A Role for Dystrophin-Associated Glycoproteins and Utrophin in Agrin-Induced AChR Clustering

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Summary

Synapse formation is characterized by the accumulation of molecules at the site of contact between pre- and postsynaptic cells. Agrin, a protein implicated in the regulation of this process, causes the clustering of acetylcholine receptors (AChRs). Here we characterize an agrin-binding site on the surface of muscle cells, show that this site corresponds to α-dystroglycan, and present evidence that α-dystroglycan is functionally related to agrin activity. Furthermore, we demonstrate that α-dystroglycan and adhalin, components of the dystrophin-associated glycoprotein complex, as well as utrophin, colocalize with agrin-induced AChR clusters. Thus, agrin may function by initiating or stabilizing a synapse-specific membrane cytoskeleton that in turn serves as a scaffold upon which synaptic molecules are concentrated.

Introduction

Sites of contact between cells are often highly specialized and serve as sites of intercellular information exchange. Chemical synaptic transmission, the major form of communication utilized in the nervous system and the basis of brain function, relies upon the arrangement of molecules in precise register at sites of interneuronal contact called synapses. The most well-characterized synapse is the peripheral synapse between nerve and muscle, the neuromuscular junction, which consists of three highly specialized components: a presynaptic nerve terminal, a postsynaptic muscle cell, and an intervening synaptic cleft that is occupied by a basal lamina. The most notable feature of the postsynaptic cell is the high concentration of chemoceptors. In the case of the mammalian neuromuscular junction, the acetylcholine receptor (AChR) is present at densities up to 10,000 molecules/μm² (Fertuck and Slepeter, 1974; Bevan and Steinbach, 1977).

It is now clear that the formation of the specialized pre- and postsynaptic structures results from a bidirectional exchange of information during development. Agrin, a component of the synaptic basal lamina, is thought to play a role in the aggregation of preexisting AChRs beneath the nascent nerve terminal. Embryonic motoneurons synthesize agrin (Magill-Solc and McMahan, 1988; Rupp et al., 1991) and transport it to the nerve terminals, where it is released at sites of contact with developing muscle fibers (Magill-Solc and McMahan, 1990; Cohen and Godfrey, 1992). In culture, exogenous agrin induces the formation of high density patches of AChR that also contain accumulations of other synaptic molecules (Nikitin et al., 1987; Wallace, 1986, 1989). Studies with recombinant protein show that agrin mediates the clustering of AChR when presented as either a cell-associated, matrix-bound form or as a soluble-truncated form (Campanelli et al., 1991; Ferns et al., 1992, 1993; Tsim et al., 1992). Finally, antibody inhibition studies suggest that agrin derived from motoneurons mediates neurite-induced AChR clustering in vitro (Reist et al., 1992).

Although the ability of agrin to induce the clustering of synaptic molecules is well documented, the mechanism by which agrin acts remains obscure. It appears that interaction of agrin with a specific receptor on the surface of the muscle cell triggers AChRs in the postsynaptic membrane to aggregate (Nastuk et al., 1991). In cultured myotubes, agrin-induced AChR aggregation occurs in the absence of protein synthesis (Godfrey et al., 1984) and is dependent upon extracellular Ca²⁺ (Wallace, 1988). Furthermore, several lines of experimentation suggest that extracellular proteoglycans are involved in the action of agrin. Exogenous heparin and heparan sulfate inhibit both nerve-induced and agrin-induced receptor clustering (Hirano and Kidokoro, 1989; Wallace, 1990), and C2-derived muscle cell lines defective in proteoglycan synthesis are significantly less responsive to recombinant agrin (Ferns et al., 1992, 1993). A role for proteoglycans is further suggested by mapping studies that localize the clustering activity of agrin to a region containing two domains homologous to heparin-binding laminin G domains (Hoch et al., 1994; Rupp et al., 1992) and by the finding that alternative splicing within one of these domains in agrin affects activity (Ruegg et al., 1992; Ferns et al., 1993).

Cell surface molecules capable of interacting with extracellular agrin, although necessary for signal transduction, are unlikely to be sufficient to explain agrin activity. AChRs present at the neuromuscular junction, as well as agrin-induced clusters, have been shown to be more resistant to detergent extraction than nonclustered receptors, suggesting that a link is made between junctional AChRs and the cytoskeleton (Podleski and Slepeter, 1988; Wallace, 1992). Cytoskeletal elements of the spectrin superfamily are thought to mediate the maintenance of distinct membrane domains in other systems that exhibit membrane protein segregation (for reviews see Bennett, 1990; Luna and Hitt, 1992; Nelson, 1992). Since the postsynaptic membrane is a composite of distinct membrane domains, including AChR-rich regions at the crests of the junctional folds and Na⁺ channel-rich domains at the troughs of the folds, it is likely that this protein distribution is at least in part controlled by interaction between a spectrin-like cytoskeleton and the segregated membrane proteins.

Several spectrin-like molecules have been localized to
at synaptic sites in normal muscle (Love et al., 1991) from the earliest times in development (Phillips et al., 1993). These spectrin-like proteins have been suggested to form a submembrane cytoskeleton that interacts with extracellular laminin indirectly via a transmembrane complex of proteins (the dystrophin-associated glycoprotein [DAG] complex) and directly with intracellular actin, thus linking the actin-based cytoskeleton to the extracellular matrix (Evasti and Campbell, 1993; Matsumura et al., 1992; Ibraghimova-Beskovnaya et al., 1992).

In the present study we describe the identification of an agrin-binding site present on the surface of muscle cells and show that this represents the binding of agrin to α-dystroglycan, a component of the DAG complex. Since this complex of proteins plays a role in linking the intracellular dystrophin or utrophin cytoskeleton (or both) to the extracellular matrix, we have investigated the potential role of the DAG complex and utrophin in agrin-mediated AChR clustering activity.

Results

An Agrin-Binding Site on Live Cells
To identify muscle cell surface proteins that mediate the AChR clustering activity of agrin, we characterized the binding of agrin to the surface of cultured muscle cells using an indirect antibody-based flow cytometry assay. Purified soluble agrin corresponding to the carboxy-terminal half of the agrin protein (C-Ag2.5k; Campanelli, 1993; Ferns et al., 1993) was incubated with live cells in suspension. Unbound agrin was washed away, and bound agrin was detected using a monoclonal antibody (MAB) against agrin, followed by a fluorescein-conjugated secondary antibody, and analyzed by flow cytometry. Using this assay, we identified a dose-dependent agrin-binding site present on the surface of C2 myoblasts, with binding detected at concentrations of agrin above 0.5 nM (Figure 1A). This binding was shown to be dependent upon agrin conformation since preincubation of agrin with the reducing agent β-mercaptoethanol resulted in a loss of staining. The dose dependence of binding is similar in C2 cells that have been induced along the pathway to myotube differentiation for 24 hr (Figure 1B). In these experiments, agrin binding begins to saturate at approximately 30 nM. Simultaneous staining with biotinylated α-bungarotoxin (α-Btx) and avidin-conjugated phycoerythrin allowed us to confirm the expression of AChR, a marker for differentiation that is not detectable on proliferating myoblasts (Bunnamo and Merlie, 1986; our unpublished data). We could therefore visualize agrin-binding sites in myoblasts versus differentiated cells in the same sample. These data demonstrate that both proliferating myoblasts and differentiated cells express agrin-binding sites.

Since we have previously shown that a variant of the C2 cell line that is defective in proteoglycan synthesis (S27; Gordon and Hall, 1989) is capable of responding to exogenous agrin (Ferns et al., 1992, 1993), we reasoned that a binding site important for the clustering activity of agrin should also be expressed on the surface of S27 cells. Therefore, we analyzed the binding of agrin to S27 myo-

the postsynaptic apparatus, including an isoform of β-spectrin (Bloch and Morrow, 1989), dystrophin (Byers et al., 1991; Love et al., 1991; Yeadon et al., 1991), and dystrophin-related protein (also called utrophin) (Khrurana et al., 1990; Ohlendeck et al., 1991). Dystrophin, which is present throughout the sarcolemma (Arahata et al., 1988; Bonilla et al., 1989; Zubrycka Gaarn et al., 1988) and also in the troughs of the junctional folds (Watkins et al., 1988; Byers et al., 1991), is the product of the gene defective in Duchenne's and Becker's muscular dystrophies (Hoffman et al., 1987, 1988) and is thought to play a role in stabilizing the muscle cell plasma membrane (for a recent review see Matsumura and Campbell, 1994). Urophin is the product of an autosomal gene related to dystrophin (Love et al., 1989; Tinsley et al., 1992) and is found concentrated
Agrin Binding Is Ca\(^{2+}\) Dependent, Inhibited by Heparin, and Parallels α-Dystroglycan Expression

To characterize further the binding of agrin to C2 and S27 cells, we performed flow cytometry binding analyses at different concentrations of Ca\(^{2+}\) and heparin, factors that have previously been shown to affect the clustering activity of agrin (Wallace, 1988, 1990). The sensitivity of agrin binding to Ca\(^{2+}\) for both C2 and S27 cells is shown in Figure 2A. The inclusion of EGTA and exclusion of Ca\(^{2+}\) from the staining media reduced staining to background levels without added agrin. Using EGTA-buffered solutions to control Ca\(^{2+}\) concentration, both C2 and S27 cells bound agrin maximally at concentrations around 5 mM, with both lower and higher concentrations giving reduced binding. Note that the peak Ca\(^{2+}\) sensitivity of both C2 and S27 binding is quite similar, suggesting that the site detected on both cell types is similar and that the differences in threshold binding may reflect differences in the level of expression of the binding site in the two cell lines. We also wanted to know whether the Ca\(^{2+}\) requirement was specific to Ca\(^{2+}\) or whether other divalent cations could support agrin binding. Inclusion of Mg\(^{2+}\) in the staining media did not restore binding in the absence of Ca\(^{2+}\). Furthermore, the addition of increasing amounts of Mg\(^{2+}\) to media containing a constant amount of agrin and Ca\(^{2+}\) resulted in a decrease in the binding (for C2 cells, 0.5 mM Ca\(^{2+}\) with 5 mM Mg\(^{2+}\) gave 30% of the binding of 0.5 mM Ca\(^{2+}\) without Mg\(^{2+}\)), demonstrating that the binding of Mg\(^{2+}\) not only fails to support agrin binding, but in fact was capable of inhibiting binding.

We next asked whether heparin could inhibit the binding of agrin to C2 cells. Figure 2B shows the binding data of a constant amount of agrin in the presence of varying amounts of heparin. Concentrations of heparin from 0.2 to 200 μg/ml are inhibitory in a dose-dependent fashion, with 50% inhibition occurring at around 1 μg/ml. The inclusion of heparin in the differentiated C2 cells stained with α-Btx showed that heparin was not deleterious to cells as heparin did not alter the level of α-Btx labeling.

Taken together, the conformation and dose-dependent binding site described above that is Ca\(^{2+}\) dependent and inhibitable by heparin is consistent with this binding site playing a role in agrin activity. Further, these characteristics are reminiscent of the binding of laminin to α-dystroglycan, a muscle cell surface protein implicated in mediating interactions between the extracellular matrix and the submembraneous cytoskeleton (Ervasti and Campbell, 1993). Given the mapping of the laminin-α-dystroglycan interaction to the G domains of laminin (Gee et al., 1993) and the presence of three G domains in the fragment of agrin used for the binding studies described above, we wondered whether this binding site might represent α-dystroglycan present on the surface of C2 and S27 myoblasts and myotubes. To begin testing this hypothesis, we analyzed the ability of an anti-α-dystroglycan MAb (II6; Ervasti and Campbell, 1993) to recognize specifically surface components of C2 and S27 cells by flow cytometry (Figures 3A and 3B). Indeed, the binding of this antibody to both cell types was detected, with the level of immunore-
activity detected on S27 cells at an antibody dilution of 1:10 (bold trace in Figure 3B) being less than that detected on C2 cells at an antibody dilution of 1:100 (bold trace in Figure 3A). This quantitative difference in cell surface immunoreactivity parallels the agrin binding data for C2 and S27 cells described above (compare Figures 3A and 3B with Figure 1).

If α-dystroglycan is the agrin-binding site, then the ability of agrin to bind cells should follow the expression of α-dystroglycan. Since dystroglycan is widely expressed (Ibraghimova-Beskovyna et al., 1992), we assessed the agrin binding (bold trace in Figure 3C) and anti-α-dystroglycan antibody binding (bold trace in Figure 3D) to CHO cells. The binding of both agrin and anti-α-dystroglycan is clearly detected relative to the controls (no agrin added or an isotype-matched control antibody, respectively). Similar results were obtained with COS cells (data not shown). These data show that agrin binding is found on a variety of cell types that express α-dystroglycan and that the level of agrin binding to C2 and S27 myoblasts parallels anti-α-dystroglycan immunoreactivity, consistent with the notion that α-dystroglycan represents the agrin-binding site detected on C2 and S27 cells by flow cytometry.

Since previous studies have shown that the interaction of laminin with α-dystroglycan in a blotting assay inhibits the binding of MAb IIH6 (Ervasti and Campbell, 1993), we asked whether staining of myoblasts with anti-α-dystroglycan MAb IIH6 was affected by the presence of agrin. Inclusion of 100 nM agrin completely eliminated the binding of MAb IIH6 to the surface of C2 myoblasts (stippled trace in Figure 3A) and S27 myoblasts (stippled trace in Figure 3B). These results suggest that agrin, like laminin, interacts with α-dystroglycan at a site that overlaps with the MAb IIH6 binding site. We therefore assessed agrin binding to C2 cells in the presence of IIH6 ascites but were unable to detect inhibition of agrin binding. Given that the agrin fragment used for these studies contains three laminin globular domains and that at least two of these domains are required for activity (Hoch et al., 1994), it is likely that agrin interacts with dystroglycan at several sites. Additionally, the binding of agrin to α-dystroglycan is likely to be stronger than the binding of IIH6, an immunoglobulin M subclass antibody. Furthermore, our ability to detect agrin binding is limited to the nanomolar range, possible as a result of our indirect assay, which might preclude addition of a sufficient molar excess of antibody to alter agrin binding.

Agrin Binds Solubilized α-Dystroglycan

To confirm independently an interaction between agrin and α-dystroglycan, we tested for agrin binding to α-dystroglycan from solubilized myotube membranes. Myotube membrane proteins were solubilized with CHAPS and incubated with agrin- or bovine serum albumin (BSA)-conjugated agarose beads, in the presence and absence of Ca\(^{2+}\). Western blot analysis of the voids and bound material is shown in Figure 4. α-Dystroglycan is bound by agrin, but not BSA, beads and only in the presence of Ca\(^{2+}\). Furthermore, this binding is inhibited by heparin (data not shown). In contrast with the quantitative removal of α-dystroglycan (Figure 4A), the protein patterns in the presence or absence of Ca\(^{2+}\) are identical in both the agrin bead voids and bound samples when assessed by Coomassie staining (Figure 4B) or by silver staining (data not shown), demonstrating the specificity of the agrin-α-dystroglycan interaction. Our inability to detect α-dystroglycan with protein-staining dyes is consistent with previous studies that showed that this protein is resistant to these staining techniques (Ervasti and Campbell, 1991).

Flow cytometry showed that the C2-derived cell line S27, as well as an independent glycosaminoglycan (GAG) chain synthesis-defective C2 derivative (S26; Gordon and Hall 1989), has Ca\(^{2+}\)-dependent agrin-binding sites and that the level of these binding sites parallels the level of anti-α-dystroglycan immunoreactivity present on the surface of these cells. We, therefore, asked whether binding of solubilized α-dystroglycan to agrin could be detected in these cell lines. CHAPS-solubilized proteins retained on the agrin- or BSA-conjugated beads, in the presence or absence of Ca\(^{2+}\), are shown in Figure 5. Again, α-dystroglycan is only retained by agrin beads and only in the presence of Ca\(^{2+}\). Interestingly, the apparent molecular weight of α-dystroglycan from these cell lines is smaller than that seen in the C2 cells. This is consistent with the previous suggestion that α-dystroglycan is a proteoglycan (Ervasti and Campbell, 1991) as these cell lines are deficient in GAG chain synthesis (Gordon and Hall, 1969). Furthermore, the alterations in the level of expression and molecular weight of α-dystroglycan in these cells are consistent with the notion that the defect in agrin responsiveness of these cell lines (Ferns et al., 1992, 1993) could be explained by aberrations in dystroglycan metabolism.
Figure 4. Agrin Binds Solubilized α-Dystroglycan

(A) Anti-α-dystroglycan immunoblot (MAb IIH6) reveals that CHAPS-solubilized C2 α-dystroglycan is retained by agrin agarose beads but not by BSA agarose beads. Starting material (30 μg) or 20% of the bound (voids) or unbound (voids) material was loaded (see Experimental Procedures). The binding to agrin agarose is only seen in the presence of Ca2+ (agrin plus). Under the conditions used in this experiment, α-dystroglycan is quantitatively removed by the agrin beads (voids agrin plus). The polydisperse immunoactivity centered around 140 kDa is consistent with the electrophoretic mobility of α-dystroglycan as previously described (Ervasti and Campbell, 1991).

(B) Coomassie staining of the total solubilized material, agrin voids and agrin beads, reveals that the retention of α-dystroglycan on agrin beads is specific. No differences in the protein patterns of the voids with or without Ca2+ (or relative to the starting material) can be seen (same amounts loaded as in [A]). Three times as much bead-associated material was loaded to emphasize the lack of Ca2+-dependent binding proteins detectable. Inability to visualize α-dystroglycan by Coomassie staining has been previously documented (Ervasti and Campbell, 1991). Size standards, indicated at the left, are prestained protein molecular weight standards (GIBCO BRL): myosin (205,000), phosphorylase B (105,000), BSA (70,000), and ovalbumin (49,000).

Figure 5. Agrin Binds α-Dystroglycan with Altered GAG Chains

Anti-α-dystroglycan immunoblot (MAb IIH6) of the bound material from solubilized S26 and S27 cells in the presence and absence of Ca2+. As with C2 cells, α-dystroglycan is retained by agrin beads, but not by BSA beads, and only in the presence of Ca2+. The smaller size of the immunoreactive species is consistent with the postulated GAG modifications of α-dystroglycan and the defects in GAG chain synthesis in these cell lines (Gordon and Hall, 1989). This blot was exposed 10-fold longer than that of Figure 3. The α-dystroglycan immunoreactivity is significantly reduced, suggesting a large reduction in α-dystroglycan expression in these cells relative to the parental C2 line. Molecular weight standards are as in Figure 4.

Colocalization of an Agrin-Binding Site, Dystroglycan, and Adhalin

Torpedo agrin has previously revealed a Ca2+-dependent binding site on the surface of C2 myotubes in culture; this site redistributes with AChRs upon agrin stimulation, but is not the AChR itself (Nastuk et al., 1991). We therefore wondered whether agrin binding to α-dystroglycan might correspond to a similar binding site. We first asked whether purified rat agrin binds C2 cells using a similar indirect antibody assay. Rat agrin, detected with an anti-agrin MAb under conditions very similar to the staining protocols employed in the flow cytometry binding studies, binds diffusely on myotubes at 4°C, but these binding sites colocalize with AChR clusters when cultures are incubated with agrin at 37°C (Figures 6A and 6B), similar to the previously described results with Torpedo agrin (Nastuk et al., 1991). The Ca2+ sensitivity and dose dependence of this binding site parallel the binding detected by flow cytometry, suggesting that these sites are, in fact, identical and therefore likely to represent agrin binding to α-dystroglycan.

If α-dystroglycan is the binding site detected on the surface of C2 myotubes in culture, then α-dystroglycan would be expected to concentrate at the sites of agrin-induced AChR clusters (Nastuk et al., 1991; unpublished data). This is, in fact, the case: the agrin-induced AChR clusters (Figure 6D) are enriched in α-dystroglycan (Figure 6C).
Figure 6. Agrin-Binding Sites, α-Dystroglycan, and Adhalin Colocalize with Agrin-Induced AChR Clusters

Anti-agrin immunoreactivity (MAb 131; shown in [A]), anti-α-dystroglycan immunoreactivity (MAb IIIb; shown in [C]), and anti-adhalin immunoreactivity (MAb IVD3; shown in [E]) colocalize with AChR clusters visualized with rhodamine-conjugated α-Btx (B, D, and F) in agrin-stimulated C2 myotube cultures.
Thus, the agrin binding to C2 cells in the flow cytometry assay and in situ likely represents the binding of agrin to α-dystroglycan present on the surface of these cells.

Previous studies have shown that α-dystroglycan is a component of a complex of proteins that traverse the plasma membrane (Ervasti and Campbell, 1991). We therefore wanted to know whether other components of this complex might show a similar distribution in agrin-treated cultures. We assessed the distribution of adhalin, a 50 kDa transmembrane component of the complex (Roberts et al., 1993), relative to AChR distribution in agrin-stimulated cells. As observed for agrin-binding sites and α-dystroglycan, adhalin immunoreactivity is concentrated at the sites of AChR clusters in agrin-treated cultures (Figures 6C and 6F).

Perturbation of Agrin Activity by Anti-α-Dystroglycan Antibodies

We attempted to block the agrin-induced AChR clustering activity by inclusion of anti-α-dystroglycan MAb IIH6 in the myotube assay media. Although we could not detect a quantitative reduction in the number of agrin-induced AChR clusters, we did notice a dramatic qualitative difference in the clusters induced by agrin in the presence of antibody relative to controls (Figure 7; see also AChR clusters in Figures 6B, 6D, and 6F and in Figures 6C and 8D). This difference in clusters is easily and routinely detected in blind experiments. In the presence of anti-α-dystroglycan antibody, the agrin-induced clusters stained weakly and displayed a fragmented appearance and are apparently comprised of a loose association of microclusters. Furthermore, areas that were not counted as clusters owing to their small size had a higher number of small microclusters. These effects of MAb IIH6 are consistent with α-dystroglycan playing a role in the activity of agrin, likely slowing or inhibiting the consolidation of microclusters into mature clusters. The inability of antibody to block clustering fully is consistent with the flow cytometry data that suggested that the interaction of agrin with α-dystroglycan is significantly stronger than the interaction of IIH6 with α-dystroglycan. The ability of IIH6 to perturb function, given the lack of agrin binding inhibition in the flow cytometry assay, might be explained in either of two ways. First, the threshold level of agrin needed to elicit clustering is 10 PM (effect shown in Figure 7), while the threshold level of agrin in the flow cytometry assay is nanomolar. Thus, at a molar ratio (antibody to agrin) of 10,000:1 (shown in Figure 7), inhibition may be achieved. Alternatively, since cell surface α-dystroglycan is not saturated with agrin under these experimental conditions, antibody-α-dystroglycan complexes may mix with agrin-α-dystroglycan complexes and exert a blocking effect.

Localization of Cytoskeletal Elements Relative to Agrin-Induced AChR Clusters

We have shown that agrin binds to a component (α-dystroglycan) of the DAG complex and that this protein, along with another component of the complex (adhalin), codistributes with AChR clusters. These results suggest that agrin may act to alter or order an internal cytoskeletal framework since dystrophin and utrophin, muscle cytoskeletal proteins belonging to the spectrin superfamily, have been shown to interact with the DAG complex intracellularly (Ervasti and Campbell, 1991; Matsumura et al., 1992). We therefore assessed the distribution of spectrin-like cytoskeletal elements relative to agrin-induced AChR clusters. As previously described, C2 myotubes do not express detectable amounts of dystrophin immunoreactivity (Phillips et al., 1993). In contrast, a rabbit polyclonal anti-peptide serum specific for utrophin recognizes intracellular structures associated with large spontaneous AChR clusters, as previously described (Phillips et al., 1993). Interestingly, agrin-induced AChR clusters are found to contain utrophin immunoreactivity (Figures 8C and 8D). Similarly, the sites of extracellular agrin binding also correspond to sites of enriched intracellular utrophin localization (Figures 8A and 8B). In contrast, the distribution of nonerythroid α-spectrin is uniform throughout the subplasma membrane cytoskeleton and is not concentrated at AChR clusters induced by agrin (Figures 8E and 8F). Thus, extracellular (α-dystroglycan), transmembrane (adhalin), and intracellular (utrophin) components of the utrophin-DAG complex codistribute with AChR clusters in response to agrin.

Discussion

We have identified a protein present on the surface of myotubes, α-dystroglycan, that interacts with agrin and presented evidence that this binding is functionally related to the activity of agrin. Furthermore, we have shown that another transmembrane component of the complex with which α-dystroglycan associates (the DAG complex) is present at agrin-induced AChR clusters. Finally, the intracellular cytoskeletal protein utrophin, which has previously been shown to associate with the DAG complex, is also localized to agrin-induced AChR clusters. Taken together, these data suggest that the binding of agrin to α-dystroglycan may serve as a functionally important linkage between the extracellular matrix and the intracellular cytoskeleton responsible for the formation, maintenance, or both of the AChR-rich postsynaptic membrane domain of the neuromuscular junction.

Several lines of evidence have been presented that demonstrate an interaction between agrin and α-dystroglycan. First, the flow cytometry binding assay reveals a binding site with the characteristics of the previously described laminin G domain-α-dystroglycan binding (Ervasti and Campbell, 1993; Gee et al., 1993). The parallel between anti-α-dystroglycan immunoreactivity and agrin binding on different cell types, taken together with the ability of agrin to inhibit the binding of anti-α-dystroglycan MAb IIH6 to the surface of C2 cells, strengthens the suggestion that this binding site represents agrin binding to native α-dystroglycan. Second, biochemical experiments provide evidence that α-dystroglycan binds agrin in a Ca²⁺-dependent and heparin-inhibitable manner when these proteins are mixed in solution, providing strong evidence that agrin interacts with α-dystroglycan.

In addition to providing evidence that agrin and α-dystro-
glycan interact biochemically, we have shown that agrin-induced clusters of AChR contain α-dystroglycan, as well as adhalin, another component of the DAG complex. Thus, in addition to being an agrin-binding protein on the surface of the myotubes, α-dystroglycan and components of the DAG complex are distributed in a fashion consistent with their playing a role in the activity of agrin.

The binding characteristics of the agrin-α-dystroglycan interaction parallel perturbations known to alter the clustering activity of agrin. Ca²⁺ has been shown to be required for agrin as well as nerve-induced AChR clustering in vitro (Wallace, 1988). Both nerve- and agrin-induced AChR clusters are inhibited by the presence of heparin (Hirano and Kidokoro, 1989; Wallace, 1990). Finally, differences in the appearance of clusters induced by agrin in the presence and absence of anti-α-dystroglycan MAb IIH6 provide evidence that the interaction between agrin and α-dystroglycan plays a functionally important role in the ability of agrin to stimulate the formation of mature clusters.

We have also demonstrated that the cytoskeletal protein utrophin is present at agrin-induced AChR clusters. Taken together with the previously described interaction of utrophin with the DAG complex (Matsumura et al., 1992), this implies that the binding of agrin to α-dystroglycan could in fact be playing an instructive role in postsynaptic cytoskeletal organization. It is possible that agrin-induced AChR aggregation is the result of the ability of agrin to initiate or stabilize a link between the internal cytoskeleton and the extracellular matrix, which in turn would serve as the scaffold upon which AChRs are concentrated. Alternatively, the multistep nature of synapse formation and agrin-induced clustering leave open the possibility that recruitment of a utrophin-based cytoskeleton to AChR clusters by agrin is a later event that mediates the consolidation of microclusters into uniform high density patches. Both nerve-induced AChR cluster formation in vivo (Steinbach, 1981) and agrin-induced cluster formation in culture (Wallace, 1992) have been shown to occur in steps, starting with the initial formation of many small clusters that then coalesce into uniform high density clusters.

The manner in which AChRs might interact with a utrophin-based cytoskeleton is not known. However, an attractive hypothesis is that other cytoskeletal components mediate such an interaction. For example, the 43 kDa protein might mediate an interaction between AChRs that results in the formation of small aggregates of AChR in the plane of the membrane. An interaction between these microclusters and utrophin, possibly mediated by the 58 or 87 kDa AChR cluster-associated proteins (Adams et al., 1993; Butler et al., 1992; Wagner et al., 1993), could

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Figure 7. A Role for α-Dystroglycan in Agrin-Induced AChR Clustering
The distribution of AChR in the absence (A) or presence (B–D) of anti-α-dystroglycan antibody (IIH6 asotes, dialyzed and diluted 10-fold) visualized with rhodamine-conjugated α-Btx reveals differences in the fine structure of agrin-induced clusters formed in the presence of antibody relative to controls (no antibody shown; equal amount of isotype-matched control asotes gives results identical to no antibody added). Note the lower intensity of α-Btx labeling in (B–D) relative to (A) and the punctate nature of the cluster areas in (B–D).
Figure 8. Specific Cytoskeletal Elements Colocalize with Agrin-Binding Sites and AChR Clusters

Anti-utrophin immunoreactivity (A and C) is found at sites of extracellular agrin binding (MAb 131 in [B]), and agrin-induced AChR clusters were visualized with fluorescein-conjugated α-Btx (D). In contrast, agrin-induced AChR clusters (F) do not have associated enhanced immunoreactivity with an anti-nonerythroid α-spectrin antibody (E). The localization of α-spectrin is uniform throughout the subsynaptic membrane cytoskeleton, demonstrating a specific interaction of utrophin with AChR cluster domains.
then consolidate microclusters into a homogeneous membrane domain at the synapse. Interestingly, the loosely associated 1–2 μm AChR aggregates induced by agrin when MAb 11H6 is present are reminiscent of the clusters formed by coexpression of the 43 kDa protein with AChR subunits in fibroblasts or oocytes (Phillipe et al., 1991; Froehner et al., 1990), suggesting that 43 kDa mediated microclusters may be consolidated by interaction with an agrin-induced utrophin cytoskeleton.

The above suggestions are consistent with data from other systems in which the generation of membrane domains is based upon the formation of linkages between internal cytoskeletal structures and extracellular ligands (for reviews see Bennett, 1990; Luna and Hitt, 1992; Nelson, 1992). Furthermore, our data raise the possibility that a common transmembrane link between internal cytoskeletal elements and matrix components could be utilized to stabilize specific membrane cytoskeletons in different places by alterations in the nature of the extracellular ligand (Figure 9). In muscle, the interaction of the DAG complex with laminin extracellularly could signal the formation of an internal dystrophin-based cytoskeleton, while the interaction of the same or a similar complex with agrin at the synapse could yield a different signal, initiating the formation of a utrophin-based cytoskeleton. The way in which ligands might convey this signal in the absence of obvious signal-transducing motifs in α-dystroglycan might be through regulating or altering the interactions among the members of the DAG complex. Alternatively, the binding of agrin could recruit novel proteins to the complex. The possibility that agrin might interact with several proteins is attractive, given the finding that at least four regions of the agrin protein contribute to clustering activity (Hoch et al., 1994).

**Experimental Procedures**

**Antibodies**

All antibodies used have been previously described: mouse ascites MAb 11H6 (α-cysteoglycan), IVD3 (adhalin), and XIXC2 (dystrophin) (Evans and Campbell, 1993); anti-α-dystroglycan affinity-purified rabbit polyclonal antibody (Phillipe et al., 1993); mouse anti-agrin MAb 131 (Hoch et al., 1994); and affinity-purified rabbit polyclonal anti-e-spectrin antibody (Marrs et al., 1993).

**Cell Culture**

Muscle cell lines for biochemical studies or flow cytometry analyses were seeded on 15 cm dishes at 8 × 10^5 cells/plate (C2 cells) or 10^6 cells/plate (S26 and S27 cells), grown for 3 days with daily feeding with growth media (Ferns et al., 1992), and then induced with 2.5% fusion media (DMEM H16, 2.5% horse serum, 2 mM L-glutamine) for 2 days for C2 cells or 2 days in 2.5% fusion media plus 1 day in 5% fusion media (DMEM H16, 5% horse serum, 2 mM L-glutamine) for S26 and S27 cells. Cells were harvested by incubation for 10 min at room temperature in 10 mM EDTA in PBS (EDTA lift). Myotubes for biochemical analysis were pelleted by centrifugation and stored at −70°C.

Muscle cell cultures for immunolocalization and antibody perturbation studies were grown as described above except that 4 × 10^5 C2 cells were plated per well in 8-well multichamber slides (Nunc).

**Flow Cytometry Binding Assay**

For flow cytometry analyses, myoblasts were harvested on day 2 or 3 and myotubes on day 4 or 5 by EDTA lift followed by two washes with divalent cation-free PBS to remove EDTA and were suspended in appropriate staining media at a concentration of 5 × 10^6 cells/ml. Agrin binding was detected by incubation of cells with the stated amount of agrin, divalent cations, and heparin (Sigma) in staining media (DMEM H16 containing 3% heat-inactivated horse serum and 10 mM HEPES [pH 7.4]) for 30 min on ice. Unbound agrin was washed away by centrifugation, and then bound agrin was detected by incubation with MAb 131 in staining media for 15 min on ice and washed, followed by fluorescence-conjugated secondary antibodies in staining media (1:50, TAGO). Stained cells were washed and resuspended in staining media containing propidium iodide and were analyzed on a FACSStar Plus system (Beckton Dickinson, Stanford Shared FACS Facility). Gating of the propidium iodide and forward scatter channels was used to eliminate dead cells and cellular debris or clumps, respectively.

CHO cells and COS cells, grown as previously described (Ferns et al., 1992), were prepared and analyzed by flow cytometry as previously described (Hoch et al., 1994).

**Binding of Solubilized Proteins**

Preparation of agrin- or BSA-conjugated beads (Affigel 10, Bio-Rad) was performed following the instructions of the manufacturer using 3 mg of protein (agrin or BSA) with 300 μl of beads in 1.5 ml of 50 mM HEPES-buffered saline overnight at 4°C. Approximately 75% of the added protein was coupled to the support as assessed by protein assay (Bio-Rad).

Membrane proteins were prepared by homogenization of frozen cell pellets in cold homogenization buffer (300 mM mannose, 0.5 mM EDTA, 1 mM MgCl₂, 50 mM Tris [pH 7.4], with PMSF) followed by centrifugation for 15 min at 1,000 × g in a JS13 rotor to remove cellular debris. Membranes were collected by centrifugation at 100,000 × g for 1 hr. The resultant pellet was washed with 0.6 M KCl in homogenization buffer for 30 min and again centrifuged at 100,000 × g for 1 hr. These KCl-washed membranes were then solubilized in 1% CHAPS in HEPES-buffered saline (150 mM NaCl, 20 mM HEPES [pH 7.2], 1 mM EDTA) for 1 hr at 4°C, and insoluble material was removed by centrifugation at 100,000 × g for 1 hr.

CHAPS-solubilized membrane proteins (150 μg) were incubated with agrin- or BSA-conjugated beads (15 μl of beads) overnight in the presence of either 5 mM Ca²⁺ or 5 mM EDTA. Proteins bound to the beads were recovered by brief centrifugation, and the unbound proteins were removed (voids), followed by two washes with binding buffer. Beads were suspended directly in gel-loading buffer, voids were
precipitated with TCA prior to addition of gel-loading buffer. Equal percentages of the void and bound fractions were separated by electrophoresis on 7.5% polyacrylamide gels and transferred to nitrocellulose or stained with CBB as previously described (Campanelli et al., 1991). Visualization of anti-iiH6 immunoreactive species on blots was carried out as previously described (Campanelli et al., 1991) using IIH6 supernatant at 1:8 dilution and anti-immunoglobulin M antibodies conjugated to HR (TAGO) followed by ECL detection according to the instructions of the manufacturer (Amersham).

**Soluble Agrin and Agrin-Induced Clusters**

Recombinant soluble agrin used in these studies was purified from a line of CHO cells stably expressing the carboxy-terminal half of agrin as previously described (Campanelli, 1993; Ferns et al., 1993). The formation of agrin-induced clusters was assessed by incubation of myotubes grown in 8-well chamber slides with agrin at 10 pM (antibody blocking studies) or 1 nM (colocalization studies) for 8–9 hr on day 5. Under these experimental conditions, the level of spontaneous AChR clusters is less than one cluster per every ten fields viewed with a 40× objective (Hoch et al., 1994). Antibody blocking studies were performed as above, with the addition of diazylated ascites (IIH6) at a dilution of 1:10.

**Immunocytochemical Localization**

Antibody staining was carried out as previously described (Campanelli et al., 1991) with the following alterations. All staining was carried out in the absence of detergents (except for cytoskeletal protein visualization; see below). Detection of bound agrin was performed by incubation of cultures with anti-agrin MAb 131 (Hoch et al., 1994) for 30 min at 4°C in 5% fusion media followed by PBS washing and fixation. Ahralin and α-dystroglycan localizations were visualized by primary antibody incubation after fixation. Detection of cytoskeletal proteins (urophin, dyrophin, α-spectrin) was obtained by inclusion of a detergent extraction step (10 min at room temperature with 1% Triton X-100 in PBS) after fixation and saline washing. Blocking buffer consisted of 3% BSA and 3% NGS in PBS. All secondary antibodies were obtained from TAGO and used at a dilution of 1:100. α-Btx labeling of AChRs was performed as previously described (Campanelli et al., 1991) with the modification that some staining protocols utilized fluorescein conjugates (TAGO). All slides were viewed with a Zeiss axiophot microscope using oil immersion lenses, as previously described (Campanelli et al., 1991).

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**References**


