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Research report

The role of alternative splicing in regulating agrin binding to muscle cells

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Abstract

The binding of agrin to the muscle cell surface can induce radical changes in the topography and physiology of the cell membrane, resulting in the organization of postsynaptic components opposite the nerve terminal. Alternative splicing of agrin mRNA yields several isoforms, which vary in their cellular expression, developmental profile, and acetylcholine receptor (AChR) clustering activity. Neurons and muscle cells express several of these agrin isoforms. To address the role of alternative splicing in regulating agrin's function, we compared the effects of splicing at the *y* and *z* sites of agrin (denoted 'Ag*y,z*'). Agrin isoforms bound differently to the myotube surface: Ag0,0 and Ag4,0 showed much higher levels of binding than Ag4,8. The artificial splice form Ag0,8 showed binding levels similar to Ag4,8. Visualization of the bound agrin after an acute incubation revealed that each isoform associated with the cell surface in a distinct pattern. These binding patterns changed following stimulation of the myotubes with Ag4,8 for 4 h (which induces the clustering of AChRs). Ag4,8 binding sites were concentrated at $>90\%$ of the induced AChR clusters, while those for Ag4,0, Ag0,8, and Ag0,0 were enriched at 70%, 50% and 25%, respectively. Together, these observations indicate that alternatively spliced forms of agrin recognize at least partially non-overlapping populations of binding sites on the cell surface, and that the eight amino acid insert is the dominant factor influencing the level of the agrin binding to the cell surface. Further, some of these populations redistribute to AChR clusters upon agrin stimulation.

Keywords: Agrin receptor; Dystroglycan; Synaptogenesis; Neuromuscular junction

1. Introduction

Efficient and rapid signal transmission between neurons and their targets relies on the marshaling of synaptic components to topographically restricted domains on the cell surface. At the neuromuscular junction, for example, AChRs are mustered in high concentrations to the postsynaptic apparatus, which in turn is aligned with neurotransmitter release sites from the presynaptic terminal [33]. To understand how these specializations are formed, maintained, and modulated, it is essential to elucidate the mechanisms that underpin these processes.

Agrin's central role in nerve–muscle synapse formation is well established [2,25]. This extracellular matrix molecule is highly concentrated in the synaptic basal lamina at both developing and mature synapses. Purified agrin is sufficient to direct the clustering of AChRs and other synaptic components on the surface of cultured myotubes [28]. Agrin expressed ectopically in mature muscle can induce virtually all aspects of postsynaptic differentiation [26]. Finally, mouse mutants lacking agrin fail to assemble a functional postsynaptic apparatus [14].

Agrin's AChR clustering activity is regulated by alternative mRNA splicing [12,31]. Two splice sites in the carboxyl-terminal half of the molecule, '*y*' and '*z*', are particularly important (denoted Ag*y,z*; Fig. 1). The introduction of an eight amino acid insert at the *z* splice site enhances agrin's clustering activity over one thousand fold. This insert is always accompanied by the presence of a four amino acid insert at the *y* splice site [20]. Only nervous tissue, and perhaps only neurons, express agrins that contain inserts at the *z* splice site [29,34].

Although splicing at the *z* site dominates agrin's AChR clustering activity, other agrin splice forms seem likely to play a role in collateral synaptogenic events such as the formation of presynaptic specializations and the localized transcription of AChR subunit mRNAs. For example, presynaptic development is aberrant in agrin knockout

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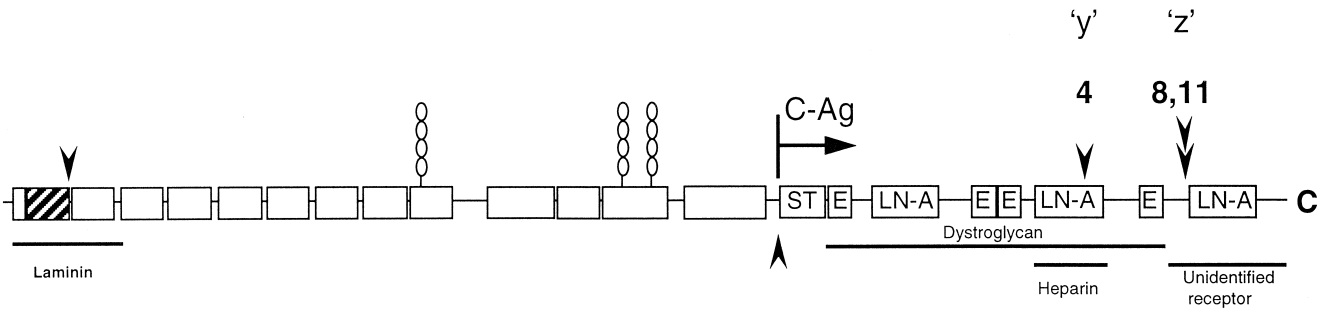

Fig. 1. Domain structure and differential splicing of agrin. Agrin's mRNA is differentially spliced at four sites (arrowheads; [11,20,36]). Bars highlight the known binding regions. The first 130 amino acids of the amino-terminal region are required for interaction with laminin [10]. The two laminin-A (LN-A) domains bind dystroglycan [21]. An extreme carboxyl-terminal region (termed c21) binds to an unidentified receptor complex on the cell surface that is distinct from dystroglycan [16]. The soluble C–Ag portion of agrin contains the molecule's clustering activity and is what we used for the experiments described here. The isoforms used in these experiments differ in the inclusion of amino acid inserts at the '*y*' and '*z*' splice sites. Isoforms are therefore denoted in the text as Ag*y,z*; for example, Ag4,8 represents the isoform that contains the 4 amino acid insert at the '*y*' splice site and the 8 amino acid insert at the '*z*' splice site. A '0' indicates that no insert was included at the splice site. Other regions: ST: serine/threonine rich; E: EGF-like; beads: likely site of glycosaminoglycan chain addition.

mutants: axons branch excessively, wander over the entire muscle, and do not make terminal specializations [14]. Cultured motor neurons adhere selectively to and make presynaptic-like contacts on fibroblasts expressing agrin on their surfaces [4]. Surprisingly, all agrin isoforms tested show equivalent activity in these assays. Further, ectopically expressed agrin can induce the transcription of AChR epsilon subunit mRNA (undoubtedly in concert with the ARIA/neuregulin system; reviewed in Ref. [13]). Again, the full range of agrin isoforms tested show this activity [22]. Finally, agrin isoforms lacking *z* inserts are synthesized by both neurons and muscle [32,34], and thus agrin derived from either of these tissues may contribute to key aspects of synaptic differentiation and maturation.

Much effort has focused on identifying receptors for agrin and understanding their interactions with agrin's isoforms. Two cell surface molecules, α-dystroglycan and MuSK, have been implicated, but neither can completely account for agrin's activity. α-Dystroglycan is the major agrin-binding protein in skeletal muscle, brain, and *Torpedo* electric organ [1,7,15,35]. Purified dystroglycan binds all agrin isoforms, but has the highest affinity for those lacking splice inserts at the *z* site [16]. Interestingly, the inclusion of the 4 amino acid insert at the *y* splice site bestows heparin sensitivity upon agrin's binding to α-dystroglycan and to the cell surface [6,16,21,30]. Dystroglycan is not essential for agrin-induced AChR clustering, although agrin mutants that do not bind dystroglycan are 100-fold less active than the native molecule [17]. Dystroglycan may also be involved in organizing the agrin-containing basal lamina at developing synapses [23,37]. The receptor tyrosine kinase MuSK is essential for agrin's AChR clustering activity [9,18]. However, MuSK does not bind to agrin directly, nor is it sufficient to mediate the cell's response to agrin. Thus, functional agrin receptors are likely to contain additional components that dictate binding selectivity for agrin isoforms and mediate the biological responses to them.

Another approach to understanding the function and mechanism of action of agrin isoforms has been to visualize their binding sites on myotubes. In previous studies, we showed that biochemically purified agrin, comprising a mixture of isoforms, binds to chick myotubes. Further, at least some of these agrin binding sites redistribute to induced AChR clusters [27]. The availability of recombinant agrin has extended these findings and shown that alternatively spliced agrin isoforms have characteristic binding properties [3], and that binding sites for isoforms with inserts at both the *y* and *z* splice sites can redistribute to AChR clusters [8,16]. However, these studies left open the contribution of splicing at the *y* site, and did not determine whether or not there might be multiple agrin isoform binding sites at induced AChR clusters.

There were two goals for this study. The first was to assess the individual and combinatorial contributions of the *y* and *z* inserts to agrin's binding to myotubes. The results show that splicing at each site regulates the binding of agrin to the cell surface. The second was to begin to investigate the relationship among the binding sites for the different agrin isoforms. We provide evidence that each agrin isoform recognizes a distinct population of binding sites on the myotube, and that these sites redistribute to characteristic extents following agrin-induced AChR clustering. Together, these findings suggest that individual inserts can act both alone and in concert to regulate qualitative and quantitative aspects of agrin's binding to the cell surface.

2. Materials and methods

2.1. Agrin and other materials

Recombinant rat agrin was produced as previously described [30]. A two-site radioimmunoassay was used to

determine relative levels of agrin present in the conditioned media of transfected COS cells [30]. Agrin was detected using the anti-agrin monoclonal antibody 131 (Stressgen Biotechnologies). Control experiments showed that this antibody did not recognize agrin produced by the chick myotubes. We used recombinant rat agrin in these studies to avoid the background signal that would arise from the anti-agrin antibodies binding to the endogenous chick agrin. To ensure that the differences in agrin isoform binding observed here did not result from species differences, we performed control experiments and found that rat agrin gave similar results to those obtained here when tested on rat myotubes. Moreover, recombinant chick agrin gave similar results when tested on chick myotubes (not shown).

2.2. Myotube culture

Myoblasts were prepared from chick embryo breast muscle at eleven days in ovo, as previously described [27,30]. Cells were grown in minimal essential medium (alpha medium; GIBCO) supplemented with 10% horse serum, 2% chick embryo extract, and 100 U/ml penicillin. The cells were plated onto polylysine- and gelatin-coated glass coverslips (Assistent) for indirect immunofluorescence studies or onto gelatin-coated 96-well Removawell plates (Immulon) for the radioimmunoassays. Cells were used 4–6 days after plating.

2.3. Radioimmunoassay

Myotubes were assayed for agrin binding in a procedure similar to that previously described [27]. Briefly, the cells were washed in HEPES-buffered minimal essential medium and incubated with one of the four agrin isoforms at a concentration of ~ 1.4 nM. The cells were washed and probed with ^{125}I-labeled anti-agrin monoclonal antibody 131. The wells were then washed, snapped apart, and then counted in a gamma counter. In order to assess Ca^{2+}-independent (background) agrin binding activity on the cells, identical assays were conducted wherein EGTA was added to all incubation and wash solutions.

2.4. Visualization of AChRs and bound rat agrin on cultured myotubes

Bound agrin was visualized on myotubes by indirect immunofluorescence as previously described [27,30]. Briefly, cells were incubated at 4°C for 30 min in normalized concentrations of agrin isoforms. Cells were then probed with anti-agrin mAb-131 followed by biotinylated horse anti-mouse IgG and rhodamine-coupled α-bungarotoxin (Molecular Probes). Cells were fixed in 1% paraformaldehyde and incubated with streptavidin-fluorescein. Cultures were postfixed in methanol, air dried, mounted on glass slides in Citifluor (Pella), and viewed under epifluorescence optics.

To induce clustering of AChRs and agrin binding sites, myotubes were incubated for 4 h with 2.5 units of Ag4,8 (~ 375 pM) at 37°C. Cells were washed with 2 mM EGTA for 30 min at 4°C, to ensure that residual Ag4,8 did not interfere with visualization of any of the other bound isoforms. Control experiments indicated that no bound agrin was detected on the cells or substrate after the EGTA wash. Cells were then probed for AChRs and bound rat agrin as described above.

2.5. Microscopy

AChR clustering and redistribution of agrin binding sites were quantified essentially as previously described [27]. Briefly, 20–30 myotube segments from at least two coverslips per isoform were randomly selected. AChR clusters > 2 μm in diameter were counted under rhodamine optics, then scored for colocalization with agrin binding sites by comparison with the same myotube segment viewed under fluorescein optics. Clusters were counted and photographed through a $63\times$ objective lens on a Zeiss Axioplan microscope.

3. Results

3.1. Binding of alternatively spliced agrin isoforms to chick myotubes

We first determined the level and distribution of binding sites for agrin isoforms on the myotube surface. To test the respective role of splicing at the *y* and *z* sites, we used a set of rat agrin splice forms that represents all possible combinations of the four and eight amino acid inserts (Ag4,8, Ag0,8, Ag4,0, and Ag0,0). Further, these isoforms embody the full range of agrin AChR clustering activity (Ag4,8 > Ag0,8 > > Ag4,0; with Ag0,0 inactive).

We quantified the binding levels of these isoforms to cultured chick myotubes by radioimmunoassay (RIA; Fig. 2A). The bound agrin was detected using an iodinated antibody that recognizes rat but not chick agrin (MAb-131) [19]. In agreement with earlier studies using biochemically purified agrin, binding was dependent upon the presence of extracellular calcium [24]. Isoforms with no inserts at the *z* splice site (Ag4,0 or 0,0) showed high levels of binding, while those with inserts (Ag4,8 or 0,8) showed much lower binding levels. The lower levels of binding of the Ag*y*,8 isoforms were unlikely to be due to denaturation or other defects of the recombinant proteins, since both agrin preparations had high levels of AChR clustering activity (data not shown). Since MAb-131 binds with similar affinity to all agrin isoforms used [30], the binding differentials observed are also unlikely to be due to differences in

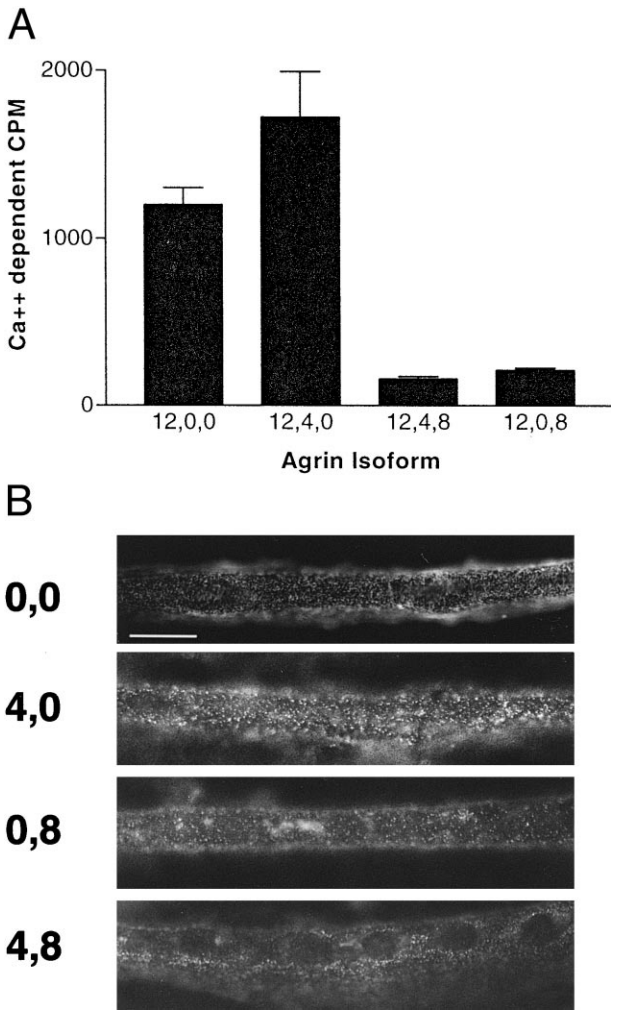

Fig. 2. Distribution and levels of binding sites of agrin isoforms on resting myotubes. (A) Agrin binding radioimmunoassay. Chick myoblasts were allowed to fuse on gelatin-coated 96-well Removawell plates. The myotubes were then assayed for agrin binding as described in Section 2. The presence of the 8 amino acid insert at the *z* splice site greatly reduced the level of binding. Values are the means of sextuplicate determinations from a representative experiment; error bars represent standard errors of the mean. (B) Indirect immunofluorescence. Myotubes were incubated with equivalent concentrations of Ag0,0, Ag4,0, Ag4,8 and Ag0,8 for 30 min at 4°C. Bound agrin was then detected with anti-agrin mAb-131 followed by biotinylated horse anti-mouse IgG and streptavidin fluorescein. The level of agrin isoform binding observed by fluorescence was in agreement with that measured by RIA (A). However, note that the micrographs shown here were each exposed to optimally display the distribution of the binding sites for each isoform. Isoforms bound to the surfaces of the cells in finely punctate patterns, but with distinct distributions. Bar = 25 μm.

antibody affinity. Thus, the presence or absence of the eight amino acid insert at the *z* splice is the principal factor regulating the level of agrin binding to the cell surface.

We next visualized the agrin isoforms' binding sites using an indirect immunofluorescence method [27]. Myotubes were incubated with agrin isoforms for 30 min at 4°C, conditions under which agrin binds but does not induce AChR clustering. In agreement with the results of the RIA, the fluorescence signal was lower for Ag*y*,8 as compared to Ag*y*,0 forms. The binding sites for all isoforms were distributed in a punctate fashion on untreated myotubes. Agrin binding sites were sometimes concentrated at the spontaneous AChR clusters on these myotubes. Because this co-localization was variable and unpredictable, we did not analyze it further (see also Refs. [8,27]). Outside of these spontaneous clusters, none of the isoforms bound in a distribution suggestive of the size and shape of AChR clusters.

Individual isoforms bound to the myotubes with a distinctive pattern and character, which were retained even

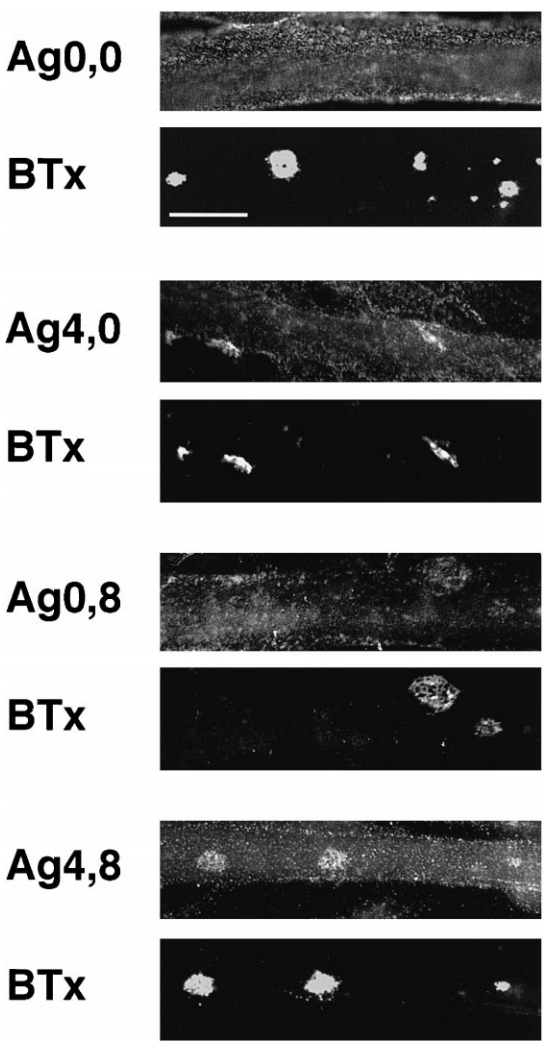

Fig. 3. AChRs and a subpopulation of agrin binding sites redistribute to clusters after agrin treatment. Chick myotubes were incubated with Ag4,8 at 37°C for 4 h. Cells were then cooled to 4°C and briefly washed in buffer containing EGTA to remove bound agrin. Agrin binding sites were detected as described in Fig. 2B. AChRs were visualized with rhodamine-coupled α-bungarotoxin. The extent of agrin isoform binding site redistribution varied. The majority of AChR clusters had co-clustered Ag4,8 and Ag4,0 binding sites (a,b) while many fewer AChR clusters had co-clustered Ag0,0 or Ag0,8 binding sites (c,d). Bar = 25 μm.

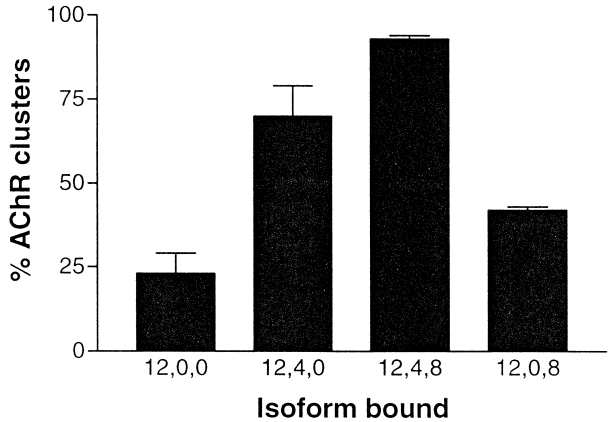

Fig. 4. Quantitation of isoform binding site colocalization with AChR clusters. The degree of colocalization of agrin binding sites with AChR clusters was determined as described in Section 2. Values are the means of two separate experiments within which at least 10 fields were counted on duplicate or triplicate coverslips; error bars represent standard errors of the mean.

when isoform concentrations were increased several-fold. The binding sites for Ag0,0 and Ag4,0 were present at the highest density (Fig. 2B). These, and the Ag0,8 binding sites were most abundant on the dorsal surface, with lower levels observed on the ventral, substrate-facing aspect. The binding sites for Ag4,0 and Ag0,0 were granular in appearance, with those for Ag4,0 being more coarse. On the other hand, binding sites for Ag4,8 and Ag0,8 were more finely granular and less abundant as compared to those for the Ag*y*,0 forms. Notably, the density of Ag4,8 binding sites was similar around the entire cell circumference. The binding sites for Ag4,8 (and to a lesser extent Ag0,8) were often excluded from membrane regions above nuclei. Such an exclusion was not observed for Ag*y*,0 binding sites.

3.2. Redistribution of agrin isoform-selective binding sites

We next asked if the distribution of the binding sites for these agrin isoforms changed after treatment of the myotubes with agrin. In these experiments cultures were incubated with Ag4,8 at 37°C for 4 h, conditions under which robust AChR clustering is induced. The bound Ag4,8 was then removed by brief incubation with EGTA at 4°C. To ensure that the EGTA treatment removed the Ag4,8 used for stimulation, some cultures were incubated with anti-rat agrin alone: no labeling was observed. The distribution of AChRs and of the binding sites for the range of agrin isoforms was then assessed. The binding sites for each agrin isoform displayed distinctive behaviors following agrin stimulation (Fig. 3). Binding sites for Ag4,8 became selectively concentrated at ~95% of the AChR clusters, and we occasionally observed a decreased level of Ag4,8 binding over the remainder of the myotubes. In contrast, the overall pattern and intensity of Ag0,0 binding was similar to untreated myotubes, and these sites were concentrated at only ~25% the AChR clusters. Ag0,8 binding sites were enriched at only ~50% of the clusters. Notably, Ag4,0 binding sites were concentrated at ~70% of the AChR clusters in these stimulated cultures. The differences in redistribution were statistically significant ($P < 0.05$, paired *t*-test). Thus, stimulation with one agrin isoform, Ag4,8, led to the redistribution of its cognate binding sites, as well as those for Ag4,0.

4. Discussion

In this study we have examined the role that alternative splicing plays in regulating agrin's binding to myotubes. Our evidence suggests that each of these isoforms recognizes a distinct set of binding sites on the muscle cell surface. We find that alternative splicing at the *z* site is the dominant variable dictating the level of agrin binding to the myotube. We also provide evidence that inclusion of the four amino acid insert at the *y* splice site confers specificity for a distinct population of binding sites on the myotube surface.

Our results show that the inclusion of the eight amino acid insert at the *z* site decreases the level of agrin binding to myotube surfaces by several-fold, irrespective of splicing at the *y* site. In contrast, isoforms without this insert (Ag4,0 and Ag0,0) bind to myotubes at high levels. We observed a similar phenomenon when bound agrin was visualized by indirect immunofluorescence: while the binding sites for the Ag*y*,0 isoforms are numerous and form coarse puncta, the Ag*y*,8 isoform binding sites are scarce and finely punctate. The basis for the difference in binding levels is not well understood, but could be related to the lower affinity that Ag*y*,8 forms have for dystroglycan [6,16,19,30].

Unlike alternative splicing at the *z* site, splicing at the *y* site does not appreciably affect the level of binding to

Table 1
Agrin isoform profiles

Agrin isoform	AChR clustering activity [2]	Dystroglycan binding [6,16,21,30]	Myotube binding	Colocalization of binding sites at induced AChR clusters
Ag0,0	−	+ + +	+ + +	+
Ag4,0	−	+ + +	+ + +	+ +
Ag4,8	+ + +	+	+	+ + +
Ag0,8	+ +	+	+	+

the myotubes. However, addition of the 4 amino acid insert at the *y* splice site changes agrin's recognition properties. The binding sites for Ag0,0 are seldom co-clustered with AChRs. On the other hand, binding sites for Ag4,0 are present at most AChR clusters (Fig. 4). Although the basis for this difference has not been elucidated, the 4 amino acid insert (KSRK) is positively charged and confers heparin binding ability to agrin. Moreover, heparin inhibits the binding of Ag4,*z*, but not Ag0,*z*, to myotubes (B.A.M. and J.R.F., unpublished results) as well as to myoblasts and to dystroglycan [6,16,30]. Thus, the addition of the 4 amino acid insert at the *y* splice site introduces a new binding region on agrin itself, and is sufficient to cause qualitative changes in the way that agrin's isoforms interact with cells.

We do not know the relationship among the sets of binding sites recognized by the agrin isoforms studied here. The agrin isoforms bind to populations of binding sites that can be distinguished on the basis of their abundance, localization, and selectivity (see Table 1). However, it is important to point out that the methods used here cannot rule out the possibility that at least some of the binding sites might be shared by more than one isoform. Future studies should reveal the extent to which the various isoforms share binding sites, as well as the composition of the sites themselves.

We have shown that Ag4,8 treatment redistributes not only its own binding sites on myotubes, but also those of Ag4,0. While it is likely that the full functional repertoire of agrin's isoforms has yet to be described, data from other studies suggest that Ag4,0 will have a specific role in synaptic differentiation [5,22]. Thus, isoform binding site redistribution could have important functional consequences, such as providing a means to increase the local concentration of specific forms of agrin at the postsynaptic anlage.

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