peaks of activity coinciding with activity released from chick cerebellum. ARIA (■), subtilisin-treated ARIA (●), and activity released by subtilisin treatment of freshly isolated cerebellum cells (▲) were analyzed sequentially on the same HPLC gel filtration column. The p185 phosphorylation activity was assayed in each fraction and normalized as the percentage of total activity per sample. The elution profiles are plotted on the same chart for comparison. 29, 12.4, and 6.5 refer to the elution positions in kD of the molecular weight standards carbonic anhydrase, cytochrome C, and aprotinin, respectively.

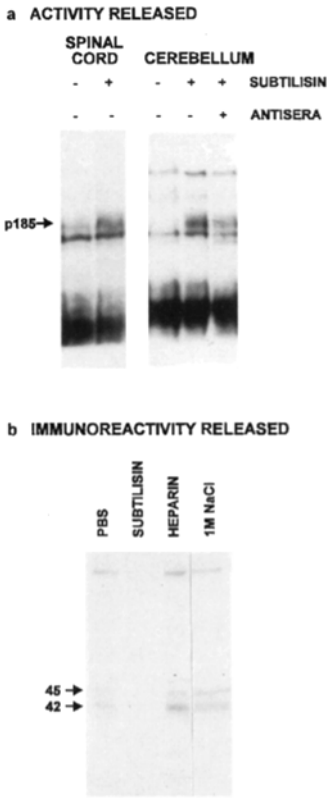

Figure 6. ARIA can be released from chick spinal cord and cerebellum. (*a*) Five million nonenzymatically isolated cells from E17 chick spinal cord and cerebellum were incubated for 20 min at room temperature in 50 μl of PBS, 1 mM $CaCl_2$ with 10 μg/ml of subtilisin. 100 μl of L6 media was added to the supernatant of each and p185 phosphorylation activity was measured by Western blot analysis. Antisera to ARIA was added to one of the subtilisin treated samples 20 min before addition to L6 cells for p185 analysis at a final dilution of 1:10. (*b*) Five million cells from cerebellum were incubated for 20 min at room temperature with either 25 μl of PBS alone, or with subtilisin (10 μg/ml), heparin (200 μg/ml). or 1 M NaCl. A Western blot was performed on the released material using an affinity-purified, anti-peptide antisera directed against the first 27 NH_2-terminal amino acids of chicken ARIA.

could be released from the surface of freshly isolated chick central nervous tissue cells by limited proteolysis. Spinal cord and cerebellum were used since in situ hybridization analysis has demonstrated high levels of ARIA mRNA expression both in spinal motor neurons and in the cerebellum (Corfas et al., 1995). Cells from embryonic day 17 spinal cord and cerebellum were isolated without protease so as to preserve the extracellular proteins and treated briefly with subtilisin. After centrifugation to remove the cells, the activity released was assayed by measuring the extent of p185 tyrosine phosphorylation in L6 muscle cells. Fig. 6 *a* shows that subtilisin treatment releases p185 phosphorylation activity from both spinal cord and cerebellum cells. Other proteases including trypsin, chymotrypsin, V8, and elastase also release this activity, suggesting that this effect is not unique to subtilisin (data not shown). Thus far, we have found no other factors that induce the phosphorylation of this 185-kD protein in L6 muscle cells and thus believe this released material to be ARIA (Corfas et al., 1993*a*).

There are four additional features of this activity released by subtilisin that suggest that it is indeed ARIA. Experiments for these studies were performed using cerebellum cells because of a greater amount of ARIA present. First, antibodies directed against hNDF, but which recognize recombinant chicken ARIA on Western blots and immunoprecipitations are able to block the released activity by ~40% (Fig. 6 *a*). Even with other antibodies directed against various forms of ARIA, only partial blockage of the released activity could be achieved perhaps due to a lowered affinity of the antibodies to the proteolytically degraded ARIA. Second, the activity released from cerebellum increases the insertion rate of acetylcholine receptors in primary chick myotube cultures by 1.6 ± 0.1-fold over nonstimulated cultures (quadruplicate samples, as described in Materials and Methods).

Third, when the activity released by subtilisin from cerebellum cells is analyzed by HPLC gel filtration chromatography, two peaks of activity elute at 12 and 4.8 kD (Fig. 5). These fragments match the size of the fragments produced by subtilisin-cleavage of recombinant chicken ARIA at 11.5 and 5.3 kD. The similarity in size and pattern of the subtilisin-cleaved recombinant ARIA and the cerebellum-released activity suggests that they are derived from a common protein backbone, and, indeed represent fragments of ARIA released from these cells lacking portions of their NH_2 termini.

Finally, Fig. 6 *b* shows that on Western blot, two bands of 42 and 45 kD are released from freshly dissociated cerebellum cells. This Western blot uses an affinity-purified polyclonal antisera made against the first 27 NH_2-terminal amino acids of ARIA (HM21). This antisera specifically recognizes recombinant forms of ARIA expressed in COS-7 cells on Western blots (data not shown). Following treatment of the cells with subtilisin, these bands disappear, which would be expected if the NH_2 terminus were degraded. However, when heparin or 1 M NaCl is added the amount of these two proteins increases by 1.9- and 2.4-fold, respectively (data obtained by densitometry). The observation that immunoreactive proteins consistent in size with ARIA are also releasable by heparin or high salt further supports the hypothesis that ARIA may be associated with the extracellular matrix of these cells through ionic interactions.

A Majority of Cell Surface ARIA Is Bound to Extracellular Matrix

The observation that ARIA can be released from the surface of cerebellum cells by heparin, high salt, and limited proteolysis, suggests that ARIA is associated with the extracellular matrix of these cells. To assess the proportion of ARIA that is matrix bound, we attempted to release ARIA from cerebellum cells by treatment with various concentrations of heparin. While detectable p185 phosphorylation activity is released from these cells at low concentrations of heparin (10 μg/ml), high concentrations (100 μg/ml) yield less detectable activity (Fig. 7 *a*). This is not unexpected because high concentrations of heparin also block ARIA-induced p185 phosphorylation. In the same experiment, chondroitin sulfate at 10 μg/ml does not release any activity, again demonstrating the specificity of the interaction.

To avoid this limitation, we measured the activity remaining after treatment of cerebellum cells with increasing concentrations of heparin. Following heparin treatment, the cells were washed to remove the heparin together with the ARIA released by the heparin. They were then resuspended with subtilisin to measure the remaining cell associated activity. Fig. 7 *b* shows that high concentrations of heparin (200 μg/ml) remove over 60% of the subtilisin-releasable activity. It also shows that neither 50 μg/ml of chondroitin sulfate nor pretreatment with 0.1% Triton X-100, a nonionic detergent, releases ARIA. The observation that a nonionic detergent does not release the activity is consistent with heparin causing a specific disruption of ionic interactions which attach ARIA to the surface of these cells, such as binding to heparin-like glycosaminoglycans expressed on extracellular matrix proteoglycans.

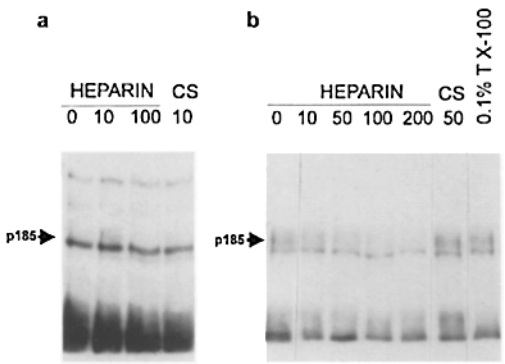

Figure 7. Release of ARIA from cerebellum cells with heparin. (*a*) 5 million chick cerebellum cells were washed and treated with 0, 10, or 100 μg/ml of heparin or 10 μg/ml of chondroitin sulfate (CS) in 50 μl PBS for 20 min on ice. The cells were removed by centrifugation and the ARIA present in the supernatant was assayed for p185 phosphorylation activity by Western blot analysis. (*b*) Cells were treated as in (*a*) for 20 min in PBS containing either 0–200 μg/ml heparin, 50 μg/ml chondroitin sulfate, or 0.1% Triton X-100. After centrifugation to remove the cells, the supernatants were removed and the cell pellets were resuspended in 50 μl of 10 μg/ml of subtilisin in PBS, 1 mM $CaCl_2$ for 20 min at room temperature. Following the reaction, the samples were centrifuged and the supernatants were assayed for p185 phosphorylation activity.

While most of the cell surface ARIA is released by high concentrations of heparin, there seems to be a small proportion of ARIA that is not releasable by heparin (Fig. 7 *b*). This could be due to a different type of cell surface interaction that is not disrupted by heparin or to the presence of a transmembrane form that requires proteolysis for release.

Discussion

ARIA Has Distinct Domains for Heparin-binding and Acetylcholine Receptor-including Activity

Our results have shown that, when the EGF-like domain of ARIA is linked to the NH_2-terminal portion including the immunoglobulin-like domain, phosphorylation of its receptor, p185, is blocked by heparin. This inhibition is selective for more negatively charged glycosaminoglycans such as heparin, heparan sulfate and dermatan sulfate, and dextran sulfate. Other less highly charged glycosaminoglycans such as chondroitin sulfate and keratan sulfate do not block activity. In contrast, the isolated EGF-like domain is fully active in the presence of heparin. These results suggest that heparin binds to the NH_2 terminus of ARIA containing the immunoglobulin-like domain and somehow prevents a successful interaction of the EGF-like domain with its receptor.

We suspect that the heparin binding site is within the immunoglobulin-like domain of chicken ARIA because this is the only region of the protein that contains a particularly high density of positively charged residues (13 out of 56 amino acids between the two conserved cysteines). This might produce a tight interaction with negatively charged glycosaminoglycans. One portion of this sequence, amino acid numbers 70–75 (TKKNRP), conforms to a consensus sequence for heparin binding found in vitronectin, apolipoproteins E and B-100, and platelet factor 4 (Cardin and Weintraub, 1989). This corresponds to a motif [X-B-B-X-B-X], where B is a basic residue and X is a hydropathic residue. Mammalian forms of ARIA (NDF or heregulins) have an additional, positively charged sequence on their NH_2 terminus in addition to the one found in their immunoglobulin-like domains. Half of the residues in the amino acids between 4–23 are positively charged (Holmes et al., 1992). However, the particular human construct we used, hNDF-$\beta1_{14\text{-}246}$, lacks the first half of this sequence, but is still effectively blocked by heparin.

Early efforts in the purification of ARIA demonstrated two peaks of activity at high and low molecular mass (Jessell et al., 1979). Later purification efforts, which included more protease inhibitors, identified only the high molecular mass 42-kD form (Usdin and Fischbach, 1986). We describe experiments here showing that while the immunoglobulin-like domain is quite sensitive to proteolysis, the EGF-like domain is relatively resistant and can be observed as a low molecular mass peak on gel filtration chromatography. One hypothesis now raised is that the low molecular mass form previously purified corresponds to an isolated EGF-like domain, generated by proteolysis. Although the EGF-like domain is sufficient for full activity, the NH_2-terminal portion containing the heparin-binding activity could modulate activity by localizing ARIA in

the extracellular matrix where it can be stored until needed.

The best studied system where heparin modulates the interaction between a receptor and its ligand activation is that of the FGF family of growth factors (Mason, 1994; Ruoslahti and Yamaguchi, 1991). Heparin, although not required for receptor activation, increases the affinity of FGF for its receptor perhaps by bridging FGF to its receptor (Mason, 1994; Roghani et al., 1994). In contrast, heparin inhibits ARIA-induced receptor phosphorylation.

ARIA Can Be Released from the Extracellular Matrix

In addition to binding heparin in vitro we also now provide evidence that ARIA may use this ability in vivo to accumulate in extracellular spaces in the central nervous system. ARIA can be released from freshly dissociated cells from chick embryonic central nervous system by either proteolysis, heparin, or high salt. Similar observations have been made on a heparin-binding protein mitogenic for Schwann cells, which may belong to the ARIA/GGF/NDF family (Ratner et al., 1988). This activity could be released from the surface of rat dorsal root ganglia cells and blocked by low concentrations of heparin (1 μg/ml).

Release of other factors thought to associate with proteoglycans in the extracellular matrix have been achieved similarly. Heparin-binding growth factors such as basic and acidic FGF, granulocyte-macrophage colony-stimulating factor, interleukin 3, and interferon gamma bind to the glycosaminoglycan portion of proteoglycans (Hardingham and Fosang, 1992), whereas other factors such as transforming growth factor-β, and leukemia inhibitory factor (cholinergic differentiation factor) bind to the polypeptide portion (Ruoslahti and Yamaguchi, 1991; Mereau et al., 1993). The heparin-binding growth factors have also been shown to be released from extracellular matrix by competition with soluble heparin or degradation of the matrix proteoglycans with protease. These two modes of releasing factors are thought to occur at sites of inflammation, by the secretion of heparin by mast cells, and in extracellular matrix remodelling, by extracellular protease activities. We favor a novel, proteolytic mechanism for the release of ARIA from the extracellular matrix that dissociates the heparin binding activity from the biologically active EGF-like domain.

The identity of the extracellular protein that binds ARIA in the central nervous system is unknown. This protein could either be a component of the extracellular matrix or the extracellular portion of a membrane-bound protein. In the peripheral nervous system, agrin, a protein known to cause clustering of acetylcholine receptors, binds to heparin and is localized in synaptic basement membranes (for review see Nastuk and Fallon, 1993). Recently, dystroglycan, a muscle cell proteoglycan associated with the dystrophin-associated glycoprotein complex, has been shown to bind to agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). This interaction also can be blocked with heparin. Heparin inhibits agrin-induced receptor clustering on myotube cultures and neuromuscular synapse formation in *Xenopus* nerve-muscle cocultures (Wallace, 1990; Saito et al., 1993). While the specific extracellular matrix binding molecule for ARIA at the neuromuscular junction is also unknown, any extracellular matrix proteoglycan expressing highly charged glycosaminoglycans, or for that matter, any negatively charged surface protein is a potential binding site.

A Model for the Storage and Release of ARIA at Synaptic Junctions

We postulate that ARIA derived from a presynaptic motor neuron leads to differentiation of the postsynaptic muscle cell at developing synapses (Falls et al., 1990). Most of our work thus far has centered on the role of ARIA in the development of the neuromuscular junction, but ARIA's presence in all cholinergic neurons in the central nervous system as well as noncholinergic neurons in both the cortex and the cerebellum suggests that it may also be a potent differentiation factor in these areas as well. Our observation here that ARIA is present and releasable from extracellular binding sites in the chick spinal cord and cerebellum by either subtilisin or heparin suggests that extracellular reservoirs of ARIA may be present in synaptic junctions and that its release could be mediated by local protease activity. One possible mechanism that incorporates these observations is presented in Fig. 8. In this model, ARIA is concentrated in the synaptic cleft by binding to negatively charged molecules, such as proteoglycans in the extracellular matrix. To get to its receptor on the postsynaptic cell, ARIA must first be released from the matrix by proteolytic cleavage of its heparin-binding domain, thus releasing the active EGF-like domain. This process could be regulated by a balance between protease activities and protease inhibitors in the extracellular matrix. One intriguing possibility is that during synaptogenesis, a

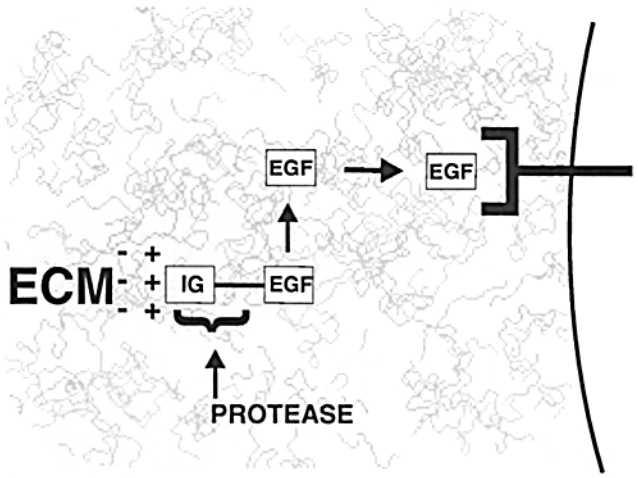

Figure 8. A model for protease-mediated release of ARIA from extracellular matrix. ARIA is secreted by neurons into the extracellular space. Once released, the immunoglobulin-like domain (*IG*) may bind to the glycosaminoglycan portion of proteoglycans in extracellular matrix (*ECM*) through ionic interactions. Once bound, it remains stored there until it is released by an extracellular protease which preferentially degrades the immunoglobulin-like domain thus releasing the active, relatively protease resistant, EGF-like domain (*EGF*). This allows the now soluble EGF-like domain to bind to its postsynaptic receptor resulting in differentiation of the postsynaptic cell.

protease is supplied by the postsynaptic cell to provide a developmentally specific signal for the release of ARIA.

Support for this model comes from several other lines of evidence. Both the central and peripheral nervous system contain extracellular proteoglycans containing glycosaminoglycans that could potentially bind ARIA (Sanes, 1989). Specifically, at neuromuscular junctions, antibodies directed against heparan sulfate stain the synaptic basal lamina (Sanes et al., 1986). Recently basal lamina preparations lacking both nerve and muscle stain for ARIA immunoreactivity (Jo et al., 1995). We have recently obtained both light and EM immunocytochemical evidence that ARIA accumulates in the synaptic cleft at neuromuscular synapses (Sandrock, A. W., Jr., A. D. J. Goodearl, Q. Yin, D. Chang, and G. D. Fischbach. 1995. ARIA is concentrated in nerve terminals at neuromuscular junctions and other synapses. *J. Neurosci.* In press., Goodearl, A. D. J., A. G. Yee, A. W. Sandrock, Jr., G. Corfas, and G. D. Fischbach. 1995. Submitted.). There is also indirect evidence that suggests that an ARIA-like activity is bound to the extracellular matrix of neurons. After axotomy, an activity found to increase local transcription of acetylcholine receptor subunits remains associated in the basal lamina of neuromuscular junctions (Goldman et al., 1991; Jo and Burden, 1992). Finally, at neuromuscular synapses, there is evidence for the existence of a thrombin-like protease that is thought to mediate synapse elimination in an in vitro system (Liu et al., 1994).

Many examples now exist where a balance between protease and protease inhibitor in the extracellular matrix regulate developmental interactions between cells, including those for the spread of tumors (for review see Alexander and Werb, 1991). Since the Neu oncogene is expressed in many breast and ovarian tumors (Peles and Yarden, 1993), ARIA-matrix interactions might also play a role in the spread of these tumors.

While we feel that this mechanism is most consistent with our data, we cannot rule out that there may be other mechansisms that regulate the release of ARIA at synaptic junctions. The intracellular processing and release of ARIA from its membrane-spanning precursor protein, proARIA, and the possibility of juxtacrine interactions of proARIA with its receptor through cell-cell contact (Massagué, 1990) offer additional ways that ARIA's effects on postsynaptic cells may be regulated.

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