Distribution of α-Dystroglycan during Embryonic Nerve–Muscle Synaptogenesis

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Abstract. The distribution of α-dystroglycan (αDG) relative to acetylcholine receptors (AChRs) and neural agrin was examined by immunofluorescent staining with mAb IIH6 in cultures of nerve and muscle cells derived from *Xenopus* embryos. In Western blots probed with mAb IIH6, αDG was evident in membrane extracts of *Xenopus* muscle but not brain. αDG immunofluorescence was present at virtually all synaptic clusters of AChRs and neural agrin. Even microclusters of AChRs and agrin at synapses no older than 1–2 h (the earliest examined) had αDG associated with them. αDG was also colocalized at the submicrometer level with AChRs at nonsynaptic clusters that have little or no agrin. The number of large (>4 μm) nonsynaptic clusters of αDG, like the number of large nonsynaptic clusters of AChRs, was much lower on innervated than on noninnervated cells. When mAb IIH6 was included in the culture medium, the large nonsynaptic clusters appeared fragmented and less compact, but the accumulation of agrin and AChRs along nerve–muscle contacts was not prevented. It is concluded that during nerve–muscle synaptogenesis, αDG undergoes the same nerve-induced changes in distribution as AChRs. We propose a diffusion trap model in which the αDG–transmembrane complex participates in the anchoring and recruitment of AChRs and αDG during the formation of synaptic as well as nonsynaptic AChR clusters.

α-DYSTROGLYCAN (αDG),[1] an extracellular peripheral membrane protein, is part of the dystrophin receptor complex, which comprises at least six proteins that are tightly associated with dystrophin or dystrophin-related protein (DRP; also called utrophin) at the cell surface in muscle, nerve, and a variety of other tissues (Ervasti and Campbell, 1991). αDG binds to the extracellular matrix proteins laminin and merosin (Douville et al., 1988; Ibraghimov-Beskrovnaya et al., 1992; Ervasti and Campbell, 1993*a*; Gee et al., 1993) and is thought to forge a structurally important link from the basal lamina surrounding skeletal muscle cells to their submembranous cytoskeleton (Ervasti and Campbell, 1993*b*; Lindenbaum and Carbonetto, 1993). Mutations in dystrophin, adhalin (a transmembrane member of the complex), or merosin can lead to muscular dystrophy (Anderson and Kunkel, 1992; Roberds et al., 1994; Sunada et al., 1994; Tomé et al., 1994; Xu et al., 1994).

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; αDG, α-dystroglycan; DRP, dystrophin-related protein; SC, spinal cord.

Immunocytochemical studies have indicated that the αDG complex and dystrophin are present over the entire surface of mature skeletal muscle cells (Ervasti and Campbell, 1991; Matsumura et al., 1992). At the neuromuscular junction, αDG is concentrated with other members of the complex, but dystrophin is replaced by DRP at the tops of the junctional folds where acetylcholine receptors (AChRs) are clustered (Ohlendieck et al., 1991*a*; Bewick et al., 1992; Bowe et al., 1994). αDG, other members of its complex, and DRP are also concentrated at agrin-induced AChR clusters on cultured muscle cells (Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994).

Agrin, in addition to causing AChRs to cluster (Godfrey et al., 1984; Nitkin et al., 1987; Campanelli et al., 1991; Ferns et al., 1992; Tsim et al., 1992), is deposited by neurites at newly forming nerve–muscle synapses (Cohen and Godfrey, 1992), and some anti-agrin antibodies inhibit nerve-induced AChR clustering (Reist et al., 1992). This evidence suggests that agrin is the primary neural agent that triggers clustering of AChRs during embryonic nerve–muscle synaptogenesis. That the αDG complex is concentrated at the mature neuromuscular junction and at agrin-induced AChR clusters implies that it too plays a role in nerve–muscle synaptogenesis. A direct role for αDG itself, as a receptor for

agrin, is suggested by the recent findings that solubilized αDG binds agrin and that this binding is influenced by the several agents that affect agrin-induced clustering of AChRs (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). More importantly, in some studies (Campanelli et al., 1994; Gee et al., 1994; but see Sugiyama et al., 1994), an anti-αDG antibody that inhibits the binding of agrin to αDG interfered with the generation of AChR clusters induced by agrin in solution.

To assess further the role of αDG in synaptogenesis, we have examined its spatial relationship to AChRs and neural agrin during synaptogenesis in cultures of nerve and muscle cells derived from *Xenopus laevis* embryos. The results indicate that αDG is present at the earliest synaptic microclusters of AChRs and agrin, that it undergoes the same nerve-induced changes in distribution as AChRs, and that it may also participate in the generation of nonsynaptic clusters of AChRs lacking agrin.

Materials and Methods

Membrane Protein Isolation

Membrane proteins were isolated from leg muscle of the frog *Xenopus laevis* or from leg and back muscles of rabbits by the protocol of Ohlendieck et al., (1991*b*). Briefly, the muscles were homogenized in a Polytron mixer (Brinkmann Instruments, Rexdale, Ontario, Canada) in 7.5 vol of homogenization buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM $MgCl_2$, 0.30 M sucrose, 0.5 mM EDTA, pH 7.0) in the presence of the following protease inhibitors: aprotinin (1 μM), leupeptin (1 μM), pepstatin A (1 μM), benzamidine (1 mM), iodoacetamide (1 mM), and PMSF (1 mM). The homogenate was centrifuged at 14,000 *g* at 4°C for 15 min. The supernatant was retained and the pellet was reextracted (and recentrifuged) in 75% of the original buffer volume. The ensuing supernatants were pooled and centrifuged at 30,000 *g* for 30 min at 4°C to pellet the heavy microsome fraction. The pellet was then resuspended in 0.6 M KCl, 0.30 M sucrose, 50 mM Tris-HCl, pH 7.4, with protease inhibitors and incubated on ice. After 30 min, this suspension was centrifuged at 142,000 *g* for 30 min at 4°C. The KCl-washed heavy microsomes (pellet) were solubilized on ice for 30 min in 1% digitonin, 50 mM Tris-HCl, pH 7.4, with protease inhibitors and centrifuged at 85,000 *g* for 30 min to pellet any insoluble material.

Western Blot Analysis

Equal amounts (20 μg) of solubilized heavy microsomes from rabbit and *Xenopus* muscle were electrophoretically separated on 7.5% SDS-PAGE gels (Laemmli, 1970), and the protein was subsequently blotted onto nitrocellulose membranes. The blots were blocked with 5% powdered skim milk in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween-20. Antibodies (mAb IIH6, anti-FP-B, and anti-FP-D) to αDG (Ibraghimov-Beskrovnaya et al., 1992) were applied in blocking buffer for 30 min at 23°C. The blots were washed repeatedly for 1 h in the same buffer without skim milk and then incubated with secondary antibody conjugated to horseradish peroxidase (Sigma Immunochemicals, St. Louis, MO). Excess secondary antibody was removed by washing for 2 h, and the bound antibody was visualized using chemiluminescence (DuPont-NEN, Boston, MA).

Cell Culture

Cultures were prepared as described previously (Cohen et al., 1994). Briefly, the culture substrate consisted of a combination of rat tail collagen (type I) and mouse tumor entactin, collagen (type IV), and laminin (ECL; Upstate Biotechnology, Inc., Lake Placid, NY), on glass coverslips. These coverslips formed the floor of the culture chamber (Anderson et al., 1977). Spinal cord (SC) and myotomal muscle were obtained from 1-d-old *Xenopus* embryos (stages 22–26; Nieuwkoop and Faber, 1967). After dissociation, SC and muscle cells were plated or stored in the refrigerator for use the next day. Cocultures were prepared by plating the SC cells 1–2 d after plating the muscle cells. All cultures were maintained at room temperature (23–25°C).

For most experiments, the culture medium consisted of 67% (vol/vol) L15, 0.25% (vol/vol) dialyzed horse serum, and 1% (vol/vol) Ultraser-G. In some experiments the medium consisted of 67% L15 and 0.1% (wt/vol) Albumax I, or 0.1% (wt/vol) albumin (fraction V; Sigma Chemical Co.), and similar results were obtained. Except for albumin, all components of the culture media were obtained from GIBCO BRL (Gaithersburg, MD). For cocultures, α-bungarotoxin or its rhodamine conjugate (Molecular Probes, Inc., Eugene, OR) was usually included in the culture medium at a concentration of 0.2 μg/ml in order to prevent contraction of innervated muscle cells. For some experiments (see Results), antibodies were added to the cultures at the same time as neurons.

Immunofluorescent Staining

Mouse ascites mAb IIH6, directed against αDG (Ervasti and Campbell, 1993*a*), was used at 1:100–1:500, and rabbit anti-agrin antiserum 36 (Godfrey, 1991) was used at 1:200–1:400. Since mAb IIH6 is an IgM, we used as controls two other mouse ascites fluids, TEPC 183 and mAb HNK-1, containing the same subclass of immunoglobulin (Sigma Immunochemicals). Another IgM, mAb HepSS-1 (Seikagaku America, Inc., Rockville, MD) was also tested. Appropriate affinity-purified secondary antibodies conjugated with fluorescein or rhodamine (Organon-Teknika-Cappel, Durham, NC; Molecular Probes, Inc.) were used at 10 μg/ml. Rhodamine-conjugated α-bungarotoxin, at 2–4 μg/ml, was sometimes included with the fluorescein-conjugated secondary antibodies to stain AChRs. The cultures were exposed to the primary and secondary antibodies for 30 min. A solution of 67% L15 and 1% (vol/vol) goat serum was used for rinsing and dilution of antibodies.

As described previously, living cultures were usually transferred to the refrigerator, stained and rinsed with refrigerated solutions, fixed with 4% formaldehyde, and processed for microscopy (Cohen and Godfrey, 1992). Alternatively, some cultures were prefixed for 15–30 min and then stained with mAb IIH6. Similar results were obtained with one exception. The immunofluorescence at large nonsynaptic clusters (see Fig. 3) sometimes appeared speckled when the cultures were stained alive, whereas when stained after fixation, it was virtually identical in appearance to the AChR fluorescence. For a few SC cultures, the staining was performed after eliminating the neurons by a combination of aggressive rinsing and treatment with 1% (vol/vol) Triton X-100 (Cohen et al., 1994).

Some living cocultures were photographed 5–7 h after plating the SC cells so that the maximum age of newly formed neurite–muscle contacts could be known (see Figs. 5 and 7). Less than 2 h later, the cultures were stained (or fixed and then stained). In estimating the maximum age of contacts that formed during this interval, we assumed that neurite growth ceased within 20 min of exposing the living cultures to the refrigerated solutions used for staining.

Because of their size, immunoglobulins have restricted access to the portion of the muscle cell surface apposed to the culture substrate. Accordingly, we excluded this lower surface of the muscle cells in assessing the distribution of the immunofluorescence. Colocalization of rhodamine and fluorescein fluorescence was evaluated directly through the microscope, using a ×63 oil immersion objective; individual sites of fluorescence were at least 2 μm in length or diameter. To analyze colocalization of individual sites <1 μm in diameter (microclusters), we used photographs at an enlargement of 1–2 mm/μm. The positions of the microclusters obtained with one of the fluorophores were marked on a transparency that was then placed on the companion photograph to determine the incidence of overlap with the contrasting fluorophore.

Results

MAb IIH6 Recognizes Xenopus Muscle αDG

As shown in Fig. 1, a band the size of αDG (156 kD) is evident in rabbit (*R*) as well as *Xenopus* (frog, *F*) skeletal muscle probed with mAb IIH6. No immunoreactivity with mAb IIH6 was detected in blots of *Xenopus* brain (data not shown) probably because of the poor reactivity of this antibody with αDG from brain and/or the low abundance of αDG in neural tissues (Douville et al., 1988; Ibraghimov-Beskrovnaya et al., 1992). In contrast with mAb IIH6, two antisera to DG fusion proteins (FP-B and FP-D) that recognize either αDG alone (anti-FP-D) or α- and βDG (anti-FP-B) failed to

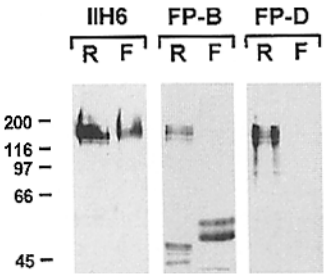

Figure 1. Western blot analysis of rabbit and frog α- and βDG. Nitrocellulose transfers of solubilized rabbit (*R*) or *Xenopus* (*F*) muscle heavy microsomes that had been separated by SDS-PAGE under reducing conditions were incubated together with mAb IIH6 (*IIH6*) or rabbit antisera to DG (*FP-B* and *FP-D*). mAb IIH6 recognizes a glycosylated region of αDG (Ervasti and Campbell, 1993). The polyclonal antisera are to fusion proteins of α- and βDG (Ibraghimov-Beskrovnaya et al., 1992). Anti-FP-B recognizes a region overlapping the cleavage point between α- and βDG, and anti-FP-D is specific for αDG. All three reagents recognize a band at 156 kD in rabbit muscle, but only mAb IIH6 binds to *Xenopus* αDG. In addition, in rabbit muscle anti-FP-B recognizes a doublet at 43 kD corresponding to βDG (Ibraghimov-Beskrovnaya et al., 1992), which is somewhat larger in *Xenopus* muscle (51–54 kD). Molecular mass markers (in kilodaltons) are shown to the left.

recognize *Xenopus* αDG, although the latter antiserum recognizes a doublet characteristic of βDG (Ibraghimov-Beskrovnaya et al., 1992) in rabbit that is somewhat larger in *Xenopus* muscle. These data indicate the specificity of mAb IIH6 for *Xenopus* αDG. They also show that the epitope recognized by mAb IIH6, which is at least in part a carbohydrate (Ervasti and Campbell, 1993a), appears to be more highly conserved across these species than portions of the protein core recognized by the anti-fusion protein antisera. Our results are consistent with data implicating these carbohydrate side chains on αDG in its binding to laminin and agrin (Ervasti and Campbell, 1993*a*; Gee et al., 1993, 1994; Campanelli et al., 1994).

Table I. Incidence of αDG Immunofluorescence at Synaptic and Nonsynaptic Clusters of AChRs

Type of AChR cluster	Number examined	Colocalized αDG immunofluorescence
Synaptic: all sizes	1,511	95.6%
Nonsynaptic: >4 μm	1,088	94.2%
Nonsynaptic: <1 μm	166	92.2%

Muscle cells were cultured for 2–3 d and neurons for 1 d.

αDG versus AChR Distribution

αDG immunofluorescence varied considerably in intensity among individual muscle cells but was detected at virtually all AChR clusters on the edge and upper surface of innervated as well as noninnervated muscle cells (Fig. 2; Table I). This colocalization was often strikingly precise and extended to the complex patterns of AChR distribution within the boundaries of large (>4 μm) nonsynaptic AChR clusters (Fig. 3 *A*; see also Fig. 8, *A* and *B*). Sometimes portions of the αDG immunofluorescence extended a few micrometers beyond the perimeter of the AChR clusters (Fig. 3 *A*, *arrows*). A high incidence of colocalization was also observed at smaller nonsynaptic AChR clusters, including AChR microclusters (Table I), but these small nonsynaptic AChR clusters were typically much less abundant than small αDG clusters (Fig. 2 *C*). When control IgMs were used instead of mAb IIH6, there was no corresponding immunofluorescence on the muscle cells (Fig. 3 *B*). One of the controls, mAb HNK-1, recognizes a carbohydrate epitope on cell adhesion molecules such as NCAM (Naegele and Barnstable, 1991) and stained the neurites brightly along their entire

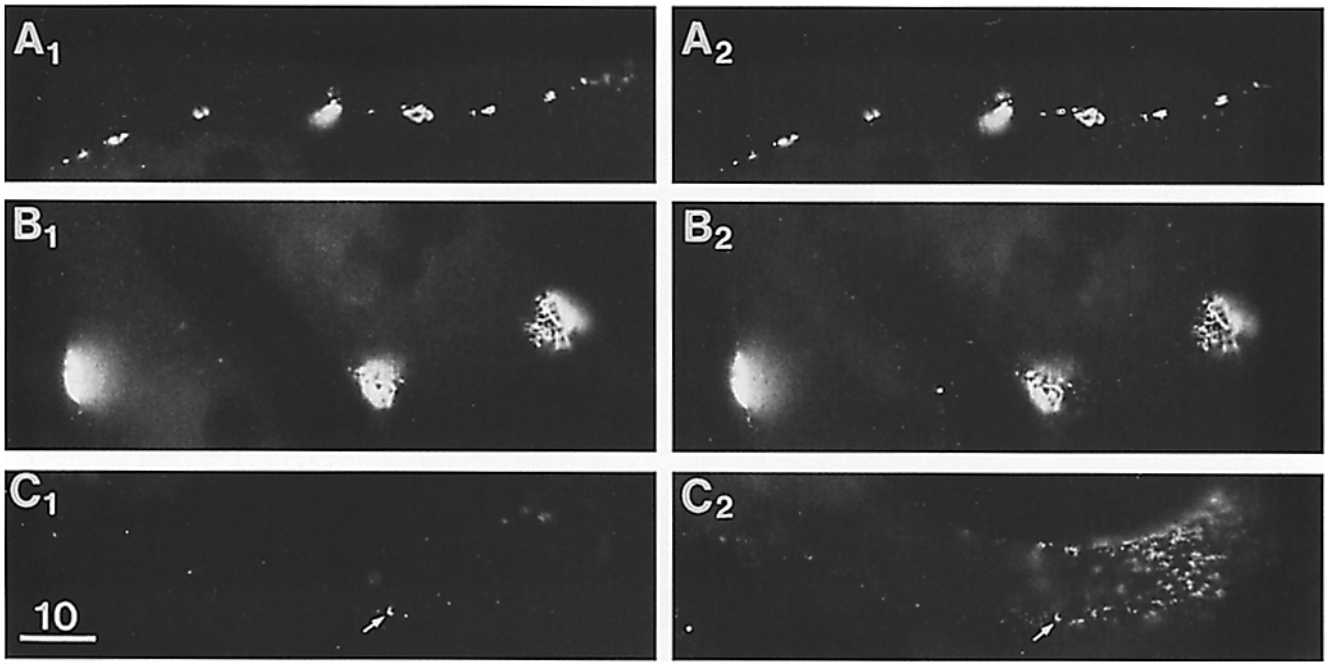

Figure 2. αDG immunofluorescence at synaptic and nonsynaptic clusters of AChRs. (A_1) AChR fluorescence along a neurite–muscle contact. (A_2) Corresponding αDG immunofluorescence, located at the synaptic clusters of AChRs. (B_1) Large clusters of AChRs on noninnervated muscle cells. (B_2) Corresponding αDG immunofluorescence, located at the same sites as the nonsynaptic AChR clusters. (C_1) AChR fluorescence. (C_2) αDG immunofluorescence, prominent near the end of a muscle cell where AChR fluorescence is sparse. The arrow in C_1 and C_2 points to the same microcluster. Bar, 10 μm.

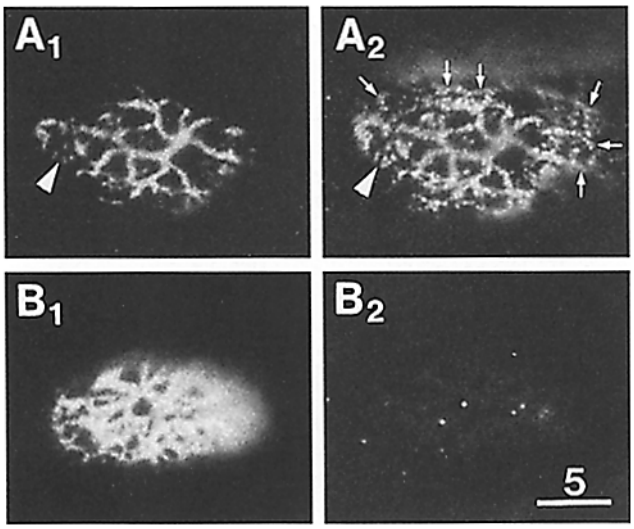

Figure 3. Precision of colocalization and specificity of αDG immunofluorescence. (A_1) Intricate pattern of AChR distribution within a large nonsynaptic AChR cluster. (A_2) αDG immunofluorescence has an almost identical pattern. The arrowhead (also in A_1) points to microclusters of colocalized AChRs and αDG. Arrows point to portions of the immunofluorescence that extend beyond the limits of the AChR cluster. (B_1) Another large nonsynaptic cluster of AChRs. (B_2) Corresponding nonspecific immunofluorescence obtained by substituting a control IgM (TEPC 183) for mAb IIH6. Bar, 5 μm.

length, similar to the pattern observed previously with another anti-NCAM antibody (Cohen et al., 1994). Together, the observations indicate a precise spatial register of αDG at virtually all sites where AChRs cluster on the surface of the muscle cells.

The patterns of narrow, aligned clusters of αDG (and AChRs) along neurite–muscle contacts (Fig. 2 *A*) were not observed on noninnervated muscle cells (Fig. 2 *B*). In addition, the αDG (and AChR) clusters along younger neurite–muscle contacts were substantially smaller (see Fig. 5). These observations indicate that innervation induces a local, time-dependent accumulation of αDG similar to the local, time-dependent accumulation of AChRs (see Anderson and Cohen, 1977). It is also known that innervation induces global changes in AChR distribution, inhibiting the formation and survival of large (>4 μm) nonsynaptic clusters of AChRs elsewhere on the muscle cell surface (Anderson et al., 1977; Moody-Corbett and Cohen, 1982). Likewise, in the current study, large nonsynaptic clusters of αDG (and AChRs) were present on most of the noninnervated muscle cells (90.2 ± 1.6%; mean ± SEM for six cultures) but were observed on very few of the innervated muscle cells (7.7 ± 2.2%). These results indicate that αDG undergoes the same nerve-induced local and global changes in distribution as previously established for AChRs.

Besides its occurrence at virtually all synaptic and nonsynaptic AChR clusters, αDG immunofluorescence was also seen elsewhere on the surface of many of the muscle cells at sites where there was little or no detectable AChR fluorescence. This additional, "AChR-free" αDG immunofluorescence usually consisted of loose arrangements of small (0.3–4-μm) clusters sometimes distributed over much of the muscle cell surface and sometimes restricted to the ends and peripheral regions of the muscle cells (Fig. 2 *C*).

αDG versus Agrin Distribution

Combined staining for αDG and for agrin revealed αDG immunofluorescence at almost all sites of agrin accumulation along neurite–muscle contacts (Fig. 4 *A*; see also Figs. 6 and 7). This finding is consistent with the recent demonstration

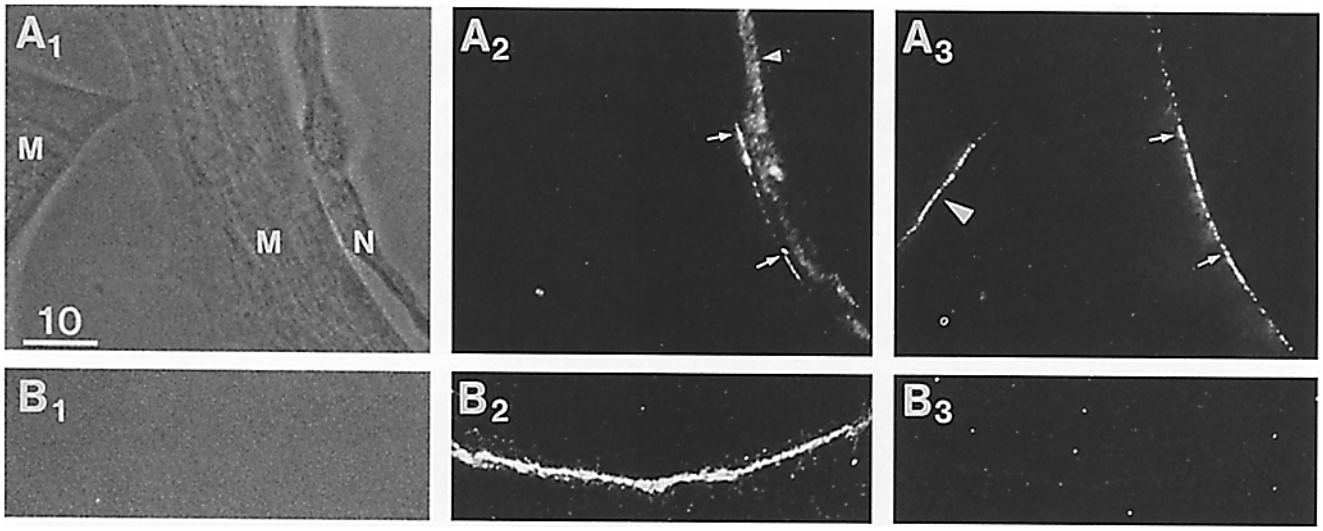

Figure 4. Comparison of agrin and αDG immunofluorescence. (A_1) Phase-contrast micrograph of a neurite (*N*) and muscle cells (*M*). (A_2) Agrin immunofluorescence at sites of neurite–muscle contact (*arrows*) and along the path of neurite–substrate contact (*arrowhead*). (A_3) αDG immunofluorescence is colocalized with, but more extensive than, the agrin along the neurite–muscle contact (*arrows*). It is also seen on the edge of another muscle cell (*arrowhead*) where there was no neurite or agrin. αDG immunofluorescence is not associated with the agrin that was deposited along the path of neurite–substrate contact. This was also the case when the neurites were eliminated before immunofluorescent staining, as shown in B_1 (phase-contrast), B_2 (agrin), and B_3 (αDG). Bar, 10 μm.

that agrin binds to αDG (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). The αDG staining on muscle cells was also more extensive than the agrin staining. Nonsynaptic sites of αDG immunofluorescence had no corresponding agrin immunofluorescence (Fig. 4 *A*$_3$, *arrowhead*), which is in line with previous findings that the muscle cells in these cultures externalize little if any agrin and that the agrin at synapses is neurally derived (Cohen and Godfrey, 1992).

Whereas αDG immunofluorescence was associated with noninnervated muscle cells, even when the cells were grown in the absence of neurons, it was never observed along neurites regardless of whether the neurons were grown with or without muscle cells. These findings are in keeping with the presence of αDG in Western blots of *Xenopus* muscle but not brain probed with mAb IIH6 and suggest that the muscle cells are the major source of αDG at neurite-muscle synapses. Further to this point, in these *Xenopus* cultures the neurites deposit agrin not only on the muscle cells they contact, but also on the culture substrate along their path of growth (Cohen et al., 1994). Although αDG was colocalized with agrin at sites of neurite-muscle contact, there was no hint of αDG immunofluorescence where the neurites deposited agrin on the culture substrate (Fig. 4 *A*$_2$, *arrowhead*). This was the case even when the staining was performed after eliminating the neurons and possible restrictions on antibody access (Fig. 4 *B*). These findings suggest that the molecular form of αDG that is on the surface of muscle cells and recognized by mAb IIH6 is neither externalized together with agrin by the SC neurites nor involved in the binding of agrin to the culture substrate, and they support the conclusion that the accumulation of αDG at synapses involves a neurite-induced redistribution of muscle αDG.

Early Accumulation of αDG at Newly Forming Synapses

If αDG participates in embryonic nerve-muscle synaptogenesis by binding neurally derived agrin as an initial step in the neurite-induced accumulation of AChRs, then it should be present at newly forming synapses before or coincident with agrin and AChRs. To test this prediction we examined the colocalization of αDG immunofluorescence at synaptic clusters of AChRs and agrin in cultures whose neurons were only 7–8 h old and along neurite-muscle contacts that were <120 min old. At such young contacts, the majority of AChR clusters and of agrin deposits are <1 μm in diameter (Cohen and Godfrey, 1992).

Fig. 5 illustrates an example from a culture stained for αDG and AChRs. Fig. 5 *A* was photographed before, and *B* after, the culture was stained and fixed. The interval between the photograph in Fig. 5 *A* and staining the culture was 95 min. Comparison of Fig. 5, *A* and *B*, reveals that during this 95-min interval, the neurite grew and established a new region of contact with the muscle cell. Fig. 5 *C* shows a series of microclusters of AChRs along the newly formed contact and along the more proximal portion of contact that was established before the initial photograph. Fig. 5 *D* shows the corresponding αDG immunofluorescence located at most of the AChR microclusters, including the most distal ones along the newly formed contact. Also apparent are some αDG microclusters where there was no detectable AChR staining. The results are summarized in Fig. 6. Together, 101

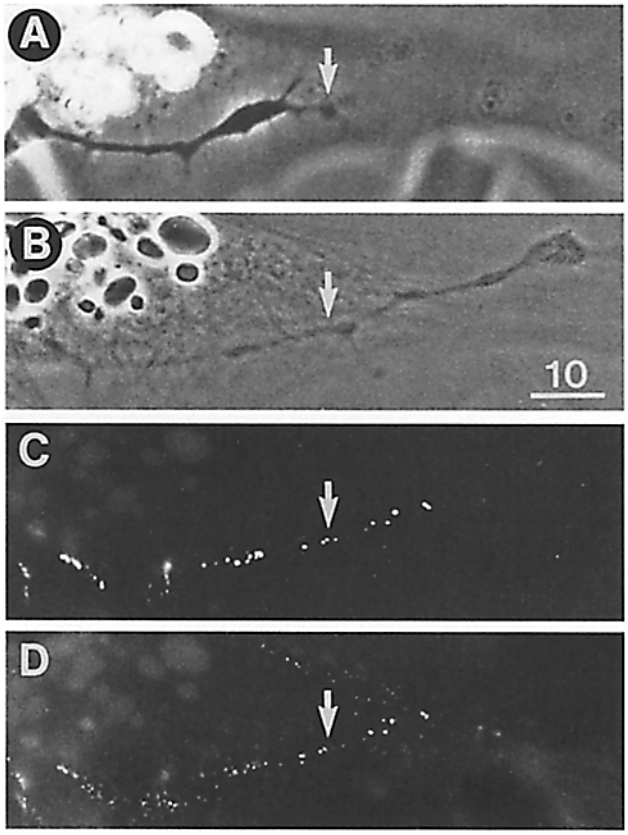

Figure 5. αDG immunofluorescence at young synaptic clusters of AChRs. (*A*) Phase-contrast micrograph of a living neurite-muscle contact. The arrow points to the end of the neurite and has the same position in *B*, *C*, and *D*. (*B*) Same field after staining and fixing. The neurite grew several micrometers during an interval of 95 min. (*C*) AChR microclusters along the neurite-muscle contact, including that portion known to be <95 min old. (*D*) αDG immunofluorescence is present at most of the AChR microclusters. Some additional microclusters of αDG are also apparent. Bar, 10 μm.

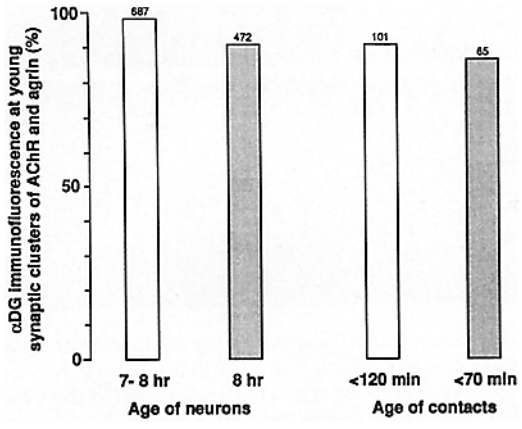

Figure 6. Incidence of αDG immunofluorescence at young synaptic clusters of AChRs (*open columns*) and agrin (*stippled columns*). The data for AChR clusters are from three 7–8-h cocultures. Within those cultures, some of the identified contacts were known to be <120 min (see Fig. 5). The data for agrin clusters are from two 8-h cocultures; some of the contacts within these cultures were <70 min. The numbers above the columns indicate how many clusters were counted. □, AChR clusters; ▨, agrin clusters.

microclusters of AChRs were examined along contacts that were established for <120 min, and 91.1% of these had overlapping microclusters of αDG immunofluorescence. This value is slightly less than the values of 97.4 and 95.6% obtained for all of the synaptic clusters of AChRs in cultures whose neurons were 7–8 h old and 1 d old, respectively.

Similar to the codistribution of AChRs and αDG at newly forming synapses, the incidence of αDG immunofluorescence at synaptic deposits of agrin was also high (Fig. 6). The values were 86.2% along neurite–muscle contacts <70 min old and 90.5% for all synaptic deposits of agrin in cultures whose neurons were 8 h old. An example of the agrin and αDG immunofluorescence along a young contact, part of which was established for <36 min, is shown in Fig. 7. The presence of αDG at most of the microdeposits of agrin is apparent. Taken together, the results indicate that αDG, neural agrin, and AChRs accumulate at synapses at precisely the same sites and with very little delay after the establishment of neurite–muscle contact.

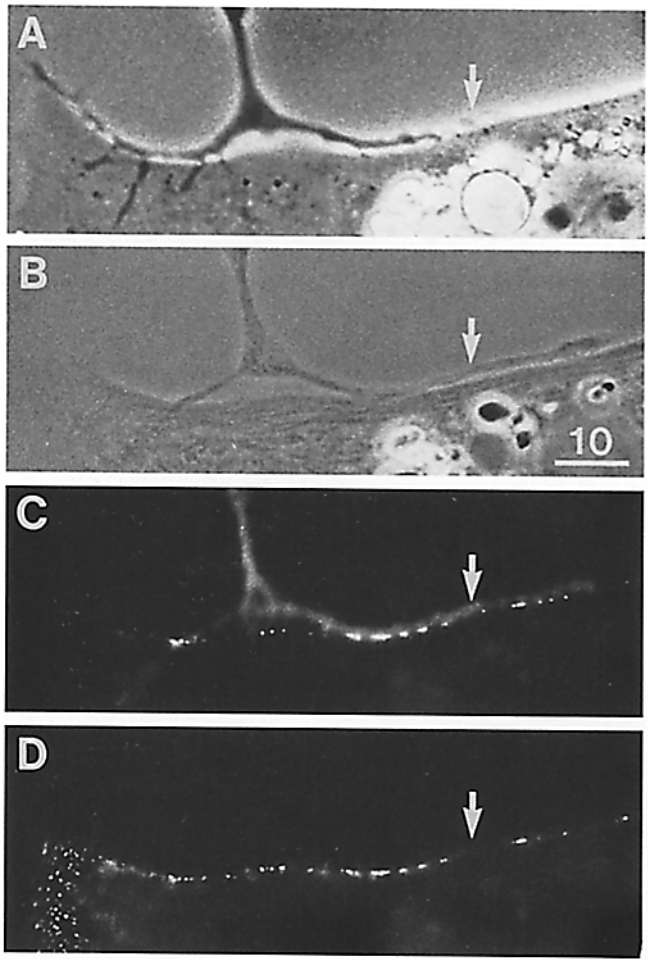

Figure 7. αDG immunofluorescence at young synaptic clusters of agrin. (*A*) Phase-contrast micrograph of a living neurite–muscle contact. The arrow points to the end of the neurite and has the same position in *B*, *C*, and *D*. (*B*) Same field after staining and fixing. The neurite grew several micrometers during an interval of 36 min. (*C*) Agrin immunofluorescence along the neurite–muscle contact, including the portion known to be <36 min old. Out-of-focus immunofluorescence is also apparent where the neurite deposited agrin on the culture substrate. (*D*) αDG immunofluorescence is present at most of the microdeposits of agrin along the neurite–muscle contact. There is additional αDG especially near the end of the muscle cell. Bar, 10 μm.

Effect of Anti-αDG Antibody on AChR Clustering

Since mAb IIH6 competitively inhibits the binding of agrin to solubilized αDG and may interfere with agrin-induced clustering of AChRs (Campanelli et al., 1994; Gee et al., 1994; but see Sugiyama et al., 1994), we tested whether the inclusion of mAb IIH6 ascites in the culture medium at dilutions of 1:10–1:100 also affects AChR clustering on embryonic *Xenopus* muscle cells. At the highest concentration, the development of the nerve and muscle cells seemed somewhat retarded, but neurite–muscle contacts formed and AChRs and agrin accumulated along these contacts. The intensity of the agrin immunofluorescence may have been reduced, but its variability even within an individual culture hinders documentation of such an effect.

A more clear-cut effect was seen at large nonsynaptic AChR clusters. In the presence of 1:10 mAb IIH6, none of the large nonsynaptic clusters on noninnervated muscle cells had the typical compact appearance of those in untreated cul-

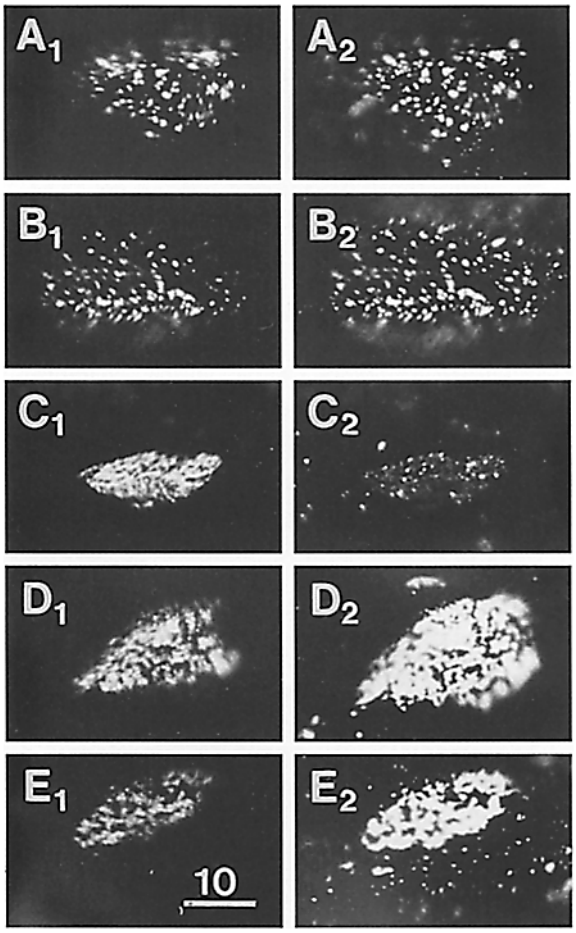

Figure 8. Effect of mAb IIH6 and other IgM mAbs on nonsynaptic AChR clusters. On the left are the AChR clusters. On the right is shown the corresponding immunofluorescence for the mAb that was included in the culture medium: 1:40 mAb IIH6 (*A* and *B*), 1:40 mAb HNK-1 (*C*), 1:40 mAb HepSS-1 (*D*), and 1:10 mAb HepSS-1 (*E*). Bar, 10 μm.

tures (see Figs. 2 *B* and Fig. 3), and at a dilution of 1:40, only 4% of the clusters (n = 200; two cultures) were judged to be compact. Instead, almost all of the clusters had a fragmented appearance. As shown in Fig. 8, A_1 and B_1, each large cluster was composed of an aggregate of small clusters (0.3–3 μm) that often occupied considerably less area than the AChR-free spaces between them. αDG was colocalized with all of the AChR-rich sites (Fig. 8, A_2 and B_2). At a dilution of 1:100, ~25% of the large nonsynaptic clusters had the same fragmented appearance, some appeared normal, and the remainder had an intermediate appearance.

Other IgM mAbs had less effect on the nonsynaptic AChR clusters, even though at the highest concentration (1:10 dilution) they appeared to retard cell development as did mAb IIH6. For example, clusters that developed in the presence of mAb HNK-1 were compact (Fig. 8 C_1), which is similar to those in untreated cultures. As noted earlier, this mAb does not stain *Xenopus* muscle cells (Fig. 8, C_2) but does stain *Xenopus* neurites brightly. On the other hand, mAb HepSS-1, directed against heparan sulfate, stains *Xenopus* muscle cells brightly and more extensively than mAb IIH6. When cultures were treated with this IgM at a dilution of 1:40, 48% of the AChR clusters (n = 100) had a compact appearance, and at a dilution of 1:10, the corresponding value was 31% (n = 100). The remainder appeared partially fragmented, but considerably less so than seen with mAb IIH6. Fig. 8, *D* and *E*, shows typical examples of this partial fragmentation as well as the corresponding HepSS-1 immunofluorescence. Whether this weak but significant effect is due to an interaction of mAb HepSS-1 with αDG or with other proteoglycans, consistent with previous data implicating glycosaminoglycans in AChR aggregation (Ferns et al., 1993; Gordon et al., 1993), remains to be determined. Taken together, the results suggest that αDG plays a role in the generation of nonsynaptic AChR clusters.

Discussion

This study has indicated that during innervation of embryonic muscle cells, the distribution of αDG is affected in the same way as AChRs (see Anderson et al., 1977; Moody-Corbett and Cohen, 1982). Locally, along the path of neurite–muscle contact, there is a neurite-induced accumulation of αDG, and globally, over the rest of the muscle cell, nonsynaptic clusters disappear because established ones disperse and new ones fail to form. Moreover, αDG is colocalized with most and perhaps all (see the following discussion) synaptic clusters and microclusters of AChRs and neural agrin, even along neurite–muscle contacts <2 h old. This close spatial-temporal accumulation of neural agrin, αDG, and AChRs during embryonic nerve–muscle synaptogenesis is consistent with the view that αDG plays a role in the initiation of synaptogenesis. The findings are thus in line with the recent demonstration that αDG binds agrin and with the evidence that the clustering of AChRs in response to diffusely applied agrin involves a corresponding clustering of αDG (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994; see also Nastuk et al., 1991). The current study also indicates that αDG is colocalized at the submicrometer level with AChRs at nonsynaptic clusters lacking agrin (Cohen and Godfrey, 1992) and that the anti-αDG mAb IIH6 affects the development of such clusters, thereby suggesting that αDG also participates in the nonsynaptic clustering of AChRs. The failure of mAb IIH6 to prevent agrin and AChR accumulation at nerve–muscle contacts may reflect a relatively low affinity of mAb IIH6, compared with *Xenopus* neural agrin, for *Xenopus* αDG and/or a relatively high local concentration of externalized neural agrin.

αDG immunofluorescence was not detected at a small percentage of AChR clusters and agrin deposits, including some 15% of the microdeposits of neural agrin along the youngest nerve–muscle contacts and some 10% of the youngest synaptic microclusters of AChRs (Fig. 6). One explanation for these results is that a small fraction of the agrin-binding sites on the surface of embryonic muscle cells are not αDG and that a small fraction of AChR clustering can occur without the participation of αDG. Alternatively some of the αDG-free microdeposits of agrin along neurite–muscle contacts may have been bound to the neurite rather than the muscle cell, since similar microdeposits of agrin are sometimes seen on growing neurites that are not in contact with muscle (Cohen and Godfrey, 1992). In addition, since mAb IIH6 is an IgM, its large size may have reduced its access to sites of neurite–muscle contact in comparison with the anti-agrin antibody and with rhodamine-conjugated α-bungarotoxin. This restricted access is probably greatest at the youngest neurite–muscle contacts, where the surface membranes are more closely apposed owing to the lack of intervening basal lamina material (Kullberg et al., 1977). Small shifts in focus between companion photographs could also have contributed to the cases in which αDG immunofluorescence was not seen at microclusters of AChRs and microdeposits of agrin. In view of these technical limitations, αDG may have been colocalized with AChRs and agrin even in those few cases in which αDG immunofluorescence was not detected. Indeed, similar analysis likewise shows a small population of synaptic AChR clusters that are free of detectable agrin immunofluorescence even though agrin is probably responsible for their formation (Cohen and Godfrey, 1992; Reist et al., 1992). Overall, our findings indicate that αDG is colocalized at the submicrometer level with most, and perhaps all, nonsynaptic and synaptic clusters of AChRs and synaptic deposits of neural agrin. This situation prevails from the onset of synapse formation and includes the smallest detectable synaptic microclusters.

Although it is an extracellular peripheral membrane protein, αDG is bound very tightly to its transmembrane complex, which in turn allows for linkages through dystrophin, or in this instance its homolog, DRP, to the actin-based cytoskeletal system (Ervasti and Campbell, 1993*a*). In fact, DRP is present at the earliest detectable synaptic clusters of AChRs (Phillips et al., 1993). Perhaps the binding of neural agrin to αDG fosters the establishment of these transmembrane linkages as well as the recruitment of AChRs and additional muscle αDG–transmembrane complex. In the case of AChRs, it has been established that those which are unclustered are mobile within the plane of the membrane (Axelrod et al., 1976), and these mobile AChRs become trapped and anchored when they enter sites of developing synaptic and nonsynaptic clusters (Anderson and Cohen, 1977; Ziskind-Conhaim et al., 1984; Kidokoro et al., 1986). Such a diffusion trap process might also account for the clustering of αDG. For example, the binding of neural agrin to αDG

might activate the establishment of a tight transmembrane link with DRP and the anchoring of the entire agrin–αDG–transmembrane complex. In turn, this anchored complex could conceivably act as a trap by establishing additional intracellular and/or extracellular linkages with mobile αDG and AChRs that encounter it. Of course, the recruitment of αDG from neighboring regions into the developing synaptic cluster would allow for additional binding of neural agrin, thereby permitting further growth of the cluster.

Such a diffusion trap model for the recruitment of AChRs and αDG does not exclude the possibility that besides binding to αDG, neural agrin may also bind with higher affinity to a less abundant receptor molecule (Sealock and Froehner, 1994; Sugiyama et al., 1994; Fallon and Hall, 1994). Sugiyama et al. (1994) found that the apparent affinity of agrin for αDG isolated by SDS-PAGE and immobilized on nitrocellulose paper does not correspond to its activity in inducing AChR clusters. This may indicate that αDG is a secondary receptor or coreceptor in the events leading to clustering. The putative higher affinity receptor might participate in triggering tyrosine phosphorylation of the AChR (Wallace et al., 1991; Wallace, 1994) and other synaptic molecules (Baker and Peng, 1993; Peng et al., 1993), thereby promoting their association with the anchored neural agrin–αDG–transmembrane complex previously discussed. Alternatively, the agrin–αDG–transmembrane complex may itself participate in triggering phosphorylation.

The described diffusion trap model might also account for the fact that neural deposits of agrin along the path of neurite–muscle contact are discontinuous, whereas they are essentially continuous along the path of neurite–substrate contact (Fig. 4; see also Cohen et al., 1994). The continuous pathways of substrate-bound neural agrin indicate that competent neurites externalize agrin along their entire path of growth. When the growth is on the surface of a muscle cell, the binding of the externalized neural agrin may be limited by the availability of αDG and the way it is recruited into developing clusters. In the diffusion trap model, as mobile αDG accumulates at developing clusters, the availability of αDG might diminish at neighboring sites along the path of neurite–muscle contact, thereby resulting in zones with few if any binding sites for the externalized neural agrin.

Unlike synaptic clusters of αDG, the nonsynaptic ones that typically develop on noninnervated muscle cells have little or no agrin associated with them and may be unoccupied with ligand (Fig. 4 *A*; see also Cohen and Godfrey, 1992). However, it is known that laminin competes with agrin for binding to αDG (Gee et al., 1994), that muscle cells produce laminin, and that diffusely applied laminin can promote AChR clustering (Vogel et al., 1983; Gee and Carbonetto, unpublished observations). Accordingly, the formation of AChR clusters at nonsynaptic sites may be triggered by the binding of laminin, rather than agrin, to αDG. Moreover, if laminin is normally bound to αDG before clustering, neural agrin may competitively displace muscle laminin from αDG during synapse formation. In any case, our results suggest that αDG participates in the formation of nonsynaptic AChR clusters. In support of this suggestion are the findings that αDG is colocalized at the submicrometer level with the AChRs of nonsynaptic clusters, that innervation has the same global effect on αDG as on AChRs (namely, an inhibition of formation of large nonsynaptic clusters), and that mAb IIH6 alters the development of the large nonsynaptic AChR clusters, causing them to be fragmented and less compact in much the same way as reported for agrin-induced AChR clusters (Campanelli et al., 1994). A much less pronounced effect was seen with an IgM mAb directed against heparan sulfate, which possibly reflects a function of other proteoglycans in AChR clustering (Anderson and Fambrough, 1983; Baynes et al., 1984; Ferns et al., 1993; Gordon et al., 1993), and no effect was seen with mAb HNK-1 (see also Sugiyama et al., 1994). Finally, αDG was precisely colocalized with AChRs at the fragmented nonsynaptic clusters. Nonsynaptic clusters also have colocalized DRP (Phillips et al., 1993). The proposed diffusion trap model for the synaptogenic accumulation of AChRs and αDG can be extended to the formation of nonsynaptic clusters of AChRs and αDG by assuming that the anchoring and trapping linkages can develop in the absence of neural agrin but may be less stable.

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