Agrin Binding to α-Dystroglycan

DOMAINS OF AGRIN NECESSARY TO INDUCE ACETYLCOLINE RECEPTOR CLUSTERING ARE OVERLAPPING BUT NOT IDENTICAL TO THE α-DYSTROGLYCAN-BINDING REGION*

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The synaptic basal membrane protein agrin initiates the aggregation of acetylcholine receptors at the postsynaptic membrane of the developing neuromuscular junction. Recently, α-dystroglycan was found to be a major agrin-binding protein on the muscle cell surface and was therefore considered a candidate agrin receptor. Employing different truncation fragments of agrin, we determined regions of the protein involved in binding to α-dystroglycan and to heparin, an inhibitor of α-dystroglycan binding. Deletion of a 15-kDa fragment from the C terminus of agrin had no effect on its binding to α-dystroglycan from rabbit muscle membranes, even though this deletion completely abolishes its acetylcholine receptor aggregating activity. Conversely, deletion of a central region does not affect agrin's clustering activity, but reduced its affinity for α-dystroglycan. Combination of these two deletions resulted in a fragment of 35 kDa that weakly bound to α-dystroglycan, but displayed no clustering activity. All of these fragments bound to heparin with high affinity. Thus, α-dystroglycan does not show the binding specificity expected for an agrin receptor. Our data suggest the existence of an additional component on the muscle cell surface that generates the observed ligand specificity.

During formation of the neuromuscular junction, motor neurons provide a number of signals that promote the differentiation of the postsynaptic membrane (1). An early signal is the secretion of specific isoforms of agrin, a large protein of the extracellular matrix, and their incorporation into the basal lamina (2). Agrin initiates the concentration of nicotinic acetylcholine receptors (AChRs)1 and other components of the lamina (2, 8). Agrin contains several EGF-like repeats. Studies of structure-function relationships indicate that a C-terminal 50-kDa fragment of rat agrin is sufficient for induction of AChR clustering. Additional deletion from either side of this recombinant fragment abolishes agrin's aggregating activity (13). In chick, a similar 45-kDa fragment is necessary for full clustering activity, and at very high concentrations, a C-terminal fragment of only 21 kDa induces AChR aggregation (14).

The mechanism by which agrin induces the formation of AChR clusters is unknown. Recently, several groups have reported that α-dystroglycan is a major binding protein for agrin on the muscle cell surface (15–18). Several characteristics of this binding support the hypothesis that α-dystroglycan serves as a functional agrin receptor. (i) Binding of agrin to α-dystroglycan is Ca2+-dependent, as is agrin's AChR aggregating activity. (ii) Binding and function of agrin are inhibited by heparin. (iii) In mutant muscle cell lines that are defective in processing of proteoglycans, a much higher concentration of agrin is needed to induce AChR clustering compared with normal muscle fibers (7, 12). Correspondingly, α-dystroglycan in these cell lines, which has a smaller apparent molecular weight, shows a reduced binding to agrin (16–18).

Early in development, α-dystroglycan is widely distributed over the muscle cell surface and becomes concentrated at developing AChR clusters upon addition of agrin (19). It is part of a complex including several transmembrane and peripheral membrane proteins that are associated with the submembrane cytoskeleton proteins dystrophin and utrophin (20, 21). Several members of this dystrophin glycoprotein complex (DGC) copurify with AChRs of the electric organ of Torpedo californica (22–25). In the extrasynaptic region, α-dystroglycan binds to laminin (26, 27). It has been suggested that the DGC forms a link between the extracellular matrix and dystrophin and thereby stabilizes the muscle fiber (28). In vertebrates, dystrophin expression is restricted to the extrasynaptic regions and the troughs of the synaptic folds. In the crests of these folds, where the AChRs are concentrated, dystrophin is replaced by its homologue utrophin (24, 29). Thus, agrin is most likely linked to utrophin via the DGC in the mature synapse, and this link may stabilize synaptic structures.

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1 The abbreviations used are: AChRs, acetylcholine receptors; G-domain, globular domain; EGF, epidermal growth factor; DGC, dystrophin glycoprotein complex; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis.
The important question of whether α-dystroglycan mediates the AChR aggregating activity of agrin alone or in conjunction with other surface components and therefore is an agrin receptor is still unanswered. In an attempt to solve this problem, several groups inhibited agrin binding to α-dystroglycan in culture by addition of a mAb against α-dystroglycan. However, the effects of agrin-induced AChR clustering are controversial: in some studies, no effects on AChR aggregation could be detected (18, 30). One group observed a significant reduction in the number of AChR aggregates (17), while another group found no change in the number of clusters, but rather a more dispersed appearance of AChR aggregates (16).

Here, we addressed this question using a different approach. We assessed the binding of previously and newly generated truncation fragments of agrin to α-dystroglycan and compared the binding pattern with the functional activity of these fragments. Our results demonstrate that α-dystroglycan does not display the ligand specificity expected for an agrin receptor.

EXPERIMENTAL PROCEDURES

Alkaline Extraction from Rabbit Skeletal Muscle Membranes—Rabbit skeletal muscle microsomes were prepared as described (21) and stored at −70°C. Alkaline extraction of proteins from rabbit skeletal muscle membranes was performed as described (27), except that iodoacetamide was omitted. Extraction was quantitative since no α-dystroglycan immunoreactivity was detected in the pellet. The alkaline extract was diluted 1:2 with 50 mM Tris, 4 mM calcium chloride, and 20 μM EGTA to pH 8–6, and the pH was adjusted to 8 at 4°C. An extract of 1.7 mg of membrane protein, containing ~10 pmol of α-dystroglycan (20, 21), was used for each binding experiment (see below).

Agrin Constructs—The agrin constructs N1, N4, N6, and C2 have been previously described (13, 31). Construct N4/C2, containing truncations from both ends, was generated by digestion of constructs N4 and C2 with AvrII. The two DNA fragments containing the N-terminal agrin domain and the C-terminal sequence of C2 as well as different parts of the pCMV vector were ligated. Correct orientation of the fragments was confirmed by digestion with SfiI/XbaI, and the integrity of the cloning sites was confirmed by recutting with AvrII. All constructs used in this study contained the 19-amino acid insert at splicing site Y and, except for N6, the 4-amino acid insert at splicing site Y (7, 10).

CO2-7 cells were transiently transfected with these agrin constructs using the DEAE-dextran method as described previously (5). Dulbecco's modified Eagle's medium was substituted for the serum-containing medium 1 day after transfection. The concentration of construct N1 in conditioned medium collected 3 days after transfection was ~50 ng/ml (12). Based on this estimate, concentrations of the other agrin constructs were determined using a dot-blot procedure (32). For agrin-containing conditioned media, this assay was linear between 5 and 100 ng of total protein.

Antibodies—Hybridoma supernatants containing mAbs HH6 and IVA4, against α-dystroglycan (28) were a gift from S. Robbers and K. Campbell (University of Iowa). A mixture of these antibodies was employed at a 1:3 dilution for immunostaining. The polyclonal antiserum A511 against agrin (10) was used at a dilution of 1:5. Anti- agrin mAbs 30, 86, 131, and 435 (13) were affinity-purified from hybridoma supernatants using protein G-Sepharose (Sigma). Alkaline phosphatase-coupled secondary antibodies were obtained from Promega. In some Western blot experiments, peroxidase-coupled secondary antibodies (Jackson/Dianova) were used.

Agrin Affinity Chromatography—For the generation of high capacity affinity beads in a batch procedure, we modified a method described by Schneider et al. (33). Briefly, 0.9 mg of anti- agrin mAb was diluted in 50 mM Tris, pH 6.7 (buffer A), and mixed with 50 μl of prewashed GammaBind Plus-Sepharose (Pharmacia Biotech Inc.), a protein G derivative, for 2 h at room temperature.

The beads were washed twice with buffer A and twice with buffer A containing 150 mM NaCl, followed by two washes with 10 mM sodium phosphate buffer, pH 6.7. GammaBind Plus-Sepharose and antibodies were equilibrated in 200 mM triethanolamine, pH 8.2, and cross-linked with 10 mM dimethyl suberimidate (Pierce) in the same buffer for 45 min at room temperature. The reaction was quenched with 200 mM ethanalmine, pH 8.2. After subsequent washes with 50 mM Tris, pH 7.4 (buffer B), containing 500 mM NaCl, with buffer B containing 150 mM NaCl, and with buffer B, the beads were mixed with agrin-containing conditioned media for 2 h. In control samples, agrin was replaced by buffer B. These and all subsequent steps were performed at 4°C.

The beads were washed with buffer B containing 150 mM NaCl and with buffer B and incubated with 1.4 ml of dialyzed aliquoted extract of rabbit muscle membranes overnight on a shaking platform. Unbound fractions were collected, and the beads were washed twice with 50 mM Tris, pH 8, 2 mM calcium chloride (buffer C) and twice with buffer C containing 150 mM NaCl. Bound α-dystroglycan was eluted by replacing Ca2+—containing buffer C with 500 μl of 50 mM Tris, pH 8, 150 mM NaCl, 20 mM EGTA for 1 h with shaking. This step was repeated once. Finally, the agrin fragments were eluted by boiling the beads in SDS-PAGE loading buffer.

Heparin-Sepharose Fast Protein Liquid Chromatography—Agrin-containing conditioned media (12 ml) were diluted 1:3 with 30 mM HEPES, pH 7.5, and concentrated in Centriplus concentrators (Amicon, Inc.). Equal volumes (4 ml) of each construct were loaded onto a 5-ml Hitrap heparin-Sepharose column (Pharmacia Biotech Inc.) at a flow rate of 18 ml/h. After collection of the flow-through fraction, the column was washed with 20 mM HEPES, pH 7.5, 50 mM NaCl, and bound agrin was eluted by applying a linear gradient ranging from 50 mM to 1 M NaCl at a flow rate of 30 ml/h. Fractions of 1 ml were collected and analyzed for agrin immunoreactivity by dot-blot assay.

Gel Electrophoresis and Western Blotting—SDS-PAGE was performed on 3–12% gradient gels or on 4–15% PhastGel (Pharmacia Biotech Inc.). Equal samples (4 ml) of each construct were loaded and electrophoresed for 45 min at 200 V. Gels were stained with Coomassie Blue and destained. The gels were scanned, and the protein bands were quantitated using the image analysis software ImageQuant (Molecular Dynamics). Proteins were transferred to nitrocellulose (Schleicher & Schuell) utilizing the procedure of Kyhse-Andersen (34). Membranes were stained with Ponceau S and subjected to Western blot analysis as described previously (5). In some experiments, the enhanced chemiluminescence detection system (Amersham Corp.) was used for peroxidase-coupled secondary antibodies. Because of the sensitivity of anti-α-dystroglycan antibodies to even moderate salt concentrations, low salt buffers containing 20 mM HEPES, pH 8, and 50 mM NaCl were used in the washing procedures for these antibodies.

AChR Aggregation Assay of Conditioned Media—Cultivation of C2 myotubes and quantification of agrin-induced AChRs were performed as described (12) with the following modification. C2 myotubes were incubated with conditioned media containing various agrin constructs for only 6 h to reduce the number of spontaneous clusters. The number of AChR clusters in at least 15 microscopic fields was quantitated for each construct.

RESULTS

We have previously generated a panel of truncated agrin fragments and characterized their ability to induce aggregation of AChRs on the surface of muscle fibers (Fig. 1A) (13). Fragments N1 and N4 showed high clustering activity, while the other fragments were inactive at equal concentrations. In this study, we used several of these fragments, which were highly expressed and efficiently secreted into the medium of transfected COS-7 cells (Fig. 1B), to identify regions of the agrin protein required for binding to α-dystroglycan and to heparin.

Since only relatively small amounts of different agrin fragments can conveniently be generated by transient transfection, we developed a two-step affinity chromatography protocol to evaluate α-dystroglycan's ability to bind to different agrin fragments. We immobilized an appropriate monoclonal antibody against agrin on protein G beads by cross-linking. In the first step, we loaded these beads with different agrin fragments. Because the epitopes of all rat agrin mAbs are known (13), it was possible to predict which agrin fragments would bind to the beads. In a second step, these agrin mAb beads were used as affinity matrices to detect possible binding of α-dystroglycan. To this end, an alkaline extract of rabbit skeletal muscle was incubated with different agrin matrices as well as a control matrix containing only mAb, but no agrin.

α-Dystroglycan bound quantitatively to N1, comprising the C-terminal half of agrin, as judged by the almost complete removal of α-dystroglycan from the unbound fraction and its detection in the eluate (Fig. 2). Binding depended on agrin since the control matrix did not retain any α-dystroglycan. Binding was also Ca2+-dependent because bound immunoreactivity could be eluted by substituting 20 mM EGTA for Ca2+ in the elution buffer (Fig. 2B). Thus, this interaction displayed...
characteristics previously described for the binding of α-dystroglycan to agrin (15–18).

In the first set of experiments, we looked at the importance of the C-terminal sequence for α-dystroglycan binding. Agrin construct C2, which lacks the last of the three laminin G homology regions, bound to α-dystroglycan to the same extent as construct N1 (Fig. 2, A and B). Comparable amounts of both fragments could be eluted from the beads (Fig. 2C). Therefore, we conclude that deletion of a C-terminal fragment of agrin, which completely abolishes agrin’s AChR clustering activity (13), did not affect its binding to α-dystroglycan.

Next, we investigated the effects of truncations from the N-terminal region of N1. For these experiments, we used a mAb whose epitope is located close to the C terminus. Agrin fragment N1, bound to mAb 86, was able to remove α-dystroglycan almost quantitatively from the muscle extract (Fig. 3A). It did not bind α-dystroglycan above the level of the antibody control under these conditions (Fig. 3B). Similarly, fragment N6, for which no AChR aggregating activity has been detected, was able to remove α-dystroglycan almost quantitatively from the muscle extract (Fig. 3B). In the presence of the other fragments, no AChR aggregating activity was detected. Analysis of agrin that remained on the beads after elution of α-dystroglycan demonstrated that comparable amounts of the three constructs had been bound to the matrices (Fig. 3C).

After prolonged development of similar Western blots, we observed weak binding of α-dystroglycan to agrin construct N4, but not to construct N6 (data not shown). This is demonstrated in Fig. 4, in which we used a more sensitive method for the detection of weak immunoreactivities on Western blots. To estimate the differences in α-dystroglycan binding between constructs N1 and N4, we adsorbed ~100 pmol of agrin fragment N4 as well as only 50, 25, and 10 pmol of N1 to mAb 30 affinity beads (Fig. 4C). Interestingly, the smallest immobilized amount of fragment N1, which would correspond to a concentration of ~7 nM in solution, removed less than half of the α-dystroglycan present in the extract (Fig. 4C). We estimate that the amount of α-dystroglycan in the binding reactions was similar to the smallest amount of N1. Even the
it with the binding site on the agrin molecule for heparin and to compare replicates proteoglycans as important for agrin-induced AChR (16–18) is inhibited by heparin. Several lines of evidence implicated proteoglycans as important for agrin-induced AChR aggregation (7, 12, 36). Therefore, we attempted to map the binding site on the agrin molecule for heparin and to compare it with the α-dystroglycan site. We assessed the retention of a small amount of N1 bound more α-dystroglycan than a 10-fold larger amount of fragment N4. We therefore conclude that unlike C-terminal deletions, truncations from the N terminus of N1 reduce agrin’s capability of binding to α-dystroglycan.

These results suggest that the second laminin G-domain, which is known to be important for agrin’s clustering activity (13, 14), as well as flanking sequences might be sufficient to mediate weak α-dystroglycan binding. To directly test this hypothesis, we generated an additional construct, N4/C2 (Fig. 1), which contained only the third and fourth EGF-like repeats as well as the two C-terminal EGF-like repeats. These regions were sufficient for low affinity binding. However, only the presence of the first laminin G-domain and the first two EGF-like repeats, as in construct C2, led to an agrin with high affinity α-dystroglycan binding similar to the largest construct, N1. The difference between low and high affinity binding is substantial since fragment N4 bound considerably less α-dystroglycan than a 10-fold smaller amount of construct N1. It is likely that the second laminin G-domain is crucial for α-dystroglycan and heparin binding as homologous domains mediate binding of laminin to α-dystroglycan and to heparin (35). The related third laminin G-domain did not influence binding at all, and the first laminin G-domain possibly had an effect on α-dystroglycan binding, but not on heparin binding. A further refinement of our analysis would require even smaller fragments of agrin. However, additional agrin truncation constructs are secreted into the medium in very low amounts insufficient for affinity chromatography (13).

Comparing regions of the agrin molecule necessary for α-dystroglycan binding and for AChR clustering activity, we found two important differences (Fig. 6): the C2 and N4/C2 constructs, lacking the C-terminal region of agrin, displayed binding, but no AChR aggregating activity. Conversely, N4, a construct lacking the first laminin G-domain and the first two EGF-like repeats, displayed normal AChR clustering activity, but significantly reduced binding to α-dystroglycan. Furthermore, in our experiments, we employed two antibodies to immobilize agrin that were known to block the AChR clustering activity of soluble agrin in culture (13); surprisingly, mAb 30 (Fig. 3) and mAb 435 (data not shown) did not prevent simul-
taneous binding of agrin to α-dystroglycan.

Two observations made in previous studies had indicated discrepancies between the ability of agrin fragments to bind to α-dystroglycan and their biological activity. First, proteolytic 70- and 95-kDa fragments of purified agrin both bound to membranes of Torpedo electric organ (37). In the 70-kDa fragment, the C terminus had been removed by proteolysis at an unknown position. Similar to our fragments C2 and N4/C2, this fragment was not able to induce AChR clustering (38). However, although the major agrin-binding protein in these membranes is α-dystroglycan (15), other proteins might have contributed to binding of the 70-kDa fragment.

Second, a neuron-specific insert of 8 amino acids (10, 11, 39) in a region close to the fourth EGF-like repeat (Fig. 1A) increases agrin’s AChR clustering activity 10,000-fold (12, 13). Sugiyama et al. (18) found that two agrin splice variants, with or without this insert, equally bound to α-dystroglycan. In their experiments as well as in our study, α-dystroglycan dissociated from β-dystroglycan and the other members of the DGC was used as a binding partner for agrin. The alkaline extraction procedure used in our experiments might have affected the characteristics of the binding of α-dystroglycan to different agrin fragments. However, this appears unlikely since a previous study demonstrated that this procedure does not affect the binding of homologous laminin α-domains of laminin to α-dystroglycan (27). In fact, binding of agrin can be detected even after SDS denaturation and renaturation of α-dystroglycan on nitrocellulose (15, 17, 18).

Interestingly, both the insert sequence and the C terminus of agrin determine a particular conformation of agrin that is defined by the presence of the epitope for mAb 86 (13). Both regions do not affect agrin binding to α-dystroglycan. Thus, this conformation, which is crucial for the clustering activity of agrin, is not required for binding to α-dystroglycan.

Taken together, our results support the idea that α-dystroglycan alone is not the functional agrin receptor. A different or additional component that generates the observed ligand specificity has to be postulated (18, 40).

Three principal scenarios can be considered for the agrin–α-dystroglycan interaction. First, α-dystroglycan may in some manner collaborate with other signaling proteins and facilitate their binding to agrin. Receptor systems that utilize auxiliary proteoglycans are known for several growth factors. For example, transforming growth factor-β first binds to an accessory proteoglycan, betaglycan, and later combines with a different receptor to form a ternary complex, which is finally converted into the signaling receptor (41). However, the concept of α-dystroglycan as an analogous auxiliary proteoglycan does not readily explain why construct C2 does not act as an inhibitor of agrin-induced AChR aggregation (13) and why the weaker binding of construct N4 does not correlate with reduced clustering activity. Therefore, our data favor two models in which the active agrin fragments N1 and N4, but not inactive fragments such as C2, bind to a yet unknown receptor other than α-dystroglycan (18).

In a second scenario, binding of agrin to this so far unidentified receptor alone is sufficient to induce AChR clustering. α-Dystroglycan does not participate in this process. Instead, it merely is a structural protein involved in stabilization of the synaptic sarcolemma. This model is entirely consistent with our data.

A more intriguing third scenario, however, would be a two-step model in which α-dystroglycan is important for the second phase of receptor aggregation. According to this model, binding of agrin to the hypothetical receptor only initiates the formation of small clusters of AChRs that are observed during the early phase of receptor aggregation both in vivo and in vitro (42, 43). α-Dystroglycan can be detected very early in these microclusters (30). Binding of agrin to α-dystroglycan could be required for a following maturation step. This binding could trigger a reorganization of the membrane cytoskeleton, for example, an association of utrophin with the DGC. Utrophin colocalizes with mature AChR clusters, but is not detectable in microclusters (44). The recruitment of utrophin or another rearrangement of the submembrane cytoskeleton could initiate the condensation of microclusters into larger aggregates. According to this model, this consolidation of AChR clusters is mediated by all α-dystroglycan-binding agrin fragments, including C2. In contrast, inactive fragments such as C2 are not expected to bind to the hypothetical receptor. Therefore, fragment C2 should inhibit neither clustering initiation nor cluster condensation. Indeed, this fragment does not block aggregation of AChRs (13). The question remains how construct N4 can induce clusters with similar efficiency compared with N1 despite its reduced binding to α-dystroglycan. A possible explanation is that agrin synthesized by muscle fibers can compensate for the diminished binding of N4 to α-dystroglycan (18).

The function of muscle-derived agrin, which does not contain an insert at splicing site Z and therefore has a low capability of binding to α-dystroglycan, could be mediated by binding of other agrin-binding proteins. Our data suggest that muscle-derived agrin can induce clusters similar to those of N1 and N4. Nevertheless, in vivo studies with transgenic mice overexpressing muscle-derived agrin are necessary to test the models that we propose. In any case,秀丽小线 does not participate in this process. Instead, it merely is a structural protein involved in stabilization of the synaptic sarclemma. This model is entirely consistent with our data.

To test these models, it will be necessary to identify and isolate additional high affinity agrin-binding proteins. Our construct N4 could be a valuable tool in this search as its binding to the signaling receptor should be less overshadowed by its binding to α-dystroglycan.

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Agrin binding to α-Dystroglycan

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