

Synapse-Associated Expression of an Acetylcholine Receptor-Inducing Protein, ARIA/Heregulin, and Its

Mammalian Muscle

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Developing motor axons induce synaptic specializations in muscle fibers, including preferential transcription of acetylcholine receptor (AChR) subunit genes by subsynaptic nuclei. One candidate nerve-derived signaling molecule is AChR-inducing activity (ARIA)/heregulin, a ligand of the erbB family of receptor tyrosine kinases. Here, we asked whether ARIA and erbB kinases are expressed in patterns compatible with their proposed signaling roles. In developing muscle, ARIA was present not only at synaptic sites, but also in extrasynaptic regions of the muscle fiber. ARIA was synthesized, rather than merely taken up, by muscle cells, as indicated by the presence of ARIA mRNA in muscle and of ARIA protein in a clonal muscle cell line. ARIA-responsive myotubes expressed both erbB2 and erbB3, but little EGFR/erbB1 or erbB4. In adults, erbB2 and erbB3 were localized to the postsynaptic membrane. ErbB3 was restricted to the postsynaptic membrane perinatally, at a time when ARIA was still broadly distributed. Thus, our data are consistent with a model in which ARIA interacts with erbB kinases on the muscle cell surface to provide a local signal that induces synaptic expression of AChR genes. However, much of the ARIA is produced by muscle, not nerve, and the spatially restricted response may result from the localization of erbB kinases as well as of ARIA. Finally, we show that erbB3 is not concentrated at synaptic sites in mutant mice that lack rapsyn, a cytoskeletal protein required for AChR clustering, suggesting that pathways for synaptic AChR expression and clustering interact. © 1995 Academic Press, Inc.

INTRODUCTION

When motor axons innervate muscle fibers, they induce formation of a specialized postsynaptic membrane, the best studied component of which is the nicotinic acetylcholine receptor (AChR). In adult muscle, AChRs are present at $\geq 10,000/\mu\text{m}^2$ in the synaptic membrane, but their density falls to $\leq 10/\mu\text{m}^2$ in extrasynaptic membrane (Salpeter and Harris, 1983; Salpeter *et al.*, 1988). The nerve appears to use at least three distinct mechanisms to achieve this striking localization. First, axons release factors that stimulate synthesis of AChRs in the synaptic portion of the muscle fiber (Chu *et al.*, 1995b). Second, nerve-evoked electrical activity represses synthesis of AChRs in extrasynaptic areas (Laufer and Changeux, 1989). Third, nerve-derived clustering factors such as agrin cause aggregation of receptors already present in the membrane, via association with the AChR-associated cytoskeletal protein, rapsyn (Bowe and Fallon,

1995; Apel and Merlie, 1995). Recent studies on all three signaling pathways are providing a view of transynaptic signaling that may be relevant to other synaptic components and to other synapses (Hall and Sanes, 1993).

Here, we focus on the pathway by which axons induce AChR expression. Studies on adult muscle have shown that this signal acts locally. RNAs that encode AChR subunits (Merlie and Sanes, 1985; Fontaine *et al.*, 1988; Goldman and Staple, 1989; Brenner *et al.*, 1990) and other synapse-specific proteins (Michel *et al.*, 1994; Moscoso *et al.*, 1995) are highly concentrated in synaptic areas. Moreover, reporter genes linked to AChR genomic fragments are selectively expressed by subsynaptic nuclei in transgenic mice, providing direct evidence for the unique transcriptional potential of synaptic nuclei (Klarsfeld *et al.*, 1991; Sanes *et al.*, 1991; Simon *et al.*, 1992). A plausible hypothesis is that a local signal released by axons acts on a receptor in the postsynaptic membrane to induce or maintain the special properties of the underlying nuclei.

A leading candidate for the nerve-derived signal is ARIA. Based on studies showing that neural extracts stimulated AChR synthesis in aneural myotubes (Cohen and Fischbach, 1977; Jessell *et al.*, 1979), an AChR-inducing activity (ARIA) was purified from chicken brain (Usdin and Fischbach, 1986) and shown to induce expression of AChR subunit genes (Harris *et al.*, 1988; Martinou *et al.*, 1991; Chu *et al.*, 1995a; Jo *et al.*, 1995). When cDNAs encoding the protein were isolated (Falls *et al.*, 1993), sequence analysis revealed that ARIA is encoded by the same gene that generates proteins that had been isolated as ligands of the neu protooncogene (heregulin or NDF; Holmes *et al.*, 1992; Wen *et al.*, 1992) and as a glial growth factor (Marchionni *et al.*, 1993). *In situ* hybridization revealed that ARIA mRNA is present in embryonic motor neurons (Falls *et al.*, 1993; Marchionni *et al.*, 1993), and immunohistochemistry showed that ARIA-like immunoreactivity is present at synaptic sites in adult muscle (Chu *et al.*, 1995a; Jo *et al.*, 1995). Thus, ARIA/hergulin is a candidate neural inducer of AChR synthesis.

Although the mechanism by which ARIA acts is unknown, its identity with heregulin and NDF suggests that its receptors are related to neu. Neu, also called erbB2 or HER2, is a receptor tyrosine kinase related to the epidermal growth factor receptor (EGFR or erbB1; Bargmann *et al.*, 1986). Additional members of this family, erbB3/HER3 and erbB4/HER4, have also been cloned (Kraus *et al.*, 1989; Plowman *et al.*, 1990, 1993a). Heregulins have been reported to bind to and activate erbB2-erbB4 in numerous cell types (Plowman *et al.*, 1993b; Carraway *et al.*, 1994; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1994; reviewed in Carraway and Cantley, 1994) and to induce tyrosine phosphorylation of both erbB2 and erbB3 in cultured myotubes (Jo *et al.*, 1995). Thus, erbB2 and/or erbB3 are candidate receptors for ARIA.

The present studies were undertaken to learn whether ARIA and erbB kinases are expressed *in vivo* at sites and times consistent with their playing roles as nerve-derived signal and muscle-associated receptor, respectively, for transcriptional activation of synaptic nuclei. We asked which erbB kinases muscle cells express and determined the distribution of ARIA and the erbB kinases in developing, adult, denervated, and mutant muscles. Several of our results suggest modifications to current views on the involvement of ARIA and erbB kinases in nerve-muscle interactions. First, synapse-associated ARIA is synthesized by muscle cells as well as by motoneurons, suggesting that ARIA may act in part by an autocrine mechanism. Second, erbB2 and erbB3 are selectively associated with the postsynaptic membrane, suggesting that the spatial restriction of the signaling pathway may reflect the localization of the receptor as well as of its ligand. Finally, the synaptic localization of erbB3 requires rapsyn, which is also necessary for the agrin-dependent clustering of AChRs. Thus, the ARIA and agrin signaling pathways may interact.

MATERIALS AND METHODS

Cloning of EGFR, erbB2, erbB3, erbB4, and ARIA Fragments

Oligonucleotide primers were designed that corresponded to cytoplasmic regions of mouse EGFR (Luetke *et al.*,

1994), rat erbB2 (Bargmann *et al.*, 1986), human erbB3 (Kraus *et al.*, 1989; Plowman *et al.*, 1990), and human erbB4 (Plowman *et al.*, 1993a). Primer sequences were chosen from regions in which the four sequences were minimally related (<30% amino acid identity for any pair). First-strand cDNA was generated by reverse transcription of RNA isolated from mouse brain and Sol 8 myotubes. The cDNA was then subjected to PCR, and amplified fragments were subcloned into Bluescript II (SK⁺) (Stratagene, LaJolla, CA). Multiple clones for each fragment were sequenced. Each mouse sequence isolated was closely related to its human ortholog but clearly distinct from the other three erbB sequences (Fig. 1). Thus, each of the four characterized human erbB genes has a clear murine counterpart.

For ARIA, primers were designed using the rat NDF sequence (Wen *et al.*, 1992), and DNA was amplified as above.

Primer sequences are as follows: EGFR 5', GATGCTGATGAGTATCTTATCCCACAGC; EGFR 3', GGTCTCTCCAGGACTGGATGCAGG; erbB2 5', TGGACA-GTACCTTCTACCGTTCCTGGA; erbB2 3', GGG-GTCTCTCGCTGTACCGCTGTAGAGGGCT; erbB3 5', TCTGGATACATGCCCATGAACCAGGGTAATCTTGG; erbB3 3', CCTTTGAGGTGTGTATCTGGCATGACATAA CC; erbB4 5', CTCTTGATGAAGAGGATTTGGAAGAT-ATGATGGA; erbB4 3', CTTGCGTAGGGTGCATTACAGCAGGAGTCATC; ARIA 5'A, TCTGGAGAGTATATGTGCAAAGTGATCAGC; ARIA 5'B, ACATCAACATCCAC GACTGGGACCAGCCATCT; ARIA 3', GCAGTAGGC-CACCACACACATGATGCC.

Animals and Cells

Adult male and timed pregnant female Wistar rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). For surgery, rats were anesthetized by intraperitoneal injection of ketamine (8.7 mg/100 g) and xylazine (13 mg/100 g). Rat lower leg muscles were denervated by unilateral section of the sciatic nerve in the upper thigh. At appropriate times thereafter, the rats were euthanized with methoxyfluorane and the tibialis anterior and extensor digitorum longus muscles were dissected. Unoperated legs served as controls.

Generation and characterization of mice with targeted mutations in the *Rapsn* (Gautam *et al.*, 1995) and *Lamb2* (s-laminin/laminin β 2) genes (Noakes *et al.*, 1995) have been described elsewhere. Heterozygotes were bred to generate homozygotes, which were identified by PCR. Wild-type and heterozygote littermates were used as controls.

Sol 8 cells (Mulle *et al.*, 1988) were maintained in log phase growth by trypsinization and replating in DMEM containing 20% fetal calf serum on gelatin-coated plates. For RNA isolation, cells were allowed to reach confluence and switched to low-serum differentiation medium, as described in Chu *et al.* (1995a). A bacterially produced recombinant heregulin fragment, rHRG β ₁₇₇₋₂₄₄ (3 nM; Holmes *et al.*, 1992), a generous gift of Mark Sliwkowski (Genentech), was added 24 hr later, and myotube cultures were harvested 48 hr later.

a

Human erbB2	1011	DDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSTRSGGDLT	1060
Mouse erbB2		DDDMGELVDAEEYLVPQQGFFSPDPALGTGSTAHRHRSSSARSGGGELT	
Human erbB2	1061	LGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPHTDP	1106
Mouse erbB2		LGLEPSEEEPPRSPLAPSEGAGSDVFDGDLAVGVTRGLQSLSPHDL	
Human erbB3	1064	GSCQESAVSGSSERCRPVSLHPMPRGCLASESSEGHVTGSEAELEQKQVRS	1113
Mouse erbB3		EACLDSSVVLGGREQFSRPI SLHP IPRGRQTSSESSEGHVTGSEAELEQKQVRS	
Human erbB3	1114	MCRSRSRSRSPRPRGDSAYHSQRHSLLLTPVTPLSPPGLEEEDVN	1157
Mouse erbB3		MCRSRSRSRSPRPRGDSAYHSQRHSLLLTPVTPLSPPGLEEEDGN	
Human erbB4	1019	AEEYLVPQAFNIPPIIYTSRARIDSNRSEIGHSPPPAYTPMSGNQFVYRD	1068
Mouse erbB4		AEEYLVPQAFNIPPIIYTSRTRIDSNR-----NQFVYQD	
Human erbB4	1069	GGFAAEQGVSVPYRAPTSTIPEAPVAQGATAEIF	1102
Mouse erbB4		GGFATQQGMPYRATTSTIPEAPVAQGATAEMF	

b

		mouse			
		erbB1	erbB2	erbB3	erbB4
human	erbB1	88	29	5	15
	erbB2	19	81	6	21
	erbB3	7	9	81	10
	erbB4	22	21	11	87

FIG. 1. cDNA probes for mouse erbB2-4. (a) Amino acid sequences of PCR products cloned from Sol 8 cells (mouse) were aligned with corresponding human sequences. Amino acid identity is indicated with vertical bars. (b) Similarity between mouse and human erbB sequences. Each mouse gene has a clear human counterpart. Accession numbers for mouse erbB2-4 sequences are L47239-41.

Immunohistochemistry

Rabbit antibodies to EGFR (sc-03), erbB2 (sc-284), erbB3 (sc-285), erbB4 (sc-283), and their respective blocking peptides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A second rabbit antibody to erbB3 was purchased from Transduction Laboratories (Lexington, KY). Antibodies to recombinant heregulin, provided by Mark Sliwkowski, were described previously (Chu *et al.*, 1995a). This antiserum was used in most studies reported here, but where noted, immunohistochemical results were confirmed with rabbit antisera to a peptide derived from the carboxy terminus of a human heregulin precursor (sc-348; Santa Cruz Biotechnology). To reduce background staining, antisera were sometimes incubated at 4°C for 2 hr with a particulate fraction from adult rat muscle. Particulate matter was removed by centrifugation before staining. Antibody to SV2 was a gift of Kathleen Buckley (Harvard Medical School), rhodamine- α -bungarotoxin was purchased from Molecular Probes (Eugene, Oregon), and fluorescein-conjugated second antibodies (goat anti-rabbit, donkey anti-sheep, and goat

anti-mouse) were obtained from Boehringer-Mannheim (Indianapolis, IN) and Sigma (St. Louis, MO).

Light microscopic immunohistochemical methods were modified from Covault and Sanes (1986). In brief, cryostat sections of unfixed muscle were incubated with antibody diluted in a solution of bovine serum albumin (10 mg/ml) in phosphate-buffered saline (PBS), either overnight at 4°C or for 3 hr at room temperature. The sections were washed, incubated with a fluorescein-conjugated secondary antibody, and washed again. Sections were mounted with paraphenyldiamine and glycerol and viewed with epifluorescence optics. To identify synaptic sites, sections were doubly stained either with rhodamine- α -bungarotoxin, which binds to acetylcholine receptors in the postsynaptic membrane, or with SV2, which stains synaptic vesicles in nerve terminals.

RNA Analysis

Myotube RNA was isolated by the method of Chomczynski and Sacchi (1987) as modified by Xie and Rothblum

(1991). Lengths of cloned PCR products used for templates were: EGFR, 328 bp, linearized with *Hind*III; erbB2, 350 bp, linearized with *Sty*I; erbB3, 350 bp, linearized with *Hind*III; erbB4, 273 bp, linearized with *Bam*HI; and heregulin, 492 bp, linearized with *Hind*III. RNA was obtained by transcription with T3 or T7 RNA polymerase (Promega, Madison, WI), using 12.5 μ M [32 P]UTP or [32 P]UTP, for *in situ* hybridization or RNase protection, respectively, to label transcripts.

RNase protection analysis was performed as described by Miner and Wold (1991). Briefly, RNA prepared from tissues or cultured cells was hybridized overnight with 5×10^9 molecules of [32 P]cRNA at 50°C in 80% formamide, 40 mM Pipes, 400 mM NaCl, and 1 mM EDTA. Samples of 300 μ l were then digested with 120 ng of RNase A (Sigma) and 300 units of RNase T1 (USB, Cleveland, OH), followed by 40 μ g proteinase K, and then electrophoresed on an 8% acrylamide 7.5 M urea gel. Finally, the gel was exposed to XAR-5 film (Kodak, Rochester, NY).

In situ hybridization was performed as described by Moscoso *et al.* (1995), using 32 P-labeled probes. The AChR ϵ subunit probe described by Moscoso *et al.* (1995) was used as a positive control. A laminin α 1 subunit (Sasaki *et al.*, 1988) probe was used as a negative control, based on RNase protection studies showing that this subunit is not expressed at detectable levels in muscle (J. Miner and J.R.S., unpublished). After sections were hybridized, slides were exposed to XAR-5 film for 3 days.

Immunoblotting

Tissues and cells were solubilized in modified Laemmli (1970) buffer. Samples were separated on a 6 or 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (0.45 mm, Schleicher & Schuell). Transfer was carried out in 96 mM glycine, 12.5 mM Tris, 20% (vol/vol) methanol for 2 hr at 400 mA. Membranes were blocked overnight at 4°C in 5% nonfat dry milk in PBS/0.5% Tween 20. Primary antibodies were diluted in 2.5% nonfat dry milk in PBS/0.5% Tween 20. Horseradish peroxidase-conjugated goat anti-rabbit (Boehringer-Mannheim) or donkey anti-sheep (Sigma) secondary antibodies were used at 1:2000. Immunoreactive species were detected using a chemiluminescence reagent according to the manufacturer's instructions (DuPont NEN, Boston, MA).

RESULTS

Expression of ARIA in Extrasynaptic Regions of Developing Muscle

We and others have shown recently that ARIA is concentrated at synaptic sites in adult muscle fibers (Chu *et al.*, 1995a; Jo *et al.*, 1995). At least some of this synaptic ARIA is likely to be associated with nerve terminals, because ARIA-like immunoreactivity sometimes extended into

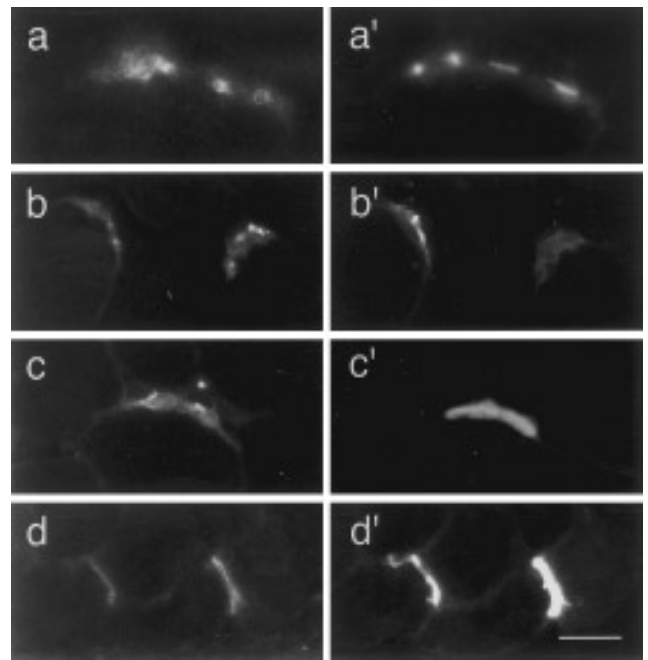


FIG. 2. ARIA is associated with nerve terminals in adult muscle. Sections of normal (a, b), 2-day denervated (c), or 12-day denervated (d) muscle was stained with antibodies to recombinant heregulin. Sections were counterstained with anti-SV2 to label nerve terminals (a') or with rhodamine- α -bungarotoxin to label the postsynaptic membrane (b'-d'). In adult muscle, ARIA is present in nerve terminals as well as in association with the postsynaptic membrane and/or basal lamina. Axotomy leads to loss of axon- and terminal-associated ARIA, but muscle-associated ARIA persists. Bar, 15 μ m for a and 40 μ m for b-d.

structures that were also labeled by antibodies to the synaptic vesicle protein SV2 (Figs. 2a and 2a') and that directly apposed the rhodamine- α -bungarotoxin-stained postsynaptic membrane (Figs. 2b and 2b'). Most of the punctate ARIA immunoreactivity was lost within 2 days of axotomy (Fig. 2c), but significant ARIA remained associated with synaptic sites for at least 12 days (Fig. 2d), indicating that some is also associated with the muscle cell surface or synaptic cleft.

Next, we asked when ARIA becomes concentrated at synaptic sites. ARIA immunoreactivity was detectable at synaptic sites by P0 (Fig. 3a). Surprisingly, however, the ARIA was not confined to synaptic sites, but extended around much of the circumference of the muscle fiber. Moreover, anti-ARIA also stained the muscle fiber cytoplasm and/or intracellular membranes. Staining of muscle fibers increased during the first several postnatal days (Fig. 3b). By P7, ARIA was concentrated at synaptic sites, but intracellular structures and extrasynaptic portions of the muscle fiber surface remained ARIA-positive (Fig. 3c). During the second postnatal week, the ratio of synaptic to extrasynaptic staining increased (Fig. 3d), until the adult pattern was achieved.

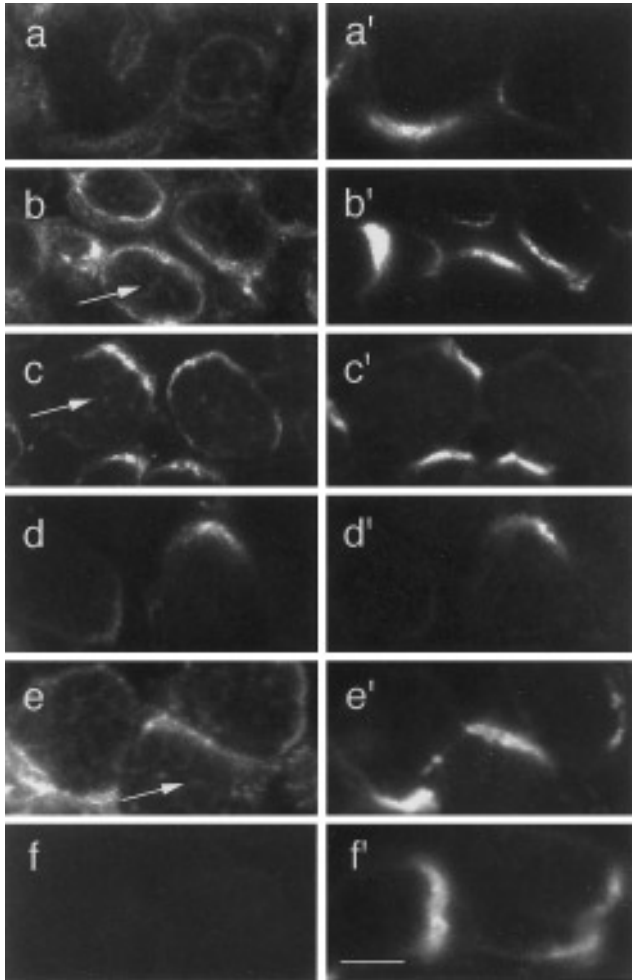


FIG. 3. Muscle-derived perisynaptic deposits of ARIA in developing muscle. Sections of rat muscle were stained with antibodies to recombinant heregulin (a–d) or with antisera to a heregulin-derived peptide (e, f) and rhodamine- α -bungarotoxin (a'–f') at P0 (a), P4 (b), P7 (c, e, f), or P14 (d). For f, the anti-peptide antisera was preincubated with the immunizing peptide. Arrows in (b), (c), and (e) indicate muscle fiber cytoplasm immunoreactivity. Staining of muscle fiber cytoplasm and of nonmuscle cells between muscle fibers (fibroblasts and/or capillary endothelium) was more prominent with the anti-peptide serum than with the anti-recombinant heregulin. Bar, 15 μ m.

Similar results were obtained with affinity-purified antisera (not shown) and with antiserum directed against a peptide derived from the predicted intracellular domain of an ARIA/hergulin precursor (Fig. 3e). Staining by the anti-peptide antiserum was completely blocked by preincubation of the antiserum with the peptide that had been used as immunogen (Fig. 3f).

These results suggested that muscle fibers synthesize ARIA. To test this possibility, we used the mouse muscle cell line, Sol 8. Sol 8 cells grow as myoblasts in rich me-

dium, then fuse to form myotubes when refed with serum-poor medium. Their advantage for the present purpose is that they are a homogeneous cell type, whereas muscle contains many cell types, including some (e.g., axons) that contain ARIA. Accordingly, we purified antibodies against the bioactive peptide heregulin $\beta_{177-244}$ as previously described (Chu *et al.*, 1995a) and used them to probe extracts of Sol 8 myotubes and brain. The antibody recognized several species in both tissues (Fig. 4a, 1). Preincubation of the antibody with heregulin peptide abolished reactivity with the three most prominent reactive proteins in muscle and the two most prominent bands in brain, thereby identifying these as ARIA-related (Fig. 4a, 2); preincubation with an unrelated peptide had no effect (Fig. 4a, 3). One of the forms in Sol 8 cells was similar in size to the ARIA-polypeptide originally isolated from chicken brain (~ 42 kDa; Falls *et al.*, 1993) and to the predominant ARIA species in P7 rat muscle and adult brain. A second ARIA species in Sol 8 cells (~ 120 kDa) was present in brain but not neonatal muscle, while a third species (~ 58 kDa) was present in neither (Figs. 4a and 4b).

Studies with recombinant fragments have shown that the EGF-like domain in ARIA is crucial for its AChR-inducing activity (Chu *et al.*, 1995a). To learn whether muscle-derived ARIA contains an intact EGF-like domain, we designed PCR primers that spanned this domain, plus two alternatively spliced regions, and used them to perform PCR on cDNA from Sol 8 myotubes. The PCR products were cloned and several clones were sequenced. Although the clones differed at the α/β and 1–5 sites, all contained an EGF-like domain and thus would be predicted to encode active ARIA (Fig. 4c). Using the β_1 fragment as a probe, we found that ARIA RNA is present in muscle at birth (Fig. 4d, 1). ARIA levels increased during the first postnatal week (data not shown) and then declined during the subsequent 2 weeks to barely detectable levels in adults (Fig. 4d). Based on these results, we conclude that muscle fibers express authentic ARIA *in vivo*.

Synaptic Localization of *erbB2* and *erbB3* in Adult Muscle

ErbB kinases are the only known receptors for ARIA/hergulin (Carraway and Cantley, 1994). We used specific antibodies to each of the four erbB kinases to assess their distribution in adult muscle. Anti-EGFR stained interstitial areas in a patchy pattern, presumably reflecting the presence of EGFR on nonmuscle cells such as fibroblasts (Fig. 5a). Synaptic sites were also sometimes EGFR-positive, but this staining was dim and inconsistent. Likewise, muscle fibers were stained weakly if at all by anti-erbB4 (Fig. 5d), even though the antibody stained known erbB4-containing structures in brain (not shown). In contrast, anti-erbB2 and anti-erbB3 selectively stained synaptic sites on muscle fibers (Figs. 5b and 5c). Their distribution precisely overlapped that of AChRs (Figs. 5b' and 5c'), indicating that the kinases were associated with the postsynaptic membrane. Staining

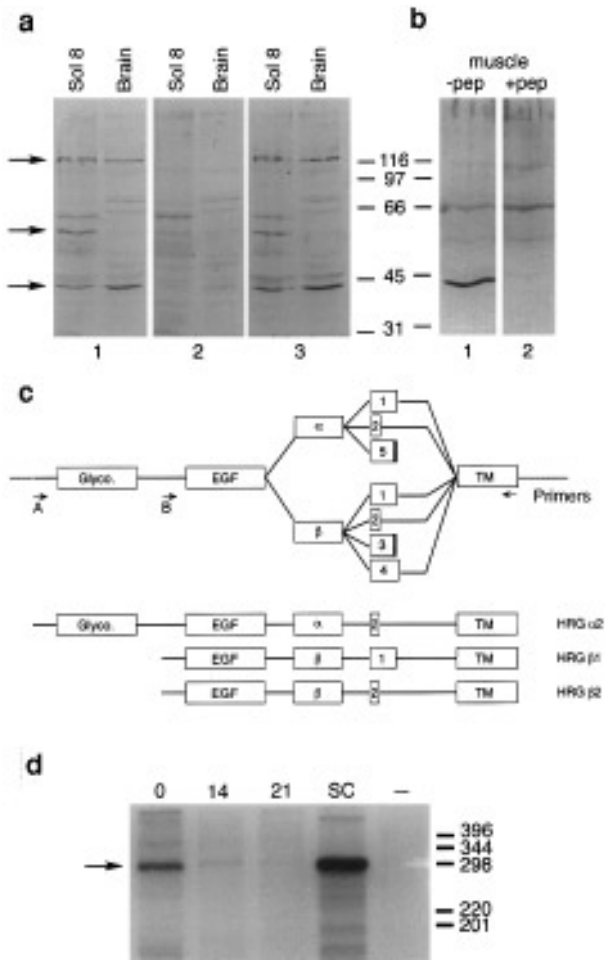


FIG. 4. ARIA protein and mRNA expression in developing muscle and Sol 8 cells. (a) Western blot analysis of Sol 8 myotube and mouse brain protein extracts (50 μ g/lane). Panel 1 was probed with anti-hergulin antibody. To identify specific bands, the anti-hergulin antibody was preincubated with either the bioactive peptide HRG β ₁₇₇₋₂₄₄ (panel 2) or with an irrelevant peptide (panel 3). Arrows on the left indicate the specific heregulin peptides of 120, 58, and 42 kDa. The 58-kDa product (middle arrow) is present in myotubes but not brain. Molecular weight markers are shown on the right. (b) Western blot of P7 rat muscle extract (50 μ g/lane) probed with anti-hergulin (lane 1) or anti-hergulin that had been preincubated with the bioactive peptide (lane 2). (c) Heregulin RNAs cloned from Sol 8 myotubes. Heregulin transcripts are alternatively spliced at several positions, as shown in the diagram at the top (modified from Ben-Baruch and Yarden, 1994). Primers used to amplify cDNAs from Sol 8 cells are indicated. Three cDNAs that were cloned are shown. (d) RNase protection assays of postnatal rat muscle, using the HRG β ₁ probe. A major protected fragment is seen in P0 muscle and spinal cord (SC). Levels of RNA decrease by P14. (—) lane, probe hybridized to yeast tRNA.

by antibodies to all four kinases was blocked by preincubation with the immunizing peptide but not with peptides that had been used to generate other anti-erbB sera (Figs. 5e

and 5f and data not shown). Identical results were obtained with each of two anti-erbB3 antibodies, one of which recognized an intracellular and the other an extracellular epitope (see Methods). Thus, erbB2 and erbB3 are concentrated at the neuromuscular junction.

As was the case for ARIA, we used Sol 8 cells to obtain evidence that muscle cells generate authentic erbB kinases. Using cRNA probes of similar length and specific activity, we detected comparable levels of erbB2 and erbB3 RNA in Sol 8 myotubes. No EGFR or erbB4 RNA was detected by RNase protection (Fig. 6a), although low levels of EGFR (but not erbB4) were detected by RT-PCR (not shown). Similar RNase protection results were obtained using untreated and ARIA-treated myotubes. EGFR and erbB4 were readily detected in tissues previously shown to express these genes (placenta and brain, respectively; Prigent *et al.*, 1992; Plowman *et al.*, 1993), ruling out the possibility that the probes were defective. Thus, *in vitro* as *in vivo*, muscle cells express relatively high levels of erbB2 and erbB3 but little or no EGFR or erbB4. Moreover, in that Sol 8 myotubes are ARIA-responsive (Chu *et al.*, 1995a), these results support the notion that erbB2 and/or erbB3 are functional ARIA receptors.

Based on these results, we immunoblotted extracts of Sol 8 myoblasts and myotubes with antibodies to erbB2 and erbB3. In myotubes, both erbB2 and erbB3 antibodies recognized proteins of 180–185 kDa, the expected sizes for these erbB kinases (Fig. 6b). ErbB2 was present at similar levels in myoblasts and myotubes, whereas erbB3 was detectable only in myotubes. Staining was blocked by preincubation of antisera with the immunizing peptide but not with a noncognate peptide (not shown). Thus, whereas both erbB2 and erbB3 are candidate ARIA receptors in myotubes, erbB2 might mediate distinct effects of heregulins on myoblasts.

In addition to the 180-kDa protein, one of the anti-erbB3 antisera also recognized a 50 kDa protein in all tissues tested. Staining was blocked by the cognate peptide, indicating that this protein shares at least one epitope with erbB3. However, the second, independently derived antiserum to erbB3, which did stain synaptic sites, recognized the 180-kDa but not the 50-kDa protein on immunoblots (not shown). Therefore, we did not characterize the 50-kDa protein further.

Distribution of erbB3 in Developing Muscle

For detailed studies of development and regulation, we focused on erbB3. AChRs cluster on developing myotubes by E14 (Chiu and Sanes, 1984), but erbB3 was not detectable by our immunofluorescence method until the day of birth (Figs. 7a and 7b). As soon as we could detect it at all, erbB3 protein was concentrated at synaptic sites. Staining of synaptic sites became more intense during the first postnatal week, but no extrasynaptic staining was observed at any age (Figs. 7c and 7d). The synaptic staining was specific in that it was blocked by incubation of the antibody with the immunizing peptide (Fig. 7e). Thus, erbB3 protein becomes

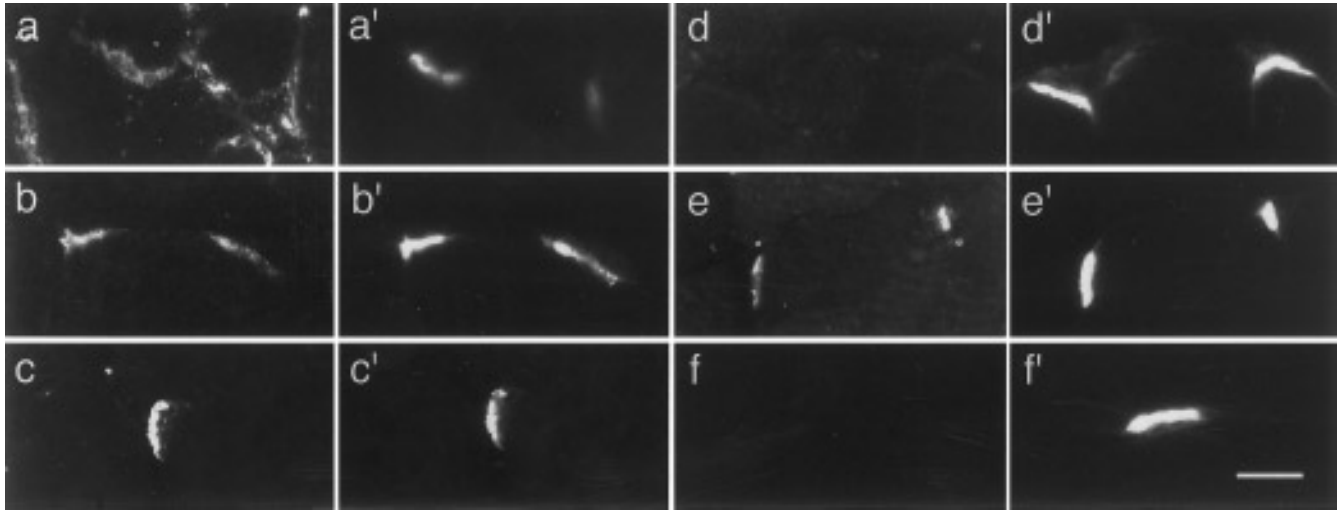


FIG. 5. Distribution of erbB proteins in adult rat muscle. Sections were incubated with specific antibodies to EGFR (a), erbB2 (b), erbB3 (c, e, f), or erbB4 (d). The sections were then stained with fluorescein-conjugated second antibodies (a–f) and rhodamine- α -bungarotoxin (a'–f'). Staining by each antibody persisted in the presence of nonspecific peptide (for example, e, erbB3 Ab + erbB2 peptide) but was inhibited by incubation with the peptide that had been used as immunogen (for example, f, erbB3 Ab + erbB3 peptide). Bar, 40 μ m.

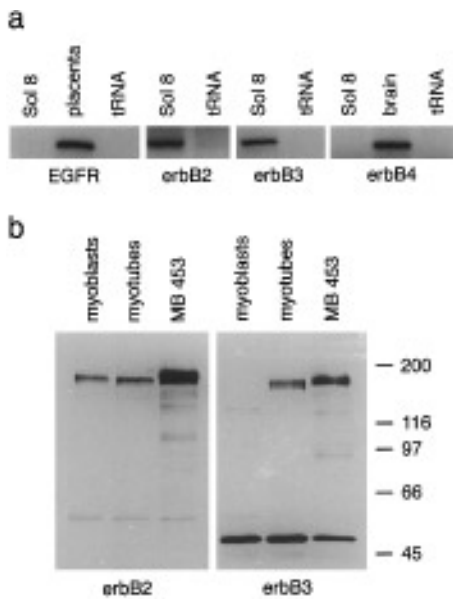


FIG. 6. Expression of erbB family kinases in Sol 8 cells. (a) RNase protection analysis. Ten micrograms of RNA from Sol 8 cells or placenta (plac) or 5 μ g of brain RNA was hybridized with probes of similar specific activities. Protected fragment sizes are: EGFR, 328 bp; erbB2, 240 bp; erbB3, 350 bp; erbB4, 273 bp. Probes were hybridized to tRNA as a negative control. Sol 8 myotubes express high levels of erbB2 and erbB3 and no detectable EGFR or erbB4 RNA. (b) Western blot analysis. Ten micrograms of Sol 8 myoblast and myotube protein was probed with antibodies specific for erbB2 or erbB3. The human breast carcinoma cell line, MDA-MB 453, which is known to express both erbB2 and erbB3 (Cailleau *et al.*, 1978), was used as a positive control (5 μ g protein per lane).

concentrated at the synapse perinatally, many days after synapses form. This pattern stands in striking contrast to that described above for ARIA (compare Figs. 3 and 7).

Because low levels of erbB3 immunoreactivity at early stages might have escaped detection, we used the sensitive RNase protection assay to ask whether erbB3 RNA was present in embryos. In fact, erbB3 RNA was present in muscle at E17 and its abundance changed little through the first postnatal week, as judged by normalization to the ubiquitously expressed eEF1 α mRNA (Fig. 7f). This result raises the possibility that erbB3 protein is present but diffusely distributed in embryonic muscle.

Mechanism of erbB3 Localization

AChRs become confined to the synaptic membrane as a result of three mechanisms: selective transcription by synaptic nuclei, activity-dependent repression of expression by extrasynaptic nuclei, and clustering of diffuse receptors via interactions with the cytoskeletal protein rapsyn (see Introduction for references). We asked whether any of these mechanisms could account for the synaptic localization of erbB3.

To seek selective transcription of erbB3 by synaptic nuclei, we performed *in situ* hybridization on longitudinal sections of intercostal muscle, in which endplates form a narrow central band. The synaptic concentration of AChR ϵ subunit mRNA is readily detectable in such sections (Fig. 8b, middle; see Moscoso *et al.*, 1995), but erbB3 RNA was diffusely distributed in intercostal muscle (Fig. 8b, top). In addition, we used RNase protection to assay synapse-free regions of diaphragm muscles, which are known to contain high and low levels, respectively, of AChR subunit mRNAs

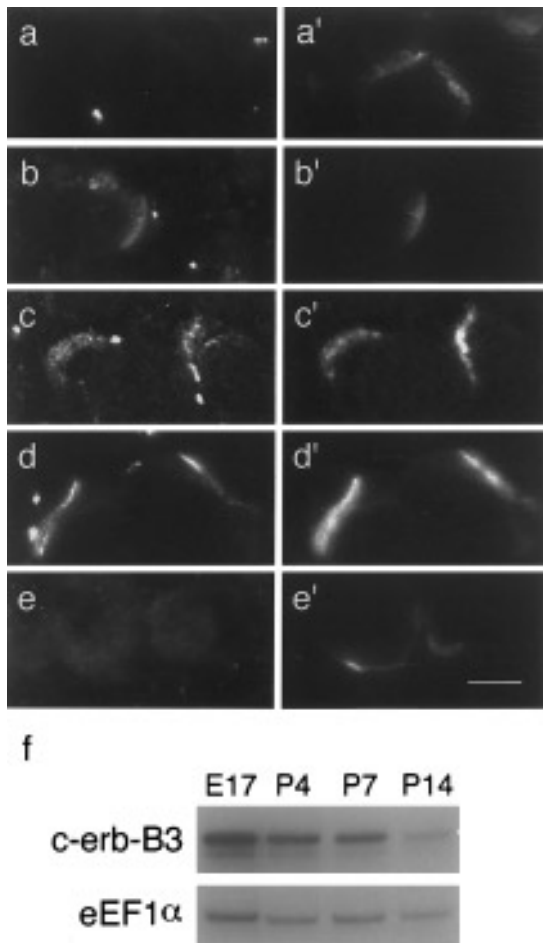


FIG. 7. Distribution of erbB3 in developing muscle. Sections of E18 (a), P0 (b, e), P7 (c), or P10 (d) rat muscle were doubly labeled with anti-erbB3 (a–e) and rhodamine- α -bungarotoxin (a'–e'). ErbB3 was concentrated at synaptic sites as early as we could detect it. Specific staining of erbB3 at early stages was confirmed by staining in the presence of specific blocking peptide (e). Bar, 15 μ m. (f) ErbB3 RNA levels in developing muscle, assayed by RNase protections in mouse muscle. Ten micrograms of muscle RNA was hybridized with the erbB3 probe for each assay. Ages are as indicated above each lane. The housekeeping gene eEF1 α was used to control for amounts of RNA.

(Merlie and Sanes, 1985). Levels of erbB3 RNA did not differ significantly between synaptic and nonsynaptic regions (Fig. 8a). Thus, erbB3 does not appear to become spatially restricted via a local transcription mechanism.

To ask whether activity represses erbB3 expression, we examined denervated muscle. ErbB3 mRNA levels changed little during the first 4 days after nerve section (Fig. 9a), a time at which AChR subunit mRNA levels had increased nearly 100-fold (Merlie *et al.*, 1984). Even after an additional week, erbB3 levels changed only a fewfold. Likewise, no erbB3 protein was detectable in extrasynaptic areas of 2- or

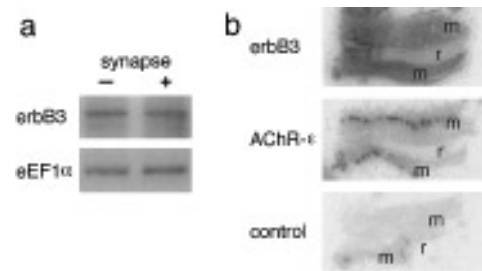


FIG. 8. Diffuse distribution of erbB3 RNA in adult muscle. (a) Synapse-rich and synapse-free regions of adult muscle were dissected and assayed for erbB3 by RNase protection. Endplate-rich regions of muscle were not significantly enriched for erbB3 RNA. (b) Sections of 3-week-old rat intercostal muscle hybridized with erbB3 (top), AChR ϵ subunit (middle), or control (laminin α 1, bottom) probes. m, muscle; r, rib. Similar results were obtained with adult muscle. Autoradiograms on X-ray film are shown.

12-day denervated muscle (Figs. 9b and 9c). These results suggest that erbB3 expression is not highly activity-dependent.

To ask whether erbB3 clustering is rapsyn-dependent, we used a rapsyn-deficient mutant mouse that we recently generated. These mice express AChRs at normal levels, but the AChRs do not form synaptic clusters (Gautam *et al.*, 1995). The animals died a few hours after birth, by which time erbB3 immunoreactivity was confined to synaptic sites in wild-type mice (Fig. 10a). In mutants, in contrast, no high

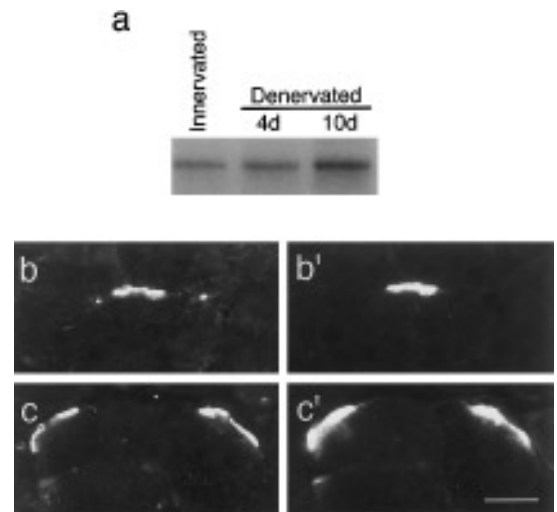


FIG. 9. ErbB3 RNA levels and protein localization change little following denervation. (a) RNA levels of erbB3 in 4- and 10-day denervated rat muscle assayed by RNase protection. (b, c) Adult rat muscles doubly labeled with anti-erbB3 and rhodamine bungarotoxin, 2 (b) or 12 (c) days after nerve section. Brightly stained endplates are observed at both times. Bar, 40 μ m.

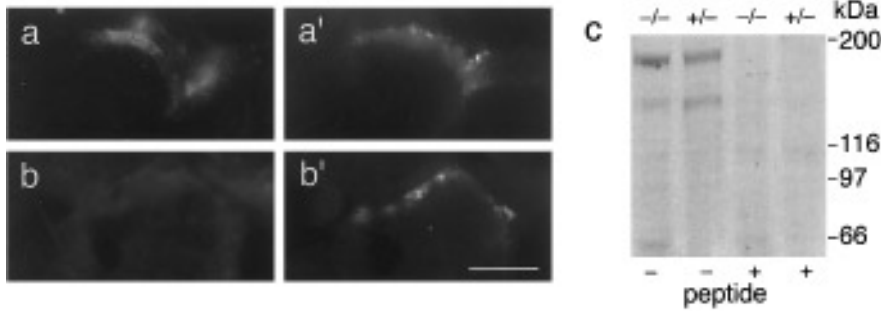


FIG. 10. Distribution of erbB3 protein in rapsyn-deficient muscle from P0 control (a) and mutant (b) mice were labeled with anti-erbB3 (a', b') and antibodies to the synaptic vesicle protein SV2 (a', b'). ErbB3 is not clustered at synaptic sites in the absence of rapsyn. Bar, 10 μ m. (c) Immunoblot analysis of muscles from control and rapsyn mutant mice, probed with anti-erbB3 in the absence or presence of the immunizing peptide.

density clusters of the erbB3 protein were detected at synapses (Fig. 10b). (Synapses were marked with SV2 in these studies, because AChR clusters were absent; see Fig. 2a.) Western blot analysis showed that similar levels of erbB3 protein were present in mutant animals and littermate controls (Fig. 10c). These results provide genetic evidence that rapsyn is not required for erbB3 gene expression, but does play a critical role in localizing erbB3 protein to the synapse. In contrast, erbB3 was normally localized in mutant mice lacking the basal lamina protein s-laminin/laminin β 2 (Noakes *et al.*, 1995; data not shown).

DISCUSSION

ARIA was discovered in a search for nerve-derived factors that stimulate muscle cells to synthesize AChRs (Usdin and Fischbach, 1986). Molecular cloning revealed that ARIA is encoded by the same gene that generates heregulin/NDF (Holmes *et al.*, 1992; Wen *et al.*, 1992). Motoneuronal somata contain ARIA mRNA (Falls *et al.*, 1993; Marchionni *et al.*, 1993), and ARIA protein is concentrated at synaptic sites in adult muscle (Chu *et al.*, 1995a; Jo *et al.*, 1995). Studies of cultured cells showed that heregulin is a ligand for the erbB family of receptor tyrosine kinases (Plowman *et al.*, 1993b; Carraway *et al.*, 1994; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1994; reviewed in Carraway and Cantley, 1994) and that myotubes contain functional erbB kinases (Jo *et al.*, 1995; see also Corfas *et al.*, 1993). A plausible hypothesis, therefore, is that developing motor axons release ARIA onto erbB-bearing myotubes, resulting in generation of a local signal that induces transcription of AChR subunit genes by subsynaptic nuclei. In the studies reported here, we asked whether ARIA and erbB kinases are expressed at times and places consistent with this hypothesis. Our tentative answer is "yes," but this affirmation is qualified by several unexpected results. First, much of the ARIA in developing muscle is made by the muscle fiber. Second, both erbB2 and erbB3 are (or become) concentrated at synap-

tic sites. Third, the distribution of erbB3 is regulated by rapsyn and, by implication, agrin. Finally, levels of both ARIA and erbB3 increase markedly in synaptic regions perinatally, several days after synaptic nuclei become transcriptionally specialized.

Muscle-Derived ARIA

Most of the intramuscular ARIA immunoreactivity we detect during early postnatal development is associated with myotubes rather than nerve terminals and is not restricted to synaptic sites. ARIA RNA is also present in muscles, arguing against the possibility that the protein is synthesized in motoneuronal somata and subsequently transferred from nerves to myotubes. In addition, cultured myotubes accumulate ARIA protein and RNA, demonstrating that ARIA is made by muscle fibers, rather than (or in addition to) other intramuscular cells, such as fibroblasts or Schwann cells. Whether the muscle contributes ARIA to the synaptic site per se is unknown, but a recent observation by Jo *et al.* (1995) suggests that it might: they showed that levels of ARIA immunoreactivity at synaptic sites were higher in denervated muscle than in basal lamina "ghosts" that survived damage to the muscle as well as to the nerve. Thus, some of the synaptic ARIA in adult muscle might be associated with the muscle membrane.

How could nerves use ARIA as a synaptic signal in the face of an excess of muscle-derived ARIA? We can envision several alternatives. One is that the muscle-derived ARIA is intracellular and has no access to its receptors, which face outwards. This seems unlikely, both because we detect surface-associated ARIA on myotubes and because only a tiny fraction of the muscle ARIA would need to be released to exceed a neural contribution. Second, axonal ARIA might induce expression of muscle ARIA, which would then amplify the signal. Indirect evidence against this possibility is the finding that application of recombinant ARIA does not significantly affect the levels of ARIA RNA or protein in Sol 8 cells (unpublished data). Third, muscle ARIA might

be biologically inactive. In fact, this explanation is now favored for agrin, a nerve-derived signal that induces AChR clustering (McMahan, 1990). Alternative splicing generates several agrin RNAs, only some of which encode biologically active protein. Most of the agrin in developing muscles is synthesized by the myotubes rather than by the motoneurons, but only the neurons express the "active" forms (Bowe and Fallon, 1995). Similarly, the ARIA gene is alternatively spliced to generate numerous RNAs, and although our PCR analysis indicates that muscle ARIA encodes the domains required for biological activity (Holmes *et al.*, 1992), it may contain additional sequences or modifications that render it incapable of inducing AChR synthesis.

Finally, it is possible that the nerve uses a distinct signal to induce expression of muscle ARIA, and ARIA subsequently induces AChR expression by an autocrine mechanism. In this context, it is noteworthy that Avila *et al.* (1995) recently demonstrated autocrine control of proliferation in a neoplastic cell line by ARIA/NDF and erbB2.

Synaptic Localization of erbB2 and erbB3

Axons can form synapses on any part of the myotube surface (Hall and Sanes, 1993), so one might expect that the location of the postsynaptic site would be determined by a localized, nerve-derived signal acting on a uniformly distributed postsynaptic receptor. Such a mechanism would be analogous to several that have been studied in other systems. In the *Drosophila* embryo, for example, the establishment of dorsoventral polarity is triggered by the binding of a ventrally produced ligand, Spätzle, to a uniformly distributed receptor, Toll (Roth, 1994). In contrast, the putative receptor erbB3 is more tightly restricted to synaptic sites than is its ligand, ARIA, in neonatal animals.

These observations raise the possibility that the spatial restriction of the ARIA signaling pathway involves localization of the receptor as well as of the ligand. It was therefore intriguing to find that the restriction of erbB3 to synaptic sites is rapsyn-dependent. Clustering of AChRs in the subsynaptic membrane, which is triggered by nerve-derived factors such as agrin, requires rapsyn: forced expression of rapsyn causes recombinant AChRs to cluster in cultured cells (Apel and Merlie, 1995), and mutation of the *Rapsn* gene prevents AChR clustering *in vivo* (Gautam *et al.*, 1995). Localization of erbB3 by a rapsyn-dependent pathway may thus provide a site at which agrin and ARIA-dependent pathways for AChR localization interact. Specifically, agrin-induced, rapsyn-dependent clustering of erbB3 might play a role in focusing a subsequent ARIA-mediated induction of AChR expression to subsynaptic nuclei.

Two Steps in the Maturation of Synaptic Nuclei

Synaptic nuclei become specialized by birth: AChR α and γ subunit RNAs are concentrated near synapses in embryos (Piette *et al.*, 1993), and transgenic mice bearing AChR genomic regulatory sequences express their transgenes at syn-

aptic sites in embryos (γ subunit; G.C.C. and J.P.M., unpublished) or by birth (δ subunit; Simon *et al.*, 1992). We find that erbB3 is not detectably concentrated at synaptic sites in embryos (Fig. 7) and that synaptic nuclei (marked by a γ -lacZ transgene) became transcriptionally specialized in rapsyn-deficient mice (Gautam *et al.*, 1995), which lack clustered erbB3 (Fig. 10). Thus, synaptic accumulations of erbB3 are not required for the initial specification of synaptic nuclei. Evidently, either diffusely distributed erbB3 (which may be present at levels below the sensitivity of our immunofluorescence method) is sufficient, or another receptor is involved.

During the first 2 postnatal weeks, synaptic nuclei cease expressing the AChR γ subunit gene and dramatically increase expression of the AChR ϵ subunit gene, resulting in a replacement of fetal AChRs (subunit composition $\alpha_2\beta\gamma\delta$) by adult AChRs ($\alpha_2\beta\epsilon\delta$; Hall and Sanes, 1993; Chu *et al.*, 1995b). Synaptic nuclei begin to express the AChR ϵ subunit gene on schedule following neonatal denervation (Brenner *et al.*, 1990; Gundersen *et al.*, 1993), indicating that subunit switching does not require the continuous presence of the nerve. Instead, it has been hypothesized that the nerve deposits a local "trace," perhaps in the basal lamina (Goldman *et al.*, 1991; Jo and Burden, 1992; Brenner *et al.*, 1992), that "imprints" synaptic nuclei. Whether the subunit switch reflects a distinct effect of the trace or an endogenous maturation remains unclear. In this context, it is interesting that synaptic deposits of erbB3 and extrasynaptic accumulations of ARIA appear in muscle fibers during the time that the γ -to- ϵ switch occurs. This correlation invites the speculation that muscle ARIA and clustered erbB3 play a role in the subunit switch. The observations that ARIA induces expression of the AChR ϵ subunit >10-fold, while inducing the AChR α , β , γ , and δ subunits \leq 2-fold in cultured muscle (Martinou *et al.*, 1991; Chu *et al.*, 1995a; Jo *et al.*, 1995), is consistent with this idea. One intriguing possibility is that a predominant role of the nerve-derived "trace" might be to localize erbB3 kinases by a rapsyn/agrin-dependent mechanism, thus localizing the ϵ -inducing effects of muscle-derived ARIA.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work. We thank J. Cunningham for assistance and M. X. Sliwkowski for recombinant heregulin and anti-heregulin antibody. This work was supported by grants from NIH to J.R.S. and J.P.M.

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Received for publication June 1, 1995
Accepted August 23, 1995