Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7369–7374, July 1996
Neurobiology

Alternative splicing of agrin regulates its binding to heparin, α-dystroglycan, and the cell surface

(synaptogenesis/glycosaminoglycans/muscular dystrophy/dystrophin-associated protein complex/neuromuscular junction)

JOHN J. O'TOOLE, KATHERINE A. DEYST, MARK A. BOWE, MARY A. NASTUK, BETH A. MCKECHNIE, AND JUSTIN R. FALLON*†

Neurobiology Group, Worcester Foundation for Biomedical Research, 222 Maple Avenue, Shrewsbury, MA 01545

Communicated by Louis M. Kunkel, The Children's Hospital, Boston, MA, April 4, 1996 (received for review December 27, 1995)

ABSTRACT **Agrin is a basal lamina molecule that directs key events in postsynaptic differentiation, most notably the aggregation of acetylcholine receptors (AChRs) on the muscle cell surface. Agrin's AChR clustering activity is regulated by alternative mRNA splicing. Agrin splice forms having inserts at two sites (*y* and *z*) in the C-terminal region are highly active, but isoforms lacking these inserts are weakly active. The biochemical consequences of this alternative splicing are unknown. Here, the binding of four recombinant agrin isoforms to heparin, to α-dystroglycan (a component of an agrin receptor), and to myoblasts was tested. The presence of a four-amino acid insert at the *y* site is necessary and sufficient to confer heparin binding ability to agrin. Moreover, the binding of agrin to α-dystroglycan is inhibited by heparin when this insert is present. Agrin binding to the cell surface showed analogous properties: heparin inhibits the binding of only those agrin isoforms containing this four-amino acid insert. The results show that alternative splicing of agrin regulates its binding to heparin and suggest that agrin's interaction with α-dystroglycan may be modulated by cell surface glycosaminoglycans in an isoform-dependent manner.**

Rapid and efficient communication at the synapse requires the precise topological arrangement of its component molecules. A prime example of such specialization is the neuromuscular junction, where there are >10,000 acetylcholine receptors (AChRs) per μm^2 of postsynaptic membrane (1). During synapse formation, agrin secreted by nerve terminals induces clustering of AChRs and several other postsynaptic elements on the muscle cell surface (refs. 2–4; reviewed in ref. 5). Later in synaptic development, agrin secreted by muscle cells also becomes localized to the synapse (6). Agrin remains stably associated with the synaptic basal lamina, where it is likely to play a role in synaptic maintenance and regeneration (7, 8).

The AChR clustering activity of agrin resides in the C-terminal half of the molecule. Agrin mRNA is alternatively spliced at three sites in this half. Splicing at two of these sites, termed *y* and *z* in rodent (and *A* and *B* in chick) have been studied in detail (9–10). These splicing events result in the insertion of a four-amino acid (aa) insert at the *y* site, and an eight- and/or an 11-aa insert at the *z* site (Fig. 1). Neurons and muscle cells express agrin both with and without the four-aa insert at the *y* site. However, inserts at the *z* site are present exclusively in neural tissue, and probably only in neurons (13).

Alternative splicing profoundly influences agrin's AChR clustering activity (10, 14). The relative order of the activities of the soluble, C-terminal portions of agrin isoforms is Ag4,19 ≅ Ag4,8 > Ag4,11 > Ag4,0 ≫ Ag0,0 (where the numbers correspond to the size of the amino inserts resulting from alternative splicing at the *y* and *z* sites, respectively; Fig. 1). Splicing at both sites is important for agrin's AChR clustering activity: (*i*) *in vivo*, inclusion of inserts at the *z* site is always accompanied by the presence of an insert at the *y* site (15); (*ii*) recombinant agrins engineered to contain only *z* inserts (e.g. Ag0,8 or Ag0,11) are less active than those with inserts at both sites (9, 10); and (*iii*) fragments of agrin corresponding to the most C-terminal 21 kDa of the protein, which include only *z* site inserts, are several hundred-fold less active than larger fragments that contain both inserts (16). Despite the distinctive tissue distributions and varying biological activities of these isoforms, the biochemical consequences of agrin's alternative splicing are unknown.

A candidate agrin receptor was recently purified from *Torpedo* electric organ postsynaptic membranes and shown to be a heteromeric complex of α- and β-dystroglycan (17, 18). β-Dystroglycan is a transmembrane glycoprotein, while α-dystroglycan is a heavily glycosylated extrinsic peripheral membrane protein that can also bind laminin and merosin (19, 20). α-Dystroglycan is the agrin binding component of this complex. The cytoplasmic tail of β-dystroglycan binds dystrophin (see below). Although several lines of evidence indicate that the α-/β-dystroglycan complex serves as at least part of the functional agrin receptor (17, 21, 22), definitive evidence for such a role is currently lacking (reviewed in ref. 23). Moreover, as judged by ligand blot overlay, all agrin isoforms bind to α-dystroglycan, although not in identical fashions (24). It is therefore important to determine if alternative splicing modulates agrin's binding to α-dystroglycan.

The α-/β-dystroglycan heteromer is a component of the dystrophin-associated protein complex, which also includes the sarcoglycans. In Duchenne muscular dystrophy, mutations in dystrophin lead to the loss of this complex from the membrane (reviewed in ref. 25). In healthy muscle, α- and β-dystroglycan probably serve to connect the dystrophin-based cytoskeleton to the basal lamina (26, 27). The disruption of this link, and the concomitant loss of the sarcoglycans, have been proposed to cause a malfunction in the sarcolemma that eventually leads to cell death (28–30). Agrin has been shown to induce the aggregation of several components of the dystrophin-associated protein complex (21). Characterizing the binding of agrin to dystroglycan, and the ensuing signaling events leading to the organization of this complex, may therefore provide important insights into the molecular pathogenesis of Duchenne and other muscular-dystrophies.

Proteoglycans, which consist of a protein core and glycosaminoglycan (GAG) side chains, are likely to play an important role in modulating agrin's biological activity. The ability of agrin to induce AChR clustering is inhibited by the GAG

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AChR, acetylcholine receptor; GAG, glycosaminoglycans; RIA, radioimmunoassay.

*To whom reprint requests should be addressed. e-mail: Justin-Fallon@Brown.edu.
†Present address: Department of Neuroscience, Brown University, 190 Thayer Street, Box 1953, Providence, RI 02912.

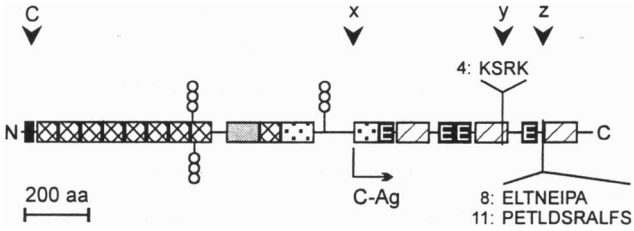

FIG. 1. Domain structure and alternative splicing of agrin. Agrin mRNA is alternatively spliced at four sites (arrowheads)(11). The aa sequences of three inserts at two positions (*y* and *z*) known to be important for agrin's AChR clustering activity are shown. Other motifs include a proposed signal sequence (filled); Kazel-type protease inhibitor domains (cross-hatched); serine/threonine-rich domains (coarse dots); a region homologous to domain III of the laminin B chain (stippled); EGF-like cysteine repeats (E); and regions homologous to the globular domains of the laminin A chain (diagonal hatching). The N-terminal half of agrin has three possible GAG addition sites (stacked circles), at least one of which bears a heparan sulfate side chain (12). The C-terminal portion of agrin, C-Ag (arrow) embodies all of agrin's AChR clustering activity. Recombinant isoforms of C-Ag were used in this study.

heparin (31). Further, variant muscle cells that are defective in the expression of proteoglycans, and perhaps other complex glycoproteins, show marked reductions in their response to agrin (14). Finally, α-dystroglycan isolated from these variants is hypoglycosylated and binds agrin poorly (24).

In this study we examined the possible link between alternative splicing of agrin and its interactions with heparin, α-dystroglycan, and the cell surface. We find that the ability of agrin to bind heparin is conferred by the presence of the four-aa insert at the *y* site, and is independent of the eight-aa insert at the *z* site. In agrin isoforms containing the four-aa insert, binding to purified α-dystroglycan and to the myoblast surface is inhibited by heparin. These results suggest that an interplay of particular agrin isoforms and endogenous GAGs may serve to regulate agrin's biological activity.

MATERIALS AND METHODS

Antibodies. Three monoclonal anti-rat agrin antibodies that recognize distinct sites on the molecule (32) were used in this study, mAb-131, mAb-247, and mAb-435 (Stressgen Biotechnologies, Victoria, BC). Control experiments showed that these antibodies bound to all the agrin isoforms used here.

Recombinant Rat Agrin Isoforms. cDNA clones encoding soluble fragments of alternatively spliced rat agrin isoforms (corresponding to the C-terminal half of the molecule) were generous gifts of Z. Hall and M. Ferns (9) (Fig. 1). The isoforms used were Ag0,0, Ag4,0, Ag4,8, and the non-naturally occurring splice form, Ag0,8. The clones were inserted in COS cell expression vectors, with the agrin coding region fused to prolactin signal sequences. COS cells were transfected using the DEAE-Dextran method, and conditioned media were harvested after three days. Relative agrin levels in the conditioned media were determined by two-site radioimmunoassay (RIA) as described below. AChR clustering activity of the recombinant agrin isoforms was determined as described previously (33).

Agrin Quantitation. Relative agrin concentrations were measured by a solid phase, two-site RIA with anti-agrin mAb-247 or mAb-435 as the capture antibody, and anti-agrin mAb-131 as the detection reagent. ^{125}I-mAb-131 was prepared using the methods described in Bowe *et al.* (17). All steps were carried out at 4°C. Immunolon 4 Removawells (Dynatech) were coated overnight with 1 μg/ml of the capture antibody, then washed in minimum essential medium, Hepes modification (Sigma). Wells were then blocked in this medium supplemented with 1% bovine serum albumin and 10% horse serum, incubated in agrin solutions for 1 hr, and washed. Wells were then incubated with 1 μg/ml ^{125}I-mAb-131 anti-agrin antibody, washed, and dried. Bound radioactivity was quantitated in a gamma counter.

Western Blot Analysis. COS cell culture supernatants containing recombinant agrin were electrophoresed under non-reducing conditions on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes. The blots were then blocked as above and incubated with 2 μg/ml anti-rat agrin antibody mAb-131. Bound mAb-131 was detected with biotinylated horse anti-mouse IgG (Vector Laboratories), and an alkaline phosphatase-based color development kit (Vectastain ABC; Vector Laboratories).

Agrin Binding to Immobilized Heparin. *Agrin depletion.* Two hundred microliters of agrin-containing supernatants from transfected COS cells were incubated with 50 μl of heparin-agarose beads (Sigma) for 2 hr at 4°C with gentle mixing. The unbound material was collected, and probed for the presence of agrin via western blotting with mAb-131. Control experiments showed that none of the agrin isoforms bound to unsubstituted agarose beads.

Agrin binding to heparin columns. Heparin-agarose columns (3 ml) were equilibrated in buffer containing 0.15M NaCl, 7 mM sodium phosphate, pH 7.4, and 0.02% NaN_3. Each column was loaded with 1 ml of agrin-containing supernatant from transfected COS cells. Columns were washed with the equilibration buffer and then eluted with a linear concentration gradient of 0.15–1.0M NaCl in 7 mM phosphate buffer. Agrin levels in the flow-through and eluted fractions were determined by two-site RIA as described above. NaCl concentrations (determined using a conductivity meter) of the assay samples were normalized to ensure consistent immunoreactivity in the RIA.

Ligand Overlay Assay and Heparin Inhibition. The ligand overlay assay was performed essentially as described (17). *Torpedo* postsynaptic membrane proteins or purified α-/β-dystroglycan complex were separated by SDS/PAGE and electroblotted onto nitrocellulose. Blots were blocked as above and incubated in normalized concentrations of the various agrin isoforms in the presence of 1 mM calcium for 3.5 hr, followed by a second layer containing 1 μg/ml ^{125}I-mAb-131. Where indicated, heparin (Sigma) was included with the agrin incubation. Bound anti-agrin antibody was quantitated on a PhosphorImager (Molecular Dynamics) and ImageQuant software by measuring the total counts within a standard-sized rectangle enclosing the α-dystroglycan band. The number of counts in an identical rectangle positioned over a negative region of the same lane was used to determine non-specific binding. Specific agrin isoform binding to α-dystroglycan in the presence of 1–1000 μg/ml heparin was expressed as percentage of binding in the absence of heparin for that particular isoform.

Agrin Binding to Cells. The quail myoblast cell line QM-7 was generously provided by P. Antin (34). Cells were grown on glass coverslips in Medium 199 (GIBCO) supplemented with 10% fetal bovine serum, 10% tryptose phosphate (Difco), 100 units/ml penicillin, 1 mg/ml streptomycin, and 2 mM L-glutamine. Living cells were rinsed with Hepes-buffered minimum essential medium and incubated at 4°C for 30 min in normalized concentrations of agrin. Where indicated, the agrin layer was supplemented with 100 μg/ml heparin. Cells were then incubated with 5 μg/ml anti-agrin mAb-131 for 30 min, followed by biotinylated horse anti-mouse IgG. In some experiments the cells were incubated with 100 μg/ml heparin for 30 min (followed by a brief wash) either before or after incubation with agrin. Cells were fixed in 1% paraformaldehyde in PBS for 5 min, incubated with streptavidin-fluorescein (Amersham), and post-fixed with absolute methanol at −20°C for 5 min. Coverslips were air dried, mounted on glass slides in Citifluor (Pella), and viewed under epifluorescence optics

with a Zeiss Axioplan microscope. For controls, sham-transfected COS cell supernatants, or an irrelevant primary antibody, were substituted for the agrin and mAb-131 layers, respectively.

RESULTS

Agrin Binding to Heparin Is Isoform Dependent. Agrin purified from *Torpedo* electric organ binds heparin, and this GAG inhibits agrin's AChR clustering activity (31). The isoform composition of this native agrin is unknown, but its high specific activity (≈5 pM) suggests that it consists predominantly of forms containing eight-aa inserts at the *z* site, along with the four-aa insert at the *y* site. In the course of characterizing the cell binding properties of a panel of recombinant agrin isoforms, we noticed that some of them were indifferent to the presence of heparin. We therefore undertook a systematic analysis and tested the heparin binding of recombinant versions of three naturally occurring isoforms: Ag0,0, Ag4,0, and Ag4,8 (where the numbers correspond to the inserts present at the *y* and *z* sites, respectively). The N-terminal half of agrin bears heparan sulfate GAG chains (12); therefore, to simplify the analysis we used recombinant fragments corresponding to the C-terminal half of agrin (Fig. 1). As expected, the highly active Ag4,8 bound heparin, as did Ag4,0. In sharp contrast, Ag0,0 failed to bind heparin (Fig. 2*A*). These results

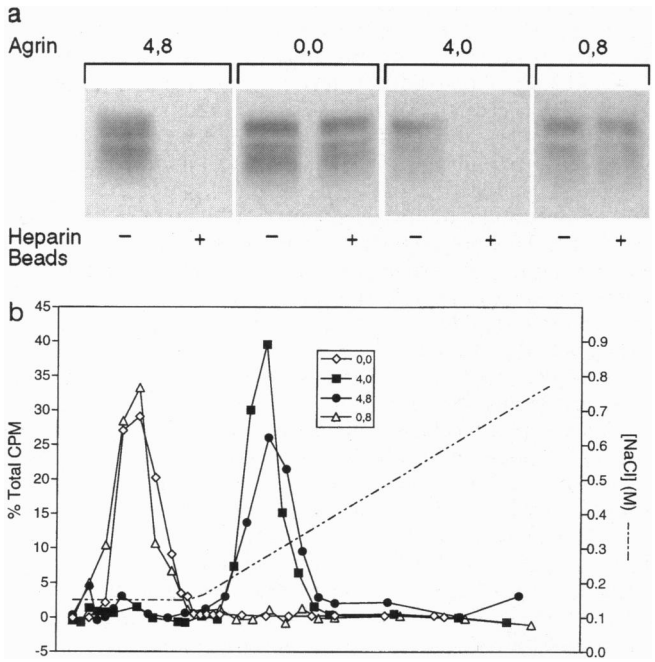

FIG. 2. Alternative splicing at the *y* site regulates agrin's binding to heparin. (*A*) Heparin-agarose beads were incubated (2 hr at 4°C) with conditioned media from COS cells expressing the indicated agrin isoforms, and then pelleted. Proteins in the starting material (−) and the bead supernatant (+) were separated by SDS/PAGE, blotted to nitrocellulose, and probed with anti-rat agrin mAb-131. Heparin beads depleted the media of those agrin isoforms containing the four-aa insert (Ag4, 8 and Ag4,0), but failed to deplete those isoforms lacking the insert (Ag0,0 and Ag0, 8). (*B*) Heparin-agarose column chromatography. Conditioned media from COS cells expressing the indicated agrin isoforms were applied to heparin-agarose columns. Columns were washed with the equilibration buffer containing 0.15 M NaCl, and then eluted with a linear 0.15–1.0 M NaCl concentration gradient (hatched line). Relative agrin concentrations for each fraction (≈0.7 ml) were determined by RIA. Ag0,0 (open diamonds) and Ag0,8 (open triangles) isoforms were not retained on the column, while Ag4,8 (filled circles) and Ag4,0 (filled squares) isoforms bound to the matrix. The peak elution of these *y*-spliced isoforms was observed at 0.28 M NaCl.

indicate that the four-aa insert confers heparin binding ability upon agrin. To determine the role of the eight-aa insert in heparin binding, we tested Ag0,8 (a splice form that does not occur naturally). This isoform did not bind heparin. Thus, the presence of the four-aa insert at the *y* site is necessary and sufficient to confer heparin-binding capability to agrin.

To obtain a quantitative measure of agrin isoform binding to heparin, we used an RIA to analyze agrin binding to heparin affinity columns (Fig. 2*B*). Ag0,0 and Ag0,8 were not retained on these columns, confirming that they do not bind heparin under these conditions. In contrast, Ag4,0 and Ag4,8 bound to the immobilized heparin, and were eluted off the column at the same salt concentration (≈0.28M NaCl). The near identical behavior of isoforms containing the four-aa insert on the one hand, and those lacking the inserts on the other, indicates that the presence (or absence) of the four-aa insert at the *y*-site is the major determinant regulating agrin's binding to heparin.

Heparin Sensitivity of Agrin Isoform Binding to α-Dystroglycan. We next sought to determine if the regulated binding of agrin to heparin was reprised in its binding to α-dystroglycan, a component of the proposed agrin receptor. Biochemically purified *Torpedo* agrin binds to α-dystroglycan on ligand overlay blots (17). Recombinant rat Ag4,8 and Ag0,0 have been reported to bind α-dystroglycan on ligand overlay blots of crude *Torpedo* membranes (24). We found that these isoforms, as well as Ag4,0 and Ag0,8, bound to purified α-dystroglycan in the absence of heparin (Fig. 3*A*). However, heparin inhibited the binding of Ag4,0 and Ag4,8, but not Ag0,0 and Ag0,8. Similar results were observed when agrin binding to crude *Torpedo* postsynaptic membranes was assessed (not shown).

We next quantified the effect of heparin on agrin isoform binding to α-dystroglycan (Fig. 3*B*). Marked inhibition of binding to α-dystroglycan was observed at 10 μg/ml heparin for forms containing the four-aa insert, while agrin forms without this insert were unaffected by 1000 μg/ml heparin, the highest concentration tested. Thus, the presence of the four-aa insert results in at least a 100-fold difference in the heparin sensitivity of agrin binding to α-dystroglycan.

Agrin Isoform Binding to Cultured Myoblasts. We next extended these *in vitro* experiments and asked whether or not agrin binding to the surface of living cells displays a similar isoform-dependent heparin sensitivity. Biochemically purified *Torpedo* agrin binds to cultured myoblasts and myotubes (33). We used the quail QM-7 cell line as a convenient source of myoblasts. We found that three of the agrin isoforms tested (Ag0,0, Ag4,0, and Ag4, 8) bound to these cells as shown by an immunofluorescence-based detection method (Fig. 4). When heparin was included with agrin in the incubation mixture, the binding of the isoforms spliced at the *y* sites (Ag4,0 and Ag4,8) was blocked, while the binding of Ag0,0 was unaffected (Fig. 4). Cells that were preincubated with heparin (and then washed) bound the three agrin isoforms in a manner indistinguishable from controls. On the other hand, postincubation with heparin completely removed bound Ag4,0 and Ag4,8, but did not alter the level of bound Ag0,0 (not shown).

In contrast to the three naturally occurring agrin isoforms tested above, we observed only very low levels of Ag0,8 binding to the QM-7 myoblasts, even at 100-fold higher concentrations than Ag0,0 (not shown). Thus, the presence of the eight-aa insert at the *z* site negatively regulates the binding of agrin to myoblasts.

We next compared the AChR clustering activity of Ag0,8 and Ag4,8 on primary chicken myotubes. In agreement with previous results, Ag4,8 induced AChR clustering, and this effect was inhibited by heparin (16, 31). Ag0,8 also induced AChR clustering on myotubes, but its specific activity was 100-fold lower than that of Ag4,8. Surprisingly, however, the Ag0,8 AChR clustering activity was sensitive to heparin (95 ± 3% inhibition in the presence of 100 μg/ml heparin; $n = 2$).

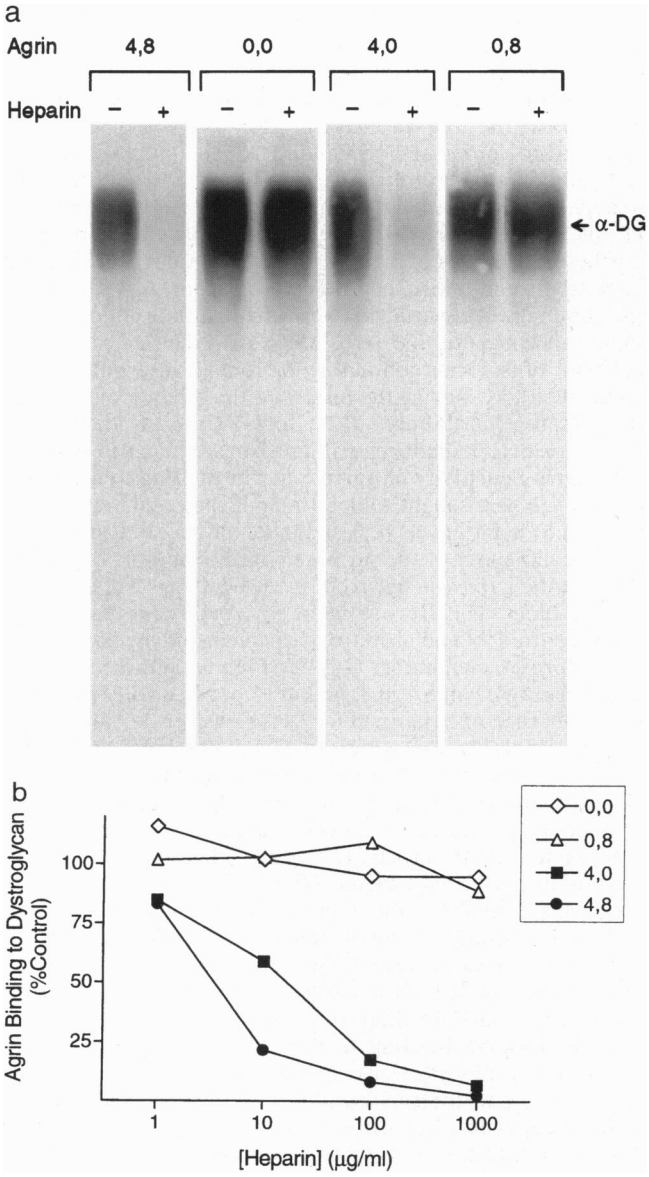

FIG. 3. Alternative splicing determines the heparin sensitivity of agrin binding to α-dystroglycan. *Torpedo* electric organ postsynaptic membrane proteins were separated by SDS/PAGE and blotted onto nitrocellulose. Blots were probed with equivalent concentrations of recombinant rat agrin isoforms alternatively spliced at the *y* and *z* sites (Ag4, 8, Ag0,0, Ag4,0, and Ag0, 8), with or without heparin, followed by ^{125}I-labeled anti-agrin antibody mAb-131.(*A*) PhosphorImager scans of agrin overlays in the absence (−) or presence (+) of 100 μg/ml of heparin. The position of the α-dystroglycan band is indicated (α-DG).(*B*) Specific agrin isoform binding to α-dystroglycan plotted as a function of heparin concentration. Heparin significantly inhibited the binding to α-dystroglycan of only those agrin isoforms that contain the four-aa insert at the *y* site.

Possible interpretations of this result are presented in discussion.

DISCUSSION

Proteoglycans, α-dystroglycan, and alternative splicing of agrin mRNA have each been shown to play a role in agrin's AChR clustering activity. The experiments reported here indicate that these features of the agrin system are linked, and suggest that they act together to regulate agrin binding to the cell. Such regulation seems likely to be an important mechanism in modulating agrin's biological action.

The most striking finding of this study is that the presence of a four-aa insert at the *y* site confers heparin binding capability to agrin. The mechanism by which this insert endows agrin with the ability to bind this GAG are unknown. Three of the four added residues are positively charged, raising the possibility that this insert may participate directly in agrin's binding to the negatively charged heparin. However, the sequence of this insert is identical in species ranging from elasmobranchs to mammals (35), suggesting that more than simple charge interactions are involved. For example, the presence of this insert could induce a conformational change in agrin that creates or exposes a heparin binding site.

These results raise interesting questions about the relationship between agrin's heparin and α-dystroglycan binding site(s). The inhibition of Ag4,x − α-dystroglycan binding (where *x* is any or no insert at the *z* site) by heparin raises the possibility that in these isoforms, the sites might be the same or overlapping. On the other hand, agrin's heparin and α-dystroglycan binding capacity can clearly be uncoupled (in Ag0,x), suggesting that these sites could be distinct. One possible model is that all agrin isoforms share a common α-dystroglycan binding site, but that exposure of this site is regulated by heparin binding (to a distinct site) in Ag4,x. Resolution of this question must await a detailed biochemical dissection of agrin's heparin and α-dystroglycan binding domains.

The binding of agrins containing the four-aa insert to heparin suggests that these isoforms may bind to GAGs or similar glycoconjugates at the synapse. The identities of such binding partners are not known. One candidate is agrin itself. Full length agrin is a heparan sulfate proteoglycan, with the GAG chains found on the N-terminal half (Fig. 1). Potential interactions between N- and C-regions could be intra- and/or inter-molecular. Binding among agrin molecules could facilitate agrin's assembly into the basal lamina, an important step in synapse maturation.

Another candidate for a synaptic GAG-like entity is α-dystroglycan. This polypeptide is highly glycosylated, and these sugar modifications are important for mediating its binding to laminin and agrin (36). Further, the α-dystroglycan expressed by a muscle cell line deficient in proteoglycan synthesis is hypoglycosylated and binds agrin much less well than wild type α-dystroglycan (24). α-Dystroglycan contains both N- and complex O-linked sugars, but there is currently no direct evidence that it has GAG chains (37). It should also be noted that α-dystroglycan may be heterogeneous. For example, previous studies have shown that agrin 0,0 and 4,8 bind to α-dystroglycan in nonequivalent manners (24).

α-Dystroglycan binds to both active and inactive agrin isoforms (24), as well as to laminin (36) and merosin (20). This apparent lack of ligand specificity does not easily fit with the proposal that α-dystroglycan serves as a functional receptor in agrin-induced AChR clustering (for discussion, see ref. 23). However, the present study indicates that the interactions between agrin isoforms and α-dystroglycan are complex. The results presented here show that agrin binding to α-dystroglycan is modulated by a glycosaminoglycan in an isoform-dependent manner. Importantly, agrin's AChR clustering activity is regulated by alternative splicing, and is attenuated in muscle cells deficient in GAG (and α-dystroglycan) synthesis (14).

The properties of Ag0,8 provide additional insights into the role of agrin's alternative splicing. This isoform binds very poorly to myoblasts, indicating that the cell binding specificity of agrin can be regulated by alternative splicing at the *z* site. This failure to bind myoblasts is unlikely to reflect defective synthesis of recombinant Ag0,8, since this isoform can induce AChR clustering on myotubes. (Similar results were obtained by Ferns *et al.* (14) using rodent myotubes, who also observed a decreased specific activity of Ag0,8). Further, we can readily detect Ag0,8 binding to differentiated myotubes, and this

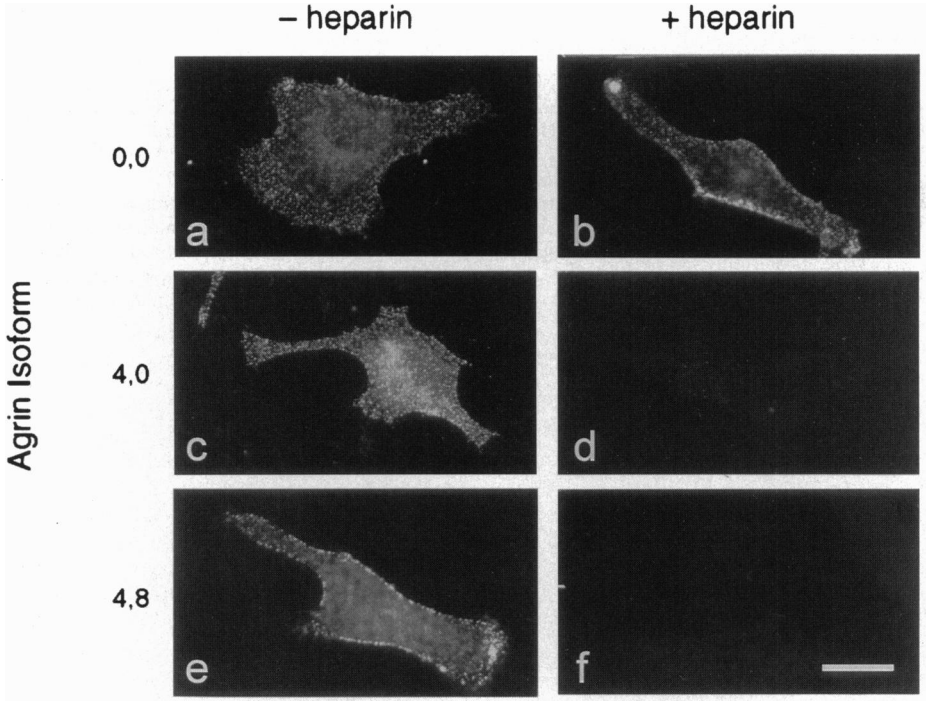

FIG. 4. Agrin isoforms bind to cell surfaces in heparin-sensitive and -insensitive manners. QM-7 quail myoblasts were incubated with equivalent concentrations of Ag0,0 (*A* and *B*), Ag4,0 (*C* and *D*), and Ag4,8 (*E* and *F*). In some cultures, heparin was added to the agrin layer at 100 μg/ml (*B*, *D*, and *F*), while others received no heparin (*A*, *C*, and *E*). Bound agrin was then detected with mAb-131 followed by biotinylated horse anti-mouse IgG and streptavidin-fluorescein. In the absence of heparin, all three isoforms bound in a fine, punctate pattern over the entire cell surface (*A*, *C*, and *E*). In the presence of heparin, binding of isoforms containing the four-aa insert at the *y* site, Ag4,0 (*D*) and Ag4,8 (*F*), was reduced to background levels. The binding of Ag0,0 was unaffected by the addition of heparin (*B*). Scale bar: 10 μm.

binding is heparin-insensitive (B.A.M. and J.R.F., unpublished observations). These results suggest that there may by a second GAG-dependent step in agrin-induced AChR clustering that is downstream of initial agrin binding. Interestingly, Mookjung and Gordon (38) have reported that endogenous chondroitin sulfate proteoglycans are important for the formation of spontaneous AChR clusters.

The present results may also contribute to our understanding of the muscular dystrophies. The α-/β- dystroglycan heteromer is a component of the dystrophin-associated protein complex (36, 39). Malfunction of this complex is thought to lead to the muscle cell death that characterizes these diseases (28). Our finding that agrin's interaction with α-dystroglycan can be regulated raises the possibility that there may be different configurations of α-dystroglycan, and perhaps distinct dystrophin-associated protein complexes, on the muscle cell surface. Elucidation of the functional and structural heterogeneity of these complexes may have important implications for understanding the molecular pathogenesis of the muscular dystrophies.

We thank Dr. H. Gordon and members of the Fallon lab for useful discussions. We are also grateful to Dr. P. Antin, and to Drs. M. Ferns and Z. Hall, for providing the QM-7 cell line and the cDNAs encoding agrin, respectively. This work was supported by grants from the Muscular Dystrophy Association, National Institutes of Health (HD23924 and MH53571), National Science Foundation (REU BIR-9423903) and fellowships from the National Institutes of Health and the American Cancer Society (M.A.B.). J.J.O was a participant in the Worcester Foundation Summer Undergraduate Training Program.

1. Fertuck, H. C. & Salpeter, M. M. (1976) *J. Cell Biol.* **69,** 144–158.
2. Wallace, B. G. (1989) *J Neurosci.* **9,** 1294–1302.
3. Reist, N. E., Werle, M. J. & McMahan, U. J. (1992) *Neuron* **8,** 865–868.
4. Cohen, M. W., Moody-Corbett, F. & Godfrey, E. W. (1994) *J. Neurosci.* **14,** 3293–3303.
5. Bowe, M. A. & Fallon, J. R. (1995) *Annu. Rev. Neurosci.* **18,** 443–462.
6. Lieth, E. & Fallon, J. R. (1993) *J. Neurosci.* **13,** 2509–2514.
7. Fallon, J. R., Nitkin, R. M., Reist, N. E., Wallace, B. G. & McMahan, U. J. (1985) *Nature (London)* **315,** 571–574.
8. Reist, N. E., Magill, C. & McMahan, U. J. (1987) *J. Cell Biol.* **105,** 2457–2469.
9. Ferns, M. J., Hoch, W., Rupp, F., Kreiner, T. & Scheller, R. H. (1992) *Neuron* **8,** 1079–1086.
10. Ruegg, M. A., Tsim, K. W., Horton, S. E. M., Kroeger, S., Escher, G., Gensch, E. M. & McMahan, U. J. (1992) *Neuron* **8,** 691–699.
11. Rupp, F., Ozçelik, T. H., Linial, M., Peterson, K., Francke, U. & Scheller, R. H. (1992) *J. Neurosci.* **12,** 3535–3544.
12. Tsen, G., Halfter, W., Kroger, S. & Cole, G. J. (1995) *J. Biol. Chem.* **270,** 3392–3399.
13. Smith, M. A. & O'Dowd, D. K. (1994) *Neuron* **12,** 795–804.
14. Ferns, M. J., Campanelli, J. T., Hoch, W., Scheller, R. H. & Hall, Z. (1993) *Neuron* **11,** 491–502.
15. Hoch, W., Ferns, M., Campanelli, J. T., Hall, Z. W. & Scheller, R. H. (1993) *Neuron* **11,** 479–490.
16. Gesemann, M., Denzer, A. J. & Ruegg, M. A. (1995) *J. Cell Biol.* **128,** 625–636.
17. Bowe, M. A., Deyst, K. A., Leszyk, J. D. & Fallon, J. R. (1994) *Neuron* **12,** 1173–1180.
18. Deyst, K. A., Bowe, M. A., Leszyk, J. D. & Fallon, J. R. (1995) *J. Biol. Chem.* **270,** 25956–25959.
19. Ibraghimov, B. O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W. & Campbell, K. P. (1992) *Nature (London)* **355,** 696–702.
20. Yamada, H., Shimizu, T., Tanaka, T., Campbell, K. P. & Matsumura, K. (1994) *FEBS Lett.* **352,** 49–53.
21. Campanelli, J. T., Roberds, S. L., Campbell, K. P. & Scheller, R. H. (1994) *Cell* **77,** 663–674.
22. Gee, S. H., Montanaro, F., Lindenbaum, M. H. & Carbonetto, S. (1994) *Cell* **77,** 675–686.
23. Fallon, J. R. & Hall, Z. W. (1994) *Trends Neurosci.* **17,** 469–473.
24. Sugiyama, J., Bowen, D. C. & Hall, Z. W. (1994) *Neuron* **13,** 103–115.
25. Ahn, A. H. & Kunkel, L. M. (1993) *Nat. Genet.* **3,** 283–291.
26. Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y. & Ozawa, E. (1994) *Eur. J. Biochem.* **220,** 283–292.

27. Jung, D., Yang, B., Meyer, J., Chamberlain, J. S. & Campbell, K. P. (1995) *J. Biol. Chem.* **270,** 27305–27310.
28. Worton, R. (1995) *Science* **270,** 755–756.
29. Boennemann, C. G., Modi, R., Noguchi, S., Mizuno, Y., Yoshida, M., Gussoni, E., McNally, E. M., Duggan, D. J., Angelini, C., Hoffman, E. P., Ozawa, E. & Kunkel, L. M. (1995) *Nat. Genet.* **11,** 266–273.
30. Noguchi, S., McNally, E. M., Ben, O. K., Hagiwara, Y., Mizuno, Y., Yoshida, M., Yamamoto, H., Boennemann, C. G., Gussoni, E., Denton, P. H., Kyriakides, T., Middleton, L., Hentati, F., Ben, H. M., Nonaka, I., Vance, J. M., Kunkel, L. M. & Ozawa, E. (1995) *Science* **270,** 819–822.
31. Wallace, B. G. (1990) *J. Neurosci.* **10,** 3576–3582.
32. Hoch, W., Campanelli, J. T., Harrison, S. & Scheller, R. H. (1994) *EMBO J.* **13,** 2814–2821.
33. Nastuk, M. A., Lieth, E., Ma, J., Cardasis, C. A., Moynihan, E. B., McKechnie, B. A. & Fallon, J. R. (1991) *Neuron* **7,** 807–818.
34. Antin, P. B. & Ordahl, C. P. (1991) *Dev. Biol.* **143,** 111–121.
35. McMahan, U. J., Horton, S. E. M., Werle, M. J., Honig, L. S., Kroger, S., Ruegg, M. A. & Escher, G. (1992) *Curr. Opin. Cell Biol.* **4,** 869–874.
36. Ervasti, J. M. & Campbell, K. P. (1993) *J. Cell Biol.* **122,** 809–823.
37. Smalheiser, N. R. & Kim, E. (1995) *J. Biol. Chem.* **270,** 15425–15433.
38. Mook-jung, I. & Gordon, H. (1995) *J. Neurobiol.* **28,** 482–492.
39. Yoshida, M., Suzuki, A., Yamamoto, H., Noguchi, S., Mizuno, Y. & Ozawa, E. (1994) *Eur. J. Biochem.* **222,** 1055–1061.