# Nerve-induced Remodeling of Muscle Basal Lamina during Synaptogenesis

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*Abstract.* To identify mechanisms that regulate the deposition of the junctional basal lamina during synaptogenesis, immunocytochemical experiments were carried out on cultured nerve and muscle cells derived from *Xenopus laevis* embryos. In some experiments successive observations were made on individual muscle cells after pulse-labeling with a fluorescent monoclonal antibody specific for a basal lamina proteoglycan. In others, old and new proteoglycan molecules were differentially labeled with antibody conjugated to contrasting fluorochromes.

These observations revealed that surface deposits of antibody-labeled proteoglycan remain morphologically stable for several days on developing muscle cells. Over the same period, however, new sites of proteoglycan accumulation formed that contained primarily those antigenic sites recently exposed at the cell surface. When muscle cells became innervated by cholinergic neurites, new proteoglycan accumulations were induced at the developing neuromuscular junctions, and these too were composed almost exclusively of recently deposited antigen.

In older muscle cultures, where many cells possessed relatively high background concentrations of antigen over their surfaces, developing neuromuscular junctions initially showed a markedly reduced proteoglycan site-density compared with the adjacent, extrajunctional muscle surface. Much of this perineural region eventually became filled with dense, nerve induced proteoglycan plaques at later stages of synapse development. Motoneurons thus appear to have two, superficially paradoxical effects on muscle basal lamina organization. They first cause the removal of any existing, extrajunctional proteoglycan from the path of cell contact, and then induce the deposition of dense plaques of recently synthesized proteoglycan within the developing junctional basal lamina. This observation suggests that the proteolytic enzyme systems that have already been implicated in tissue remodeling may also contribute to the inductive interaction between nerve and muscle cells during synaptogenesis.

D URING embryonic development, early cell proliferation is eventually followed by a period of extensive differentiation that involves both the biosynthesis of specialized gene products, and their assembly into the variety of specialized organelles that characterize different cell lineages. This final process is expressed with particular extravagance during the development of the nervous system, when growing neurons establish elaborate networks and form synaptic connections with appropriate target cells. At present, however, we know very little about the molecular basis of the cellular interactions that regulate either nerve growth or the localized differentiation that occurs when developing neurons eventually make contact with their targets. The vertebrate neuromuscular junction, by virtue of its size and abundance, has long been a useful source of information about the structure and function of chemical synapses, and may also serve as a useful model for understanding the mechanisms that regulate synapse development.

The neuromuscular junction is a region of morphological and chemical specialization for both nerve and muscle cells, and is known to include components of direct relevance for synaptic transmission, such as a high concentration of acetylcholine receptors (AChRs)[1] and acetylcholinesterase (AChE) (21, 24, 41), as well as other substances whose physiological roles remain uncertain. These latter substances include antigens unique to synaptic vesicles (14, 18, 32, 60), postsynaptic elements of the muscle cytoskeleton (13, 15, 28, 31), and a variety of components concentrated in the junctional basal lamina (5, 23, 56). While it is clear that the development of this organelle must depend upon a local exchange of regulatory signals during synaptogenesis, little is known about the molecular basis of neuron–target interaction, or of the cellular processes responsible for the orderly assembly of synaptic components. It has been shown, however, that growing cholinergic neurons cause a rearrangement of mobile AChRs within the sarcolemma, leading to their aggregation within the developing postsynaptic membrane, and to the disappear-

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[1] *Abbreviations used in this paper:* AChE, acetylcholinesterase; AChR, acetylcholine receptor; αBGT, α-bungarotoxin; DME, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; HSPG, heparan sulfate proteoglycan; TRITC, tetramethylrhodamine isothiocyanate.

ance of any existing aggregates of extrajunctional AChRs (3, 4, 7, 26). Likewise, motor neurites also induce the accumulation of both AChE (53) and a heparan sulfate proteoglycan (HSPG) within a specialized, junctional basal lamina (5, 7). This inductive process appears to be dependent upon some action that is unique to cholinergic neurons (20, 35), but independent of the relatively well-understood electrical and contractile events associated with synaptic activity (3, 4, 7).

In an effort to understand how the motoneuron induces the accumulation of synaptic components such as the AChR, several reports have described the preparation of soluble and particulate factors which can increase the number of AChR aggregates on embryonic muscle cells in culture (19, 23, 30, 33, 34, 40, 50, 55, 65), and suggested that the accumulation of junctional AChR might be induced by specific, AChR aggregation-promoting factors released by the motor neurite. Also important has been the discovery that the development of "junctional" AChR aggregates in regenerating adult muscle can be induced by contact with the original, synaptic basal lamina (16). In fact, AChR aggregates consistently appear to be associated with congruent, localized specializations of muscle basal lamina that are characterized by the presence of a high concentration of an HSPG (5, 7, 10) as well as other antigens (10, 22, 42, 54, 56). Furthermore, both junctional AChR aggregates (13, 15, 28, 31), and those that occur at ectopic sites on non-innervated muscle (12, 17, 48), appear also to be associated with corresponding morphological and biochemical specializations of the sarcolemma and adjacent cytoskeleton. Taken together, these observations suggest that the accumulation of AChR at the developing neuromuscular junction may be dependent upon a nerve-induced assembly of a transmembrane complex of structural proteins that includes basal lamina constituents such as HSPG, as well as elements of the muscle cytoskeleton.

It should be stressed that during development of the neuromuscular junction in cell culture, where the time-course of the relevant physiological events can be observed directly, the accumulation of AChR in the postsynaptic membrane is coupled to the disappearance of extrajunctional AChR aggregates, actually leading to a net decrease in the number of AChR clusters on many cells (see references 3, 4, and 7). Furthermore, extrajunctional AChR clusters can develop spontaneously on denervated muscle cells in vivo (37) as well as in culture (3, 4, 25, 61), and can also be induced by factors not obviously related to synapse development (46, 47). It is thus possible that a variety of exogenous agents can either focally perturb the organization of the transmembrane complex with which AChR aggregates are associated, or change the biosynthesis and degradation rates of its constituent structural proteins, indirectly affecting the number and distribution of AChR clusters. These actions need not be representative of the physiological mechanisms through which motoneurons regulate AChR organization during synapse development. It is therefore important to directly examine the mechanisms through which innervation regulates the assembly of the junctional basal lamina, since this organelle (a) forms part of the transmembrane complex that accompanies AChR aggregates, and (b) can itself induce synaptic differentiation in both nerve and muscle.

This study examines the development of the junctional basal lamina in a number of immunocytochemical experiments, using a monoclonal antibody specific for an HSPG

that is concentrated both in the specialized junctional basal lamina, and at other sites often associated with extrajunctional AChR clusters (5). It has already been shown that cholinergic motoneurons induce the deposition of this HSPG in a number of dense postsynaptic plaques that remain virtually congruent with the developing junctional aggregates of AChRs (5, 7). During synaptogenesis, AChRs dissociate from stable extrajunctional deposits containing this HSPG, and aggregate adjacent to the new, organized junctional basal lamina developing at the site of nerve–muscle contact (7).

These experiments have identified two distinct mechanisms that contribute to changes in proteoglycan distribution during synapse development. Existing HSPG deposits on the muscle cell surface have been found to remain morphologically stable for extended periods. Over this time, new accumulations of HSPG form both spontaneously and in response to innervation, primarily through localized increases in the rate of deposition at the cell surface of recently synthesized HSPG molecules. Furthermore, the deposition of newly synthesized HSPG at the developing neuromuscular junction appears to be preceded by a nerve-induced elimination of pre-existing surface HSPG along the path of nerve–muscle contact. These observations indicate that the developing cholinergic nerve causes the replacement of extrajunctional basal lamina with a junctional lamina containing a high HSPG concentration, a process that has interesting implications for an understanding of the mechanisms that regulate synaptic differentiation.

## Materials and Methods

### Preparation of Cultures

Procedures for the preparation of primary cultures of *Xenopus laevis* myotomal muscle cells, with or without neural tube cells from the same embryos, have already been described in detail (4, 7, 35). Briefly, the myotomal musculature and neural tubes of stage 24–26 (44) embryos were isolated by sterile dissection in the presence of 1 mg/ml collagenase (type I, Worthington Biochemical Corp., Freehold, NJ). Muscle was dissociated in trypsin-EDTA (GIBCO Laboratories, Grand Island, NY) and plated onto collagen-coated glass coverslips in 60% (vol/vol) Hepes-buffered Dulbecco's modified Eagle's medium (DME) (GIBCO Laboratories) supplemented with 0.5–5% (vol/vol) fetal bovine serum. After 1–3 d at room temperature to allow cell attachment and spreading, the cultures were maintained at 10°C for periods up to 6 wk before use.

Neural tubes (see above) were dissociated in trypsin-EDTA, and added to established muscle cultures in medium (see above) supplemented with 10 μM d-tubocurarine (Sigma Chemical Co., St. Louis, MO) to prevent innervated cells from contracting, and thereby retracting from the substratum. Nerve growth was most prolific for the next 2 d (at 22–24°C). Thereafter, some nerve growth continued, but many of the original neurites gradually retracted or degenerated.

### Preparation of Fluorescent Antibody and Toxin Probes

α-Bungarotoxin (αBGT) was isolated from the lyophilized venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, FL) and conjugated with the fluorescent dye, tetramethylrhodamine isothiocyanate (TRITC, Research Organics Inc., Cleveland, OH) as described previously (2), and the singly labeled dye-toxin derivative was isolated by ion-exchange chromatography (51). Fluorescent toxin was later diluted to 10 μg/ml in DME supplemented with 5% (vol/vol) fetal bovine serum, and sterilized by passage through 0.2-μm pore fluorocarbon filters (Millipore/Continental Water Systems, Bedford, MA).

The preparation and characterization of monoclonal antibody (2AC2), specific for a frog basal lamina HSPG, has been described previously (5). Procedures for the preparation of fluorescent antibody conjugates have also been described in detail (5). Solutions of fluorescent antibody (5 μg/ml) were prepared in DME containing 5% (vol/vol) fetal bovine serum, and sterilized by filtration through 0.2-μm pore Millipore membranes (see above).

## Fluorescent Staining of Cultured Muscle Cells

Cultures were routinely stained by incubation with fluorescent toxin (10 μg/ml) for 45 min at 22–24°C, and with fluorescent antibody (5 μg/ml) for 60 min. In experiments where old and new proteoglycan molecules were to be differentially stained with contrasting fluorochromes, antibody labeled with fluorescein isothiocyanate (FITC) (Molecular Probes Inc., Junction City, OR) was used in the initial exposure. This was followed by extensive rinsing in culture medium to achieve at least a billion-fold dilution of the fluorescent antibody over a period of 30–45 min. The original FITC–antibody stain was amplified 2–3 d later by a further exposure to FITC-labeled goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) that had been diluted 1:100 (vol/vol) with culture medium, and filtered through 0.2-μm pore Millipore membranes. After rinsing, residual anti-mouse IgG binding sites were quenched by another, similar exposure to 10 μg/ml FITC-labeled mouse IgG from the MOPC-21 myeloma. New proteoglycan sites were then stained by exposure of the cultures to TRITC-labeled antibody (see above), and the cultures were mounted in 60% (vol/vol) L15 medium (GIBCO Laboratories) containing 0.5% (vol/vol) fetal bovine serum. This latter precaution was essential because of fluorescent (in FITC optics) components present in DME.

## Fluorescence Microscopy

Fluorescently stained, living cultures were examined on a Zeiss WL microscope equipped for incident illumination with narrow-band filter combinations selective for FITC and TRITC, as described previously (5, 7). Micrographs were prepared on Ilford HP5 film developed for 18 min at 20°C in Ilford Microphen developer. Exposure times for fluorescence micrographs ranged from 15–30 s.

In some experiments individual, stained muscle cells were examined at intervals of 2–3 d to determine whether reorganization of the antibody-labeled proteoglycan had occurred. Such cells were relocated on the basis of microscope stage coordinates and sketches of the adjacent muscle cells.

## Video Densitometry of Fluorescence Micrographs

To estimate the local variation in antigen site density on the muscle cell surface more objectively, some fluorescence micrographs were analyzed by digital video densitometry. For this analysis, the original negative images of fluorescence micrographs were illuminated by diffuse light, and recorded by a Bosch video camera. The video signal was then digitized and stored in the frame memory of an IBAS interactive image analysis computer (Carl Zeiss Canada Ltd., Toronto, Ontario). The digitized image consisted of a 512 × 512 pixel matrix, with the signal intensity in each pixel assigned one of 256 possible binary gray levels. These gray values were then converted into their corresponding optical density equivalents. Densitometric data was recovered by converting the signal from each pixel into different colors, corresponding to different ranges of optical density. Such a representation permits the distribution of the fluorescence signal recorded in the original micrograph to be displayed as a false-color map (see Fig. 7), substantially increasing the ability to discriminate and measure subtle changes in image intensity. An even more precise representation of local differences in antigen site density could also be obtained by plotting portions of the digitized images stored in frame-memory as densitometric scans through relevant regions of the micrographs (see Fig. 8).

## Results

Immunocytochemical experiments with muscle cells developing in culture have already shown that motor neurites induce a gradual appearance and growth of specialized postsynaptic sites on the muscle surface near the path of nerve contact (3, 4, 6, 7, 26, 35). These sites include dense deposits of a basal lamina HSPG and adjacent aggregates of AChRs (5, 7). The junctional AChR accumulations have been shown to form, at least in part, through an aggregation of mobile AChRs which were dispersed throughout the sarcolemma prior to the establishment of nerve–muscle contact (3, 4, 7). Since AChR aggregates are invariably associated with dense accumulations of HSPG (5), it is possible that junctional HSPG deposits also form through similar processes of migration and aggregation on the cell surface. Alternatively, HSPG molecules could be restricted in their mobility by adhesive interactions with one another, or with other immobile elements of the extracellular matrix or plasma membrane. This would indicate that quite different mechanisms are involved in the regulation of proteoglycan and AChR organization.

This question has been examined in two ways. In some experiments, muscle surface HSPG was pulse-labeled with a specific monoclonal antibody (2AC2) that had been conjugated with the fluorescent dye, tetramethylrhodamine isothiocyanate (TRITC). The pattern of fluorescent staining on individual muscle cells was then examined as a function of time. In other experiments, HSPG molecules that became exposed on the muscle cell surface at different times were differentially stained with monoclonal antibody that had been labeled with contrasting fluorochromes[2] (see Materials and Methods).

## Stability of Proteoglycan Organization on Aneural Muscle Cells

In a previous report, fluorescent monoclonal antibody (2AC2) was used to determine time-dependent changes in the distribution of muscle surface HSPG at developing neuromuscular junctions in cell culture (7). Skeletal muscle cells were shown to undergo a gradual change in HSPG organization, which involved the development of new HSPG deposits along the path of nerve–muscle contact. The same experiments also revealed the appearance and growth of new extrajunctional HSPG deposits on both innervated and aneural muscle cells. In these earlier experiments, the cultures were maintained in the presence of a low concentration (~100 ng/ml) of fluorescent antibody to ensure that recently synthesized HSPG would become labeled upon its exposure at the cell surface.

A goal of the present experiments, however, was to determine whether existing deposits of HSPG on the muscle surface could undergo time-dependent changes in organization as is known to occur with AChR (3, 7). Eight nerve-free muscle cultures were therefore pulse labeled with TRITC-labeled antibody, and then washed extensively (see Materials and Methods) to remove residual traces of antibody that might bind to proteoglycan molecules subsequently deposited on the muscle cell surface. In two additional cultures, unlabeled antibody (5 μg/ml) was included in the medium to block new antigen sites, thus ensuring that residual traces of the fluorescent antibody would not artifactually contribute to any changes in the original pattern of the stain. Fifty individual muscle cells were then selected, and photographed in both fluorescence and phase-contrast optics. 2 or 3 d later, the same muscle cells were relocated (see Materials and Methods) and the pattern of the fluorescent stain again recorded. Enlarged fluorescence micrographs were compared to determine which changes, if any, had occurred in the distribution of the original, stained HSPG molecules. An example of the results of such an experiment are presented in Fig. 1.

Obvious morphological changes occurred on most of the cells examined between the second and fifth day after plating of dissociated muscle cells. The most conspicuous changes

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[2] As a homogenous molecular preparation specific for a single species-specific immunological determinant on the HSPG core protein (5), the monoclonal antibody can be expected to bind in stoichiometric amounts to its macromolecular antigen, and to sterically hinder further antibody binding at the same sites. The antibody (2AC2) used for these experiments has already been shown (a) to be specific for a frog basal lamina HSPG (5), and (b) not to inhibit the nerve-induced accumulation of HSPG during synaptogenesis (7).
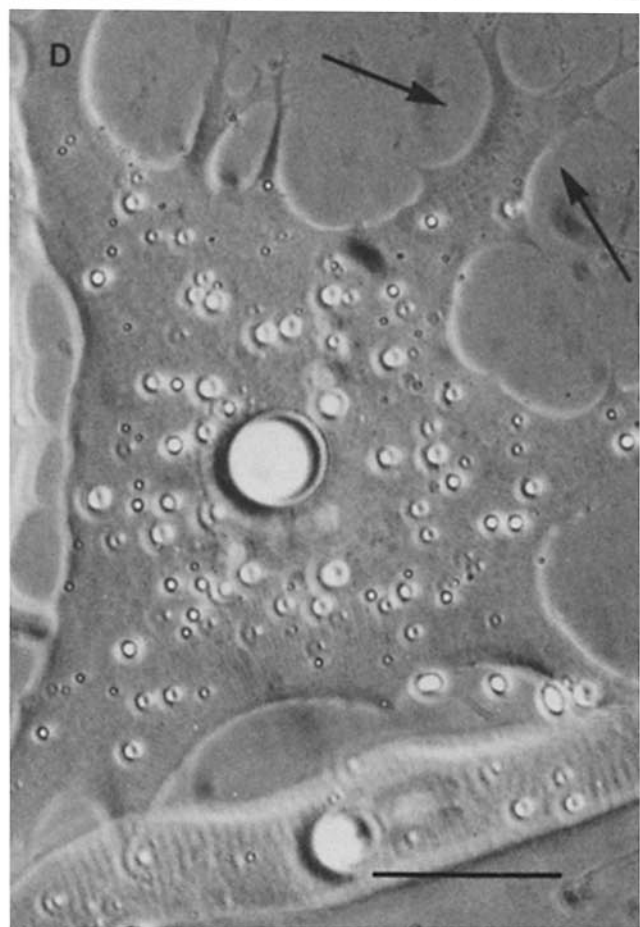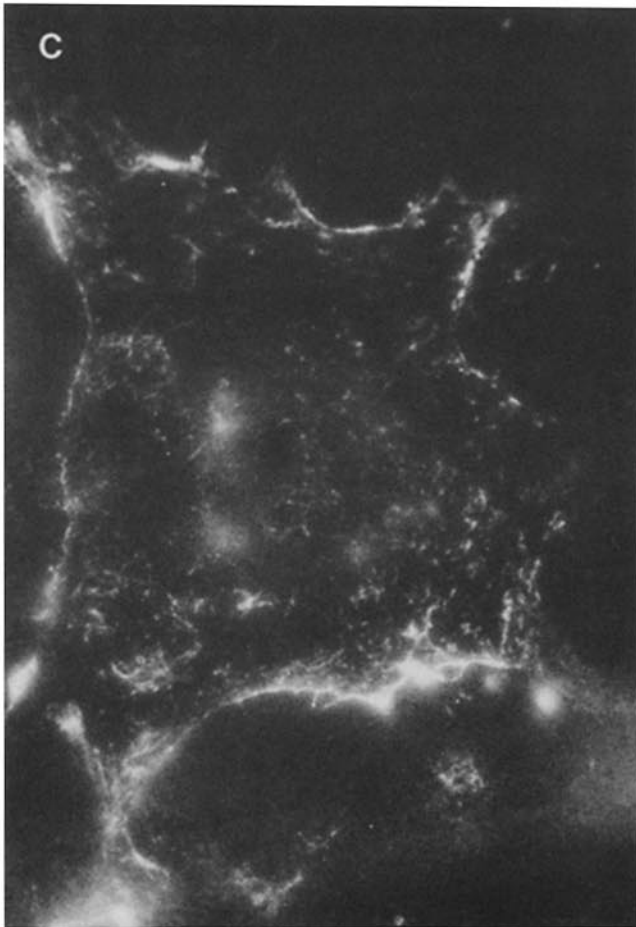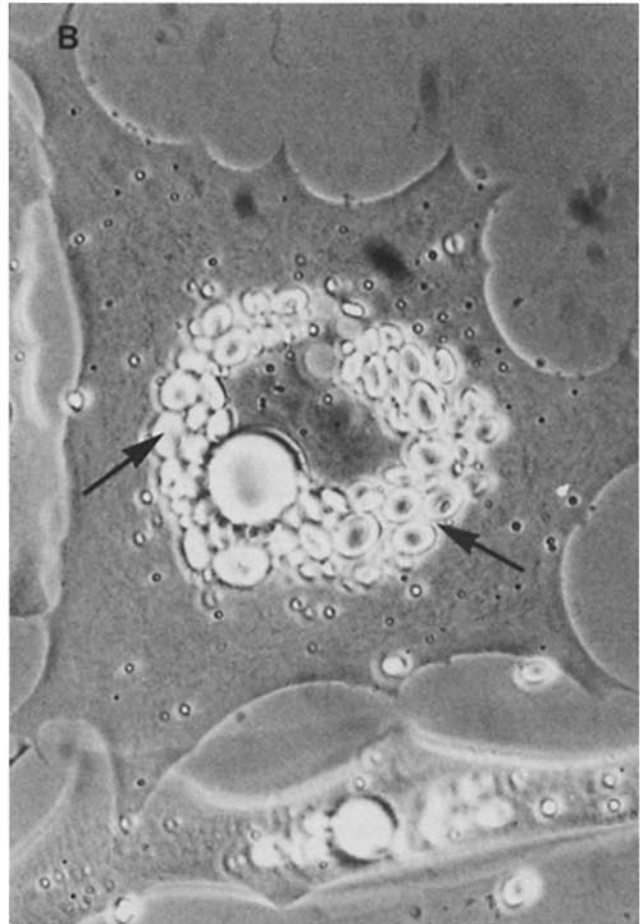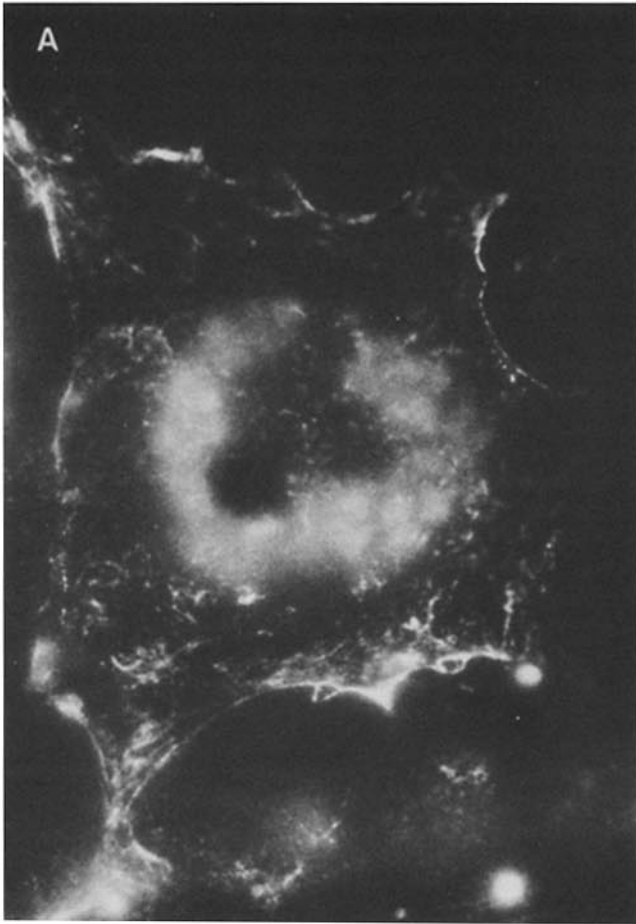
were found in the shape of the cell perimeter, usually as a result of the formation and growth of new pseudopodal extensions (compare Fig. 1, b and d). In addition, there were also changes in the number and distribution of cytoplasmic organelles visible in phase-contrast optics. These included the disappearance or shrinkage of cytoplasmic yolk platelets, the development of striations, and appearance of scattered cytoplasmic granules.

In spite of these obvious changes, there were few detectable changes in the distribution of the fluorescent stain on the cell surface. Essentially all the sites of fluorescent staining recorded at the initial observation were still present 3 d later, and appeared virtually identical in shape and size. When minor changes did occur in the shape or dimensions of individual antigen plaques, they were invariably restricted to the edges of the cell (see Fig. 1), and presumably reflected the exposure of stained HSPG deposits that were already present at the initial observation, but had remained outside the original plane of focus. Therefore, even though this monolonal antibody (2AC2) has been shown not to prevent the appearance and growth of new HSPG deposits on the developing muscle cells in this preparation (7), new HSPG deposits did not become stained under these experimental conditions. It is therefore unlikely that new HSPG deposits appeared through the rearrangement of HSPG molecules that were previously dispersed over the cell surface. This can be contrasted with essentially identical experiments using TRITC-labeled αBGT, which have revealed that new extrajunctional AChR accumulations form through the aggregation of AChR units within the sarcolemma (3, 7).

## Differential Staining of New and Old Proteoglycan Deposits on Aneural Muscle Cells

As described above, successive observations of antibody-labeled HSPG on the muscle surface revealed that little or no change occurred in the morphology of individual, stained proteoglycan deposits over a 3-d period in culture. These experiments did not exclude the possibility that new HSPG deposits had formed in the interval between observations, but were composed of recently synthesized HSPG molecules that would have remained invisible under these experimental conditions.

To test this possibility, 10 3-d-old muscle cultures were exposed to fluorescent monoclonal antibody (2AC2), this time labeled with FITC. The cultures were rinsed extensively (see Materials and Methods) and allowed to develop for 2 more d. They were then further stained with FITC-conjugated goat anti-mouse IgG, followed by exposure to FITC-labeled IgG from the MOPC-21 myeloma (see Materials and Methods). This last step was carried out to block residual anti-mouse IgG binding sites remaining on the cells after exposure to the goat antibody. Finally, new HSPG antigenic sites that had become exposed on the cell surface since the initial incubation

with monoclonal anti-HSPG were stained with TRITC-labeled monoclonal antibody (2AC2). These procedures ensured that the original antibody remaining on cell surfaces would be intensely stained with two additional layers of FITC-conjugated antibody, while new HSPG would be labeled with the contrasting fluorochrome TRITC.[2]

When the living muscle cultures were examined in the fluorescence microscope with optics selective for each fluorochrome, it was thus possible to compare the distributions of new and old HSPG molecules on the same muscle cells (Fig. 2). In many cases these observations indicated that individual HSPG deposits on the muscle surface were almost uniformly labeled with both fluorochromes (data not shown). However, numerous examples were also found in which HSPG plaques were intensely labeled with TRITC, but not with FITC[3] (Fig. 2). This was most conspicuous at the tips of individual pseudopodal extensions, which were commonly labeled only with TRITC (compare Fig. 2, a–c). Such examples, in which stained HSPG deposits were visible only in TRITC optics, cannot be explained either by the presence of a residue of FITC-labeled antibody in the culture medium after the initial antibody exposure, or by redistribution of the original FITC-labeled antibody. To explain this observation as a methodological artifact, it would be necessary to postulate that conspicuous local differences exist in the dissociation rate of adjacent HSPG–antibody complexes on the same cell surface. However, such localized losses of fluorescent antibody would have been detected by experiments in which the pattern of antibody staining was examined over time on individual cells (see above). It is therefore reasonable to conclude that existing proteoglycan deposits on the muscle cell surface remained morphologically stable, and that new proteoglycan deposits were composed almost exclusively of antigenic sites that had recently become exposed at the cell surface.

## Differential Staining of Proteoglycan on Innervated Muscle Cells

These experiments have demonstrated that existing surface deposits of basal lamina HSPG remain morphologically stable for several days, even during obvious changes in cell size and shape. During this period, however, new proteoglycan deposits form; these are composed primarily of molecules only recently exposed at the cell surface. Together, these observations strongly suggest that the complex surface organization of muscle basal lamina proteoglycan, which consists of dense plaques and fibrils separated by expanses of much lower antigen site-density, is the result of a locally targeted deposition of newly synthesized material.

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[3] Different sets of cultures varied in the relative abundance of FITC and TRITC staining, suggesting that the rate of HSPG deposition was variable between individual cells, and separate sets of cultures. In all cases, however, there were striking examples where TRITC-labeled HSPG plaques and fibrils were devoid of FITC staining, and less frequent examples where FITC label predominated.

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Figure 1. Stability of existing basal lamina HSPG deposits on a muscle cell developing in a nerve-free culture. The 2-d-old culture was pulse labeled with fluorescent monoclonal antibody, then examined immediately (A and B) and after 2 more d of differentiation in vitro (C and D). Note the absence of change in the distribution of the fluorescent stain (compare A and C) despite notable changes in cell morphology, including the growth of new pseudopodal extensions (see arrows in D) and the disappearance of autofluorescent perinuclear yolk platelets (see arrows in B). Note, however, that stained regions occasionally appeared at cell edges due to exposure of antibody-labeled proteoglycan deposits that were originally outside the plane of focus. Bar, 30 μm.
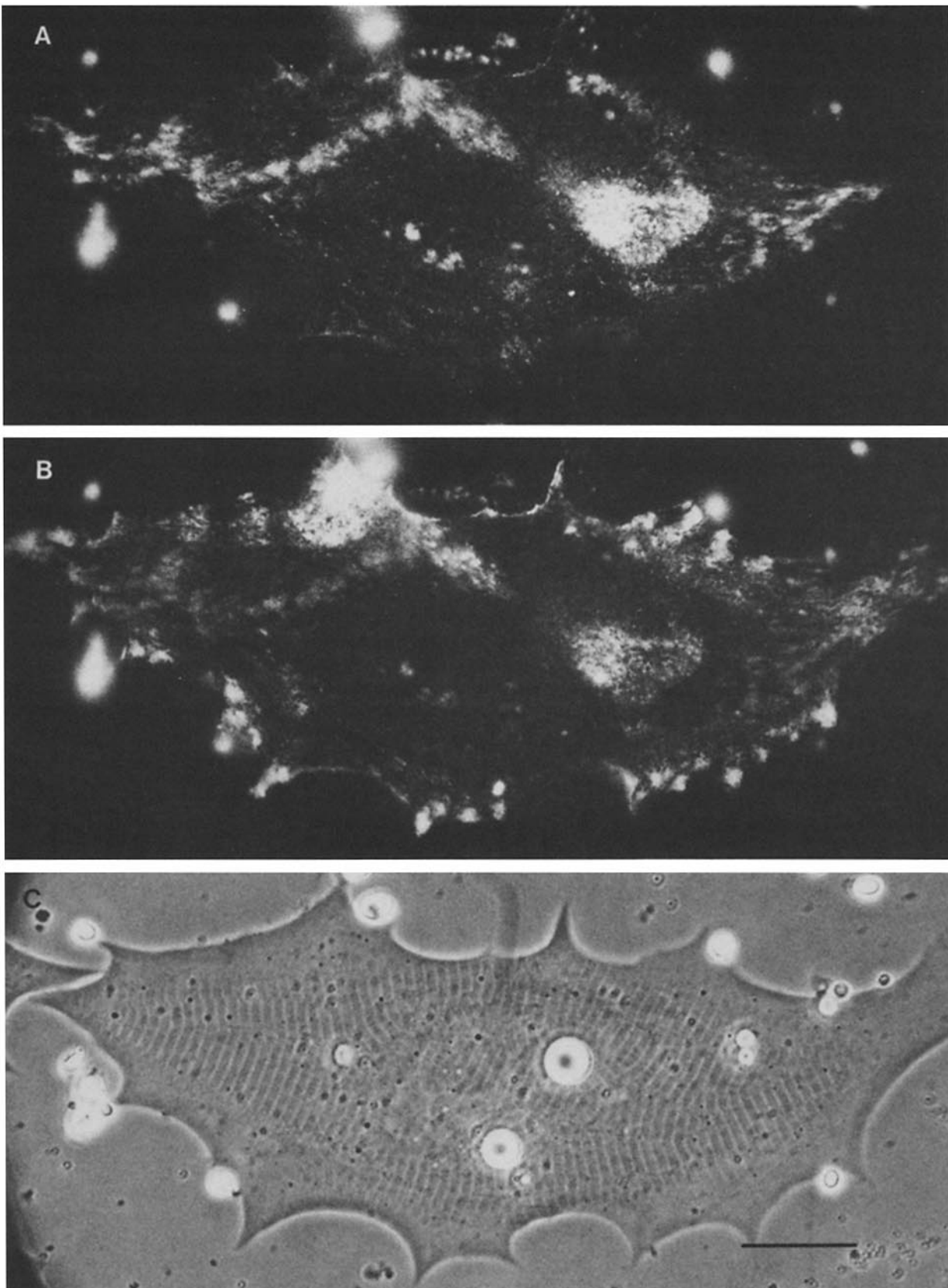
*Figure 2.* Differential distribution of old and new proteoglycan deposits on the muscle surface. Nerve-free muscle cultures were pulse labeled with FITC-conjugated monoclonal antibody, and then allowed to differentiate for an additional 2 d (see also Fig. 1). The cultures were then counterstained with TRITC-labeled antibody (see Materials and Methods) and examined alive in the fluorescence microscope. (*A*) FITC staining associated with older proteoglycan deposits. (*B*) TRITC staining of recently deposited HSPG on the surface of the muscle cell, shown in phase-contrast optics (*C*). Note the striking preponderance of new proteoglycan deposits (*B*) in regions of growth at the cell perimeter, and their scarcity at the older proteoglycan deposits seen in *A*. Bar, 20 μm.

Previous studies in this system have already shown that developing cholinergic nerve fibers can induce the formation of new HSPG deposits along paths of nerve–muscle contact (7), and that these deposits are associated with virtually congruent aggregates of junctional AChR (5). To determine whether this also reflected a preferential deposition of newly synthesized proteoglycan at sites of nerve–muscle contact, experiments were carried out in which HSPG molecules present on the cell surface before and after innervation were stained with contrasting fluorochromes. The procedure was similar to that described above, except that dissociated neural tube cells from Xenopus embryos were added to 15 muscle cultures immediately after an initial exposure to FITC-labeled antibody (2AC2). 2 d later, the intensity of the initial stain was again augmented with further FITC-antibody (see above), and the cultures were then exposed to either TRITC-labeled αBGT or TRITC-labeled antibody (2AC2).

Previous experiments have shown that the junctional accumulations of AChR that develop in this system are invariably associated with almost congruent HSPG deposits (5). Under the present experimental conditions, however, intensely stained junctional AChR aggregates were visible along many paths of nerve–muscle contact, but were devoid of visible FITC–antibody staining (see below). When TRITC-labeled antibody (2AC2) was used instead of fluorescent toxin, it became clear that new HSPG deposits had in fact formed along the paths of nerve–muscle contact (Fig. 3), but were composed almost exclusively of antigen which had become exposed at the cell surface after the addition of neural tissue.[4] These observations indicate that the developing cholinergic nerve fibers had induced a localized deposition of recently synthesized HSPG adjacent to the site of cell contact.

## Proteoglycan Deposition at Synapses in Older Muscle Cultures

The observations described so far have indicated that cholinergic nerve fibers can induce a localized deposition of recently synthesized HSPG at the developing neuromuscular junction. However, this result raised an unexpected question. As described above, the non-innervated muscle cells present in these cultures already had surface deposits of HSPG before the establishment of nerve–muscle contact, and these deposits remained morphologically stable for periods of several days. This initially led to an expectation that junctional HSPG plaques would consist primarily of recently deposited HSPG, with occasional contributions from older HSPG accumulations that were fortuitously present in the path of the growing nerve fibers. However, staining of this latter form was not observed[4] (data not shown).

Previous studies of this preparation (5, 7) have shown that the developing muscle surface has a complex proteoglycan organization that is composed of dense HSPG plaques and fibrils, separated by expanses of cell surface with little or no detectable staining (see also Figs. 1–3). As the cells mature in culture, there is a gradual increase in the size and number of dense HSPG deposits, and a corresponding increase in the

background level of diffuse HSPG stain (5, 7). The organization of basal lamina HSPG on older muscle cells in culture thus approaches that observed on adult muscle fibers, where the diffuse extrajunctional HSPG site density is ~19% of that at the neuromuscular junction (5). To determine whether this greater abundance of surface HSPG might affect the devel-
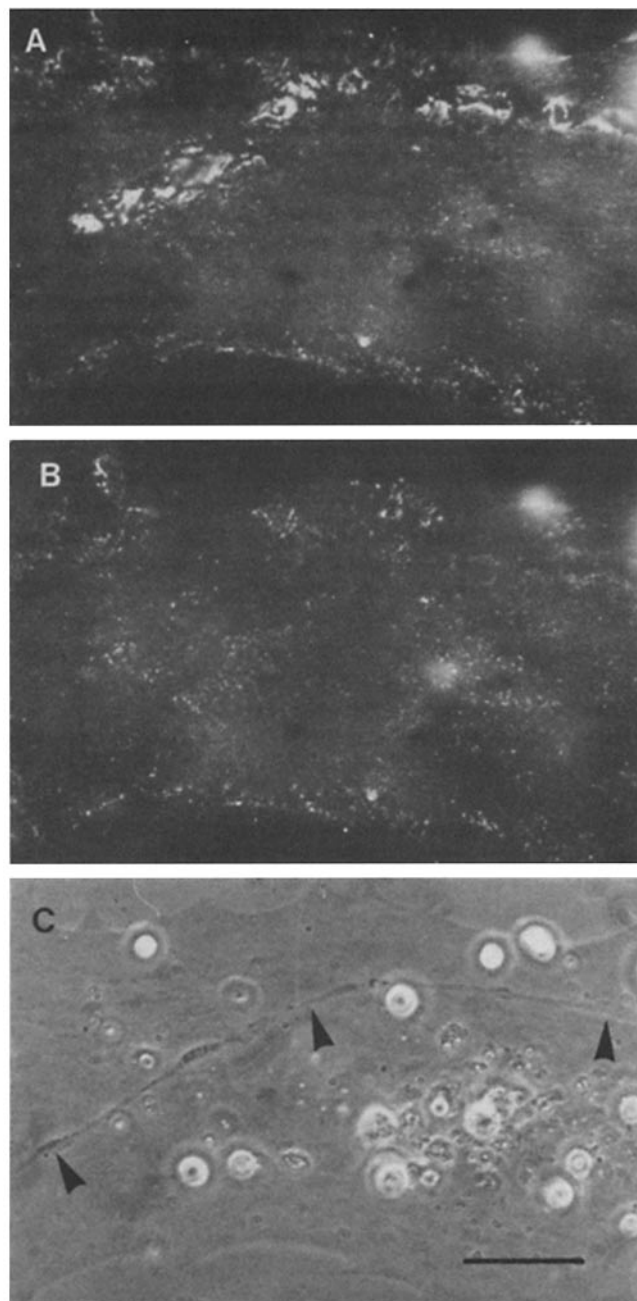


Figure 3. Preferential deposition of new proteoglycan at a neuromuscular junction developing in culture. Experimental conditions were similar to those in Fig. 2, except that dissociated neural tube cells were added to the muscle cultures immediately after pulse-labeling with FITC-conjugated monoclonal antibody. New proteoglycan deposits were counterstained with TRITC-labeled antibody 2 d later. Note that the junctional proteoglycan deposits (A) present along the nerve fiber (see arrowheads in C) were labeled only with TRITC and not with FITC (B), indicating that the nerve had induced a local deposition of new HSPG along the path of cell contact. Bar, 20 μm.

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[4] In preliminary experiments, occasional examples were found where the HSPG plaques at sites of nerve–muscle contact were stained by both fluorochromes. This was not observed in later experiments in which more extensive rinsing was carried out after the original exposure to FITC-labeled antibody (see Materials and Methods).
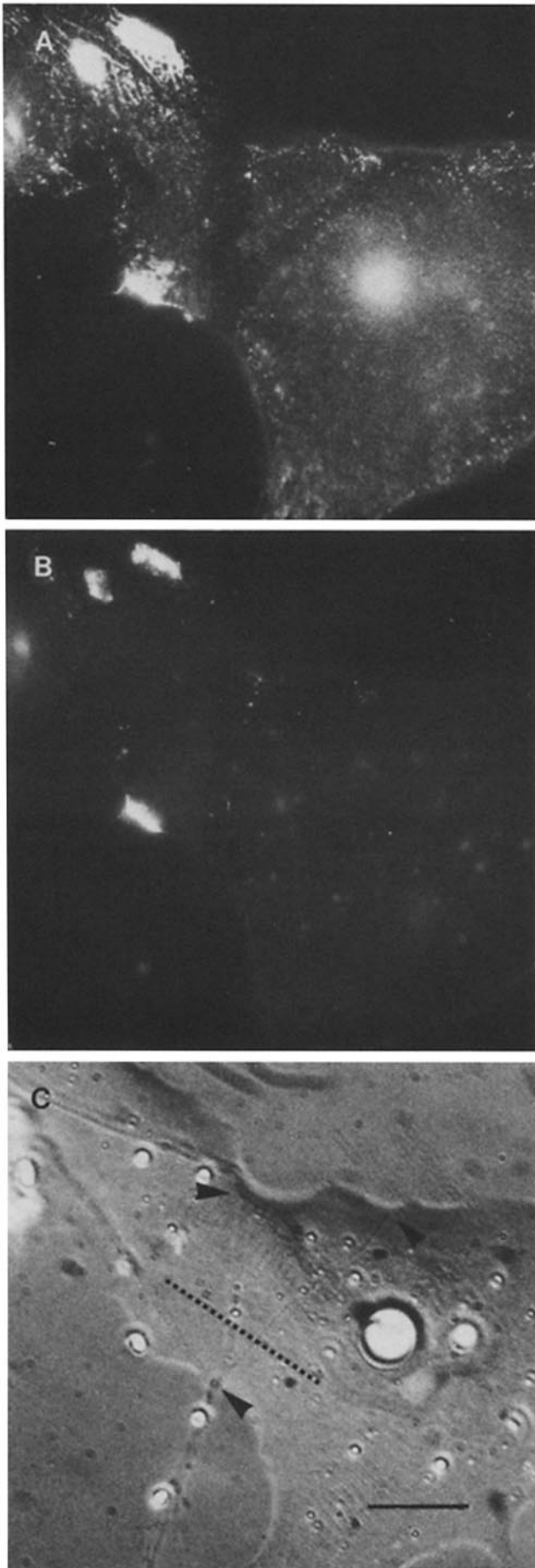
opment of junctional proteoglycan deposits, dissociated neural tube cells were added to 10 older muscle cultures that had been maintained for 4–5 wk at 10°C (see Materials and Methods). The resulting cultures, which again had nerve–muscle contacts visible in phase-contrast optics, were stained with FITC-labeled antibody and TRITC-labeled αBGT after 1–3 d of nerve growth. As expected (see reference 5), characteristic junctional aggregates of AChRs could be observed along many paths of nerve–muscle contact, and were invariably associated with almost congruent, densely stained plauqes of HSPG. This was true both for the small AChR accumulations characteristic of early stages of synapse development, and for the larger patches and bands of AChR at more mature neuromuscular junctions (see also references 6, 7, 35). However, an unexpected difference was evident on some of the older muscle cells which showed high background levels of extrajunctional HSPG staining. On these cells there was an abrupt drop in fluorescence intensity near the region of nerve–muscle contact (Fig. 4, a and b). This appeared as dark band of relatively low antigen site density, cutting through the more brightly stained HSPG deposits present over the extrasynaptic cell surface.[5] This pattern of antigen staining was most conspicuous on those muscle cells that had only rudimentary evidence of junctional AChR staining (Fig. 4), but was also common on cells with more elaborate patches of junctional AChR (Fig. 5). In these latter cases, however, the perineural region of reduced antigen density was interspersed with intensely stained antigen plaques, usually associated with corresponding junctional AChR aggregates. At advanced stages of junctional AChR accumulation (see references 7 and 35), the zone of low antigen density was difficult to discern, and remained visible only in the occasional gaps which separated adjacent bands of intensely stained junctional basal lamina (data not shown).

To obtain a more objective view of antigen organization at these developing sites of nerve–muscle contact, the negative images of some fluorescence micrographs were also analyzed by video densitometry. Portions of fluorescence micrographs containing sites of nerve–muscle contact were recorded with a video camera coupled to an IBAS interactive image analysis computer (see Materials and Methods). The system had previously been calibrated with filters of known optical density. This allowed the data recorded in the fluorescence micro-

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[5] Similar results were also observed with monoclonal antibodies 2BDS, 2AA5, and 2BA5 (5), which recognize at least two further epitopes on the HSPG core protein (data not shown).

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Figure 4. Removal of muscle surface HSPG from paths of nerve–muscle contact during synapse development. Older muscle cells that showed relatively high background levels of HSPG staining (compared to those in Figs. 1–3) were stained with FITC-labeled antibody (A) and TRITC-labeled αBGT (B) after 1 d of culture with dissociated neural tube cells. Note the reduced intensity of proteoglycan staining (A) along the path of nerve–muscle contact shown in phase-contrast optics (see arrowheads in C). At these early stages of synapse development, little AChR accumulation (B) can be detected near the site of cell contact, and AChRs remain associated with dense extrajunctional plaques of HSPG (compare A and B). This phenomenon is analyzed more quantitatively in Fig. 7. Densitometric scans of the images in A and B (along the broken line in C) are shown in Fig. 8. Bar, 30 μm.
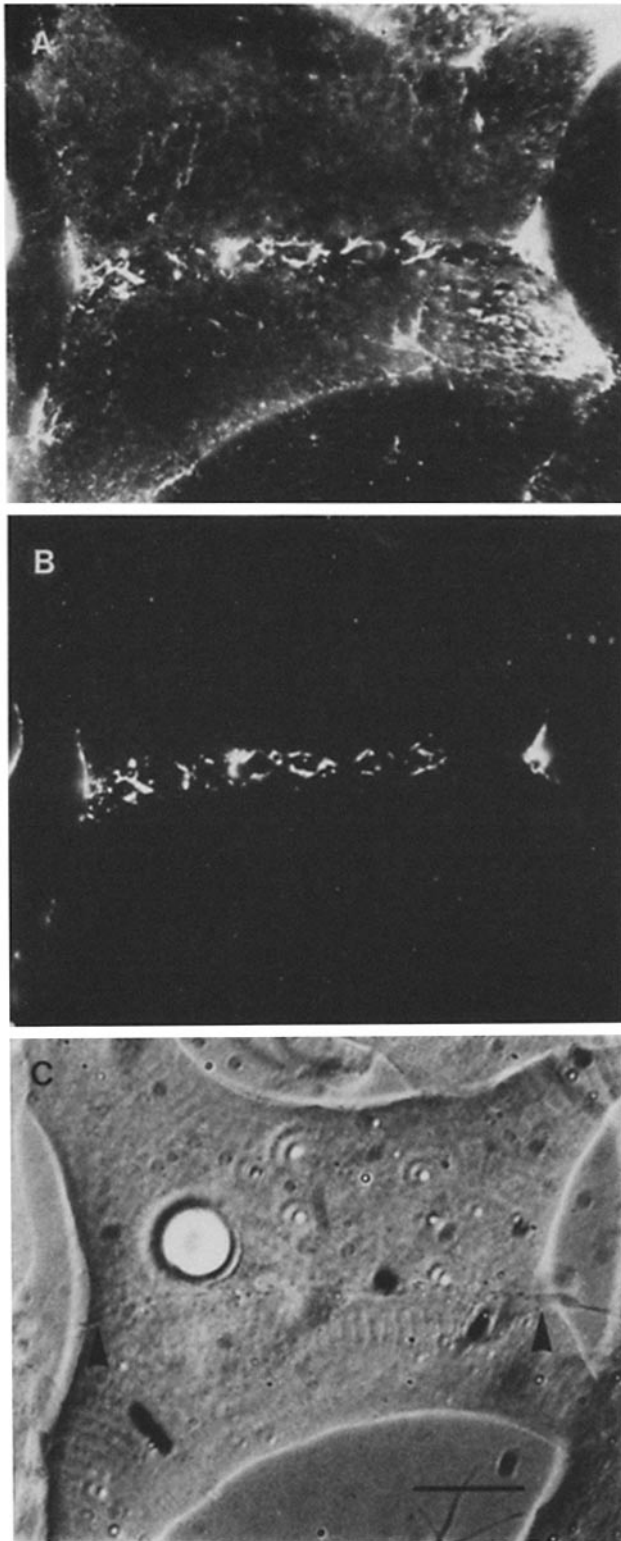
*Figure 5.* Proteoglycan organization at a later stage of synapse development. The culture of old muscle cells was stained as in Fig. 4, after 2 d of nerve growth. Note that intensely stained HSPG plaques (*A*) have become associated with each of the junctional AChR aggregates (*B*) along the path of nerve–muscle contact visible in phase-contrast optics (see arrowheads in *C*), leaving only short expanses of cell contact with a reduced HSPG site density similar to that shown in Fig. 4*A*. Bar, 30 μm.

graphs to be presented as false-color maps that more quantitatively reflect the local differences in antigen (or toxin) site-density. The results of such an analysis of the cell in Fig. 4 are shown in Fig. 6, using a color look-up-table in which increasing image intensity corresponds to increasing color-temperature. Using this method of analysis, it became evident that the path of nerve–muscle contact was associated with a region of reduced antigen site-density (Fig. 6*A*), and that there was no corresponding drop in the density of αBGT sites along the same paths of nerve–muscle contact (Fig. 6*B*).[6] This comparison is illustrated more quantitatively in Fig. 7, which shows densitometric scans across the path of nerve–muscle contact obtained from the images shown in Fig. 6, *A* and *B*. Taken together, these observations confirm that the paths of nerve–muscle contact were associated with localized regions of muscle surface containing a reduced antigen site-density.

The presence of a reduced antigen concentration at sites of nerve–muscle contact suggested that some developing nerve fibers might have eliminated existing surface deposits of basal lamina HSPG adjacent to the path of cell contact. This possibility was confirmed in further experiments where extrajunctional HSPG present on the aneural muscle cells was again selectively stained with FITC-labeled antibody immediately before plating neural tube cells (see above). 2 d later, after the development of neuromuscular junctions, the cultures were counterstained with TRITC-labeled antibody or TRITC-αBGT to reveal junctional aggregates of HSPG and AChR. A total of seven cultures was used in each of these experiments. Under these conditions local regions of reduced HSPG staining were associated with virtually all paths of nerve–muscle contact that showed both extensive staining of junctional AChR, and significant levels of extrajunctional HSPG (Fig. 8). In those cultures counterstained with TRITC-labeled antibody to selectively reveal newly deposited HSPG, it was clear that the plaques of dense, HSPG-containing junctional basal lamina were also associated with paths of reduced FITC staining similar to those shown in Figs. 4 and 8 (data not shown).

These discrete bands of reduced antigen site density were not seen in control, nerve-free muscle cultures (data not shown), indicating that developing nerve fibers had caused the elimination of existing HSPG deposits. In fact, since these bands of reduced antigen density were seen even at early stages of synapse development, before the appearance of extensive AChR aggregates and their associated deposits of HSPG (Figs. 4 and 6), these observations indicate that the nerve-induced removal of extrajunctional HSPG precedes the subsequent formation of junctional HSPG plaques in the region of cell contact. This removal presumably explains the unexpected absence of older HSPG accumulations at the developing neuromuscular junctions in this system (see above).

Though further study will be required to characterize this nerve-induced elimination of muscle surface HSPG more systematically,[7] the present experiments have provided another significant observation. It has already been shown that

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[6] This observation also indicates that the apparent decrease in HSPG site density shown in Figs. 6*A* and 7*A* was unlikely to have been an artifact caused by the more complex membrane geometry along the path of nerve–muscle contact.
[7] The inability of noncholinergic neurons to cause HSPG elimination will be examined in a subsequent, more detailed study.
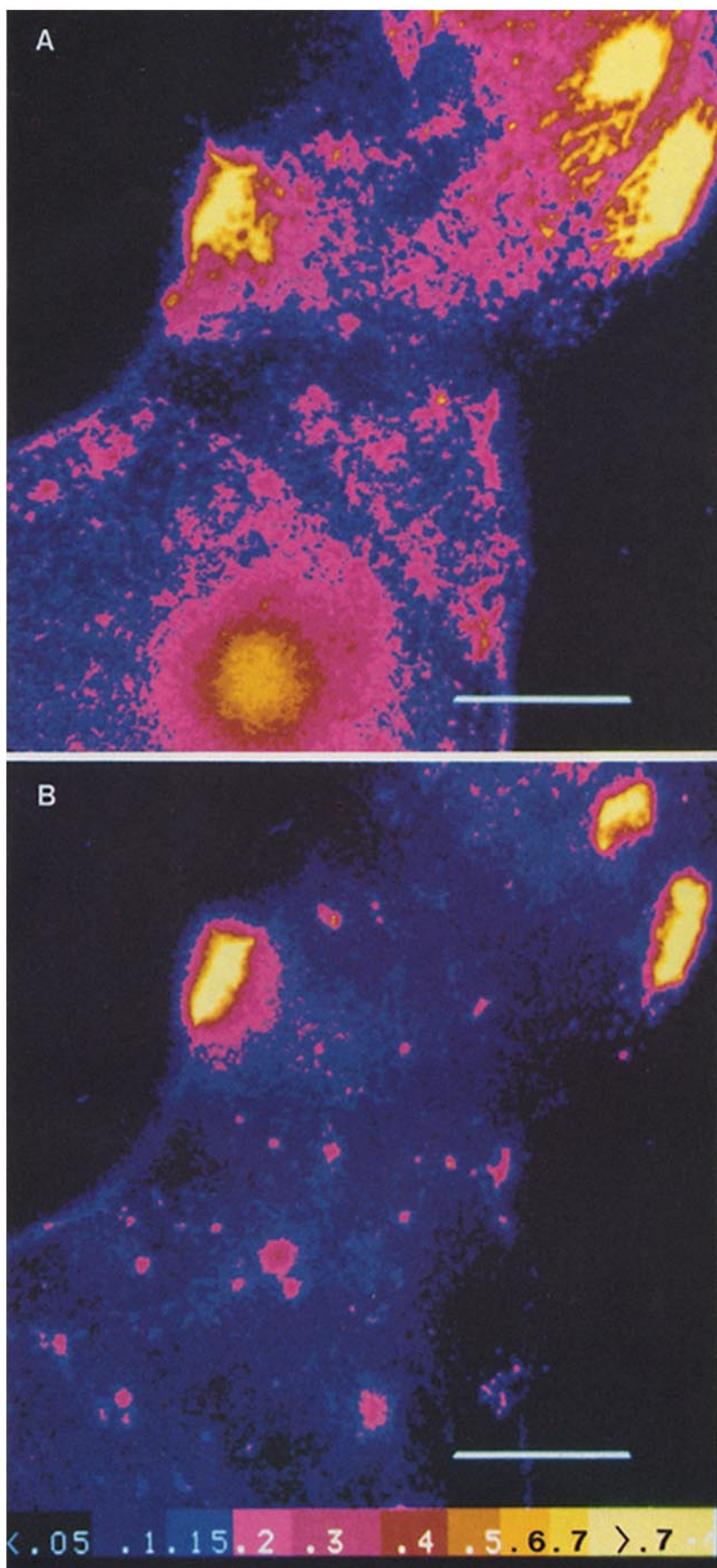
*Figure 6.* Quantitative analysis of fluorescence micrographs (shown in Fig. 4) by digital video densitometry. Portions of the original negatives used in the preparation of Fig. 4, *A* and *B*, were analyzed with a densitometric video camera connected to an IBAS image analysis computer (see Materials and Methods). The digital image was stored as a $512 \times 512$ pixel matrix, and has here been presented as a false-color map where increases in the absorbance of the photographic emulsion are represented by increases in color-temperature. Note the reduced image density, corresponding to a reduced antigen site density, along the path of nerve contact (marked by arrowheads in Fig. 4*C*) in *A*, and the absence of a corresponding reduction in AChR staining in *B*. The color values assigned to each range of optical density (absorbance) are shown below the image in *B*. Bars, 20 μm.
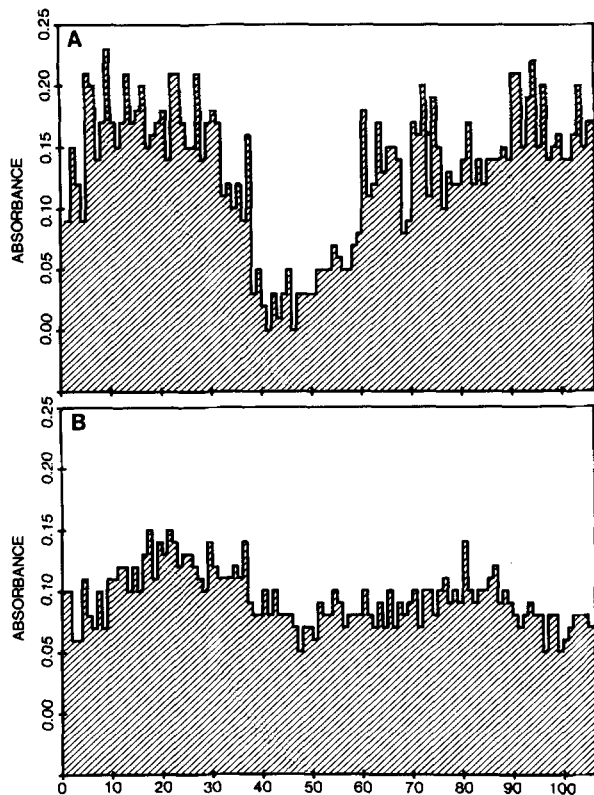
*Figure 7.* Densitometric scans through the path of nerve–muscle contact shown in Figs. 4, *A* and *B*, and 7, *A* and *B*. Densitometric values stored in video frame-memory have been plotted along the dotted scan-line shown in Fig. 4*C*. Vertical bars represent the absorbance (optical density) values associated with every third pixel. Note that a reduced image density is associated with the path of nerve–muscle contact in staining for HSPG (*A*), but not in the corresponding view of AChR distribution (*B*).[6]

a substantial fraction (~40%) of the nerve–muscle contacts in these cultures show no detectable spontaneous synaptic activity, and do not induce AChR accumulation (35). These properties are shared by neurites derived from noncholinergic neurons of dorsal root and sympathetic ganglia (35), indicating that the ability to induce AChR accumulation is probably limited to cholinergic neurons. In view of such observations, it is significant that most of the paths of nerve–muscle contact, which showed no AChR accumulation after 2 d in culture, were not associated with detectable bands of reduced antigen site density, even when adjacent regions of the muscle surface had high levels of background staining (data not shown). Furthermore, regions of nerve growth over the extracellular matrix deposited on the substratum by occasional islands of epithelial cells, which also contain HSPG, did not reveal similar evidence of localized antigen degradation (Fig. 9). This indicates that the nerve-induced elimination of basal lamina HSPG occurs at the developing neuromuscular junction (Figs. 4, 5, and 8), but is not simply a consequence of nerve growth, or even the establishment of nerve–muscle contact. It is therefore more likely to reflect a nerve-induced remodeling of the muscle cell surface during synaptogenesis.

## Discussion

This study has used fluorescent derivatives of αBGT and a monoclonal antibody (2AC2) to analyze the mechanisms

responsible for the elaboration of a chemically specialized basal lamina at the developing neuromuscular junction. Previous experiments have already demonstrated that neither of these probes prevents the accumulation of AChR or HSPG at developing neuromuscular junctions in this system (3, 7). The results of this study indicate that quite different cellular mechanisms are responsible for changes in the organization of AChR and HSPG, even though their distributions show a striking degree of congruence on both aneural and innervated muscle cells (5). New accumulations of AChR have been found to develop via a reorganization of mobile AChR units within the plasma membrane (3, 4, 7). In contrast, new accumulations of basal lamina HSPG consist of antigenic sites only recently exposed at the cell surface, while older HSPG deposits remain relatively stable in size and morphology. The appearance of a dense junctional accumulation of HSPG is also associated with the formation and growth of discrete plaques of a new, organized basal lamina adjacent to the path of nerve–muscle contact (7, 66). Based upon these results, it is reasonable to conclude that the motoneuron induces a localized deposition of recently synthesized HSPG at the region of nerve–muscle contact. However, the ability to target the site of deposition of new HSPG is clearly a property that is endogenous to the developing muscle cell, and is not itself dependent upon innervation. This indicates that an appropriate, developing nerve fiber either (*a*) is able to specify new sites of deposition for HSPG molecules synthesized by the muscle cell, or (*b*) itself secretes HSPG that becomes incorporated in the developing junctional basal lamina. This ability of muscle cells to generate great differences in their local rates of HSPG deposition at the cell surface is remarkable, but difficult to explain in mechanistic terms since the processes that regulate the transport of recently synthesized products to the cell surface still remain poorly understood.

It has already been shown in this system that innervation by cholinergic motoneurons induces the aggregation of mobile AChR in a perineural region adjacent to the site of nerve–muscle contact, and a corresponding disappearance of extrajunctional AChR accumulations (3, 4, 7). A number of investigations have subsequently identified both soluble and insoluble factors which can stimulate the formation of new AChR aggregates on non-innervated muscle cells in culture, suggesting that the developing motor nerve might release analogous AChR aggregation-promoting factors during synaptogenesis. In keeping with this line of reasoning, it would be possible to propose that the developing nerve either releases HSPG, or other factors which selectively cause recently synthesized muscle HSPG to become deposited at the site of innervation.

It is worth noting in this regard that the list of exogenous factors that promote AChR aggregate formation on aneural muscle cells is already substantial, including various proteins of $M_r$ ~1,700 (33), $M_r$ ~75,000 (23, 30), $M_r$ 86,400 (sciatin) (40), $M_r$ 200,000 (8), and $M_r$ ~1,000,000 (laminin) (65), together with ascorbate ($M_r$ 176) (34), particulate matrix extract (31), polylysine-coated latex beads (47), and strong electric fields (46). It is not yet known whether these factors all represent novel molecular entities, or include proteolytic fragments of the large structural proteins that are already well-established components of extracellular matrices. Additional studies will be required to determine whether any of these substances act as neurotrophic AChR-aggregating factors during synapse formation.
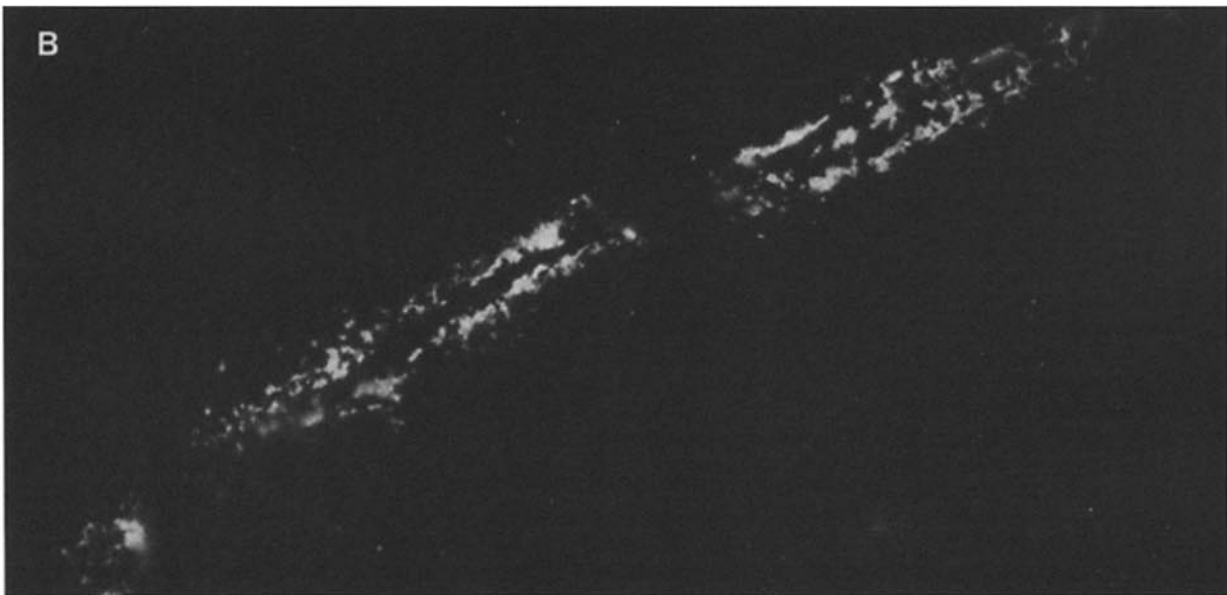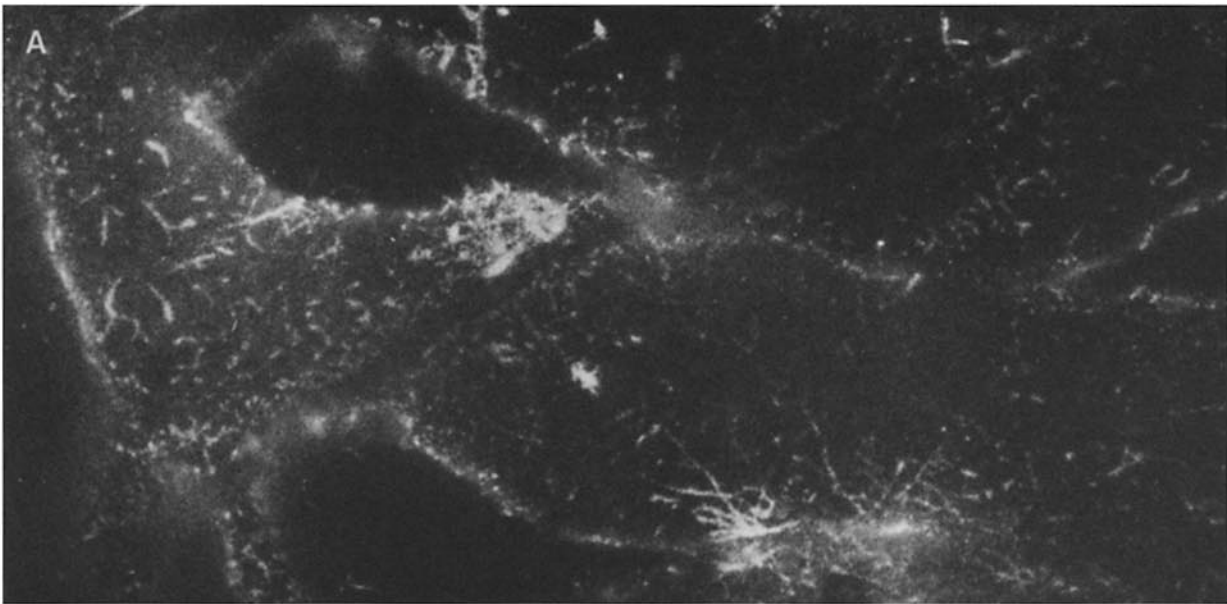
*Figure 8.* Removal of extrajunctional HSPG deposits at a developing neuromuscular junction. Extrajunctional HSPG deposits on old muscle cells were pulse stained with FITC-antibody immediately before plating neural tube cells (as in Fig. 3). 2 d later developing neuromuscular junctions were again revealed by staining with TRITC-αBGT. Note that, under these experimental conditions, junctional AChR aggregates (*B*) were not associated with stained HSPG plaques in *A* (compare with Fig. 5), and that old HSPG deposits (*A*) appear to have been removed along the path of nerve–muscle contact visible in phase-contrast optics (see arrowheads in *C*). Bar, 30 μm.
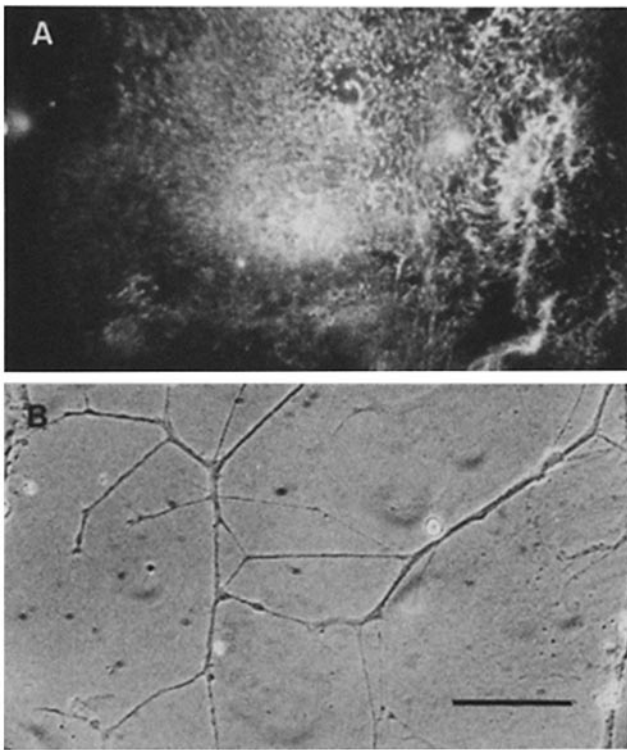
*Figure 9.* Failure of developing neurites to remove antigenic sites from HSPG-containing extracellular matrix deposited on the collagen substratum by nonmuscle cells. (*A*) HSPG staining associated with matrix deposited by epithelial cells that contaminate cultures of *Xenopus* myotomal muscle. These had degenerated prior to the addition of neural tube cells. (*B*) Phase-contrast view of the field seen in *A*, showing the paths of nerve growth over the substratum. Note the absence of etched regions (*A*) associated with sites of nerve-substrate contact in *B*. Bar, 30 μm.

It is already established, however, that AChR aggregates are consistently associated with corresponding surface specializations of both the basal lamina and cytoskeleton (5, 10, 12, 13, 15, 17, 22, 23, 28, 31, 48, 56), and can be induced by the specialized, junctional basal lamina during muscle regeneration (16). The production of a specialized basal lamina that could be evoked by a variety of physiological and nonphysiological stimuli may therefore be a necessary precursor for AChR aggregation.

The results of this study show that at least one other process contributes to the nerve-induced formation of the junctional basal lamina during synapse development. Before inducing the formation of new HSPG deposits along the path of cell contact (see above), the nerve first causes the elimination of existing HSPG from the adjacent portion of the muscle surface. This elimination can be detected at very early stages of synapse formation, before the accumulation of junctional HSPG plaques and their associated aggregates of AChR (see Figs. 4 and 5). Since new HSPG plaques and AChR aggregates can also form spontaneously on aneural muscle cells (3, 5, 7, 10, 25, 37, 61), this nerve-induced elimination of muscle surface HSPG is the earliest detectable change in muscle surface organization during synaptogenesis, and represents the only such change that is unique to cells with nerve contacts. This suggests that the nerve-induced elimination of muscle basal lamina HSPG may represent a particularly revealing phenomenon.

It may initially seem paradoxical that the developing nerve fiber causes the elimination of HSPG from the muscle surface, since the junctional basal lamina is later characterized by an unusually high local concentration of this proteoglycan (5, 7). However, it should be noted that the junctional basal lamina appears to be specialized, relative to the extrajunctional lamina present over the remainder of the muscle fiber, in a variety of substances other than HSPG. Immunohistochemical techniques have, in fact, suggested that some antigens may even be unique to the extrajunctional basal lamina (54). Also, like other basement membranes that are stabilized by covalent cross-linkages (see reference 36), the muscle basal lamina shows a remarkable degree of structural stability, and will survive for extended periods after the degeneration of the muscle fiber (57). To assemble a chemically unique junctional basal lamina, it may therefore be essential to first eliminate HSPG, along with any existing extrajunctional lamina components. The nerve-induced removal of extrajunctional basal lamina HSPG may thus reflect an appropriate initial phase, prior to the development of a junctional basal lamina. Indeed, inability to eliminate a stable, extrajunctional basal lamina could inhibit the development of ectopic synapses in adult muscle, resulting in a preferential reinnervation of the existing junctional basal lamina (see reference 57).

In addition to this obvious role of clearing the muscle surface of any inappropriate extrajunctional basal lamina components, the initial nerve-induced elimination of muscle HSPG has other interesting implications for an understanding of the molecular mechanisms that mediate synapse development. These derive from the fact that there are well-established precedents for interpreting the biochemical mechanisms responsible for a localized elimination of basal lamina components by an invasive cell. The elimination or modification of organized complexes of extracellular matrix proteins is commonly mediated by localized processes of proteolytic degradation (for reviews see 11, 39, 43, 44, 52, 58, 62). The archetype of such processes is found in the lysis of the fibrin clot by the proteinase plasmin. In fact, the enzyme systems which regulate the formation and lysis of fibrin also contribute to processes of tissue remodeling (39, 43, 52). Based upon these precedents, it is reasonable to suggest that the nerve-induced elimination of muscle surface HSPG is also brought about by a localized release or activation of proteinases at the site of innervation. It has already been shown that both sensory and sympathetic neurites secrete proteinases in culture (38, 49), primarily in the vicinity of the growth cone (38), and that one of the proteinases released by growing nerve fibers (49) has a pattern of substrate selectivity very similar to a gelatinase, also secreted by cultured fibroblasts (1, 29, 59, 63, 64, 67), that has been shown to have proteoglycanase activity (27, 29, 67). Developing nerve fibers are thus able to secrete enzymes that could account for the nerve-induced elimination of muscle HSPG observed in this study.

Even more interesting implications may be drawn from the observations that HSPG elimination occurred as an initial phase in the development of the neuromuscular junction, but could not be detected at other sites of nerve–muscle contact or nerve–substrate contact, where the intensity of HSPG staining should have permitted its visualization. This latter observation, which will require confirmation in further detailed studies, is presumably related to the fact that ~40% of the nerve–muscle contacts in this preparation also do not form cholinergic synapses with muscle, or induce the aggre-

gation of AChRs (20, 35). This apparent coupling of HSPG elimination with its subsequent deposition into dense plaques of junctional basal lamina may indicate that the proteolytic enzyme systems responsible for HSPG elimination also contribute to the formation of the junctional basal lamina. A localized proteolysis at the site of cell contact, either by its direct action on cell surface components, or indirectly by the generation of active proteolytic fragments, could regulate a variety of cytoplasmic mechanisms such as the reorganization of cytoskeletal proteins and the vectoring of secretory products from the Golgi apparatus, resulting in localized chemical differentiation at the cell surface. It has also been shown that a calcium-dependent metalloproteinase (or gelatinase, see above) similar to that released by developing neurites in cell culture (49), can both degrade proteoglycans and activate the procollagenases required for the deposition of soluble, secreted procollagens into the insoluble supramolecular complexes that exist in extracellular matrix (64). In fact, for an orderly removal and reassembly of the muscle basement membrane, there must be a precisely regulated activation and inhibition of several specific proteinases, required to degrade both HSPG and the other structural proteins of basal lamina (such as laminin and type IV collagen), and perhaps to activate procollagen proteinases that could contribute to the assembly of a new type IV collagen matrix in the developing junctional basal lamina.[8] Since the junctional basal lamina appears to function as a local regulatory signal, inducing focal synaptic differentiation in both nerve and muscle cells (16, 57), this would imply that the inductive interaction between neurons and their target cells could ultimately be mediated by proteinase–cascade reactions reminiscent of those already implicated in blood clotting and tissue remodeling.

A useful characteristic of these types of enzymatic cascade reactions is their ability to use an array of proteinases, activators, and inhibitors to produce a common activating signal that is initiated only under specific conditions (for a more detailed discussion of these issues, see references 43, 44, 52, and 58). It is thus conceivable that highly specific molecular mechanisms of cell–cell recognition could regulate a localized activation of a proteinase–cascade reaction common to a wide range of neuron–target cell systems, leading in each case to localized synaptic differentiation. Furthermore, if the proteinases involved in synaptic transmission (9), or other components of such a system, were also released locally by the nerve terminal, analogous inductive mechanisms could also couple the level of synaptic differentiation to the amount of synaptic activity throughout the life of the organism.

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