Introduction

Interactions between cells that govern the formation of specialized intercellular contact sites are critically important during synaptogenesis. Although our understanding of this process in the central nervous system is just beginning, much has been learned about the synapse formed between nerve and muscle, the neuromuscular junction (NMJ). It is now clear that the formation of the mature NMJ results from a bi-directional exchange of information molecules between the presynaptic motorneuron and the postsynaptic muscle cell (Hall and Sanes, 1993). While muscle-derived growth factors are thought to affect the growth and survival of motorneurons (Oppenheim, 1989; Koliatsos et al., 1993), the influence of the motorneuron upon the postsynaptic muscle cell is pleiotropic: ranging from effects upon gene expression to localized redistribution of existing muscle cell proteins. Molecules that have been implicated in the regulation of this motorneuron-induced differentiation include ARIA (a member of the neuregulin family of differentiation molecules; Falls et al., 1993), the neuropeptide CGRP (New and Mudge, 1986) and the extracellular matrix molecule agrin (McMahan, 1990). Presumably, these and other molecules act in concert to regulate formation of the mature NMJ.

Several lines of evidence suggest that agrin mediates local aspects of postsynaptic apparatus differentiation. Exogenously applied agrin has the ability to direct the clustering of postsynaptic muscle cell molecules: including intracellular, transmembrane and extracellular matrix molecules (Nitkin et al., 1987; Wallace, 1986, 1989). The most well-characterized activity of agrin is its ability to induce the redistribution of the acetylcholine receptor (AChR). Initially AChRs are diffusely distributed on the surface of the muscle but, through development, they become localized specifically at synaptic sites (Bevan and Steinbach, 1977). Antibody inhibition studies (Reist et al., 1992) and cell culture studies using recombinant agrin (Ferns et al., 1992, 1993) are consistent with the notion that neurally derived agrin mediates this AChR accumulation at synaptic sites.

While most tissues including muscle express agrin, more highly active forms are specifically expressed in nervous system tissue as a result of alternate splicing (Ferns et al., 1992; Hoch et al., 1993). Two of these sites (denoted Y and Z; Fig. 1A) alter agrin function. The presence of a 4-amino acid (AA) insert at the Y site increases activity tenfold and the presence of an 8-AA insert at the Z site enhances AChR clustering approximately 1,000 fold (Ferns et al., 1993). Splicing is coordinately regulated between the Y and Z sites: inclusion of the 4-AA-encoding exon at the Y site is necessary for inclusion of exons at the Z site (Hoch et al., 1993), suggesting that a concerted effect of both splice site insertions is required for optimal biological function.

Although the activity of agrin has been well characterized, its mechanism of action remains obscure. Several lines of evidence implicate muscle cell proteoglycans in transduction of the agrin signal. First, the ability of agrin to induce clustering of AChRs is blocked by the addition of exogenous heparin: likely due to an interruption of agrin interaction with a muscle proteoglycan that mediates agrin activity. To further test this hypothesis, we have compared the ability of differentially active agrin isoforms to interact with a model component of proteoglycans, heparin, as well as with the putative proteoglycan α-dystroglycan. We demonstrate that an alternately spliced exon (encoding the sequence lysine, serine, arginine, lysine: Y site) is necessary for agrin-heparin interactions. We also show that alternate splicing at another site (Z site) dramatically affects interaction of α-dystroglycan with agrin. We propose a model in which multiple distinct domains of agrin interact with both protein and sugar moieties of α-dystroglycan. The isoform-specific binding of agrin to α-dystroglycan is consistent with a functional role for this interaction during synaptogenesis.

Key words: synapse formation, agrin, proteoglycan, dystroglycan, heparin
cell surface heparan sulfate proteoglycan (Wallace, 1990). Second, muscle cell lines deficient in glycosaminoglycan (GAG) synthesis have reduced responsiveness to exogenously added agrin (Ferns et al., 1992, 1993). Addition of soluble agrin to two independent GAG mutant cell lines results in clustering of AChRs only when 10-1000 fold more agrin is applied as compared to the parental line. Taken together, these results imply that an agrin receptor is a proteoglycan, and alterations in the carbohydrate moieties affects the receptor-agrin interaction. Alternatively, a proteoglycan may play an auxiliary role in agrin activity. For example, proteoglycans form a part of the basic fibroblast growth factor (bFGF) receptor complex: serving to concentrate or present bFGF to a second transmembrane receptor (Rapraeger et al., 1991; Yayon et al., 1991). Finally, it is possible that proteoglycan-dependent matrix interactions are essential for the stabilization of molecules clustered by agrin.

The biological activity of agrin has been localized to the carboxyl terminal \(50 \times 10^3 (M_r)\) of the agrin protein (Hoch et al., 1994; N4 construct, Fig. 1). Although a smaller fragment of agrin has been reported to retain clustering activity, it does so only at concentrations 800-fold higher than the \(50 \times 10^3 (M_r)\) fragment (Gesemann et al., 1995). Thus, biological activity is mediated by a relatively large, structurally diverse protein, which includes the Y and Z splicing sites, epidermal growth factor (EGF)-like repeats and regions homologous to the G-domain of laminin. In laminin, G-domains are involved in the binding of laminin to heparin, a component of proteoglycans (Ott et al., 1982; Jackson et al., 1991; Skubitz et al., 1988). Together with the evidence implicating proteoglycans in agrin activity (see above), the presence of G-domains in the active fragment of agrin suggests that a direct interaction between agrin and a muscle cell proteoglycan is involved in clustering activity.

A putative muscle cell surface proteoglycan, \(\alpha\)-dystroglycan, has been shown to bind agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). \(\alpha\)-dystroglycan is present at agrin-induced clusters of AChRs and, in some experiments, antibodies against \(\alpha\)-dystroglycan alter agrin’s clustering activity (Campanelli et al., 1994; Gee et al., 1994; see also Sugiyama et al., 1994). \(\alpha\)-dystroglycan is one component of a complex of proteins (dystrophin glycoprotein complex: DGC; Campbell and Kahl, 1990; Ervasti et al., 1990; Yoshida and Ozawa, 1990) that span the plasma membrane of muscle cells. This complex associates with the intracellular cytoskeletal components dystrophin (Ervasti and Campbell, 1991; Suzuki et al., 1992) and utrophin (Matsumura et al., 1992): presumably throughout the plasma membrane, and at neuromuscular junctions respectively. \(\alpha\)-dystroglycan also interacts with the matrix component laminin via G-domains (Ibraghimov-Beskrovnaya et al., 1992; Gee et al., 1993). Thus, the DGC can mediate interactions between the extracellular matrix and the membrane cytoskeleton. The finding that agrin also interacts with \(\alpha\)-dystroglycan raises the possibility that the function of the DGC may be general: mediating several different matrix-cytoskeleton interactions.

In the present study, we have investigated the interaction of agrin with heparin (a model proteoglycan component) and \(\alpha\)-dystroglycan. Our results suggest that the second laminin G-domain of agrin mediates agrin-heparin interaction, with the alternate splice site found in this domain (Y site) conferring a majority of the binding activity. We do not detect any agrin-heparin binding differences as a result of splicing at the Z splice site, although this site is of critical importance for agrin activity. In contrast, the binding of agrin to \(\alpha\)-dystroglycan is greatly affected by splicing at the Z splice site, suggesting that this interaction is important for agrin activity.

**MATERIALS AND METHODS**

The agrin constructs used in this study were derived from previously published clones (Campanelli et al., 1991; Ferns et al., 1992, 1993; Hoch et al., 1994). The deletion constructs N6 and C2, as well as the N1 constructs containing the 4-AA insert at the Y position have been described (Hoch et al., 1994). N1 constructs lacking the 4-AA insert at the Y position were produced by exchanging the three prime end (5′ to polylinker sequence) of full-length agrin (without the 4-AA insert at the Y position) with the various inserts at the Z position: Ferns et al., 1993) with the 3′ end of N1 4,8 (Ferns et al., 1993; Hoch et al., 1994). N1 constructs used for stable transfection of CHO cells were first subcloned into pRe/RSV (Stratagene) using unique 5′ (ClaI) and 3′ (NcoI) restriction sites present in the polylinker.

Culture of CHO (Campanelli et al., 1991), COS (Hoch et al., 1994) and C2 cells (Campanelli et al., 1994) was performed as described previously. Stable CHO cell lines were obtained by obtaining calcium phosphate transfection, followed by selection with G418 (Campanelli et al., 1991) and limiting dilution cloning. Transient transfection of COS cells was performed by the DEAE dextran method as described previously (Hoch et al., 1994), with cells washed and refed serum-free medium 24 hours after transfection. Conditioned media from transiently transfected COS cells was collected 2 days after switching to serum-free medium. Conditioned medium from stable CHO cell lines was prepared by feeding half confluent plates with serum-free media followed by 48 hours of growth. All conditioned media was filtered (0.2 μm) prior to use for western blotting or chromatography. Preparation of C2 membrane proteins and western blotting was performed as previously described (Campanelli et al., 1994).

Monoclonal antibodies against agrin (33, 86, 131; Hoch et al., 1994) were obtained as purified ascites from Stressgen. Anti-hemaglutinin epitope antibody (12CAS) was obtained as an ascites from BABCO. Anti-\(\alpha\)-dystroglycan antibody (IIH6: ascites) was generously supplied by Kevin Campbell and Steven Robards. The rabbit polyclonal antibody against the carboxy-terminal half of agrin (anti-agrin L) has been described previously (Campanelli et al., 1991).

Heparin-affinity chromatography was carried out using a Pharmacia FPLC. Hitrap heparin columns (1 ml) were obtained from Pharmacia. All columns were run at a flow rate of 1 ml per minute and 2 ml samples were loaded. Linear gradients were formed between two buffers (A and B) by Pharmacia controller LCC 500 plus using two pump P 500s. A Pharmacia Frac 100 fraction collector was used to collect each 1 or 1.5 ml fractions as stated.

Purified agrin was isolated from CM of agrin-transfected cells. CHO cell lines expressing agrin N1 4,0 and N1 4,8 were adapted to growth in suspension and cultured in SFM II media (GIBCO) according to manufacturers guidelines. Conditioned media from 3-6 liters of cells grown for 4 days was clarified by centrifugation and filtration to remove cells and cellular debris (all subsequent steps were carried out at 4°C). Soluble proteins were precipitated by addition of PEG 8000 to a final concentration of 20%. Precipitated material was collected by centrifugation, resuspended in 175 mM Tris pH 7.5 and applied overnight with recirculation to a Jacalin agarose (Vector Labs) column. Unbound proteins were washed away with greater than 20 column volumes of 175 mM Tris and bound agrin eluted by addition of 175 mM Tris containing 0.2 M melibiose. Jacalin eluate was applied to an affigel-heparin (Biorad) column overnight with recirculation, washed with 80 mM NaCl/20 mM Hepes and bound agrin...
RESULTS

Characterization of agrin expressed in CHO and COS cells

The carboxyl-terminal half of the agrin protein encodes the information necessary for AChR clustering activity. This region also contains the two sites of alternative splicing (Y and Z site) which regulate agrin function (Fig. 1A). We have produced recombinant protein in two mammalian cell lines, COS and CHO, corresponding to this region of agrin in order to characterize the interaction of agrin with potential agrin receptors. Fig. 1 shows a schematic of the agrin proteins used, along with western blot characterization using several different antibodies. Stable CHO cell lines were used to express N1 agrin isoforms containing the 4-AA insert at the Y splicing site but varying at the Z splicing site (N1 4,0, N1 4,8, N1 4,11 and N1 4,19; Hoch et al., 1994; also denoted C-Ag in Ferns et al., 1993), and the minimal fragment of agrin necessary for full activity (N4 4,19; Hoch et al., 1994). Agrin N1 constructs that lack the 4-AA insert at the Y position (N1 0,0, N1 0,8, N1 0,11 and N1 0,19) were produced by transient expression in COS cells. Western blot analysis of the conditioned media from these cell lines is shown in Fig. 1B,C. The electrophoretic mobility and size heterogeneity of agrin, whether produced by COS or CHO cells, is identical, suggesting that these two mammalian cell lines do not differentially process the recombinant agrin. Anti-agrin monoclonal antibodies 131 and 33 recognize all Z splice site variants equally well, with all cell lines expressing a diffuse band of immunoreactivity (predominantly two bands of approximately 105 and 120×10^3 M_r (Fig. 1B, C), likely reflecting heterogeneity in the carbohydrate content of the translated product, consistent with previous observations (Ferns et al., 1993; Hoch et al., 1994).

Alternative splicing, which affects agrin activity, also alters the conformation of the agrin protein as revealed by reactivity with anti-agrin monoclonal antibody 86 (Hoch et al., 1994). We have performed western blot characterization using this antibody to further explore the relationship between alternate splicing and agrin conformations. In contrast to the results with the 131 and 33 antibodies, the 86 antibody reacts best with agrin containing the 8-AA insert at the Z position (Fig. 1B). This finding is consistent with our previous characterization using live and paraformaldehyde-fixed cells (Hoch et al., 1994). However, we also observed that the 86 antibody is capable of reacting with N1 0,0 protein when large quantities of this isoform are used (Fig. 1B lane 2 versus lane 1 and 6, and data not shown). These results are consistent with the notion that the epitope recognized by antibody 86 results from a favored rather than absolute conformation induced by the alternative splicing. The reactivity of this antibody with agrin isoforms on immunoblots after exposure to the reducing agent β-mercaptoethanol suggests that the conformation of agrin responsible for this epitope is not maintained by the predicted disulfide bonds in this part of the protein. Finally, the selectivity of this monoclonal antibody is independent of splicing at the Y position (reacting equally well with N1 0,8 and N1 4,8: data not shown).

We also expressed three deletion constructs, which contain different complements of G-domains (N4, N6 and C2; Fig. 1A). All of the constructs contain the 4-AA insert at the Y position (except N6, which does not contain this region of the agrin protein), and the 19-AA insert at the Z position. These constructs also contain sequences encoding the hemagglutinin (HA) epitope at the amino terminus allowing the use of the monoclonal antibody 12CA5 to visualize the agrin isoforms (Field et al., 1988; Hoch et al., 1994). Fig. 1D shows that these constructs are produced in the appropriate size and reactivity with both the monoclonal antibody directed against the HA epitope tag and a polyclonal antibody directed against a fusion protein of the C-terminal half of agrin (agrin anti-L antibody; Campanelli et al., 1991).

Structural requirements of agrin necessary for the agrin-heparin interaction

Several observations suggest that proteoglycans, and specifically heparan sulfate proteoglycans, are important in the clustering activity of agrin. Therefore, we assessed the ability of differentially active splice variants of agrin to interact with heparin as a first step in defining the role that the carbohydrate moieties of proteoglycans play in the interaction of agrin with the muscle cell surface. We assayed the binding of recombinant agrin isoforms to heparin columns. In order to make quantitative comparisons of agrin binding, the NaCl and Ca^{2+} sensitivity of these interactions was determined (see methods).

Fig. 2 shows dot blot analysis of agrin binding to heparin columns eluted with a gradient of NaCl. All of the deletions tested, except for the N6 protein, bind heparin under the conditions used. Furthermore, the elution of these agrin isoforms are very similar, occurring in the region of 520 to 600 mM NaCl (large arrow). The slightly earlier elution of N1 4,19 is due to experimental variability; this shift relative to the other N1 constructs was not consistently observed (detailed later, see Figs 3, 4, 5). Taken together, these results indicate that the second laminin G-domain, the third EGF-like repeat, or both, constitute an essential component of the heparin-binding site in agrin.

The Y splicing site of agrin occurs within the second laminin G-domain. Interestingly, this site consists of the sequence-lysine, serine, arginine, lysine (KSRK). Thus, inclusion of this insert results in the addition of three basic residues in a region of agrin that is implicated in interactions with acidic proteoglycans (Fig. 3, Rupp et al., 1992). Therefore, we wondered if splicing at the Y site might alter the heparin-binding characteristics of the agrin proteins. To test this hypothesis, we generated agrin isoforms with and without the 4-AA insert at the Y position, expressed these isoforms transiently in COS cells (Fig. 1B, C) and analyzed the binding of the secreted agrin to heparin columns (Fig. 3). In contrast to the N6 construct (Fig. 2), small but detectable amounts of agrin lacking inserts at the Y position were found to bind heparin columns (small arrow), although the quantities bound were significantly less than for agrin isoforms.
The X splicing site, which is present in the N1 construct, has been omitted for simplicity since no effect of this site has been observed on agrin activity (Ferns et al., 1993). The Y (no insert or 4-AA insert) and Z (no insert, 8-, 11-, or 19-AA inserts) splicing sites are shown with the sequences below and above the N1 construct, respectively. All of the constructs contain sequences encoding the signal sequence from bovine prolactin at the amino terminus as previously described (Ferns et al., 1993; Hoch et al., 1994). Constructs N4, N6 and C2 also contain sequences encoding the hemagglutinin epitope between the prolactin sequence and the agrin-coding sequence (Hoch et al., 1994). (B) Western blot analysis of conditioned media (CM) from CHO cell lines expressing Z splice site agrin variants. Three different anti-agrin monoclonal antibodies (131, 86, 33; Hoch et al., 1994) and the anti-HA antibody (12CA5) were used to probe parallel gels (7.5% polyacrylamide) of equal amounts of trichloroacetic acid (TCA) precipitated CM. Lane 1 contains untransfected CHO cell CM, lanes 2 through 5 contain N1 4,0, N1 4,8, N1 4,11 and N1 4,19 respectively, lane 6 contains N4 4,19. All N1 isoforms are recognized by the 131 antibody (large arrowhead at right, M, ~115x10^3). Lack of reactivity of the N4 protein with this antibody (small arrowhead at right) is consistent with the mapping of the epitope recognized by this antibody (Hoch et al., 1994). The 86 antibody recognizes agrin isoforms containing inserts at the Z splice site and weakly recognizes the N1 4,0 protein (note that the amount of N1 4,0 on the gel is greater than the amount of N1 4,8 as determined using antibody 131 or 33). The 33 antibody recognizes all N1 proteins as well as the N4 4,19 protein. Anti-HA recognizes the only epitope tagged protein run, N4 4,19. Arrows at the left indicate the migration of molecular weight standards (GIBCO BRL prestained: Mr = 203x10^3, 105x10^3, 70x10^3, 43x10^3). (C) Western blot analysis using anti-agrin 131 antibody demonstrates Y splice site variant isoforms migrate identically to Z splice site variants whether synthesized by transiently transfected COS cells or stably transfected CHO cells. Equal amounts of TCA-precipitated conditioned media was run on a 10% polyacrylamide gel. Lanes 1 through 10 are as follows: Vector only transfected COS, COS N1 0,0, COS N1 0,8, COS N1 0,11, COS N1 0,19, COS N1 4,0, COS N1 4,8, Y, CHO N1 4,8, CHO N1 4,19, untransfected CHO. The position of the size standards (same as in B) are indicated by arrowheads at the left. (D) Western blot analysis of deletion constructs. Nitrocellulose transfers of 10% gels were probed with either anti-agrin L polyclonal antibody (Campanelli et al., 1991) or the anti-HA antibody. Lanes 1 through 6 are as follows: COS N6 -19, CHO N4 4,19, COS C2 4,19, COS N1 4,0, COS N1 4,8, vector only transfected COS. The polyclonal, directed against the carboxyl terminal half of agrin, recognizes all agrin variants. The anti-HA antibody recognizes only the epitope tagged constructs (N6, N4, C2; lanes 1,2,3). Molecular weight markers are indicated as in B and C.

Heparin binding of agrin isoforms varying at the Y position is consistent with the suggestion that the second laminin G-domain of agrin plays an essential role in the interaction of agrin with heparin. However, these results do not rule out the possibility that other regions common to the C2 and N4 constructs (as well as N1) are also required for heparin binding. All of the deletion constructs that bind heparin contain the Z splicing site. Since the Z site is known to modulate agrin activity (Ferns et al., 1992, 1993), we next assessed whether splicing at this position affects the interaction of agrin with heparin when the 4-AA insert is present in the Y position (Fig. 4). Western blot analysis of fractions eluting in the linear region of the gradient (chosen based on the presence of immunoreactivity detected by dot blot analysis) reveal that the
The majority of all other agrin proteins bound the heparin columns and eluted in the range of 520 to 600 mM NaCl (arrow). The top N1 4,8 was from the stable CHO cell line, while the second N1 4,8 was from transiently transfected COS cells; no difference was detected as a result of the different cell lines.

NaCl sensitivity of agrin binding to heparin is independent of inserts at the Z position and confirms the dot blot procedures. The small (one fraction) difference seen between the N1 4,11 and N1 4,19 versus the N1 4,0 and N1 4,8 was further investigated by running shallower gradients in the region of agrin elution. This did not reveal any significant difference in the salt sensitivity of agrin binding to the heparin columns as a function of Z splice site identity (data not shown).

Agrin-induced clustering of AChRs is known to depend upon Ca$^{2+}$ (Wallace, 1988). Furthermore, the interaction of agrin with the cell surface protein α-dystroglycan has been shown to be Ca$^{2+}$ dependent (Bowe et al 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Since α-dystroglycan may contain heparin-like sugars, we asked whether the concentration of Ca$^{2+}$ might affect the interaction of agrin isoforms with heparin. Fig. 5 shows the eluted agrin

![Fig. 2](image2.png)  
**Fig. 2.** Agrin constructs containing the second G-domain bind heparin. Fractions from Heparin column chromatography (see Materials and methods) were dot blotted with monoclonal antibody 131 (N1 proteins) or anti-HA (C2, N4 and N6 proteins). The graph at top shows the NaCl gradient which was run (1 ml fractions were collected). N6 −,19 was the only agrin protein that did not bind heparin: eluting with the flowthrough (arrowhead).

![Fig. 3](image3.png)  
**Fig. 3.** The Y splice site regulates heparin binding. Fractions from heparin column chromatography were dot blotted with monoclonal antibody 131 as described in Fig. 2. The majority of the agrin protein containing the 4-AA insert at the Y splice site (N1 4,0 and N1 4,8) bound to the heparin columns and eluted in the range of 520 to 600 mM NaCl (large arrow). In contrast, all agrin isoforms lacking the 4-AA insert at the Y splice site bound to heparin only weakly: the majority of the protein is found in the flow though (arrowhead). Compared to the N6 −,19 protein, however, a detectable amount of these isoforms did bind, eluting at 290 mM NaCl (small arrow; compare to Fig. 2).

![Fig. 4](image4.png)  
**Fig. 4.** The Z splice site variants all bind heparin with equal affinity. Fractions from heparin column chromatography were TCA precipitated, run on 7.5% polyacrylamide, transferred to nitrocellulose and probed with monoclonal antibody 131. A gradient from 0.1 M NaCl to 2 M NaCl (containing 2.5 mM CaCl$_2$) was run and 1 ml fractions were collected. Only fractions surrounding the region of immunoreactivity as detected by dot blot analysis are shown. The two N14,8 immunoblots represent a comparison of N14,8 agrin obtained from CHO and COS cells. All agrin N1 proteins with the 4-AA insert at the Y position eluted in the same region of the gradient (595-635 mM NaCl, arrowheads on right) independent of the identity of the insert in the Z position. Only the region of the gel from the beginning of the separating gel to the 70 KD standard is shown (molecular weight standards indicated at left, arrowheads: $M_r$=203×$10^{3}$, 105×$10^{3}$ and 70×$10^{3}$).
isoforms, varying at the Z position, when binding and elution were performed without added Ca\(^{2+}\). Similar to the results in the presence of 2.5 mM Ca\(^{2+}\) shown in Fig. 4, all agrin isoforms varying at the Z position eluted at comparable concentrations of NaCl. Furthermore, the NaCl concentration necessary for elution of all isoforms in the absence of added Ca\(^{2+}\) was similar although slightly less than the NaCl concentration which eluted agrin in the presence of Ca\(^{2+}\) (575-610 mM versus 595-635 mM, respectively). This suggested that agrin-heparin interactions are not Ca\(^{2+}\) dependent.

As a further test for Ca\(^{2+}\) dependence of agrin-heparin binding, we performed heparin-affinity chromatography with the addition of 10 mM EDTA to the elution buffer. In this case, elution of bound agrin would be a function of both increasing NaCl as well as decreasing Ca\(^{2+}\). Dot blot analysis of this experiment reveals that agrin elutes at the same NaCl concentration (575-625 mM), even though this corresponds to 2.4 mM Ca\(^{2+}\) and 0.6 mM Ca\(^{2+}\). Thus, decreasing Ca\(^{2+}\) had no effect upon the agrin-heparin interaction. Finally, the potential for a Ca\(^{2+}\) dependence of agrin-heparin interaction was probed in the following manner. Agrin was bound to heparin columns under conditions of low salt and eluted with a gradient of decreasing Ca\(^{2+}\) (from 2.5 mM Ca\(^{2+}\) to 5 mM EGTA; Fig. 6B). Under these conditions, the agrin was not eluted. Subsequent elution of the columns with 2 M NaCl and 5 mM EGTA resulted in the release of bound agrin. Taken together, these results show that the second G-domain of agrin mediates a Ca\(^{2+}\)-independent interaction with heparin.

**Binding of \(\alpha\)-dystroglycan to Z position agrin variants**

In order to assess the interaction of agrin with \(\alpha\)-dystroglycan, and to quantitatively compare the binding of \(\alpha\)-dystroglycan to different agrin isoforms, we coupled purified agrin to columns and performed agrin-affinity FPLC with solubilized muscle cell membrane proteins. We have purified two N1 agrin isoforms (N1 4,0 and N1 4,8). In bioassays of AChR clustering, there is a 1,000-fold difference between the concentration of these two isoforms needed to elicit activity (Ferns et al., 1993). Fig. 7A shows Coomassie staining and western blot analysis of the purified agrin, which was coupled to Hitrap columns and used for FPLC analysis of muscle cell \(\alpha\)-dystroglycan binding (see Materials and Methods). CHAPS solubilized C2 muscle cell membranes were applied to the agrin columns, and the bound proteins eluted with a gradient of NaCl followed by elution with high salt plus 10 mM EDTA. Fractions were separated on polyacrylamide gels and reacted with anti-\(\alpha\)-dystroglycan antibody (Fig. 7B). Although \(\alpha\)-dystroglycan binding to N1 4,0 and N1 4,8 eluted at the same point in the gradient, this position corresponds to a NaCl concentration equivalent to the elution of these isoforms when only NaCl is varied (Figs 2-5). N1 4,0 and N1 4,8 agrin isoforms were applied to heparin columns and eluted with a decreasing gradient of Ca\(^{2+}\) only (load buffer 100 mM NaCl/2.5 mM Ca\(^{2+}\), elution buffer 100 mM NaCl/5 mM EGTA). Neither agrin isoform eluted in the gradient. Subsequent elution with 2 M NaCl plus 5 mM EGTA (dashed line) resulted in the release of bound agrin.
troglycan bound to both agrin isoforms, the sensitivity of this interaction to salt was vastly greater for the 8-AA insert containing isoform when compared to the isoform without an insert at the Z position. Elution of α-dystroglycan from the N1 4,8 column occurred at approximately 500 mM NaCl while α-dystroglycan was retained on N1 4,0 columns in the presence of 2 M NaCl and was only eluted by the addition of 10 mM EDTA.

In order to confirm the Ca2+ sensitivity of the agrin-α-dystroglycan interaction, agrin columns were run with an elution buffer containing both NaCl and EDTA such that elution would result from both increasing NaCl and decreasing Ca2+ (same gradients as run for the heparin columns shown in Fig. 6). Fig. 8A shows anti-α-dystroglycan dot blots of the fractions obtained using sequential NaCl and EDTA elution (different agrin columns and C2 membrane preparation than shown in Fig. 7B), whereas Fig. 8B shows the profiles resulting from the combined NaCl and Ca2+ gradient. Although the elution of α-dystroglycan from N1 4,8 is only slightly changed (a one fraction difference), α-dystroglycan elutes significantly earlier from the N1 4,0 column when compared to the NaCl only gradient (compare Fig. 8A and B). Thus, in contrast to agrin-heparin binding (Figs 4, 5), the Z splice site dramatically alters Ca2+-dependent binding of α-dystroglycan to agrin.

DISCUSSION

Interaction of neurally derived agrin with muscle cell components is likely to induce the localized accumulation of synaptic molecules which occurs during synapse formation. Previous
studies suggest that α-dystroglycan, the only known agrin-binding protein, mediates agrin’s role in synaptogenesis. Heparin can block the interaction of agrin to α-dystroglycan as well as the activity of agrin (Wallace, 1990; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Furthermore, glycosaminoglycan-deficient muscle cell lines (S26 and S27 cells) show reduced responsiveness to agrin as well as reduced binding of agrin to α-dystroglycan produced by these cells (Ferns et al., 1992, 1993; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Together, these results suggest that α-dystroglycan binds agrin via sugar residues and that this interaction is involved in clustering activity. Alternatively, the effects of heparin and the results of agrin activity in S26 and S27 cells may reflect the involvement of a heparin-containing component on the surface of muscle cells that is necessary for agrin function, but distinct from α-dystroglycan. In this paper, we have presented results that suggest that the interaction of agrin with α-dystroglycan is very different from the interaction of agrin with heparin. Since we show that the alternate splicing that affects agrin activity regulates the binding of agrin to both heparin and α-dystroglycan, both interactions may be involved in synaptogenesis, although possibly representing separate muscle cell components.

Z splice site-dependent conformation is independent of Y splice site

We have used several monoclonal antibodies that recognize different epitopes in the agrin protein to investigate splice-dependent conformations of agrin. One of the monoclonal antibodies, anti-agrin 86, interacts with a conformation-sensitive epitope dependent upon alternative splicing at the Z splice site (Hoch et al., 1994). We find that the reactivity of this antibody with agrin varies with the activity level of agrin isoforms generated by splicing at the Z site (Fig. 1B; Hoch et al., 1994), but is independent of alternate splicing at a second site (Y splice site) that also affects agrin activity. The most parsimonious interpretation of these new findings is that insertions at the Z splice site favor a disulfide-bond-independent conformation, and that the Y and Z splice sites are likely to alter structurally independent regions of the agrin protein. Interestingly, coordinate expression of inserts at the Z site with inserts at the Y site in vivo (Hoch et al., 1993) suggests that the biological function of the domains affected by these inserts is linked. Further, activity assays (Ferns et al., 1992, 1993) and antibody blocking studies (Hoch et al., 1994) suggest that both of these regions of agrin are involved in clustering activity. Our finding that these inserts alter structurally independent domains of agrin suggests that either multiple agrin-cell surface interactions are necessary for clustering activity, or that a single cell surface receptor for agrin interacts with multiple domains of the agrin protein.

Structural requirements mediating agrin heparin binding

Several conclusions can be drawn from our results with heparin-affinity chromatography. First, the series of agrin deletions that we have tested suggest that the second laminin G-domain of agrin plays a dominant role in the binding of agrin to heparin (Fig. 2). While involvement of a G-domain is not unexpected given the mapping of laminin heparin interactions to the G-domains of laminin (Ott et al., 1982; Skubitz et al., 1988), our deletion constructs leave open the possibility that the third EGF-like repeat could also be involved in heparin binding. However, experiments using chick agrin detected heparin binding of an agrin fragment lacking the third EGF repeat (Gesemann et al., 1995). Furthermore, data with isoforms lacking the 4-AA insert at the Y position (Fig. 3), which is contained within the second laminin G-domain, argue for a primary role of this domain in agrin-heparin interaction. Interestingly, a peptide containing this sequence of basic residues (KSRK) has previously been shown to be, in itself, ineffective in binding agrin (Skubitz et al., 1991). Thus, the context of the 4-AA insert is likely to be very important for heparin binding, consistent with a hierarchy of contributions to heparin binding in the agrin protein: laminin G-domain 2 represents an essential component of the binding with the sequence KSRK present in this domain contributing a majority, though not all, of the binding activity.

It is also worth noting that the forms of agrin that yield detectable heparin binding all contain two laminin G-domains (this study; Gesemann et al., 1995). Thus, the interaction of agrin with heparin could reflect a combination of laminin G-domain number two, along with interactions mediated by a second G-like domain (either G 1 or G 3). This raises the possibility that the binding of agrin to a cell surface glycosaminoglycan-containing protein could be multivalent. Alternatively, divalent agrin could mediate dimerization of heparin-containing cell surface proteoglycans.

Interestingly, agrin has been demonstrated to be a heparan sulfate proteoglycan itself (Tseng et al., 1995). The role of heparan sulfate modification to agrin is unknown but is unlikely to mediate the agrin-α-dystroglycan interaction since the putative glycosaminoglycan addition sites are in the amino terminus. Heparin modifications of agrin could be involved in organizing other yet unidentified heparin-binding proteins at the synapse.

Agrin-α-dystroglycan interaction is Ca2+ dependent and altered by Z splice site insertions

We have characterized the binding of two agrin isoforms differing by only eight amino acids at the Z splice site to α-dystroglycan. These isoforms display a 1,000-fold difference in clustering activity when assayed as a soluble molecule on myotubes in vitro (Ferns et al., 1993). We therefore predicted that, if α-dystroglycan-agrin interactions were important for agrin activity, these isoforms would differentially interact with α-dystroglycan. This is in fact the case as the data demonstrate that the more active form of agrin, with the eight amino acids at the Z splice site, bound α-dystroglycan less tightly than the less active form of agrin (no insert in the Z splice site, Figs 7, 8). While the results clearly show a large difference in activity is paralleled by a large difference in α-dystroglycan binding, the simple model of stronger binding reflecting greater activity seems not to be the case. These data are consistent with the possibility that the magnitude of binding strength is critically important for agrin activity (see below).

We have presented two lines of evidence suggesting that the interaction of agrin with α-dystroglycan must be substantially mediated by interactions different from heparin binding. First, although agrin-α-dystroglycan binding is Ca2+ dependent (Fig. 8: Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), we found no evidence that the interac-
tion of agrin with heparin required Ca\(^{2+}\) (Figs 5, 6). Second, splicing at the Z site does not affect agrin-heparin binding but greatly affects agrin-α-dystroglycan interaction (Figs 4, 5, 7, 8). These results suggest that heparin-like sugar moieties, if present on dystroglycan, represent a small fraction of the interaction with agrin.

However, several lines of evidence support the notion that α-dystroglycan is in fact a proteoglycan. The isolated protein shows resistance to proteolysis, tight association with cell membranes and heterogeneous mobility on gels after enzymatic deglycosylation (Ervasti and Campbell, 1991). Furthermore, α-dystroglycan synthesized by muscle cell lines deficient in glycosaminoglycan synthesis (S26 and S27 cells) migrates as a smaller protein (Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). These findings, taken together with the reduced binding of agrin to α-dystroglycan produced by S26 and S27 cells and the ability of heparin to block agrin-α-dystroglycan binding (Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), are consistent with the idea that a component of agrin-α-dystroglycan binding is heparin mediated. Thus, it seems likely that the interaction between agrin and α-dystroglycan is composed of multiple components, mediated by the protein backbone of α-dystroglycan and carbohydrate moieties.

Other workers have compared the ability of agrin isoforms differing at both the Y and Z position (identical to N1 0,0 and N1 4,8) to interact with α-dystroglycan in a nitrocellulose blot overlay assay (Sugiyama et al., 1994). In these experiments, it was reported that the N1 0,0 isoform was better able to compete the binding of the N1 4,8 isoform to α-dystroglycan, whereas the N1 4,8 was less effective at competing the binding of N1 0,0 to α-dystroglycan. This leads to the suggestion that N1 0,0 bind α-dystroglycan better than N1 4,8. Our results with agrin isoforms varying at the Z position are consistent with this finding; α-dystroglycan bound to N1 4,0 was more resistant to salt elution than α-dystroglycan bound to N1 4,8 (Figs 7, 8). However, we found that isoforms lacking inserts at the Y position bound heparin poorly (Fig. 3), and suggested above as the interaction of agrin with α-dystroglycan is likely to have a heparin component. Thus, while our data pertaining to the Z splice site are consistent with the blot overlay assays, our data with the Y splice site suggest that N1 0,0 might bind α-dystroglycan less well than N1 4,8 as a result of variation at the Y position.

Models of agrin-α-dystroglycan interaction

The formation of the postsynaptic apparatus involves the localized concentration of synaptic molecules. In other systems where membrane proteins are concentrated at specific sites, the mechanism of localization is thought to involve the regulation of membrane protein interaction with membrane cytoskeletal elements (Bennet, 1990; Luna and Hilt, 1992; Nelson, 1992). In the case of the neuromuscular junction, the AChRs appear to be at least in part differentially distributed by a similar mechanism of cytoskeletal interaction. AChRs at the NMJ are resistant to extraction by Triton X-100 (Podleski and Salpeter, 1988). Furthermore, the AChRs found in clusters produced by agrin in vitro are also resistant to Triton X-100 extraction (Wallace, 1992, 1994). These results suggest that localization of the AChR at the mature NMJ is, at least in part, due to an interaction with the cytoskeleton, possibly reflecting cytoskeletal regulation mediated by the DGC via agrin-α-dystroglycan binding.

Our results suggest several possible models for the role of agrin-α-dystroglycan interactions in the organization of the postsynaptic apparatus. The ability of agrin to direct the reorganization of the cytoskeleton may underlie agrin-induced clustering of AChRs. If this is so, it is possible that strong binding of agrin to α-dystroglycan would inhibit the ability of agrin to mediate cytoskeletal rearrangements: reorganization may necessitate a series of transient associations rather than highly stable binding. Strong binding may also reflect fundamental differences in the way different agrin isoforms interact with α-dystroglycan. The different conformations revealed by the 86 antibody, which correlate with activity and depend upon the Z splice site, could underlie an altered orientation of agrin functional domains: the spatial organization of which might mediate differences in binding and activity. For example, the high affinity of agrin N1 4,0 for α-dystroglycan could reflect multivalent interactions with a single α-dystroglycan, while the weak interaction of agrin N1 4,8 with α-dystroglycan might result from a conformation incompatible with multivalent interactions.

Secondly, agrin activity may reflect the binding of agrin to more than one muscle cell component. Although no other agrin-binding proteins have yet been identified, it is possible that the strong interaction of some agrin isoforms with α-dystroglycan could inhibit interaction of agrin with another receptor, or that agrin isoforms with inserts at the Z position may exhibit stronger binding for another putative agrin receptor. The orientation of agrin domains relative to each other (see above) might also mediate differences in activity if multiple agrin receptors are necessary for activity: isoform-specific conformations could spatially organized cell surface receptors differentially.

Finally, it is possible that the different interaction of agrin splice variants with α-dystroglycan reflects the binding of the different isoforms to different sites on α-dystroglycan. It is interesting to speculate that multiple binding sites might help to explain specificity in matrix protein-α-dystroglycan interactions. Both laminin and agrin (present in the matrix) can interact with α-dystroglycan, while both dystrophin and utrophin can interact with intracellular components of the DGC complex. If α-dystroglycan interacts specifically with the different matrix proteins as well as with isoforms of these proteins, binding could transduce a differential effect to the DGC complex which might underlie differential interactions with intracellular cytoskeletal structures. This could result in a specific utrophin-based cytoskeleton, which clusters and localizes AChRs at synaptic sites.

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