

# Muscle-Specific Agrin Isoforms Reduce Phosphorylation of AChR $\gamma$ and $\delta$ Subunits in Cultured Muscle Cells

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The accumulation of nicotinic acetylcholine receptors (AChRs) at neuromuscular synapses is triggered by agrin, a protein that is synthesized by both nerve and muscle. Nerve-derived agrin, which contains an amino acid insert at a conserved splice site in the carboxy-terminal part of the protein, induces AChR aggregation and causes tyrosine phosphorylation of the AChR  $\beta$  subunit. In contrast, agrin isoforms synthesized by muscle cells lack such an insert and have no effect on AChR distribution. In order to identify possible functional roles of muscle-derived agrin we have analyzed further the effect of various fragments of recombinant agrin on AChR phosphorylation. A carboxy-terminal fragment of muscle agrin, c95<sub>A0B0</sub>, reduced AChR  $\gamma$  and  $\delta$  subunit phosphorylation when added to C2C12 myotubes in culture. Although c95<sub>A0B0</sub> had no effect on AChR  $\beta$  subunit phosphorylation when added alone, it inhibited AChR  $\beta$  subunit phosphorylation and AChR aggregation by the nerve-specific agrin isoform c95<sub>A4B8</sub>. We conclude that muscle-derived agrin can influence, both directly and indirectly, AChR phosphorylation. Such changes may play a role in the formation, maintenance, or function of the neuromuscular junction.

## INTRODUCTION

Differentiation of the vertebrate skeletal neuromuscular junction requires structural rearrangements of both pre- and postsynaptic components. A large body of evidence suggests that the formation of the postsynaptic apparatus is triggered by agrin, a ~400-kDa multidomain heparan sulfate proteoglycan that is bound in the synaptic basal lamina (McMahan, 1990; Bowe and Fallon, 1995; Denzer *et al.*, 1995; Gautam *et al.*, 1996; Jones *et*

*al.*, 1996, 1997; Meier *et al.*, 1997; Rimer *et al.*, 1997; Cohen *et al.*, 1997).

A crucial step in the formation of the postsynaptic apparatus is the accumulation of acetylcholine receptors (AChRs) into high-density aggregates in the postsynaptic membrane of the muscle cell (Hall and Sanes, 1993). When added to myotubes in cell culture, agrin induces the formation of specializations at which AChRs and other components of the postsynaptic apparatus accumulate (McMahan, 1990; Bowe and Fallon, 1995). Agrin's AChR-aggregating activity lies in the ~95-kDa carboxy-terminal portion of the protein, which contains three domains homologous to the globular (G) domains of the laminin  $\alpha$  chain (Tsim *et al.*, 1992; Gee *et al.*, 1994; Gesemann *et al.*, 1995). This region is characterized by two conserved splice sites, designated as A and B in chick and y and z in rodent. Amino acid inserts at these sites regulate the ability of agrin to induce AChR aggregation (Ruegg *et al.*, 1992; Rupp *et al.*, 1992; Ferns *et al.*, 1992, 1993; McMahan *et al.*, 1992). Agrin isoforms with inserts at both splice sites, in particular agrin<sub>A4B8</sub>, are very potent inducers of AChR aggregation on cultured muscle cells (Ferns *et al.*, 1993; Gesemann *et al.*, 1995). Motor neurons express high levels of such isoforms (Tsim *et al.*, 1992; Hoch *et al.*, 1993; Ma *et al.*, 1994, 1995). Muscle cells, on the other hand, synthesize agrin isoforms that lack inserts at one or both splice sites (agrin<sub>A4B0</sub>, agrin<sub>A0B0</sub>); these isoforms are weakly active or inactive in AChR aggregation assays when added in a soluble form to cultured myotubes (Ruegg *et al.*, 1992; Hoch *et al.*, 1993; Ma *et al.*, 1994; Gesemann *et al.*, 1995). Although the role of neural agrin in triggering postsynaptic differentiation at developing neuromuscular junctions is well established (Bowe and Fallon, 1995; Gautam *et al.*, 1996), the function of muscle agrin remains unclear.

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Muscle agrin codistributes with AChRs during early differentiation of *Torpedo* electrocytes (Cartaud *et al.*, 1996) and accumulates at sites of nerve- or agrin-induced AChR aggregation in developing muscles and in myotubes in culture (Fallon *et al.*, 1985; Olwin and Hall, 1985; Reist *et al.*, 1987; Fallon and Gelfman, 1989; Lieth *et al.*, 1992; Lieth and Fallon, 1993). Further, muscle agrin, when presented on the surface of transfected cells or together with heparin-binding growth associated molecule (HB-GAM) shows AChR-aggregating activity (Ferns *et al.*, 1992, 1993; Daggett *et al.*, 1996). Such observations suggest that muscle-derived agrin may participate in synaptic differentiation. On the other hand, muscle-derived agrin is not seen at ectopically located postsynaptic specializations experimentally induced by nerve-derived agrin in innervated adult muscle (Meier *et al.*, 1997; Cohen *et al.*, 1997), indicating that under such conditions at least some of neural agrin's effects do not require accumulation of muscle-derived agrin.

Muscle agrin strongly binds  $\alpha$ -dystroglycan (Sugiyama *et al.*, 1994; Gesemann *et al.*, 1996).  $\alpha$ -Dystroglycan is a component of the dystrophin glycoprotein complex, which is thought to reinforce the myofiber membrane to provide the structural stability required to withstand cycles of contraction and relaxation (Henry and Campbell, 1996). The dystrophin complex also accumulates at agrin- and nerve-induced AChR aggregates. Indeed, based on its ability to bind agrin,  $\alpha$ -dystroglycan was proposed to function as the muscle agrin receptor mediating AChR aggregation (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994). However, agrin isoforms that do not cause AChR aggregation bind to  $\alpha$ -dystroglycan with higher affinity than do isoforms that induce AChR aggregation (Sugiyama *et al.*, 1994; Gesemann *et al.*, 1996) and fragments of agrin that lack the binding domain for  $\alpha$ -dystroglycan still induce AChR aggregation (Gesemann *et al.*, 1996; Hopf and Hoch, 1996; Meier *et al.*, 1996). Thus, binding of agrin to  $\alpha$ -dystroglycan is not required for AChR aggregation. On the other hand, laminin has been shown to induce AChR aggregation on cultured myotubes by a mechanism that involves  $\alpha$ -dystroglycan (Sugiyama *et al.*, 1997; Montanaro *et al.*, 1998), suggesting that the dystrophin complex may play a secondary role in synapse formation and/or stabilization (Phillips *et al.*, 1993; Campanelli *et al.*, 1994; Cohen *et al.*, 1995).

Experiments on cultured myotubes suggest that agrin-induced AChR aggregation involves tyrosine phosphorylation of the AChR  $\beta$  subunit (Wallace *et al.*, 1991; Baker and Peng, 1993; Qu and Haganir, 1994; Wallace, 1994, 1995; Meier *et al.*, 1995; Ferns *et al.*, 1996). Neural agrin isoforms, which induce AChR aggregation, cause tyro-

sine phosphorylation of the AChR  $\beta$  subunit, while muscle agrin isoforms do not (Meier *et al.*, 1996). Two protein tyrosine kinases have been identified that might mediate agrin-induced AChR  $\beta$  subunit phosphorylation: the receptor protein tyrosine kinase MuSK, which appears to form part of the muscle receptor for neural agrin (DeChiara *et al.*, 1996; Glass *et al.*, 1996; Apel *et al.*, 1997), and src-like kinases, which may be activated by MuSK (Swope and Haganir, 1993, 1994; Fuhrer and Hall, 1996; Fuhrer *et al.*, 1997).

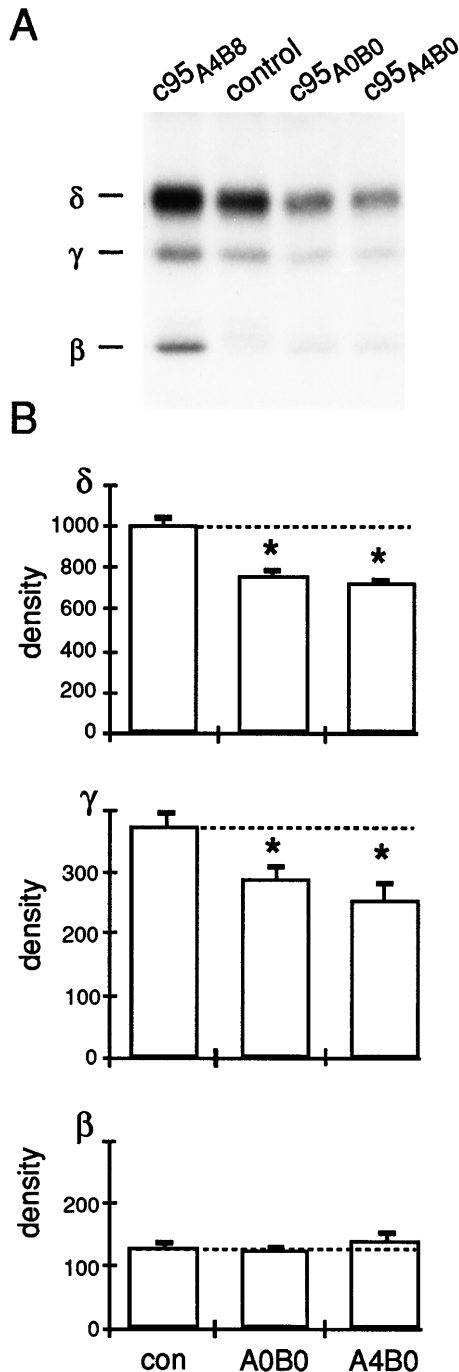
All subunits of the nicotinic AChR can be phosphorylated on tyrosine and/or serine residues (reviewed in Haganir and Miles, 1989), and phosphorylation not only causes AChR aggregation, but appears to regulate a variety of other receptor properties as well (Levitan, 1994). As a step toward identifying the function of muscle agrin, we sought to compare quantitatively the effect of fragments of recombinant nerve and muscle agrin isoforms on AChR phosphorylation. Here we report that muscle agrin c95<sub>A0B0</sub> specifically reduced the level of AChR  $\gamma$  and  $\delta$  subunit phosphorylation when tested on cultured C2C12 myotubes. Although agrin<sub>A0B0</sub> had no effect on AChR  $\beta$  subunit phosphorylation when tested alone, it inhibited the effect of neural agrin<sub>A4B8</sub> on AChR  $\beta$  subunit phosphorylation and AChR aggregation. This indicates an interplay of muscle and neural agrin isoforms at the level of AChR subunit phosphorylation that might play a role in the formation, maintenance, or function of the neuromuscular junction.

## RESULTS

### **Muscle-Specific Agrin Isoforms Reduce Phosphorylation of AChR $\gamma$ and Subunits**

As a step toward understanding the role of muscle agrin at the neuromuscular junction, we have analyzed the effect of muscle agrin on AChR phosphorylation in cultured C2C12 myotubes. Myotubes were incubated in medium containing [<sup>32</sup>P]orthophosphate and treated with various isoforms of a ~95-kDa recombinant C-terminal fragment of chick agrin that contains the A and B splice sites (Ruegg *et al.*, 1992; Gesemann *et al.*, 1995, 1996). AChRs were purified from the treated myotubes, the subunits separated by SDS-polyacrylamide gel electrophoresis, and the incorporation of radioactive phosphate measured by autoradiography. As expected from previous results (Meier *et al.*, 1995, 1996), an agrin isoform with the splice site composition of neural agrin, c95<sub>A4B8</sub>, induced phosphorylation of the AChR  $\beta$ ,  $\gamma$ , and  $\delta$  subunits (Fig. 1A). On the other hand, up to 200 nM of two muscle agrin isoforms, c95<sub>A0B0</sub> and c95<sub>A4B0</sub> did not

change the level of AChR  $\beta$  subunit phosphorylation, but specifically decreased the [ $^{32}$ P] content of the AChR  $\gamma$  and  $\delta$  subunits (Figs. 1A and 1B). The effect of c95<sub>A0B0</sub> on AChR  $\gamma$  and  $\delta$  subunit phosphorylation was only obvious at concentrations at or above 100 nM. Thus, the effects of muscle agrin isoforms on phosphorylation of AChR subunits are distinct from the changes caused by the neural agrin isoform c95<sub>A4B8</sub>.



### Time Course of the Reduction of AChR $\gamma$ and $\delta$ Subunit Phosphorylation by Muscle Agrin

We next analyzed the time course of the reduction in AChR  $\gamma$  and  $\delta$  subunit phosphorylation by c95<sub>A0B0</sub>. The effect of muscle agrin c95<sub>A0B0</sub> on AChR  $\gamma$  and  $\delta$  subunit phosphorylation was obvious after 3 h of incubation (Fig. 2A), the shortest time interval tested. The maximum effect, an approximately 40% reduction in the level of phosphorylation for both AChR  $\gamma$  and  $\delta$  subunits, was reached after 8 h (Fig. 2B). Prolonged incubation (15 h) had no additional effect. The extent and time course of the reduction in phosphorylation was similar for both subunits. The time course appears to be slower than that for neural agrin-induced tyrosine phosphorylation of the AChR  $\beta$  subunit and is comparable to the time course of receptor aggregation (Ferns *et al.*, 1996).

To test whether the decrease in AChR  $\gamma/\delta$  phosphorylation might be a consequence of a reduction in the number of AChRs, we determined AChR content by  $\alpha$ -[ $^{125}$ I]bungarotoxin binding. Following a 10-h incubation with 100 nM c95<sub>A0B0</sub>, the level of specific  $\alpha$ -[ $^{125}$ I]bungarotoxin binding did not differ from cultures incubated in control medium (Fig. 2C). Likewise, the rate of AChR degradation was unchanged (data not shown). Thus, the effect of muscle agrin on AChR  $\gamma$  and  $\delta$  subunit phosphorylation was not caused by a change in the number of AChRs or their turnover rate.

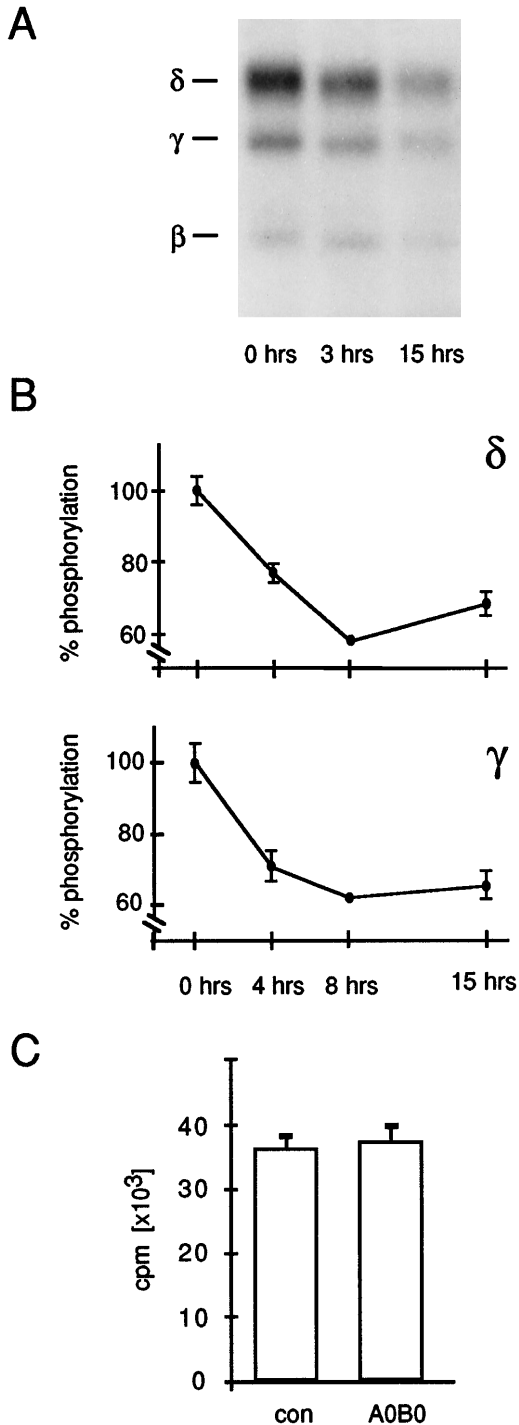
### Muscle Agrin Antagonizes the Effects of Neural Agrin on AChR Phosphorylation and Aggregation

Both muscle- and nerve-derived agrin accumulate at neuromuscular junctions (Bowe *et al.*, 1994) and muscle

**FIG. 1.** Muscle-specific agrin isoforms reduce phosphorylation of the AChR  $\gamma$  and  $\delta$  subunits. (A) Autoradiogram of AChRs isolated from C2C12 myotube cultures prelabeled overnight with [ $^{32}$ P]orthophosphate and analyzed by SDS-PAGE. Compared to controls, incubating cultures for 4 h with 200 nM of the muscle specific agrin isoforms c95<sub>A0B0</sub> and c95<sub>A4B0</sub> reduced phosphorylation of the AChR  $\gamma$  and  $\delta$  subunits. In contrast, 20 nM of the nerve-specific agrin isoform c95<sub>A4B8</sub> induced phosphorylation of all three AChR subunits ( $\beta$ ,  $\gamma$ , and  $\delta$ ). (B) Quantitative analysis of agrin-induced changes in AChR  $\beta$ ,  $\gamma$ , and  $\delta$  phosphorylation in C2C12 myotubes prelabeled with [ $^{32}$ P]orthophosphate and incubated for 3–4 h with 200 nM c95<sub>A4B0</sub> (A4B0) or 200 nM c95<sub>A0B0</sub> (A0B0). Control cultures (con) were incubated in parallel in normal medium. Data from different experiments were combined by normalizing the results of each experiment to the  $\delta$  subunit of control myotubes in normal medium (=1000; see Meier *et al.*, 1995). Data are mean  $\pm$  SEM;  $N = 6$  (con),  $N = 3$  (A4B0),  $N = 6$  (A0B0). Dashed line indicates level of subunit phosphorylation in control cultures. \*Differs significantly from control,  $P < 0.05$  (one-way ANOVA, Tukey-Kramer HSD test).

agrin has been shown to shift the dose–response curve for neural agrin-induced AChR aggregation two- to threefold to higher concentrations (Gesemann *et al.*, 1996). Accordingly, we analyzed the effect of muscle agrin on the level of AChR phosphorylation and aggregation in myotubes stimulated with neural agrin. For

these experiments, C2C12 myotubes were treated with a saturating amount of neural agrin c95<sub>A4B8</sub>, either alone or in combination with an excess of muscle agrin c95<sub>A0B0</sub>. Incubation of myotubes for 15 h with 100 pM c95<sub>A4B8</sub> increased the phosphorylation of AChR  $\beta$ ,  $\gamma$ , and  $\delta$  subunits and caused AChR aggregation, as expected (Fig. 3). Addition of muscle agrin antagonized the effects of neural agrin: phosphorylation of the AChR  $\gamma$  and  $\delta$  subunits was blocked, phosphorylation of the AChR  $\beta$  subunit was inhibited by ~60%, and AChR aggregation was inhibited by ~30% (Fig. 3).



### Shorter Agrin Fragments and Laminin Do Not Reduce AChR $\gamma$ and $\delta$ Phosphorylation

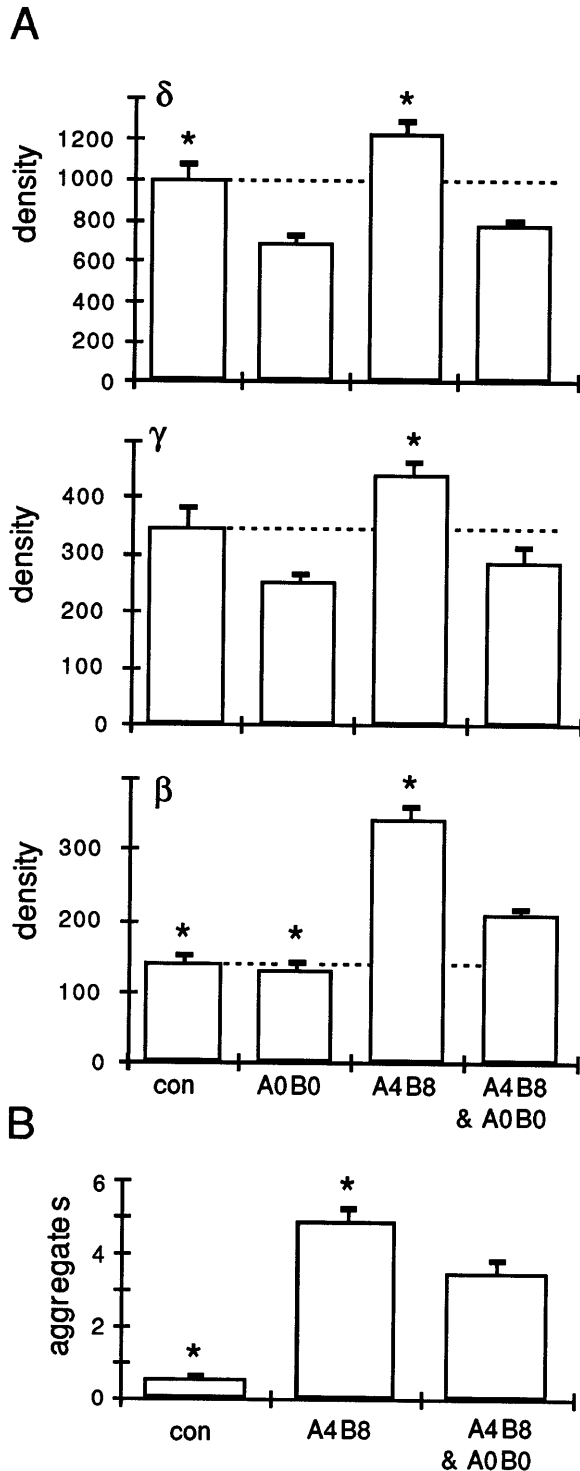
Different binding properties have been assigned to distinct but partially overlapping regions of the C-terminal portion of agrin, including binding to  $\alpha$ -dystroglycan, to heparin, and to the receptor mediating AChR aggregation (Gesemann *et al.*, 1996). For example, a minimal agrin fragment comprising the third laminin G-like domain with the eight amino acid insert at the B-splice site does not bind to  $\alpha$ -dystroglycan or heparin, but does induce AChR aggregation and  $\beta$  subunit phosphorylation (Gesemann *et al.*, 1996; Meier *et al.*, 1996). To determine if the effect on AChR  $\gamma/\delta$  phosphorylation seen with muscle agrin was associated with one of the known functional domains of agrin, we measured the effect of short agrin fragments on AChR  $\gamma/\delta$  phosphorylation. At 200 nM, agrin isoforms comprising the second laminin G-like domain with or without the 4 amino acid insert at the A splice site (G2<sub>A0</sub> and G2<sub>A4</sub>), had no effect on AChR  $\gamma/\delta$  phosphorylation (Fig. 4). The same was true for the c21<sub>B0</sub> fragment, comprising the

**FIG. 2.** Time course of c95<sub>A0B0</sub>-induced reduction of AChR  $\gamma$  and  $\delta$  phosphorylation. (A) Autoradiogram of AChRs isolated from cultures preincubated for 2 h with medium containing [<sup>32</sup>P]orthophosphate and stimulated with 200 nM c95<sub>A0B0</sub> for 0, 3, or 15 h. The time of exposure to [<sup>32</sup>P]containing medium was the same for all conditions tested. (B) Quantitative analysis of the time-course of muscle agrin-induced reduction of AChR  $\gamma$  and  $\delta$  subunit phosphorylation. C2C12 myotube cultures were stimulated with 100 nM of c95<sub>A0B0</sub> agrin isoforms for the indicated period of time, the level of subunit phosphorylation determined by densitometry and compared to that of AChRs from control cultures (0 h), which was set as 100%. Data are mean  $\pm$  SEM; error bars are shown where they exceed the size of the data point.  $N = 11$  (0 h),  $N = 3$  (4 h),  $N = 4$  (8 h),  $N = 7$  (15 h). (C) Number of AChRs, measured by specific binding of  $\alpha$ -[<sup>125</sup>I]bungarotoxin. Compared to control cultures (con), stimulation with 100 nM c95<sub>A0B0</sub> (A0B0) for 10 h did not influence the number of AChRs. Data in counts per minute (cpm) is mean  $\pm$  SEM ( $N = 3$ ).

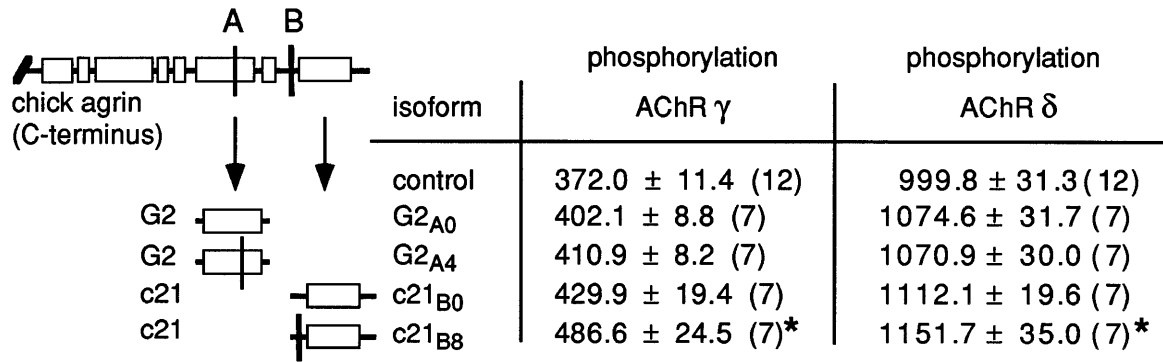
third laminin G-like domain without an amino acid insert at the B splice site. Agrin fragment c21<sub>B8</sub>, which is sufficient to cause AChR aggregation and  $\beta$  subunit phosphorylation (Gesemann *et al.*, 1995, 1996; Meier *et al.*, 1996), increased the level of AChR  $\gamma$  and  $\delta$  subunit

phosphorylation. Thus, with or without amino acid inserts, isolated laminin G-like domains did not mimic the reduction in AChR  $\gamma/\delta$  phosphorylation observed with c95<sub>A0B0</sub>. We conclude from this experiment that neither binding to heparin, as observed for G2<sub>A4</sub>, nor binding to the agrin receptor mediating AChR aggregation, as observed for C21<sub>B8</sub>, alone can account for the effect of the muscle agrin fragment c95<sub>A0B0</sub> on phosphorylation of the  $\gamma$  and  $\delta$  subunits.

Muscle agrin isoforms also bind to  $\alpha$ -dystroglycan (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994). To determine if binding of c95<sub>A0B0</sub> to  $\alpha$ -dystroglycan might cause reduction in AChR  $\gamma$  or  $\delta$  subunit phosphorylation, we tested whether laminin-1, another binding partner of  $\alpha$ -dystroglycan (Ibraghimov-Beskrovnya *et al.*, 1992; Gee *et al.*, 1993; Gesemann *et al.*, 1996), could mimic the effect of agrin c95<sub>A0B0</sub>. Incubation of C2C12 myotubes with laminin did not reduce the level of phosphorylation of AChR  $\gamma$  and  $\delta$  subunits (Table 1). In contrast, laminin induced a dose-dependent increase in the level of phosphorylation of all three AChR subunits. However, the increase in AChR phosphorylation was accounted for by a laminin-induced increase in AChR content. For example, incubation of C2C12 myotubes with 25 nM laminin caused a 25% increase in phosphorylation of AChR  $\beta$ ,  $\gamma$ , and  $\delta$  subunits (Table 1).  $\alpha$ -[<sup>125</sup>I]Bungarotoxin binding revealed a similar ~33% increase in the total number of AChRs. Thus, while muscle agrin reduced AChR  $\gamma$  and  $\delta$  subunit phosphorylation without changing the number of receptors on the myotube surface, laminin increased the number of surface receptors without any apparent change in the specific activity of receptor phosphorylation.



**FIG. 3.** Muscle-specific agrin c95<sub>A0B0</sub> reduces AChR phosphorylation and aggregation in cultures treated with nerve-specific agrin c95<sub>A4B8</sub>. (A) Quantitative analysis of agrin-induced changes in AChR  $\beta$ ,  $\gamma$ , and  $\delta$  phosphorylation as determined by densitometric analysis of autoradiograms. Myotube cultures were incubated for 15 h with 100 pM c95<sub>A4B8</sub> (A4B8), 100 nM c95<sub>A0B0</sub> (A0B0), or both agrin isoforms (A4B8 and A0B0). AChRs were isolated and the level of phosphorylation of each subunit compared to that in control cultures (con). Data are mean  $\pm$  SEM,  $N = 6$ . Dashed line indicates level of subunit phosphorylation in control cultures. \*Differs significantly from "A4B8 and A0B0,"  $P < 0.05$  (one-way ANOVA, Tukey-Kramer HSD test). (B) The muscle-specific agrin isoform c95<sub>A0B0</sub> partially inhibits the formation of AChR aggregates induced by nerve-specific agrin c95<sub>A4B8</sub>. Myotube cultures were incubated overnight with 100 pM c95<sub>A4B8</sub> (A4B8) or 100 pM c95<sub>A4B8</sub> and 100 nM c95<sub>A0B0</sub> (A4B8 and A0B0), AChRs labeled with rhodamine- $\alpha$ -bungarotoxin, and aggregates counted in 45 myotube segments from 3 culture dishes. Data, expressed as number of aggregates per myotube segment, are mean  $\pm$  SEM. \*Differs significantly from "A4B8 and A0B0,"  $P < 0.05$  (one-way ANOVA, Tukey-Kramer HSD test).



**FIG. 4.** Analysis of changes in AChR  $\gamma$  and  $\delta$  phosphorylation induced by individual laminin G-domain fragments of chick agrin. Schematic representations identify fragments of the carboxy-terminal part of chick agrin and isolated G-domains (Gesemann *et al.*, 1995, 1996). The presence or absence of the 4 amino acid insert in the second G-domain (G2<sub>A4</sub>, G2<sub>A0</sub>) and the presence or absence of the conserved 8 amino acid insert of the third G-domain (c21<sub>B8</sub>, c21<sub>B0</sub>) are indicated by vertical bars. <sup>32</sup>P-Pre-labeled C2C12 myotubes were treated for 4 h with 200 nM of each of the G-domain fragments and levels of AChR  $\gamma$  and  $\delta$  phosphorylation determined by densitometric analysis of autoradiograms. Control cultures (con) were incubated in parallel in normal medium. Data from different experiments were combined by normalizing the results of each experiment to the  $\delta$  subunit of control myotubes in normal medium (=1000). Data is mean  $\pm$  SEM (N). \*Differs significantly from control,  $P < 0.05$  (one-way ANOVA, Tukey-Kramer HSD test).

## DISCUSSION

The observations that muscle-derived agrin is concentrated at neuromuscular junctions and at neural agrin-induced AChR aggregates on myotubes in culture suggests that muscle agrin could play a role in synapse formation, maintenance, or function. Our study supports this hypothesis by demonstrating that muscle-derived agrin isoforms influence AChR phosphorylation. Unlike neural agrin, which increases phosphorylation of the  $\beta$ ,  $\gamma$ , and  $\delta$  subunits, muscle agrin specifically decreases phosphorylation of the  $\gamma$  and  $\delta$  subunits.

Muscle agrin is clearly not required for formation of AChR aggregates. For example, neural agrin still induces AChR aggregation on myotubes from agrin-deficient mice (Gautam *et al.*, 1996) or on agrin-deficient C2C12 cells (Pun *et al.*, 1997), and muscle agrin does not

accumulate at ectopic postsynaptic specializations induced by neural agrin on adult muscle fibers (Meier *et al.*, 1997). Likewise, changes in AChR  $\gamma$  and  $\delta$  subunit phosphorylation are not required for neural agrin-induced AChR aggregation. Neural agrin-induced increases in  $\gamma$  and  $\delta$  subunit phosphorylation can be blocked with the serine protein kinase inhibitor H-7 (Wallace, 1994) without preventing AChR aggregation. Neural agrin-induced tyrosine phosphorylation of the AChR  $\beta$  subunit, on the other hand, correlates closely with AChR aggregation. Preventing such an increase with staurosporine or herbimycin blocks AChR aggregation (Wallace, 1994; Ferns *et al.*, 1996), and, as shown here, reducing the increase in  $\beta$ -subunit phosphorylation by addition of muscle agrin also reduces the extent of receptor aggregation.

One might speculate that AChR  $\gamma$  and  $\delta$  phosphorylation is involved in the stabilization or maintenance of

**TABLE 1**  
Laminin-Induced Changes in AChR Phosphorylation and Content

Treatment	AChR subunit phosphorylation				$\alpha$ -[ <sup>125</sup> I]BGT binding sites	
	$\beta$	$\gamma$	$\delta$	N	cpm	N
Control	143.2 $\pm$ 9.8	395.0 $\pm$ 12.6	1000.0 $\pm$ 25.6	5	36,405 $\pm$ 1,803	3
Laminin						
10 nM	186.0 $\pm$ 8.6	473.1 $\pm$ 12.0	1,202.6 $\pm$ 20.4	7	n.d.	
25 nM	201.3 $\pm$ 10.5	532.0 $\pm$ 14.4	1,259.3 $\pm$ 39.9	3	48,655 $\pm$ 2,262	3

*Note.* C2C12 cultures were stimulated for 15 h with 10 or 25 nM EHS-laminin. AChR phosphorylation was determined from autoradiograms of receptors isolated from <sup>32</sup>P-labeled cultures. AChR content was measured by specific binding of  $\alpha$ -[<sup>125</sup>I]bungarotoxin. Data is expressed as mean  $\pm$  SEM; n.d.: not determined.

receptor aggregates. Consistent with such an hypothesis is the finding that, in myotubes treated with neural agrin, AChRs that are more firmly attached to the cytoskeleton, as judged by resistance to detergent extraction (Prives *et al.*, 1982; Stya and Axelrod, 1983; Podleski and Salpeter, 1988; Wallace, 1992, 1995), are more heavily phosphorylated on  $\gamma$  and  $\delta$  subunits than are more readily extracted receptors (Meier *et al.*, 1995). On the other hand, if myotubes are treated with phorbol ester to activate protein kinase C, then phosphorylation of the AChR  $\gamma$  and  $\delta$  subunits is increased, yet AChR aggregates disperse (Ross *et al.*, 1988; Wallace, 1988). Clearly it would be of interest to analyze directly the stability of AChR aggregates under conditions that specifically promote or prevent phosphorylation of the AChR  $\gamma$  and  $\delta$  subunits.

AChR phosphorylation appears to affect other properties of AChRs, such as desensitization (Huganir and Miles, 1989). cAMP-dependent changes in phosphorylation influence the rate of receptor turnover in myotubes (Shyng *et al.*, 1991; O'Malley *et al.*, 1993; Xu and Salpeter, 1995), although it is not known whether this is due to phosphorylation of AChRs or other proteins. Agrin was shown to slow slightly the rate of AChR degradation in a heterologous expression system (Phillips *et al.*, 1997). However, at concentrations that are optimal for inducing AChR aggregation, agrin partially purified from *Torpedo* electric organ was shown to have little effect on the number or degradation rate of AChRs (Godfrey *et al.*, 1984). Here we show that, even at the relatively high concentrations that decrease phosphorylation of the AChR  $\gamma$  and  $\delta$  subunits, muscle agrin did not cause a change in the number of cell surface AChRs or their rate of degradation.

#### **Role of Identified Domains within Agrin in Reducing AChR $\gamma$ and $\delta$ Subunit Phosphorylation**

A striking finding in the present study is that removal of the 8 amino acid insert from the B splice site converts agrin from a form that increases phosphorylation of the  $\beta$ ,  $\gamma$ , and  $\delta$  subunits to one that decreases phosphorylation of the  $\gamma$  and  $\delta$  subunits. Together with the observation that the c21<sub>B8</sub> fragment is sufficient to increase phosphorylation of the  $\beta$ ,  $\gamma$ , and  $\delta$  subunits this suggests that the presence of an insert at the B splice site affects the conformation of agrin in at least two ways. First, the presence of a B insert enables agrin to combine with the receptor that mediates aggregation and phosphorylation of the  $\beta$ ,  $\gamma$ , and  $\delta$  subunits. This change in conformation must occur in the immediate vicinity of the B splice site, since the same receptor can be activated by the small c21<sub>B8</sub> fragment, but not c21<sub>B0</sub>. Second, presence of

the B8 insert must change the conformation of more distant portions of the agrin protein, preventing agrin from causing a decrease in  $\gamma$  and  $\delta$  phosphorylation. Hoch *et al.* (1994) likewise concluded that the presence of an insert at the B splice site results in a distributed conformation change in the agrin protein, based on the location of the epitope recognized by a splice variant-specific monoclonal antibody.

The precise domains of agrin that cause decreased phosphorylation of the AChR  $\gamma$  and  $\delta$  subunits have yet to be identified. Isolated G2 domains had no effect on AChR  $\gamma$  and  $\delta$  subunit phosphorylation. Further, binding to heparin, conferred by the 4 amino acid insert at the A splice site within the G2 domain (Gesemann *et al.*, 1996; O'Toole *et al.*, 1996; Campanelli *et al.*, 1996; Hopf and Hoch, 1997), did not appear to have any additional effect; c95<sub>A0B0</sub> and c95<sub>A4B0</sub> are equally effective at reducing  $\gamma$  and  $\delta$  subunit phosphorylation. Thus, muscle agrin-mediated reduction in phosphorylation of the  $\gamma$  and  $\delta$  subunits requires domains of agrin larger than the isolated laminin G-like domains.

#### **Muscle Agrin Binds to $\alpha$ -Dystroglycan**

Results of previous experiments have led to the suggestion that the dystrophin glycoprotein complex, which includes  $\alpha$ - and  $\beta$ -dystroglycan, might be involved in signal transduction during synapse formation and/or maintenance. For example, components of the dystrophin-associated glycoprotein complex accumulate at sites of agrin-induced AChR clusters (Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994; Cohen *et al.*, 1995; Meier *et al.*, 1997). Further, Grb2, an adapter protein containing SH2- and SH3-domains (Lowenstein *et al.*, 1992), which is thought to link receptor protein tyrosine kinases with the Ras signaling pathway (Ridley and Hall, 1992; Matuoka *et al.*, 1993), has been found to be associated with  $\beta$ -dystroglycan in muscle (Yang *et al.*, 1995) and with phosphorylated AChRs in *Torpedo* electric organ (Colledge and Froehner, 1997). However, in *Torpedo* there is no evidence for an interaction between Grb2 and  $\beta$ -dystroglycan (Colledge and Froehner, 1997), and the Grb2-binding motif in the  $\delta$  subunit of *Torpedo* AChR is not conserved in the  $\delta$  subunits of other species. Moreover, there is currently no known ligand for  $\beta$ -dystroglycan that might be capable of stimulating Grb2 and thereby activating the Ras pathway.

The findings presented here raise the possibility that AChR  $\gamma/\delta$  subunit phosphorylation might be regulated by muscle-derived agrin isoforms binding to the  $\alpha/\beta$ -dystroglycan complex. The concentration of c95<sub>A0B0</sub> required to reduce AChR  $\gamma/\delta$  phosphorylation ( $\geq 100$  nM) is similar to the concentration required for binding

of agrin c95<sub>A0B0</sub> to  $\alpha$ -dystroglycan on cultured myotubes (Gesemann *et al.*, 1996). Binding studies have shown that the minimal domain required for binding of agrin to  $\alpha$ -dystroglycan comprises the two laminin-like G-domains, G1 and G2 (Gesemann *et al.*, 1996; Hopf and Hoch, 1996; Sugiyama *et al.*, 1994). Although the third G-domain (which is included in c21 fragments) and its associated B splice site are not required for binding to  $\alpha$ -dystroglycan, the presence of an insert at the B site decreases the avidity with which c95-fragments of agrin bind to  $\alpha$ -dystroglycan. As shown here, c95<sub>A0B0</sub>, which contains all three G-domains, reduces AChR  $\gamma/\delta$  subunit phosphorylation, while none of the individual G-domains tested (G2 and c21 fragments) had any effect. Thus, the characteristics of the interaction of agrin with  $\alpha$ -dystroglycan resemble those for reduction of AChR  $\gamma$ - and  $\delta$ -subunit phosphorylation.

It is interesting to note that there is a significant discrepancy between the apparent affinity for binding of agrin c95<sub>A0B0</sub> to  $\alpha$ -dystroglycan on myotubes (100–200 nM, Gesemann *et al.*, 1996) and to pure  $\alpha$ -dystroglycan *in vitro* (1–2 nM; Gesemann *et al.*, 1998). It seems likely that this difference arises from the presence of endogenous ligands for  $\alpha$ -dystroglycan, such as laminin and endogenous muscle agrin, in the extracellular matrix (ECM) surrounding myotubes in culture that compete with exogenous c95<sub>A0B0</sub> for binding. Even if present at low absolute levels, the effective local concentration of such competing endogenous ECM-associated ligands could be quite high at the myotube surface, and thus shift to much higher concentrations the apparent affinity for exogenous c95<sub>A0B0</sub>. Conversely, depending on their relative position within the ECM, low absolute levels of full-length muscle agrin, which is an integral component of the ECM, could have a sufficiently high effective local concentration to saturate even relatively low affinity binding sites on adjacent molecules of  $\alpha$ -dystroglycan.

If binding of muscle agrin to  $\alpha$ -dystroglycan regulates AChR  $\gamma$  and  $\delta$  phosphorylation, then laminin, which also binds to  $\alpha$ -dystroglycan (Ibraghimov-Beskrovnya *et al.*, 1992; Gee *et al.*, 1993; Gesemann *et al.*, 1996), might be expected to reduce AChR  $\gamma$  and  $\delta$  phosphorylation. To the contrary, we found that laminin-1 had no apparent effect on the extent of AChR phosphorylation, but increased the number of surface AChRs. The increase in the number of surface AChRs on C2C12 myotubes induced by soluble laminin, reported here for the first time, may reflect a role of laminin in the differentiation of cultured myotubes mediated by activation of integrin receptors (Olwin and Hall, 1985; Vogel *et al.*, 1983; Song *et al.*, 1992; Vachon *et al.*, 1996). Thus, even if binding of laminin to  $\alpha$ -dystroglycan tended to reduce AChR  $\gamma/\delta$

phosphorylation as does muscle agrin, such an effect may be masked by the induction of AChR synthesis.

Unlike muscle agrin, laminin does have effects on the distribution of  $\alpha$ - and  $\beta$ -dystroglycan, and in some species on the distribution of AChRs as well. For example, laminin induces AChR aggregation on cultured C2C12 myotubes by an  $\alpha$ -dystroglycan-dependent mechanism (Sugiyama *et al.*, 1997; Montanaro *et al.*, 1998). This pathway does not require phosphorylation of either MuSK or the AChR  $\beta$  subunit. In *Xenopus* myotubes, on the other hand, although laminin induces aggregation of  $\alpha$ - and  $\beta$ -dystroglycan, AChRs do not aggregate (Cohen *et al.*, 1997). Such results suggest that laminin, and perhaps muscle agrin as well, may interact with a variety of muscle receptors that activate distinct signaling pathways influencing the distribution of AChRs. Thus, the formation of the postsynaptic apparatus is likely to be a complex and highly regulated event. For example, ECM components, such as laminin and muscle agrin, that are induced to accumulate together with AChRs by neural agrin are, in turn, likely to participate in and/or regulate the further recruitment of AChRs and other postsynaptic components, acting through diverse signaling pathways some of which involve  $\alpha/\beta$ -dystroglycan.

In summary, the results presented here support the idea that muscle-derived agrin might have several roles in synapse formation, maintenance, or function. Muscle agrin is likely to be a significant structural component of the synaptic basal lamina. Our results suggest that, in addition, muscle agrin stimulates an intracellular signal transduction pathway, perhaps by binding to  $\alpha$ -dystroglycan, that leads to changes in AChR phosphorylation that may influence receptor aggregation or function.

## EXPERIMENTAL PROCEDURES

**Agrin and laminin.** The recombinant chick agrin fragments and isoforms used in this study were expressed and purified as described by Gesemann *et al.* (1995, 1996). Natural mouse laminin isolated from Engelbreth-Holm-Swarm (EHS) sarcoma was purchased from Gibco BRL. This preparation consists predominantly of laminin-1 (Kleinman *et al.*, 1982).

**Cell cultures and aggregation assays.** C2C12 cells were grown in collagen-coated 35-mm tissue culture dishes in proliferation medium until they reached confluence. Cells were subsequently changed to differentiation medium as described earlier (Meier *et al.*, 1995). After 4 to 6 days, cells had fused and were used for AChR aggregation and phosphorylation assays. For aggregation assays, cultures were incubated with agrin

isoforms for 16 h. AChRs were labeled with  $4 \times 10^{-8}$  M rhodamine- $\alpha$ -bungarotoxin (Molecular Probes, Eugene, OR), and AChR aggregation was determined by counting the number of receptor aggregates per 300- $\mu$ m myotube segment.

**Measurement of AChR phosphorylation.** AChR phosphorylation was assayed as previously described (Meier *et al.*, 1995, 1996). Briefly, C2C12 cultures were rinsed with MEM without sodium phosphate (Gibco BRL) supplemented with 1 mg/ml bovine serum albumin (RIA grade, Sigma Chemical Co.), 20  $\mu$ g/ml conalbumin (type II, Sigma Chemical Co.), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, followed by a 16-h incubation in the same medium containing 0.25 mCi/ml [ $^{32}$ P]orthophosphate ([ $^{32}$ P]H $_3$ PO $_4$  in H $_2$ O, ICN Biomedicals Inc., Costa Mesa, CA). Agrin isoforms were added to this culture medium at the designated concentrations for the indicated time. AChRs were labeled with biotinylated  $\alpha$ -bungarotoxin (Wallace *et al.*, 1991; Meier *et al.*, 1995) and toxin-AChR complexes were solubilized by incubation for 10 min on ice in extraction buffer (20 mM sodium phosphate buffer, pH 7.4, supplemented with 5 mM EDTA, 5 mM EGTA, 50 mM sodium fluoride, 40 mM sodium pyrophosphate, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, 1 mM PMSF, 1% [w/v] Triton X-100 and 0.25% [w/v] deoxycholate). Extracts were cleared and toxin-AChR complexes purified on streptavidin-conjugated agarose beads. Beads were washed thoroughly and eluted into SDS sample buffer at room temperature. AChR subunits were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels. Gels were fixed, dried, and exposed to preflashed autoradiography film (Hyperfilm-MP, Amersham, Arlington Heights, IL) and autoradiograms were analyzed by densitometry as previously described (Wallace, 1994). AChR  $\beta$ ,  $\gamma$ , and  $\delta$  subunits were identified by their position relative to prestained molecular weight markers and by criteria described previously (Meier *et al.*, 1995). Densitometric analyses were carried out from autoradiography films or directly from dried gels using a PhosphoImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Data from different experiments were combined by normalizing the results of each experiment to the  $\delta$  subunit of control myotubes in normal medium (=1000).

**Measurement of AChR turnover and number.** Cultures were changed to serum-free medium [DME-F12 supplemented with 1 mg/ml bovine serum albumin (RIA grade, Sigma Chemical Co.), 20  $\mu$ g/ml conalbumin (type II, Sigma Chemical Co.), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin] and experimental cultures were treated for 10 h with 100 nM c95<sub>A0B0</sub> or 25 nM

laminin. Control and experimental cultures were then incubated for 1 h with  $2 \times 10^{-8}$  M  $\alpha$ -[ $^{125}$ I]bungarotoxin (Amersham) to label surface AChRs. After washing in serum-free medium and adding agrin and laminin at concentrations as above, 1 ml medium was added and 100- $\mu$ l samples were taken after 4, 8, and 13 h incubation at 37°C to determine AChR turnover-rates. Residual AChRs were extracted with 1 M NaOH at the end of the experiment. AChRs were quantitated for each fraction by gamma counting after correction for nonspecific binding.

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