A Muscle Cell Variant Defective in Glycosaminoglycan Biosynthesis Forms Nerve-induced but Not Spontaneous Clusters of the Acetylcholine Receptor and the 43 kDa Protein

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Myotubes of the C2 mouse muscle cell line form clusters of ACh receptors (AChRs) at apparently random sites along their length when cultured alone, and near sites of nerve-muscle contact when cocultured with neurons. We find in aneural cultures that myotubes of a C2 variant, S27, which is defective in glycosaminoglycan synthesis, express the AChR on their surface, but do not form clusters. S27 cells in aneural cultures also express the 43 kDa protein but do not cluster it. The altered distribution of laminin and collagen IV on the surface of S27 myotubes suggests that the basal lamina is abnormal. Neither the addition of exogenous proteoglycans or conditioned medium from wild-type C2 cells, nor the growth of S27 cells on substrates rich in basal lamina elements caused clusters to appear on S27 myotubes in aneural cultures. When cultured with primary neurons, however, S27 myotubes formed large clusters of the AChR near sites of neurite contact. The clusters were coincident with patches of the 43 kDa protein. Prelabeling experiments indicate that at least some AChRs in the clusters arise through aggregation. Although *Torpedo* agrin induces AChR clusters on C2 myotubes, it does not do so on S27 cells. Our experiments suggest that the spontaneous formation of clusters of AChRs and the 43 kDa protein in aneural cultures of myotubes depends upon the normal synthesis of muscle proteoglycans, and that nerve-induced clustering does not. Thus, there appear to be multiple mechanisms for the formation of AChR clusters.

[Key words: muscle, mouse, proteoglycan, glycosaminoglycan, ACh receptor, 43 kDa protein, agrin]

During the development of the neuromuscular junction, ACh receptors (AChRs) on the muscle membrane become clustered near sites of nerve contact. As the junction matures, the clusters increase in size and receptor density to form the adult postsyn-

aptic membrane (Salpeter, 1987). Myotubes cultured with neurons also form clusters of AChRs near sites of neurite contact; when cultured alone they form spontaneous clusters (Fischbach and Cohen, 1973; Sytkowski et al., 1973). The formation of both aneural and nerve-induced clusters appears to involve interactions of the AChR with molecules in the extracellular matrix and the underlying cytoskeleton. The extracellular matrix protein agrin causes clustering of AChRs when added to non-innervated myotubes in culture (Godfrey et al., 1984; McMahan, 1990), and AChR clustering induced in cultured muscles by nerves is blocked by antibodies to agrin (Reist et al., 1992). Clustering of the AChR expressed in nonmuscle cells can also be induced by the coexpression of the 43 kDa protein, a peripheral membrane protein associated with the AChR on the cytoplasmic surface of the postsynaptic membrane (Froehner et al., 1990; Phillips et al., 1991). Other molecules in the extracellular matrix and cytoskeleton may also play roles in forming AChR clusters (Froehner, 1991).

Heparan sulfate proteoglycan, a component of the basal lamina, is closely associated with aggregated AChRs in chick and *Xenopus* muscles, both at the adult end-plate and in cultured, aneural myotubes (Anderson and Fambrough, 1983; Bayne et al., 1984). To investigate the role of proteoglycans in synaptogenesis at the neuromuscular junction, we have recently made genetic variants of the C2C12 mouse muscle cell line that are defective in the synthesis of sulfated proteoglycans (Gordon and Hall, 1989). One of these variants, S27, differentiates in culture to form myotubes that express AChRs and the 43 kDa protein on their surfaces. We report here that although wild-type C2 myotubes spontaneously form clusters of AChR and 43 kDa protein, S27 myotubes form spontaneous clusters of neither. The addition of soluble *Torpedo* agrin, which induces AChR cluster formation in C2 myotubes, does not promote AChR cluster formation in S27 myotubes, but large clusters of AChR and 43 kDa protein are formed when S27 cells are cocultured with primary neurons.

Materials and Methods

Muscle cultures. Culture methods for C2 and S27 cells were as described in Gordon and Hall (1989). Cells were propagated at low density in growth medium (GM) consisting of Dulbecco's Modified Eagle's Medium with 1 gm/liter (5.6 mM) glucose (DMEM) supplemented with 20% fetal bovine serum and 0.5% chick embryo extract. Cultures at about 80% confluence were made to differentiate by changing the medium to differentiation medium (DM) consisting of DMEM supplemented with 2–5% horse serum without chick embryo extract.

Cells were grown for immunocytochemical experiments by culturing them on 22 mm square coverslips that had been flamed in 200 proof ethanol. Typically, $1-4 \times 10^4$ cells were plated per coverslip in wells of a six-well plate. Cultures were fed GM on the next day, and then switched

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to DM on the following day when cultures were approximately 80% confluent. They were fed DM daily thereafter.

Nerve-muscle cocultures. Explanted and dissociated mouse spinal cord cells were cultured as described previously (Lupa and Hall, 1989; Lupa et al., 1990). Briefly, spinal cords were taken from mice between embryonic day (ED) 12 and postnatal day 7. The spinal cords were chopped with a sterile razor blade to produce 1 mm³ fragments that were either cultured as spinal cord explants or dissociated by mechanical trituration and grown as isolated cells (spinal cords from ED 12–14 mice). The spinal cord cells grew equally well in GM or DM.

Ciliary ganglia (CG) were dissected from ED 8–10 chicks and incubated for 20 min at 37°C in Ca²⁺/Mg²⁺-free PBS with 0.5 mg/ml glucose and 2.5% trypsin. Quenching of the protease was accomplished by adding an equal volume of GM, and the ganglia were spun down in a tabletop centrifuge. The resuspended tissue could then be cultured either as whole ganglia explants, or mechanically dissociated by trituration and grown as isolated cells. When culturing dissociated CG cells, a 1–2 hr preplating was usually included to reduce the number of non-neuronal cells. The CG cells survived longer if the medium was supplemented with 2.5% chick eye extract, which was prepared by homogenizing eyes from ED 18 chicks in cold PBS, centrifuging the resultant extract, and filter-sterilizing the supernatant.

Conditions for culturing spinal cord and ciliary ganglion cells with C2 and S27 myotubes were identical to those described previously (Lupa and Hall, 1989; Lupa et al., 1990). C2 or S27 myoblasts were grown as described above and neuronal cells were added either at the time of the switch to DM or after 2–3 d of fusion. The cocultures were then treated with cytosine arabinoside for 24 hr to block proliferation of dividing cells. Fresh medium was supplied every 1–2 d. 3T3 and primary Schwann cells were cocultured with S27 cells in C2 GM and DM. 3T3 cells were obtained from the University of California, San Francisco, Cell Culture Facility; Schwann cells were the generous gift of Dr. Greg Lemke (Salk Institute).

Immunological reagents. Two different antibodies against neurofilament protein (NF) and synaptic vesicle antigens were used. A monoclonal antibody (mAb) to the 160 kDa subunit of bovine NF (Shaw et al., 1984) was obtained from Boehringer-Mannheim (Indianapolis, IN). This monoclonal antibody recognizes a single 145 kDa band in immunoblots of mouse sciatic nerve (Lupa and Hall, 1989). A polyclonal antibody against chicken brain NF was acquired from Advanced Immunochemical Services. Two mAbs against the 43 kDa protein, designated 1234 and 1579 (Froehner, 1984), were provided by Dr. S. Froehner (University of North Carolina). For immunolocalization, fresh culture supernatant from hybridoma cells secreting these antibodies was mixed 1:1 and applied directly to fixed cultures of C2 and S27 myotubes that had been permeabilized with 0.1% saponin. For immunoblots, antibodies to laminin were obtained from E-Y Laboratories (San Mateo, CA); to fibronectin, from Calbiochem (San Diego, CA); to collagen IV, from Collaborative Research (Bedford, MA) and from Biotest (Kenilworth, ME); and to creatine kinase, from Ventrex Laboratories (Woburn, MA). Nonimmune serum was obtained from Cappel (Malvern, PA). Polyclonal secondary antibodies were purchased from Cappel and conjugated to aminomethylcoumarin acetic acid (AMCA) according to the method of Wessendorf et al. (1987). ¹²⁵I-labeled donkey antibodies to rabbit IgG were supplied by Amersham (Arlington Heights, IL).

Fluorescence staining. α -Bungarotoxin (α -BuTx) was conjugated with tetramethylrhodamine according to the method of Ravdin and Axelrod (1977). Acetylcholine receptors were stained in living cultures with 50 nM rhodamine-conjugated α -BuTx (rho-BuTx) for 1 hr at 37°C. After washing with PBS, the cells were fixed in 2–3% paraformaldehyde in PBS (pH 7.3) for 20 min at 4°C. For staining with antibodies to intracellular neuronal antigens, saponin (0.075%) was added to the fixation medium. Following another PBS rinse, the coverslips were incubated in 0.1 M glycine for 30 min. Preparations were then permeabilized by treatment with 0.1% saponin/PBS for 10 min, and incubated in PBS/10% fetal bovine serum/4% bovine serum albumin for 15 min to inhibit nonspecific binding of antibodies. Immunolabeling was carried out by incubating with the primary antibody for 1 hr at room temperature or overnight at 4°C, followed by washing with PBS and then a 1 hr incubation of the fluorescein isothiocyanate (FITC)- or AMCA-coupled secondary antibody. Coverslips were mounted on glass slides with a glycerol-based mountant containing *para*-phenylenediamine (Platt and Michael, 1983) and viewed with a Leitz microscope. Control incubations were done with either normal rabbit serum or affinity-purified mouse IgG at a concentration of 1 μ g/ml substituted for the first antibody. No staining above background was observed in these cases.

Intracellular and surface distribution of the AChR. The method of Gu et al. (1990) was used to compare the relative distributions of AChR between internal and surface compartments in C2 wild-type and S27 cells. Briefly, cells were plated at 1.5×10^5 per 35 mm dish, fed with GM on the following day, and switched to DM on the second day with daily feeding thereafter. After 3 d in DM, cultures were labeled with 1 ml of 10 nM ¹²⁵I- α -BuTx for 1.5 hr. Cultures were washed with ice-cold PBS and extracted in buffer containing 1% Triton X-100. Aliquots were then incubated with or without an additional 7.4 nM ¹²⁵I- α -BuTx and subsequently with Sepharose beads conjugated to mAb 88B, which recognizes the δ -subunit of the AChR (Froehner et al., 1983). After 2 hr, the Sepharose beads were washed and counted in a gamma counter. The beads from incubations without additional toxin provided measures of surface AChR, while those from incubations with additional toxin provided measures of total AChR.

Immunoprecipitation and immunoblotting. The 43 kDa protein was immunoprecipitated according to the method of Musil et al. (1988, 1989). Briefly, myotube cultures of C2 and S27 cells were grown in 60 mm dishes and labeled with 0.25 mCi/ml Tran-³⁵S label in medium containing 20 μ M cysteine and methionine for 2 hr. Cells were harvested and extracted in buffer containing 0.5% SDS and 10 mM N-ethyl maleimide (NEM). Extracts were boiled for 3 min, and DNA was removed by shearing through a 27 gauge needle followed by centrifugation. Samples were incubated with or without mAbs 1234 and 1579, which recognize the 43 kDa protein (LaRochelle and Froehner, 1987; LaRochelle et al., 1989), and then with Sepharose beads conjugated to rabbit antibodies against mouse IgG. The beads were washed extensively and then boiled in SDS sample buffer [1.75% SDS, 62.5 mM Tris (pH 6.8), 5% (w/v) glycerol, and 0.0013% (w/v) bromophenol blue]. The supernatants were diluted in sample buffer to normalize for total counts incorporated, and equal volumes were run on a 10% polyacrylamide gel. The gel was then autoradiographed, and the 43 kDa bands were quantitated on an LKB Ultrosan XL scanning densitometer. Parallel cultures were assayed for surface binding of ¹²⁵I- α -bungarotoxin and for total protein.

Immunoblotting was carried out on extracts of C2 and S27 cultures that had reached comparable levels of differentiation as assessed by visual inspection. The cultures were rinsed three times in PBS and harvested by incubation in 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA 2–5 min at 37°C to detach cells and cell-associated matrix from substrate. This material was then centrifuged at 1000 rpm for 5 min and the resultant pellet homogenized by vortexing in extraction buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM benzamide, 1 mM N-ethylmaleimide, 1 mM sodium tetrathionate, 20 μ g/ml aprotinin, 20 μ g/ml leupeptin, and 400 μ M phenylmethylsulfonyl fluoride). After incubation at 4°C for 30 min with periodic vortexing, an aliquot was removed for determination of protein concentration using a dye-binding assay (Sedmak and Grossberg, 1977). The remaining extract was added to an equal volume of 2 $\times$ SDS sample buffer, boiled, and stored at –20°C.

Protein-matched aliquots from paired C2 and S27 cultures were then reduced with 5% β -mercaptoethanol and subjected to SDS polyacrylamide gel electrophoresis, and the proteins were then electrophoretically transferred to nitrocellulose sheets (Towbin et al., 1979). The nitrocellulose sheets were blocked 2–16 hr in PBS containing 0.3% Tween 20 and 5% bovine hemoglobin (“blocking solution”), and then incubated with antisera (diluted as indicated) to collagen IV (1:250–1:500), fibronectin (1:500–1:1000), laminin, or creatine kinase, or with a nonimmune serum (all 1:500) in blocking solution and incubated for 2–4 hr at room temperature with agitation. They were then washed five or six times for 10 min with PBS containing 0.3% Tween 20. Whole donkey ¹²⁵I-labeled anti-rabbit IgG antiserum was then added to the blots diluted 1:500 in blocking solution and incubated 2 hr at room temperature with agitation. The blots were then washed five or six times for 10 min in PBS with 0.3% Tween 20, air dried, and exposed to either Kodak X-Omat or Amersham Hyperfilm-MP x-ray film at –70°C for 1–22 d.

The expression level for each antigen blotted was determined by optically scanning the major band in each lane using an Ultrosan XL Laser Densitometer (Pharmacia-LKB Biotechnology, Piscataway, NJ), and subtracting the background for each lane from the maximum absorbance (where the background was defined as the mean of the first 16 data points). Ratios were calculated only from lanes that met the following criteria: (1) the maximum absorbance fell within the range over which the response of the scanning system was linear (0–3.0 absorbance units), (2) the maximum absorbance was greater than 1.75 times the background, and (3) the maximum absorbance was greater than 10 times

Figure 1. AChR distribution on the surface of C2C12 wild-type (*A*) and S27 myotubes (*B*) in culture. Receptors were visualized by incubating unfixed cultures with rho-BuTx. Receptor clustering is frequent on C2 myotubes and nonexistent on S27 myotubes. All photographic procedures used were identical for *A* and *B*. Scale bar, 100 μ m.

the difference between the background absorbance and the minimum absorbance. For scans meeting these criteria, the maximum absorbance minus the background for each lane was determined and the ratio of this figure from lanes containing a C2 extract to the figure from the paired S27 extract was determined. The mean and standard error of these ratios was then calculated. In control experiments in which increasing amounts of a C2 myotube extract were immunoblotted with antibodies to laminin, the expression level of laminin increased linearly with the amount of total protein loaded when assessed by the method described above.

Materials. Ascorbic acid, calcitonin gene-related peptide, heparin, heparan sulfate (from bovine kidney), chondroitin sulfate A (from bovine trachea), chondroitin sulfate C (from shark cartilage), dermatan sulfate (from porcine skin), and hyaluronic acid (from human umbilical cord) were obtained from Sigma Chemical Co. (St. Louis, MO). Matrigel was obtained from Collaborative Research (Bedford, MA). Bovine corneal endothelial matrix was generously provided by Dennis Fujii (Sterling Research Group, Malvern, PA). ARIA was a gift of Douglas Falls and Gerald Fischbach (Harvard University), and partially purified agrin was given to us by Bruce Wallace and U. J. McMahan (Stanford University).

Results

S27 cells fail to cluster AChRs

Cells of the S27 variant of the C2C12 muscle cell line, a variant that is defective in proteoglycan biosynthesis, differentiate and fuse to form large myotubes that express creatine phosphokinase and AChRs (Gordon and Hall, 1989). Although S27 myotubes express AChRs on their surface, they do not form spontaneous receptor clusters (Fig. 1). When C2 and S27 cells cultured in DM for 2–4 d were compared after staining with rho-BuTx, the C2 cultures contained many AChR clusters, but none were seen in S27 cultures. In a typical experiment, in randomly chosen fields viewed in a Zeiss 63 $\times$ lens, there were 5.0 ± 1.0 clusters per field in C2 cultures (mean $\pm$ SEM) and none in parallel S27

cultures. Very rarely, AChR clusters were seen in cultures of S27 cells at long times (>5 d) after fusion. We have previously reported that the amount of AChR on the surface of S27 cells is approximately the same as that of wild-type cells (Gordon and Hall, 1989). To determine if AChRs are accumulated in an intracellular compartment of S27 cells, we determined the relative distribution of AChRs in surface and internal compartments (see Materials and Methods). The values obtained for the two cell types were similar. In C2 cultures, 86% of the AChRs were on the surface; the corresponding value for S27 cells was 81%.

Because S27 cells are defective in the production of sulfated proteoglycans, we attempted to induce AChR clusters by providing them with exogenous glycosaminoglycans (GAGs) or proteoglycans. Cells were grown either on precoated substrates or in medium containing commercially available GAGs or proteoglycans (heparan sulfate, chondroitin sulfates A and C, dermatan sulfate, hyaluronic acid). In no case were AChR clusters found on S27 myotubes. Similarly, conditioned medium from cultures of wild-type C2 cells failed to induce AChR clusters on S27 myotubes. More complex or organized extracellular matrix components such as Matrigel, a matrix produced by EHS cells, or the extracellular matrix secreted by bovine corneal endothelial cells were also not effective in stimulating S27 cells to form AChR clusters, nor was addition of ARIA (Usdin and Fischbach, 1986), ascorbic acid (Knaack and Podleski, 1985), or rat calcitonin gene-related peptide (Fontaine et al., 1986; New and Mudge, 1986), agents that have been reported to increase the number of AChRs expressed by myotubes.

We also attempted to mimic the genetic lesion in proteoglycan biosynthesis by interfering with the biosynthesis of GAGs in wild-type C2 cells. C2 cells grown in medium containing either chlorate (20 mM) or *p*-nitrophenyl- β -xyloside (1 mM), which interfere with GAG chain sulfation (Humphries and Silbert, 1988) or initiation (Robinson and Sindahl, 1981), respectively, fused into multinucleated myotubes and clustered AChRs in a manner that was indistinguishable from the control C2 cells. In parallel cultures, 20 mM chlorate reduced $^{35}\text{SO}_4$ incorporation into GAGs to 1% of control values, and 1 mM *p*-nitrophenyl- β -xyloside caused a decrease in the proportion of cell-associated GAGs and a corresponding increase in soluble GAGs (Gordon and Hall, 1989).

The 43 kDa protein in S27 myotubes

We examined S27 cultures for the presence of the 43 kDa protein, a peripheral membrane protein closely associated with AChR clusters on the cytoplasmic side of the membrane. This protein is of particular interest, as recent experiments have shown that coexpression of the 43 kDa protein and the AChR can cause AChRs in nonmuscle cells to cluster (Froehner et al., 1990; Phillips et al., 1991). Immunoprecipitation experiments in metabolically labeled cells showed that the amount of the 43 kDa protein in S27 cells was comparable to that seen in C2 cells (Fig. 2). When normalized to the amount of protein in each culture, the ratio of 43 kDa protein in S27 cells to that in C2 cells was 1.4 while the ratio of surface AChR in parallel cultures was 0.86. The pattern of immunofluorescence of the 43 kDa protein in C2 and S27 cells, however, differed dramatically. In cultures of C2 myotubes, patches of 43 kDa staining were frequently observed (Fig. 3*A,B*), always in conjunction with AChR clusters, as previously reported (LaRoche et al., 1989). Patches of 43 kDa protein were not seen in S27 cells (Fig. 3*C,D*); rather, a

Figure 2. Immunoprecipitation of 43 kDa protein from cultures of differentiated C2 (lane 2) and S27 (lane 4) cells. Extracts of cultures labeled with ^{35}S -labeled amino acids were immunoprecipitated as described in Materials and Methods, and aliquots, normalized for total incorporation of label into protein, were analyzed by PAGE. Lanes 1 and 3 are controls in which the primary antibodies were left out. The prominent upper band in all lanes corresponds in molecular weight to actin.

diffuse distribution of both AChR and 43 kDa staining evenly covered the myotube surface.

Basal lamina components show reduced staining at the surface of S27 cells

We investigated whether S27 cells had normal amounts of two extracellular matrix components that are often associated with AChR clusters: laminin and collagen IV. When myotube cultures were extracted and immunoblotted with specific antisera for laminin or collagen IV, the amounts of each in S27 cells were similar to, or larger than, the amounts found in C2 cells (Figs. 4, 5). When the distribution of laminin was examined by immunofluorescence, however, an altered pattern of staining was seen in S27 cultures. In both C2 and S27 cultures, antibodies to laminin showed diffuse, filamentous staining to matrix material around and between the cells (Fig. 6) similar to that reported by Kuhl et al. (1982, 1984) on chick myotubes in culture. In addition, C2 myotubes showed punctate staining on the surface of the cells. Often the cell surface was speckled with small patches of stained material, and large dense patches were seen associated with clusters of AChRs (Fig. 6B). In S27 cultures, in contrast, the myotube surface was almost completely devoid of punctate staining; neither small nor large patches of laminin

were seen (Fig. 6C,D). Similar differences between the distribution of collagen IV on C2 and S27 cells were seen (not shown). In the case of both collagen IV and laminin, there was a lack of material associated with the cell surface, such that the myotubes appeared almost completely dark after immunostaining (Fig. 6D). These results show that although S27 myotubes synthesize components of the extracellular matrix other than proteoglycans, and release them into the medium, they are not retained at the cell surface.

Coculture of nerves with S27 cells induces AChR clusters

When C2 cells are cultured with explants of mouse spinal cord or chick CG, many AChR clusters are observed near sites of neurite contact (Lupa et al., 1989; also see Fig. 8). The nerve-induced AChR clusters (NARCs) are usually associated with extracellular matrix and cytoskeletal proteins normally found at adult neuromuscular junctions (Lupa et al., 1989). We found that both mouse spinal cord and chick CG cells were also able to induce AChR clusters in S27 myotubes (Fig. 7). These clusters often outlined the course of the neurite for tens of micrometers along the myotube surface. In both C2 and S27 cultures, clusters were not always confined to the site of nerve-muscle contact, but could be detected up to 100 μm away. This was particularly true in cultures with nerve explants in which many clusters often ringed the vicinity of the explant. The nerve-induced clusters found in both C2 and S27 cells were always associated with clusters of 43 kDa protein (Fig. 8) and often with clusters of laminin (not shown).

The nerve explants added to the muscle cultures contained Schwann cells and fibroblasts in addition to neurons. To determine if these cells were capable of inducing AChR clusters, S27 cells were cocultured with primary cultures of mouse fibroblasts, with cells of the 3T3 fibroblast cell line, and with Schwann cells derived from sciatic nerve. In none of these experiments were clusters seen. Thus, the clustering of AChRs in the cocultures must be mediated by neurons.

To see if neurons caused AChRs already in the membrane to be recruited into the clusters, cultures of S27 cells were labeled with a saturating concentration of rho-BuTx prior to addition

Figure 3. AChRs and the 43 kDa protein in C2 and S27 myotube cultures. AChRs were visualized with rho-BuTx (A, C) and the 43 kDa protein by immunolocalization (B, D) in C2 wild-type (A, B) and S27 (C, D) myotubes, as described in Materials and Methods. In C2 myotubes, the AChR and 43 kDa protein are clustered, but in S27 myotubes they are not. Photographic processing was identical for A and C, and also for B and D. Scale bar, 50 μm .

Figure 4. Protein-matched extracts of C2 and S27 myotubes immunoblotted with antibodies against laminin (LN), fibronectin (FN), collagen IV (C IV), creatine kinase (CPK), or a nonimmune rabbit antiserum (NI). Lanes C show extracts from C2 myotubes; lanes S show extracts from S27 myotubes. The arrows mark 200 kDa on the immunoblots for laminin, fibronectin, and collagen, 45 kDa for the CPK immunoblot, and 200 kDa (top arrow) or 45 kDa (bottom arrow) for the nonimmune serum blot. Immunoblots were performed as described in Materials and Methods.

of nerve cells. After 18–72 hr, the cultures were labeled with FITC-BuTx, fixed, and stained with an antibody to label the neurons (see Materials and Methods). As shown in Figure 9, NARCs viewed at early times of coculture (18–36 hr) stained brightly with rho-BuTx, but staining with FITC-BuTx was barely visible (not shown). Thus, the clusters must be largely formed by the lateral migration of AChRs that were present in the membrane before addition of neurons.

S27 cells do not cluster AChRs in response to Torpedo agrin

The ability of the nerves to cause aggregation of AChRs in S27 cells suggested that the clusters might arise through the action

of agrin (McMahan, 1990). When purified *Torpedo* agrin was added to C2 myotubes, AChR clusters were induced with a dose-response curve (not shown) that was similar to that seen with chick myotubes. Incubation of cultures with 10 U/ml agrin (Godfrey et al., 1984) for 16 hr resulted in an approximately twofold increase in the number of AChR clusters seen in random fields. In contrast, *Torpedo* agrin was completely ineffective in inducing clusters in S27 cultures (Fig. 10).

The effect of nerve cells on proteoglycan synthesis in muscle cells

The induction by neurons of AChR clusters on the proteoglycan-deficient variant S27 could occur because the neurons induce an increase in the synthesis of sulfated proteoglycans in the muscle cells. Coculture of S27 cells with neurons, however, caused only a small increase in the incorporation of ³⁵S-sulfate into proteoglycan that could probably be attributed to incorporation into the neurons themselves (Table 1). Although a local effect on proteoglycan synthesis is difficult to exclude, the nerves do not appear to affect the overall synthesis of proteoglycans by the cultured S27 cells.

Neurons could also induce AChR clusters on S27 myotubes by themselves depositing a proteoglycan locally. To investigate this possibility, we used chick-specific antibodies to laminin and to a heparan sulfate proteoglycan that is concentrated at adult neuromuscular junctions (Anderson and Fambrough, 1983). In cocultures of chick CG neurons and S27 myotubes, these antibodies should recognize only the proteins secreted by the chick neurons. Although antibodies to both proteins stained primary cultures of chick myotubes, we were unable to detect staining in cultures of chick neurons with S27 myotubes. These results are consistent with those reported by Swenarchuk et al. (1990). Furthermore, coculture in medium containing 1 mM *p*-nitrophenyl- β -xyloside or 20 mM Na chlorate had no significant effect on the induction of NARCs. The percentage of neurites inducing NARCs on C2 cells, measured as described in Lupa et al. (1990), was $53 \pm 13\%$ in the absence of these agents, and $43 \pm 7\%$ (*p*

Figure 5. Expression levels of several basement membrane proteins and of creatine kinase (CPK) in C2 and S27 myotubes. Immunoblots were performed on C2 and S27 myotube extracts and the results were quantitated by scanning densitometry as previously described. The solid bars indicate the level of expression per unit of total cellular protein for each antigen in C2 as a fraction of its expression level in S27. Error bars represent SEM.

Figure 6. Laminin and AChR distribution in C2 and S27 myotube cultures: visualization of AChR with rho-BuTx (*A, C*) and immunolocalization of laminin (*B, D*) in differentiated cultures of C2C12 wild-type (*A, B*) and S27 (*C, D*) myotubes. Laminin shows two different distributions in wild-type cultures (*B*): a fibrous extracellular distribution and a punctate distribution on the surface of myotubes. The outlines of the myotubes can be seen in *A* as the surface on which receptor is expressed. Comparison of *C* and *D* shows that the cell is devoid of punctate staining. Most of the staining is of fibrous, extracellular structures. Photographic processing was identical for *A* and *C*, and also for *B* and *D*. Scale bar, 100 μ m.

> 0.2) in their presence; the corresponding figures for S27 co-cultures were $31 \pm 4\%$ and $26 \pm 7\%$ ($p > 0.5$).

Discussion

The S27 variant was isolated after mutagenesis of the parental C2 line by a screen that detected reduced incorporation of ^{35}S -sulfate into GAGs and proteoglycans (Gordon and Hall, 1989). The incorporation of both ^{35}S -sulfate and ^3H -glucosamine into proteoglycans in S27 cells was less than 25% of that seen with wild-type cells, suggesting that synthesis of the carbohydrate backbone of the GAG chain is defective; no evidence was found that a deficit in core protein or GAG chain initiation was responsible for the defect (Gordon and Hall, 1989). More recent experiments have provided direct evidence that the GAG chains from S27 cells are shorter than those of wild-type cells; both heparan sulfate and chondroitin sulfate proteoglycans appear to be affected (Bowen et al., unpublished observations).

S27 cells differentiate to form myotubes and express normal or slightly reduced levels of surface AChR, creatine phosphokinase and acetylcholinesterase (Gordon and Hall, 1989; Inestrosa and Hall, unpublished observations), all of which are markers for muscle cell differentiation. In addition, we show here that S27 cells make approximately normal amounts of the basal lamina components laminin and collagen IV and of the 43 kDa protein. The results are consistent with the idea that the primary biochemical defect in S27 cells is in the synthesis of GAGs.

The most striking phenotype of the S27 cells is that they do not form spontaneous AChR clusters. Although AChR clusters are frequent in C2 wild-type cultures, they were virtually never seen in cultures of S27 cells (Fig. 1). Froehner et al. (1990) and Phillips et al. (1991) have found that AChRs expressed in *Xenopus* oocytes or in a quail fibroblast cell line are not clustered unless the 43 kDa protein is also expressed. When the 43 kDa protein is expressed alone in these cells, it also forms clusters. In S27 cells, in contrast, both the 43 kDa protein and the AChR are present, but neither is clustered. The lack of 43 kDa clusters

is reminiscent of previous findings from our laboratory in which we found that the 43 kDa protein fails to cluster in variant muscle cells in which AChR expression is drastically reduced (LaRochelle et al., 1989). The failure of spontaneous clusters of the 43 kDa protein and of the AChR to form in S27 muscle cells suggests that required cofactors for clustering that are present in *Xenopus* oocytes or quail fibroblasts are missing in S27 cells, or, alternatively, that clustering can be actively inhibited in muscle cells by factors not present in nonmuscle cells.

The failure of spontaneous AChR clustering in S27 cells could be related, either directly or indirectly, to their inability to produce normal GAGs. Several previous lines of experiments have suggested that proteoglycans may be involved in AChR clustering. Proteoglycans are associated with AChR clusters in both chick and *Xenopus* myotubes from the time of their earliest appearance (Anderson and Fambrough, 1983; Bayne et al., 1984); moreover, heparin and heparan sulfate partially inhibited the nerve-induced formation of clusters on *Xenopus* myocytes, suggesting that similar endogenous molecules might participate in AChR cluster formation (Hirano and Kidokoro, 1989). To test the idea that proteoglycans are involved in AChR cluster for-

Table 1. Incorporation of $^{35}\text{SO}_4$ into GAGs of C2 and S27 cultures

Culture	10^4 cpm per 35 mm dish
C2	
Without neurons	43.4 ± 2.7
With neurons	40.2 ± 1.1
S27	
Without neurons	5.28 ± 0.12
With neurons	6.84 ± 0.48
Neurons alone on Matrigel	3.24 ± 0.90

Cells were cultured in $^{35}\text{SO}_4$ and the GAGs precipitated from cell extracts as described in Gordon and Hall (1989). Mouse spinal cord neurons were cultured on Matrigel as described in Materials and Methods. Each value represents the mean $\pm$ the SEM of three cultures, with two determinations from each.

Figure 7. Neurite-associated AChR clusters on S27 myotubes. A chick CG neurite labeled with a polyclonal antibody against NF (*A*) is associated with an AChR cluster (*B*). Scale bar, 20 μ m.

mation, we have tried to inhibit the formation of AChR clusters in wild-type C2 cells using exogenous GAGs or agents that cause the synthesis of modified GAGs. Although these experiments were unsuccessful, they do not rule out a possible role for proteoglycans, as the precise effects of the agents on the extent and pattern of GAG formation are unknown. We were also unable to stimulate clustering in S27 cells by adding conditioned medium from C2 cultures, or by growing them on extracellular matrices from various sources. Thus, in our experiments, we have been unable to establish a direct link between the defect in GAG synthesis and the defect in AChR clustering. In other experiments, we have shown that when quail muscle cells are allowed to fuse with S27 cells, the hybrid myotubes that are formed have AChR clusters (Gordon et al., 1992). The clusters are invariably near the quail nuclei and are usually associated with proteoglycan produced by the quail nuclei. These results are consistent with a model in which proteoglycan pro-

duced by the quail nuclei is locally deposited and is associated with basal lamina elements that mediate receptor clustering.

If proteoglycans do play a role in AChR clustering, one possibility is that they stimulate clustering directly. Another possibility is that they anchor other components of the basal lamina that are responsible for clustering. An attractive idea, for example, is that they help mediate the effects of agrin on muscle cells. Their role in clustering could be analogous to the suggested role of proteoglycans in the action of two growth factors, basic fibroblast growth factor (Rapraeger et al., 1991; Yayon et al., 1991) and transforming growth factor- β (Lopez-Casillas et al., 1991; Wang et al., 1991). In each case, the proteoglycan, a component of the extracellular matrix, forms a low-affinity component of the receptor system that concentrates the growth factor or assists its binding by the high-affinity receptor. Nastuk et al. (1991) have recently described a high-affinity receptor for agrin that appears to be a membrane-bound protein.

Agrin is a basal lamina protein that was originally purified from *Torpedo* electric organs on the basis of its ability to increase the number of AChR clusters in chick myotubes (Godfrey et al., 1984). The protein, which has recently been cloned (Rupp et al., 1991; Tsim et al., 1992), is a large protein with multiple protease inhibitor and epidermal growth factor-like domains. Because agrin causes AChR clusters to appear within several hours of application even in the absence of protein synthesis (Godfrey et al., 1984), the clusters are thought to arise by aggregation of preexisting AChRs in the muscle cell membrane. The AChR clusters are associated with heparan sulfate proteoglycan, and the action of agrin on chick myotubes can be inhibited by exogenous GAGs (Wallace, 1990).

Our experiments show that *Torpedo* agrin also increases the number of AChR clusters in C2 myotube cultures (Fig. 9); *Torpedo* agrin fails to induce AChR clustering, however, in cultures of S27 cells. Preliminary experiments suggest that this failure may arise from the inability of S27 myotubes to bind *Torpedo* agrin (H. Gordon, Z. Hall, and J. Fallon, unpublished observations). The S27 cell line is not unresponsive to all forms of agrin, however. In the rat, several forms of agrin are generated by RNA splicing; four of these differ in the presence or absence of two short contiguous segments, one of 8 amino acids and one of 11 amino acids (Rupp et al., 1992). Although all of the splice variants are active on C2 cells, only those containing the 8 amino acid insert are active on S27 cells. These results suggest either that the proteoglycan promotes the activity of only certain forms of agrin or that some forms are so potent that they can act even in the absence of proteoglycan. These results leave unexplained, however, the failure of purified *Torpedo* agrin to act on S27 cells. Although the inserts that the purified *Torpedo* agrin contains are not known, the preparation resembles the rat agrin form containing the 8 amino acid insert in being active on chick myotubes (Ferns et al., 1992).

Although S27 myotubes are unable to support the spontaneous clustering of AChRs or the 43 kDa protein, clusters of both were readily formed when the myotubes were cocultured with mouse spinal cord neurons or with chick CG cells. The effect appears to be specific for neurons, as cultures of Schwann cells or fibroblasts, the two other major cell types in spinal cord explants, were inactive when cultured alone with S27 myotubes. How the neurons induce the formation of clusters is unclear. They do not increase the overall synthesis of proteoglycans by the muscle, nor were we able to obtain evidence for local secretion of proteoglycan by the nerve. One possibility is that the

C2

S27

Figure 8. Neurite-associated AChR clusters colocalize with clusters of 43 kDa protein on C2C12 wild-type (*left*) and S27 (*right*) myotubes. Nerve muscle cocultures were immunostained to reveal NFs, AChRs, and 43 kDa protein as described in Materials and Methods. Neurites from a mouse spinal cord explant (*left*) or a chick CG neurite (*right*) were immunostained with antibodies to NF followed by AMCA-conjugated secondary antibodies (*top*). In both cell types, the nerve-associated AChR clusters stained with rho-BuTx (*middle*) colocalize precisely with 43 kDa protein (*bottom*) as revealed by immunostaining with anti-43 kDa primary antibodies and fluorescein-conjugated secondary antibodies. Scale bar, 15 μ m.

Figure 10. *Torpedo* agrin increases the frequency of AChR clusters on chick primary, mouse primary, and C2C12 wild-type myotubes but has no effect on the absence of clustering on S27 myotubes. Fields for primary chicken and primary mouse muscle cultures were observed through a Zeiss 25 $\times$ lens, and fields for C2 and S27 cultures were observed through a Zeiss 63 $\times$ lens. Hatched bars represent control without agrin. Solid bars represent 10 U/ml agrin.

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Figure 9. Neurite-associated AChR clusters on S27 myotubes are formed by lateral diffusion and trapping of receptors present in the membrane. Receptors were labeled with a saturating concentration of rho-BuTx prior to addition of chick CG neurons to the culture. *Top*, A chick CG neurite immunostained with an antibody to NF, followed by AMCA-conjugated secondary antibodies. *Bottom*, AChRs stained with rho-BuTx on the cell surface prior to addition of neurons are clustered at sites of neurite-myotube contact. Scale bar, 30 μ m.

nerves secrete a form of agrin whose activity does not depend on proteoglycan synthesis by the muscle. Alternatively, agrin may be secreted as part of a preformed complex with proteoglycans or other molecules that we do not detect. In any case, a strong implication of our work is that the mechanisms by which spontaneous and NARCs are formed are different.

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