Extracellular Matrix Organization in Developing Muscle: Correlation with Acetylcholine Receptor Aggregates

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ABSTRACT Monoclonal antibodies recognizing laminin, heparan sulfate proteoglycan, fibronectin, and two apparently novel connective tissue components have been used to examine the organization of extracellular matrix of skeletal muscle in vivo and in vitro. Four of the five monoclonal antibodies are described for the first time here.

Immunocytochemical experiments with frozen-sectioned muscle demonstrated that both the heparan sulfate proteoglycan and laminin exhibited staining patterns identical to that expected for components of the basal lamina. In contrast, the remaining matrix constituents were detected in all regions of muscle connective tissue: the endomysium, perimysium, and epimysium.

Embryonic muscle cells developing in culture elaborated an extracellular matrix, each antigen exhibiting a unique distribution. Of particular interest was the organization of extracellular matrix on myotubes: the build-up of matrix components was most apparent in plaques overlying clusters of an integral membrane protein, the acetylcholine receptor (AChR). The heparan sulfate proteoglycan was concentrated at virtually all AChR clusters and showed a remarkable level of congruence with receptor organization; laminin was detected at 70–95% of AChR clusters but often was not completely co-distributed with AChR within the cluster; fibronectin and the two other extracellular matrix antigens occurred at ~20, 8, and 2% of the AChR clusters, respectively, and showed little or no congruence with AChR. From observations on the distribution of extracellular matrix components in tissue cultured fibroblasts and myogenic cells, several ideas about the organization of extracellular matrix are suggested. (*a*) Congruence between AChR clusters and heparan sulfate proteoglycan suggests the existence of some linkage between the two molecules, possibly important for regulation of AChR distribution within the muscle membrane. (*b*) The qualitatively different patterns of extracellular matrix organization over myotubes and fibroblasts suggest that each of these cell types uses somewhat different means to regulate the assembly of extracellular matrix components within its domain. (*c*) The limited co-distribution of different components within the extracellular matrix in vitro and the selective immune precipitation of each antigen from conditioned medium suggest that each extracellular matrix component is secreted in a form that is not complexed with other matrix constituents.

The surface of adult vertebrate skeletal muscle fibers is regionally specialized, both at tendon-attachment sites and at the neuromuscular junction. While the mechanisms that control the formation and maintenance of such specialized regions are still not clearly understood, attention has been focused recently on the possible influence of extracellular matrix on muscle surface organization. The extracellular matrix surrounding each muscle fiber can be divided into two obvious morphological layers (see reference 40). Most proximal to the sarcolemma is a felt-like band, ~10–15-nm thick,

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often termed the basal lamina. Surrounding this is a complex zone composed of various connective tissue fibrils, which together constitute the reticular lamina. At the neuromuscular junction the basal lamina, but not the reticular lamina, extends into the synaptic cleft between nerve and muscle. While morphologically indistinguishable from the remaining basal lamina, this junctional region appears to be both structurally and functionally specialized. At least some of the acetylcholinesterase found at neuromuscular junctions is bound to synaptic basal lamina (5, 17, 33). Moreover, studies of regenerating nerve and muscle have suggested that elements of the synaptic basal lamina can induce both the differentiation of regenerating nerve terminals (40) and the accumulation of the acetylcholine receptor (AChR)[1] within the postsynaptic membrane (3, 8). When immunohistochemical techniques were employed to compare synaptic and extrasynaptic basal lamina, some antigenic determinants were detected in both regions, some were detected only in extrasynaptic regions, and some appeared to be limited to synaptic basal lamina (39, 41). Since most of the synaptic antigens have been defined only by serological criteria, their biochemical identities remain unknown. It also remains to be determined how such antigenic differences might be involved in any influence of extracellular matrix on muscle membrane organization.

It is worth noting that conventional immunological studies of extracellular matrix are subject to several methodological limitations. Extracellular structures are composed of an array of macromolecules, many of which adhere strongly to one another. Isolation of the extremely pure antigens required for immunization, or for immunoadsorption, thus poses formidable problems. It is inevitably difficult to rigorously determine how extensively antibodies to immunogenic contaminants contribute to immunocytochemical staining. This becomes particularly relevant when such contaminants represent uncharacterized components that cannot be isolated for use as immunoadsorbants. Hybridoma techniques allow one to circumvent this problem and obtain homogenous antibodies specific for single antigenic determinants.

In the present study, therefore, we have used monoclonal antibodies to examine the organization of five different components of muscle extracellular matrix, both in vivo and in cultured embryonic muscle. Our observations indicate that individual matrix elements assume ordered distributions both in the intact tissue and upon embryonic cells developing in culture. This becomes particularly obvious when the distribution of individual matrix components is compared with that of AChR clusters in the sarcolemma. Such experiments indicate that the distribution of each matrix constituent shows some correlation with that of AChR clusters on the myotube surface, but that this varies considerably in extent. Most impressive is the close correspondence between the distribution of AChR and elements of the muscle basal lamina, particularly a heparan sulfate proteoglycan. The virtually congruent organization of proteoglycan and AChR clusters suggests that linkages between these two molecules may contribute to the organization of AChR in the postsynaptic membrane.

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor, α-BTX, α-bungarotoxin; FITC, fluorescein isothiocyanate; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; and TRITC, tetramethylrhodamine isothiocyanate.

MATERIALS AND METHODS

Hybridoma Production and Preparation of Monoclonal Antibodies: Hybridomas-33, -39, and -35 were generated using spleen cells from mice immunized with fragments of 14-d chick embryo leg muscle. The tissue was homogenized in PBS and insoluble material was washed several times by centrifugation and resuspension in PBS. The final pellet was resuspended in an equal volume of PBS, emulsified with complete Freund's adjuvant (1:1[vol/vol]) and injected into BALBc/J mice (0.2 ml final volume per mouse, given intraperitoneally). Mice were boosted 1 mo later with a similar preparation in incomplete Freund's adjuvant and, after 3 d, the spleens were removed for cell fusion.

The immunogen used to generate hybridoma-31 consisted of a muscle extract prepared by methods similar to those used to extract laminin from the Engelbreth-Holm-Swarm (EHS) sarcoma (46). Leg muscle from one dozen 18–19-d chick embryos was homogenized in 3.5 M NaCl, 50 mM Tris, pH 7.5, containing 5 mM *N*-ethylmaleimide (NEM), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Insoluble material was sedimented and then washed three times in the above buffer. The resulting pellets were then suspended in extraction buffer consisting of 0.5 M NaCl, 50 mM Tris, 5 mM NEM, 1 mM PMSF, washed again, resuspended in 50 ml of similar buffer, and extracted with stirring overnight at 4°C. The resulting extract was concentrated approximately fivefold in dialysis tubing placed on polyvinyl pyrrolidone and then emulsified 1:1 (vol/vol) with complete Freund's adjuvant. 1 mo after injection (see above) the mice were boosted with 0.5 ml of similar extract, and fusions were carried out three days later.

SP2/0 myeloma cells were used for all fusions and the hybridization procedure was essentially that described by Kennett et al. (24). Hybridoma cells, selected by their ability to grow in hypoxanthene-aminopterin-thymidine medium, were cloned in soft agar. Before and after cloning, the hybridomas were screened for antibody production. In some experiments, ^{125}I-labeled rabbit anti-mouse Fab was used to detect monoclonal antibody bound to exposed antigen in live muscle cultures. Alternatively, immunofluorescence was employed to assay monoclonal antibody binding to live cells or frozen-sectioned muscle.

Isotypes of the antibodies were determined by subunit analysis of metabolically labeled antibody on SDS PAGE. All of the antibodies used in the present studies were IgG. Each hybridoma was injected intraperitoneally into pristane-primed BALBc/J mice and IgG was subsequently purified from ascitic fluid by ammonium sulfate precipitation and ion exchange chromatography on DE52 (Whatman Laboratory Products Inc., Clifton, NJ).

Antifibronectin monoclonal antibody (B3) has been previously described by Gardner and Fambrough (16). This IgG is specific for avian fibronectin (both cellular and plasma forms) and does not cross-react with horse serum fibronectin (a component present in our culture medium).

Antisera: An antilaminin antiserum was generously provided by Drs. Hynde K. Kleinman and George R. Martin (National Institute of Dental Research, Bethesda, MD). This serum was raised in sheep which had been immunized with mouse laminin isolated from the EHS tumor, and its specificity had previously been tested via enzyme linked immunoassays (36). The serum was known to cross-react with chicken laminin.

A rabbit anti-chicken collagen antiserum, provided to us by Drs. R. Rotundo and M. Chiquet (Carnegie Institution of Washington, Baltimore, MD), was also used in some experiments.

Preparation of Labeled Conditioned Medium and Immunoprecipitation of Labeled Antigen: Chick muscle cultures containing both fibroblasts and well-differentiated myotubes were labeled with either (*a*) 100 μCi/ml [^{35}S]methionine (1,100 Ci/mmol, Amersham Corp., Arlington Heights, IL), (*b*) 100 μCi/ml [^{3}H]glycine (20 Ci/mmol, Amersham Corp), and 100 μCi/ml [^{3}H]proline (40 Ci/mmol, New England Nuclear, Boston, MA) or (*c*) 200 μCi/ml [^{35}S]sodium sulfate (0.9 Ci/mmol, New England Nuclear). Isotope was added to carrier-free Eagle's minimal essential medium supplemented with 10% horse serum and 2% embryo extract. In some cases 0.05 mg/ml ascorbate and 0.05 mg/ml β-aminopropionitrile were also included. Cells were labeled overnight at 37°C. After this, the conditioned medium was collected, diluted 1:1 with borate-buffered saline, pH 7.8, supplemented with 2 mM PMSF, 5 mM EDTA, and 5 mM NEM, and centrifuged at 20,000 *g* for 15 min at 4°C to remove cellular debris.

To precipitate labeled antigen, monoclonal antibody (usually 10 μg) was added to 1 ml diluted conditioned medium in a 1.5-ml microfuge tube. After 1–2 h, 80 μl of rabbit anti-mouse IgG (Cappel Laboratories, Inc., Cochranville, PA) was added to each tube and the mixture was incubated an additional hour at room temperature, followed by overnight at 0°C. In immunoprecipitations using sheep antiserum, rabbit anti-sheep IgG (Cappel Laboratories, Inc.,) was used as second antibody. The immune precipitates were collected by centrifugation and washed four to five times by suspension in borate-buffered saline

and centrifugation. Protease inhibitors (see above) were included in the borate-buffered saline except when this might have interfered with subsequent enzyme digestions. The washed pellets were dissolved in sample buffer for electrophoresis, either before or after treatment with collagenase or glycosaminoglycan lyases (see below).

Enzyme Digestions: Collagenase digestions were done with purified bacterial collagenase (2,600 U/ml Advanced Biofactures, Lynbrook, NY). For digestion of metabolically labeled conditioned medium, 65 U of collagenase in 50 mM Tris, pH 7.4, with 10 mM calcium acetate was added per milliliter, and digestion was carried out in the presence of 7 mM NEM and 2 mM PMSF for 1–4 h at 37°C. The medium was then either analyzed by SDS PAGE or used for immunoprecipitations. For digestion of immune precipitates, the pellets were resuspended in 50 μl collagenase (2,600 U/ml in the same buffer as above) and incubated at 37°C for 4 h. Deoxycholate (2 μl of a 1% solution) was then added to each tube and the samples were precipitated with 2–3 vol of ice-cold trichloroacetic acid before analysis via SDS PAGE.

For analyses of the basal lamina antigen precipitated by monoclonal antibody-33, degradation was attempted with several glycosaminoglycan lyases. These included *Proteus vulgaris* condroitinase ABC (E.C. 4.2.2.4) (44), *Flavobacterium heparinum* heparinase (E.C. 4.2.2.7) (30) *Pseudomonas* keratanase (keratan sulfate 4-galactopyranosyl glycanohydrolase), and *Streptomyces* hyaluronidase (E.C. 4.2.2.1) from Siekagaku Kogyo Co. (Miles Laboratories, Elkhart, IN). An extract of heparinase from Dr. A. Linker (courtesy of Dr. G. Martin) was also used. Stock solutions were prepared in 0.125 M Tris HCl, pH 6.8, containing 10 mM $CaCl_2$, at 0.1 mg/ml heparinase, and at 0.2 mg/ml for all others. Immunoprecipitates were resuspended in 40 μl of enzyme solution and digested at 37°C for 4 h.

Analysis of Labeled Products by SDS PAGE: SDS PAGE was carried out on 1.5-mm thick (3–15, 3.5–15, or 5–15% acrylamide) gradient running gels with 3% stacking gels. The buffer systems of Laemmli (26) were used. Immunoprecipitates and conditioned medium were prepared for electrophoresis by addition of concentrated sample buffer, to yield final concentrations of 2% SDS, 10% glycerol, 0.125 M Tris-HCl, pH 6.8, 0.1 M dithiothreitol. Samples were boiled for 5 min unless otherwise specified. After electrophoresis, slab gels were fixed and stained with Coomassie Brilliant Blue. To detect labeled bands the gels were impregnated with 2,5-diphenyloxazole (6, 28) or Enhance (New England Nuclear), dried, and exposed to Kodak XAR film (with or without preflashing) at −70°C. Apparent molecular weights of cell products were estimated by comparison with standards including: fibronectin (M_r = 220,000), β-galactosidase (M_r = 116,000), phosphorylase *a* (M_r = 95,000), BSA (M_r = 68,000), pyruvate kinase (M_r = 57,000), ovalbumin (M_r = 43,000), and desoxyribonuclease (M_r = 31,000).

Cell Cultures: Primary cultures of chick myogenic cells were established from 11-d embryonic leg muscle. Muscle masses were dissected out, mechanically dissociated, and filtered to produce a suspension of mononucleate cells. Cells were cultured in Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% horse serum and 2% embryo extract in culture dishes coated with rat-tail collagen. Chick muscle-derived fibroblasts were obtained from similar suspensions by plating the cells for 1 h at 37°C in dishes without collagen. Adherent cells were grown to confluence and passaged four or five times before use. No myotubes were apparent in these latter cultures.

Fluorescence Microscopy: To analyze monoclonal antibody binding to adult skeletal muscle, we froze tissue in liquid nitrogen and cut cryosections (~8 μm thick) at −20°C. Sections were incubated for 10 min in PBS containing 10% horse serum, then for 30 min with 10 μg/ml monoclonal antibody in PBS with 10% horse serum. After several 5-min washes the sections were exposed to a 1:200 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Cappel) in the same medium. In some cases muscle tissue pinned in paraffin-coated plates was pretreated with tetramethyl rhodamine isothiocyanate–conjugated α-bungarotoxin (TRITC-αBTX) and washed extensively before freezing. This procedure allowed visualization of motor end-plates within muscle sections (1).

To analyze monoclonal antibody binding to cell cultures, we plated cells on collagen-coated glass coverslips. Living cells were incubated for 30 min at room temperature with 10 μg/ml monoclonal antibody, in Leibovitz L-15 medium with 10% horse serum, then washed several times and incubated with a 1:200 dilution of FITC-conjugated goat anti-mouse IgG. In studies comparing the distributions of antigen and AChR, cultures were treated with TRITC-αBTX in L-15 medium containing 10% horse serum for 30 min before addition of first antibody. Cells were viewed live or after fixation in 2% formaldehyde, as described by McLean and Nakane (32). With both frozen sections and cultured cells, control experiments were carried out (*a*) using IgG from the P3 × 63 myeloma line, and (*b*) omitting either first or second antibody. All specimens were viewed on a Zeiss fluorescence microscope equipped for epiillumination with narrow-band selective filter combinations for FITC and TRITC. Micrographs were prepared using Kodak Tri-X film developed at 20°C for 18 min in Acufine developer.

RESULTS

Characterization of Antigens

Similar strategies were used to define the target antigen of each monoclonal antibody. Initial analyses were carried out to determine the distribution of the antigens, both in cell cultures and in intact muscle tissue. Indirect radioimmunoassay (data not shown) revealed that each of the antibodies employed in the present study recognized antigen that was exposed in unfixed muscle cultures, and was therefore externally situated. Indirect immunofluorescent staining further demonstrated that in culture, each antigen was associated not only with cell surfaces but also with organized structures well removed from cells. This was in contrast to findings with antibodies against membrane antigens (13, 14, 48) where staining was limited to the cell surface. Examination of antigen distribution in frozen sections of intact muscle also revealed the presence of the antigens in various regions of the extracellular matrix.

For biochemical analyses, metabolically labeled antigen that had been secreted by cells into their culture medium was used. The use of conditioned medium as a source of soluble antigen was of particular value, since it allowed us to circumvent the denaturation commonly involved in extraction of poorly soluble components from extracellular matrix. After isolation from conditioned medium by immunoprecipitation, individual antigens were analyzed by SDS PAGE followed by fluorography. Further analysis included testing of the sensitivity of the isolated antigens to various hydrolytic enzymes.

Biochemical Identifications

ANTIGEN-31—LAMININ: When antibody-31 was used to isolate [^{35}S]methionine-containing antigen from metabolically labeled conditioned medium, and the antigen was then analyzed via SDS PAGE, the predominant polypeptides had apparent molecular weights of 200,000 and close to 400,000 (Fig. 1). These polypeptides are similar in molecular weight to mouse laminin polypeptides (46) and identical to the dominant species precipitated from chick muscle conditioned medium by a sheep antiserum against mouse laminin (Fig. 1). These observations, together with the results of immunocytochemical analysis of antigen distribution in muscle sections (see below), strongly suggest that antibody-31 recognizes chicken laminin.[2] Additional experiments (data not shown) demonstrated that the precipitated polypeptides were not degraded by bacterial collagenase.

In addition to the major high molecular weight bands, at least two lower molecular weight components were also precipitated by both the monoclonal antibody and the laminin

[2] It may be worth noting that when (unlabeled) mouse laminin was analyzed on 3.5–15% acrylamide gels, its constituent polypeptides showed slightly higher apparent molecular weights than corresponding chicken laminin subunits. When the same antiserum that was used in immunoprecipitations from chicken conditioned medium was used to precipitate [^{35}S]methionine-labeled laminin from the conditioned medium of mouse muscle cultures, subunits corresponding in molecular weight to the unlabeled mouse laminin were obtained (data not shown). The monoclonal antibody appeared to be specific for chicken antigen and did not precipitate mouse laminin.

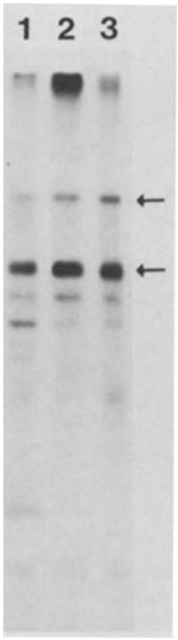


FIGURE 1 Immunoprecipitations from culture medium containing [^{35}S]methionine-labeled secreted products. Muscle cultures were labeled for ~24 h and the conditioned medium was used for double antibody immunoprecipitations. Products were reduced, run on 3.5–15% SDS polyacrylamide gels, and examined by fluorography. Lane *1*, immunoprecipitation using sheep antilaminin antiserum; lanes *2* and *3*, immunoprecipitations using 20 and 10 μg, respectively, of antilaminin monoclonal antibody-31. Arrows mark positions of major bands at 200,000 and slightly less than 400,000 mol wt.

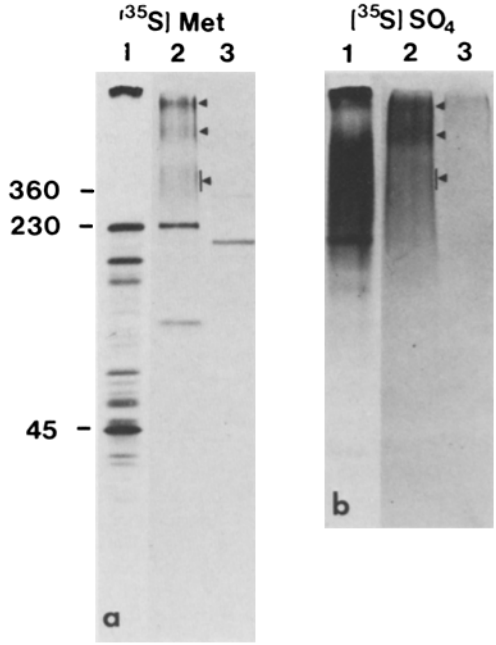


FIGURE 2 Immunoprecipitations from culture medium containing [^{35}S]methionine- (*a*) and [^{35}S]sulfate- (*b*) labeled secreted products. Muscle cultures were labeled for ~24 h and the conditioned medium was either analyzed directly or used for double antibody precipitations. Products were reduced, electrophoresed on 3–15% gradient SDS polyacrylamide gels, and analyzed by fluorography. (*a*) Lane *1*, profile of [^{35}S]methionine-labeled secreted products in unfractionated conditioned medium; lane *2*, immunoprecipitation using monoclonal antibody-33; lane *3*, immunoprecipitation of chicken laminin from the same conditioned medium using monoclonal antibody-31. Values at left are molecular weight × 10^{-3}. (*b*) Lane *1*, profile of [^{35}S]sulfate-labeled secreted products in unfractionated conditioned medium; lane *2*, immunoprecipitation using monoclonal antibody-33, lane *3*, immunoprecipitation from the same conditioned medium using antilaminin antibody-31. Arrowheads mark position of antigen-33.

antiserum (Fig. 1). It remains to be determined whether these represent proteolytic fragments of the major bands or other components that were associated with secreted laminin and thus precipitated as a complex from conditioned medium (22, 25). It should also be noted that the antilaminin antiserum precipitated several additional polypeptides not seen in immunoprecipitations with monoclonal antibody.

ANTIGEN-33—A HEPARAN SULFATE PROTEOGLYCAN: Monoclonal antibody-33 was also used to immunoprecipitate metabolically labeled antigen from the conditioned medium of chick muscle cultures (see Materials and Methods). The results of such an immunoprecipitation from [^{35}S]methionine-labeled conditioned medium are shown in Fig. 2*a*. For comparison, the results of immunoprecipitation of chicken laminin from the same conditioned medium (using antibody-31) are also shown. Whereas control IgG from the MOPC P3 × 63 myeloma line precipitated no detectable label (data not shown), monoclonal antibody-33 precipitated an array of high molecular weight components which appeared as diffuse bands with apparent molecular weights of 550,000 or less.[3] These bands represent minor secreted components which are not obvious in the unfractionated conditioned medium (lane *1*).

The heterodispersity and high molecular weight of these bands suggested that the major component in the precipitate might be a proteoglycan. Proteoglycans may be distinguished from other extracellular matrix components by the large amounts of sulfated carbohydrate they usually contain (19, 37). Accordingly, confluent chick muscle cultures were incubated with [^{35}S]sulfate (see Materials and Methods) and the conditioned medium was analyzed. When the secreted products labeled with [^{35}S]sulfate were examined after SDS PAGE on 3–15% gradient gels, much of the incorporated label appeared to be associated with components too large to enter the gel (see also reference 2). The remaining label (Fig. 2*b*) was distributed through several broad, poorly resolved bands in the upper third of the resolving gel. Immunoprecipitation from sulfate-labeled medium with antilaminin antibody-31 (Fig. 2*b*) or antibody-39 (not shown) yielded no bands with detectable levels of labeled sulfate. Likewise, control antibody from the MOPC P3 × 63 myeloma line did not precipitate labeled material. In contrast, antibody-33 precipitated a complex of broad, poorly resolved bands with electrophoretic mobilities similar to those obtained from [^{35}S]methionine-labeled conditioned medium (compare Fig. 2, *a* and *b*). This

[3] Antibody-33 immunoprecipitates also contained [^{35}S]methionine-labeled polypeptides of apparent molecular weights of 230,000 and 95,000 (Fig. 2*a*) that were without detectable sulfate label under our experimental conditions (Fig. 2*b*). These polypeptides were identical in electrophoretic mobility to fibronectin and a minor co-precipitant also isolated from [^{35}S]methionine-labeled conditioned medium with antifibronectin antibody-B3. Furthermore, overexposed fluorographs of fibronectin immunoprecipitates, analyzed on the same SDS PAGE system, revealed the presence of heterodisperse minor bands of the same electrophoretic mobility as the major species precipitated by antibody-33 (data not shown). Since monoclonal antibodies B3 and 33 clearly recognize different antigens (see also immunocytochemical observations), it is most likely that a small proportion of each antigen exists as a complex in conditioned medium and can thus be precipitated by either antibody. This is not entirely unexpected since antigen-33 appears to be a heparan sulfate proteoglycan (see below) and fibronectin is known to bind both heparin and heparan sulfate (29, 38, 49). (Antibody-33 does not recognize SDS-denatured antigen and could not be used in Western blots.)

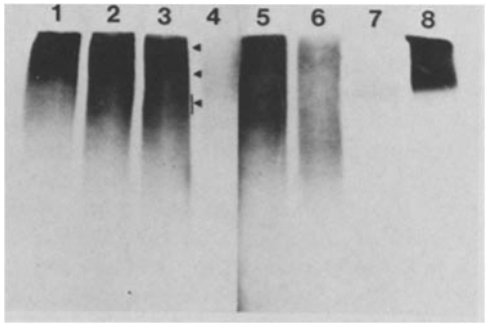


FIGURE 3 Effects of different glycosaminoglycan lyases and reduction on electrophoretic mobility of [^{35}S]sulfate-labeled proteoglycan, precipitated by monoclonal antibody-33. Lanes *1–7* show reduced antigen immune precipitated from [^{35}S]sulfate-labeled conditioned medium, subjected to enzyme digestion, and analyzed on 3.5–15% gradient gels. Lanes *1* and *2*, controls incubated without enzyme; lanes *4* and *7*, after incubation with 0.1 mg/ml heparinase (see Materials and Methods); lane *3*, after 0.2 mg/ml chondroitinase ABC; lane *5*, after 0.2 mg/ml keratanase; lane *6*, after 0.2 mg/ml hyaluronidase; lane *8*, control incubated without enzyme and analyzed without reduction. Note virtual removal of [^{35}S]sulfate-label by heparinase, but not chondroitinase or keratanase. Note also the conspicuous increase in electrophoretic dispersity brought about by reduction. The arrows in lane *3* represent [^{35}S]methionine-labeled antigen from Fig. 2.

observation indicates that antigen-33 is a large, sulfated glycoprotein, most likely a proteoglycan.

As both the immunocytochemical (see below) and immunochemical observations indicated notable similarities between antigen-33 and an amphibian basal lamina antigen recognized by antibody-2AC2, we carried out further biochemical characterization using a method of selective glycosaminoglycan lyase degradation already tested in the amphibian system. These enzymes selectively degrade the different classes of glycosaminoglycan side chains that characterize each species of proteoglycan. Control experiments demonstrated that whereas commercial preparations of hyaluronidase and keratanase exhibited detectable levels of protease contamination, the chondroitinase ABC and two separate heparinase preparations did not (2). In experiments where [^{35}S]sulfate-labeled immunoprecipitates were treated with the various enzymes before electrophoretic analysis (Fig. 3), both heparinase preparations removed virtually all of the label from antigen-33. In contrast, chondroitinase ABC and keratanase had little effect. As expected from its known protease contamination, hyaluronidase notably reduced the amount of label. Furthermore, when [^{35}S]sulfate-labeled antigen-33 was analyzed without reduction (Fig. 3, lane *8*) each of the lower molecular weight bands (Fig. 2*a*, lane *2*, and 2*b*, lane *2*) disappeared, leaving only the highest molecular weight band.[4] These observations are virtually identical to those obtained with the analogous *Xenopus* basal lamina proteoglycan identified by monoclonal antibody-2AC2 (2). On the basis of these observations, it is reasonable to conclude that antigen-33 is also a heparan sulfate proteoglycan.

ANTIGEN-39: When antibody-39 was used to isolate its [^{35}S]methionine-labeled antigen from conditioned medium

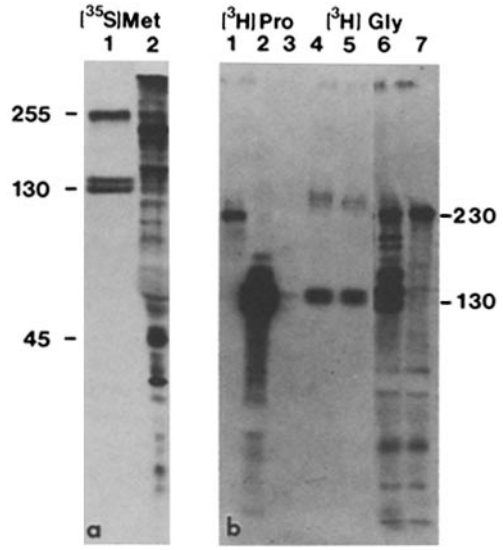


FIGURE 4 SDS PAGE analyses of [^{35}S]methionine- (*a*), and [^{3}H]-proline-, and [^{3}H]glycine- (*b*) labeled secreted products. Muscle cultures were labeled for ~24 h and aliquots of conditioned medium were either used for double antibody immunoprecipitations, analyzed directly, or subjected to collagenase digestion. Samples were reduced, electrophoresed on either 5–15% (*a*) or 3.5–15% (*b*) gradient SDS polyacrylamide gels, and analyzed by fluorography. (*a*) Lane *1*, immunoprecipitation using monoclonal antibody-39; lane *2*, profile of [^{35}S]methionine-labeled secreted products. (*b*) Lane *1*, control immunoprecipitation using antifibronectin monoclonal antibody-B3; lane *2*, immunoprecipitation using a rabbit anti-chicken collagen antiserum; lane *3*, immunoprecipitation identical to that in lane *2*, but treated with bacterial collagenase; lane *4*, immunoprecipitation using monoclonal antibody-39; lane *5*, immunoprecipitation identical to that in lane *4*, but treated with bacterial collagenase; lane 6, profile of [^{3}H]proline- and [^{3}H]glycine-labeled secreted products in unfractionated conditioned medium; lane *7*, [^{3}H]proline- and [^{3}H]glycine-labeled condition medium that has been subjected to treatment with bacterial collagenase.

(Fig. 4*a*, lane *1*), a doublet of relatively well-resolved bands with apparent molecular weights of 130,000 and 140,000 were precipitated together with another polypeptide with an apparent molecular weight of 255,000. Because its distribution in frozen-sectioned muscle was similar to that described for type III collagen by Duance et al. (12), further experiments were undertaken to determine whether or not antigen-39 might also be collagenous. Conditioned medium, containing either [^{35}S]methionine- or [^{3}H]proline- and [^{3}H]glycine-labeled secreted products, was treated with purified bacterial collagenase before immunoprecipitation. Under conditions where several collagenous components in the conditioned medium were degraded (Fig. 4*b*, lanes *6* and *7*), antigen-39 appeared unaffected. Instead, immunoprecipitation from the collagenase-treated medium yielded antigen identical in electrophoretic mobility to that obtained from mock-digested medium (data not shown). In other experiments the antigen was subjected to collagenase digestion after immunoprecipitation. Conditioned medium from cells incubated with [^{3}H]proline and [^{3}H]glycine (in the presence of β-aminopropionitrile and ascorbate) was used in these experiments. Control experiments were carried out using a rabbit antiserum to chicken collagen. As shown in Fig. 4*b* (lanes *2* and *3*), collagenase treatment digested virtually all of the labeled bands precipitated by the rabbit antiserum. However, the treatment had no effect on

[4] This suggests that the proteoglycan antigen-33 in muscle-conditioned medium had undergone limited proteolysis, but that fragments remained joined owing to intramolecular disulfide binding (2).

precipitated antigen-39 (Fig. 4 *b*, lanes *4* and *5*). These results indicate that the antigen is unlikely to be collagenous. It is also clear that the antigen is distinct from the other matrix components examined in this study.

ANTIGEN-35: Like the antigen recognized by antibody-39, antigen-35 appears to be an abundant substance which is distributed throughout muscle connective tissue (see below). It accumulates on both the cells and substratum of myogenic cultures. Immunoprecipitation performed with antibody-35 (from [^{35}S]methionine-labeled conditioned medium) yielded a complex series of bands upon SDS PAGE (data not shown). This pattern was clearly distinct from those related to other antigens in this study. To date, antigen-35 has not been further characterized biochemically, and it has been included in the present study primarily to illustrate the conspicuous differences in surface organization seen among the various constituents of muscle extracellular matrix (see below).

Distribution of Antigens In Vivo

When frozen-sectioned adult chicken muscle was stained with monoclonal antibodies against a heparan sulfate proteoglycan (antibody-33) or laminin (antibody-31), followed by FITC-conjugated goat antibody to mouse IgG, a continuous ring of fluorescence was observed around each muscle fiber (Fig. 5, *a* and *b*). This staining appeared to be closely associated with the fiber surface and spaces devoid of staining were frequently observed between muscle fibers. Furthermore, staining was not evident in the connective tissue of the epimysium which surrounds the muscle as a whole, or the perimysium, which surrounds fascicles of muscle fibers. However, stain was associated with perineurial sheaths of intramuscular nerve bundles, the endoneurium around axons, and with intramuscular blood vessels and capillaries. This pattern of staining corresponds exactly to the distribution of basal lamina antigens (39) within skeletal muscle tissue, as well as the distribution of basal lamina viewed at the ultrastructural level (for review see reference 23).

In contrast, when antifibronectin monoclonal antibody B3, followed by FITC-conjugated goat anti-mouse IgG antibody, was used to stain muscle sections (Fig. 5 *c*), the resulting fluorescence extended throughout the endomysium between individual muscle fibers and into both the perimysium and epimysium. In all regions the staining had a characteristic patchy appearance. In addition, fluorescence was associated with capillaries and larger blood vessels (Fig. 5 *c*), perineurial sheaths, and the endoneurium around myelinated axons (not shown).

The antigen organizations revealed by antibodies-39 and -35 in frozen sectioned muscle were distinct from each of the other matrix components. Like fibronectin, antigen-39 (Fig. 5 *e*) was present in endo-, peri-, and epimysium. Unlike fibronectin, however, the staining did not appear diffuse or patchy. Rather, bright bands of fluorescence surrounded muscle fibers. Intense staining was also evident throughout the perimysium and epimysium. Antigen-35 (Fig. 5 *d*), on the other hand, exhibited a more delicate staining pattern which at higher magnification (not shown) appeared as a meshwork of fine fibrils. The antigen was present in all regions of muscle connective tissue.

When intact muscle was pretreated with TRITC-αBTX and then stained with monoclonal antibody and FITC-conjugated anti-mouse IgG antibody, it was possible to examine the distribution of antigen at motor end-plates. At this level of analysis, both the proteoglycan and laminin appeared to extend into the synaptic region. Furthermore, the intensity of proteoglycan fluorescence at neuromuscular junctions appeared higher than in extrajunctional regions (4). This increased staining intensity most likely reflects an increased concentration of antigen at the neuromuscular junction, as was observed for an analogous basal lamina proteoglycan at the frog neuromuscular junction (2). In contrast, laminin staining (with antibody-31) appeared to be enriched only slightly, if at all, in junctional regions labeled with TRITC-αBTX (data not shown). It should also be noted that under these experimental conditions, some of the fibronectin occasionally appeared to co-distribute with labeled αBTX. However, this co-localization was not consistent, and no evidence for any preferential accumulation of fibronectin was seen at neuromuscular junctions. The resolution of this form of immunocytochemistry is insufficient to determine rigorously whether staining was associated with the synaptic cleft or with other connective tissue adjacent to the neuromuscular junction. Fibronectin may, however, in addition to its location in reticular connective tissue, also be present in muscle basal lamina (39). In contrast to the above observations, neither antigen-39 nor antigen-35 appeared to extend into the synaptic cleft or show obvious accumulation in the synaptic region (data not shown).

Antigen Organization on Cultured Myotubes

The preliminary experiments described above have confirmed that elements of the extracellular matrix of skeletal muscle exist in ordered structures, distinguishable even at the level of the light microscope. At present, however, very little is known about the cellular mechanisms responsible for the formation of this elaborate extracellular complex, and the relation of various matrix components to developing membrane specializations needs to be further explored. We have thus employed the described monospecific antibodies to examine the elaboration of extracellular matrix during the development of myogenic cells in culture. In these experiments we were most interested in determining (*a*) whether the isolated muscle cells in culture would also elaborate orderly structures containing each of these identified matrix components, and (*b*) whether any specialized structures of the extracellular matrix might show correspondence to the clusters of AChR which are known to develop on cultured muscle even in the absence of innervation (15, 18, 45).

To these ends, myogenic cultures were examined after exposure to monoclonal antibody, followed in each case by FITC-conjugated goat anti-mouse IgG. In some experiments TRITC-αBTX was used to reveal correlations between the organizations of extracellular matrix antigens and plasma membrane AChR. The binding of the antimatrix antibodies to unfixed cultures (see Materials and Methods) did not cause any detectable antigen redistribution. In control experiments where fixation with 2% paraformaldehyde (32) was carried out before staining, the cultures exhibited fluorescent patterns indistinguishable from those seen upon labeling live cells. However, such treatment did notably reduce the intensity of staining in some cases. For the present analyses, cultures were examined at approximately 96 h postplating, a stage when myotubes were well developed and fibroblasts within the cultures were still subconfluent.

After immunofluorescent staining the extracellular matrix antigens examined in this study showed several common features of organization. In each case, the antigen was organized into discrete, brightly stained surface structures separated by expanses of conspicuously lower antigen-site density (see Figs. 6–9). Discrete regions of apparently high antigen density were present over both the surface of myotubes and adjacent regions containing cells of fibroblastic morphology. These antigen-containing matrix deposits consisted of both plaques and fibrils and varied greatly in size and morphology.

Despite these similarities in organization of the different antigens, the overall pattern of staining with each antibody

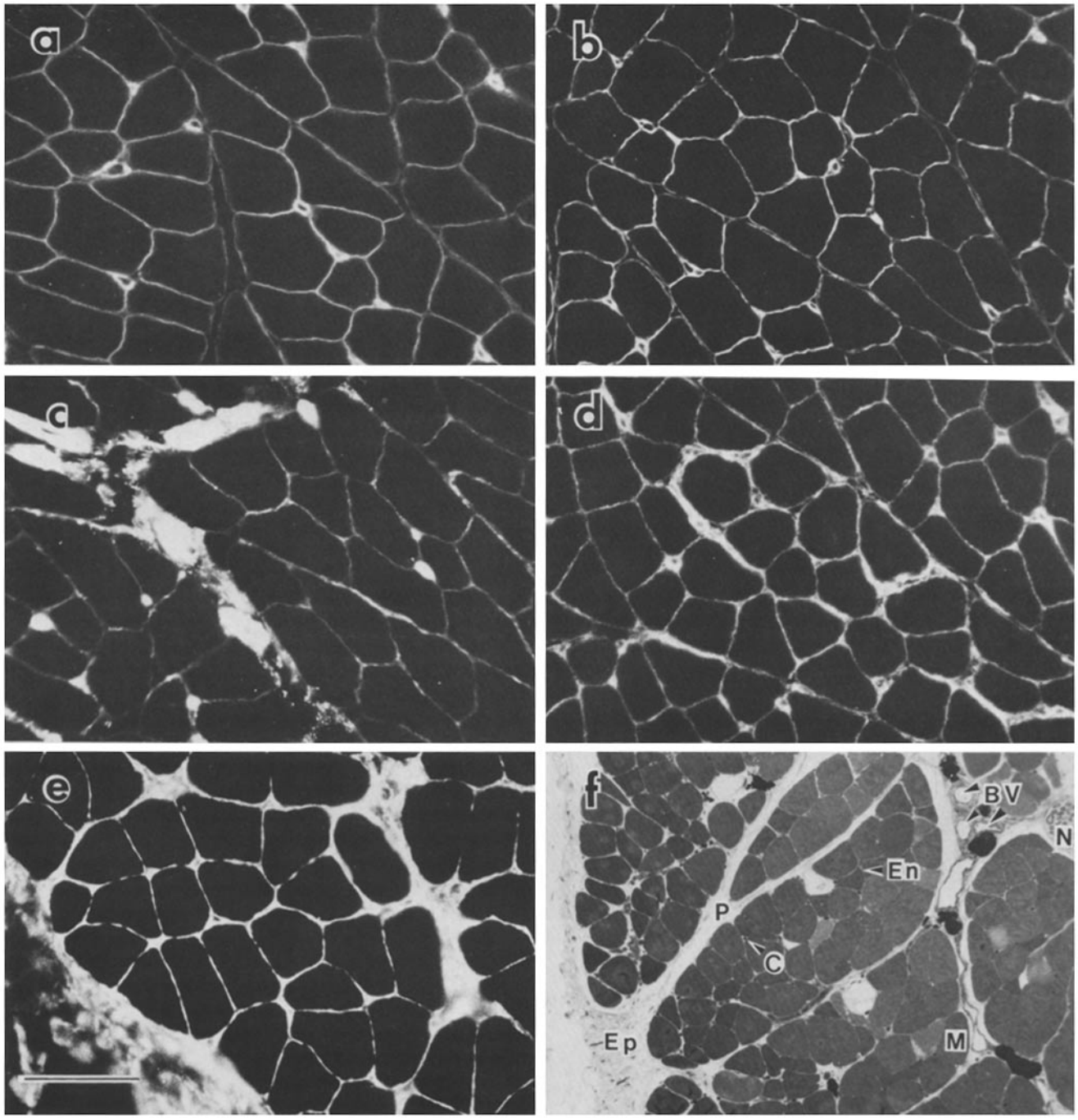


FIGURE 5 Distribution of extracellular matrix antigens in chicken skeletal muscle. Unfixed frozen cross sections of adult chicken skeletal muscle were incubated with (*a*) anti-heparan sulfate proteoglycan antibody-33; (*b*) antilaminin antibody-31; (*c*) antifibronectin antibody-B3; (*d*) antibody-35; and (e) antibody 39. The sections were then stained with a fluorescein-conjugated second antibody. A 1-μm cross section of Epon embedded chicken skeletal muscle, stained with Toluidine blue and shown at low magnification (*f*) is also included to illustrate muscle histology. Anti-heparan sulfate proteoglycan and antilaminin stain the basal lamina around muscle fibers and capillaries. Note the lack of staining in the connective tissue of the perimysium. Fibronectin is detected both in the endomysium around individual muscle fibers and in the perimysium around bundles of muscle fibers. Note the intense staining associated with capillaries and larger blood vessels. Antigens-35 and -39 extend throughout muscle connective tissue. Epimysium (*Ep*); perimysium (*P*), endomysium (*En*); capillary (C); muscle fiber (*M*); blood vessels (*BV*); and nerve (*N*). Bar, 50 μm. × 392.

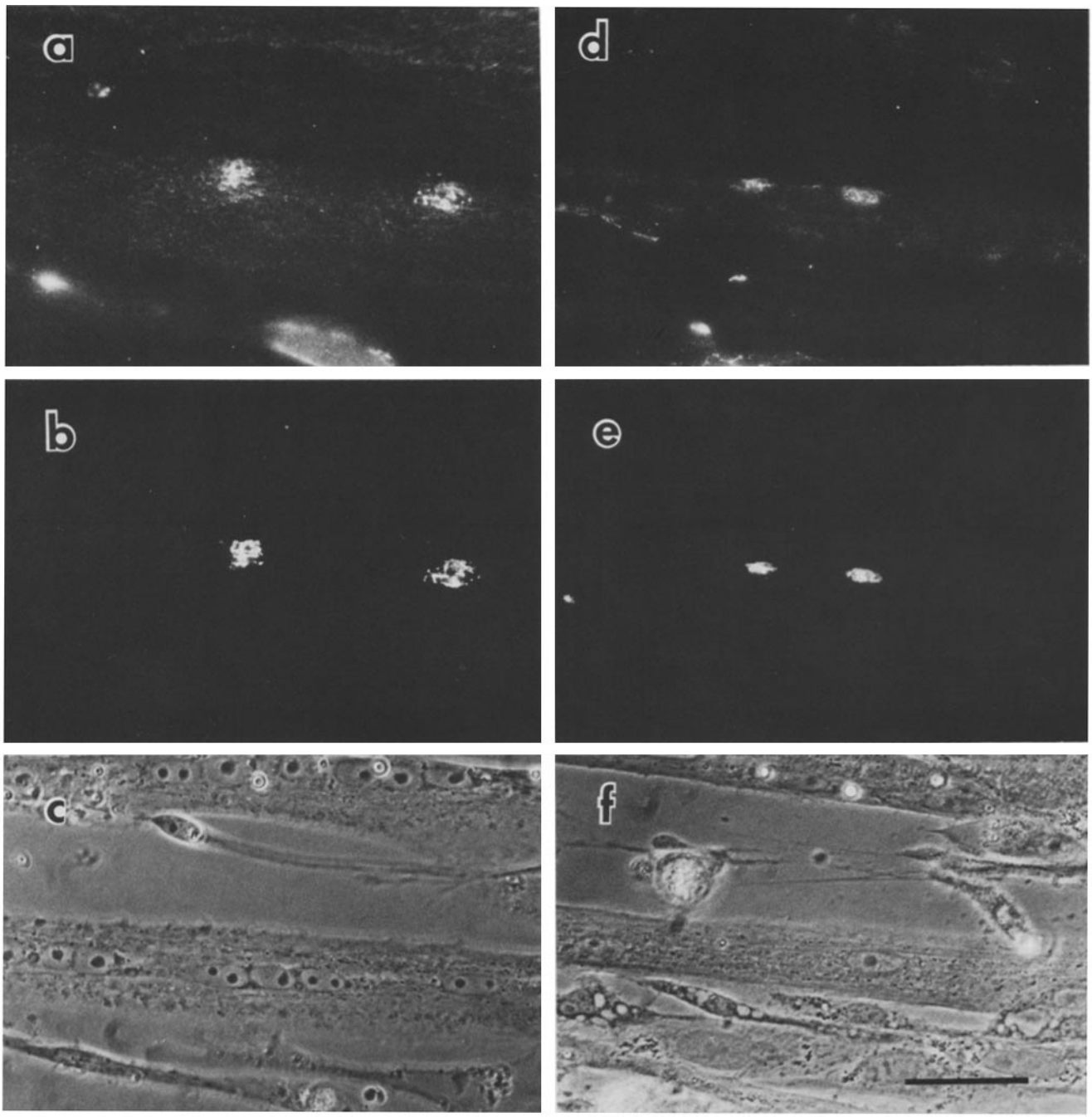


FIGURE 6 Localization of antigens at AChR clusters on cultured chick myotubes. Unfixed cultures were stained with TRITC-αBTX, then monoclonal antibody and a fluorescein-labeled second antibody. *a* and *d*, staining with monoclonal antibodies against heparan sulfate proteoglycan (antibody-33) and laminin (antibody-31), respectively; *b* and *e*, same fields as *a* and *d*, showing rhodamine fluorescence at AChR clusters. *c* and *f*, phase-contrast micrographs of the same fields. Bar, 30μm. × 617.

was unique. This was particularly evident when cultures were counter-stained with TRITC-αBTX, thus revealing the presence of complex AChR aggregates within the muscle plasmalemma (1). Under these conditions it was obvious that there were correlations between the organization of extracellular matrix, particularly basal lamina, and the adjacent plasmalemma. AChR clusters were found in most cases to be associated with discrete plaques containing high concentrations of both laminin (Figs. 6, *d–f* and 7, *a–c*) and a heparan sulfate proteoglycan (6, *a–c*). In the latter case, the morphological organizations of AChR and antigen were virtually congruent (Fig. 6, *a–c*), though additional regions of proteoglycan staining commonly occurred without corresponding AChR clusters. While some congruence between receptor distribution and laminin accumulations was seen (Figs. 6, *d–f* and 7, *a–c*), the correspondence was not as striking as that observed with the heparan sulfate proteoglycan. Often laminin did not completely co-distribute with AChR within the cluster area (Fig. 7, *a–c*).

Unlike the two basal lamina antigens described above, fibronectin was not detected at most AChR clusters. Nonetheless, some receptor patches did have associated fibronectin (see below). When this occurred (Fig. 7, *d–f*), some general correspondence in the size and shape of the receptor cluster

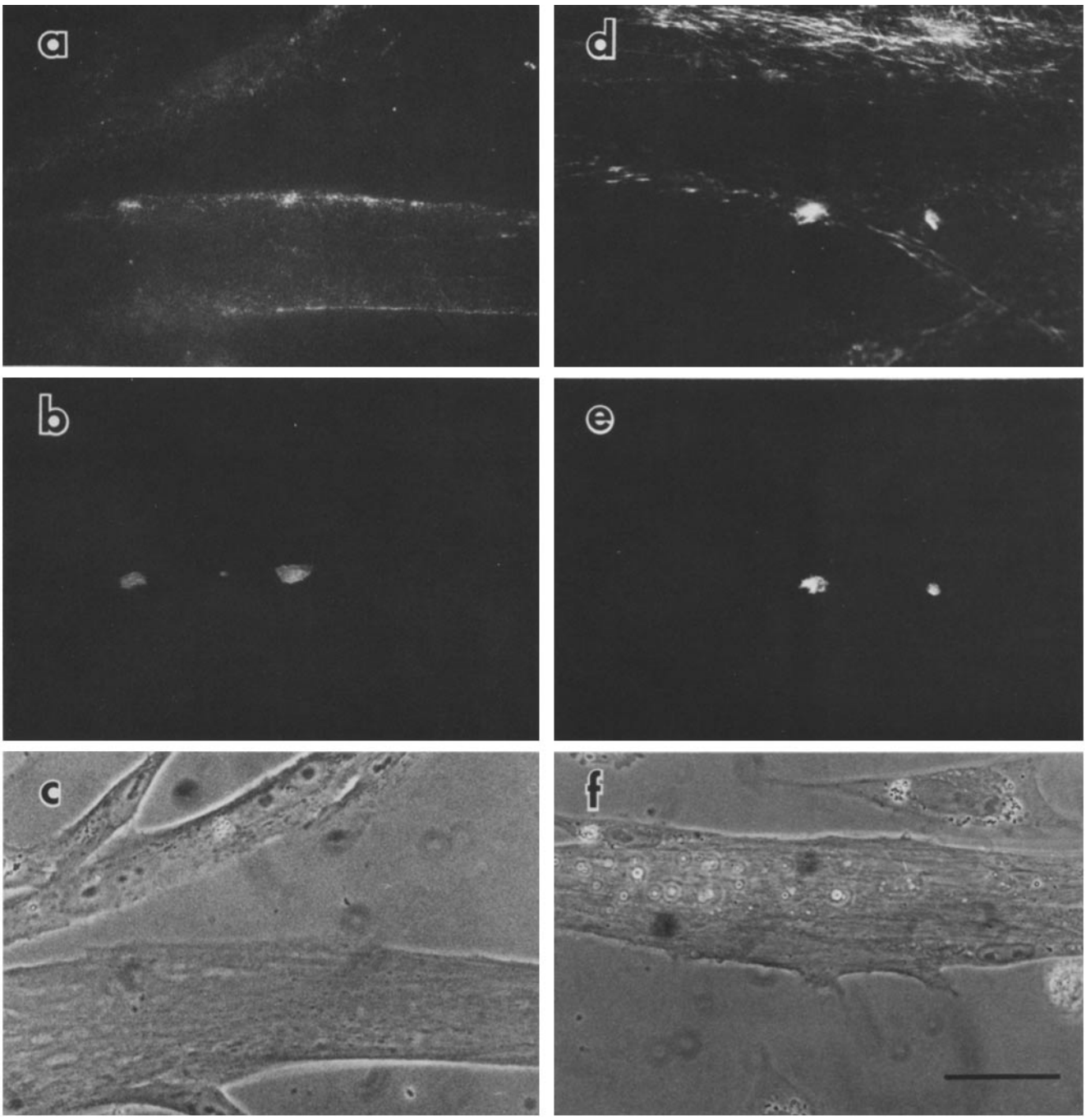


FIGURE 7 Localization of antigens at AChR clusters on cultured chick myotubes. Unfixed cultures were incubated with TRITC-αBTX, then monoclonal antibody and fluorescein-conjugated second antibody. *a* and *d*, staining with antilaminin antibody-31 and antifibronectin antibody-B3, respectively. *b* and *e*, same fields as *a* and *d*, but viewed with rhodamine optics to show TRITC-αBTX at AChR clusters. *c* and *f*, phase-contrast micrographs of the same fields. Bar, 30 μm. × 617.

and antigen patch was often evident. However, the congruence between fibronectin and AChR was even less than that observed with laminin. Most receptor clusters also lacked detectable levels of antigen-39 (Fig. 8, *a–c*), and in those few cases where antigen and receptor accumulations coincided (Fig. 8, *d–f*), there appeared to be little if any homology between the distributions of the two components.

Although the staining of myotubes with antibody-35 tended to be fairly uniform (and usually appeared as a stippling of very short fibrils), occasional discrete regions of more concentrated staining were present and sometimes these regions correlated with AChR clusters (Fig. 9, *d–f*). Most receptor clusters, however, were not associated with discrete accumulations of antigen-35 (Fig. 9, *a–c*).

Degree of Correlation Between Extracellular Matrix Antigens and AChR Clusters

In early experiments it became clear that discrete specializations containing each extracellular matrix antigen developed on the surfaces of cultured myotubes and that some of these coincided with clusters of AChRs. It was also evident

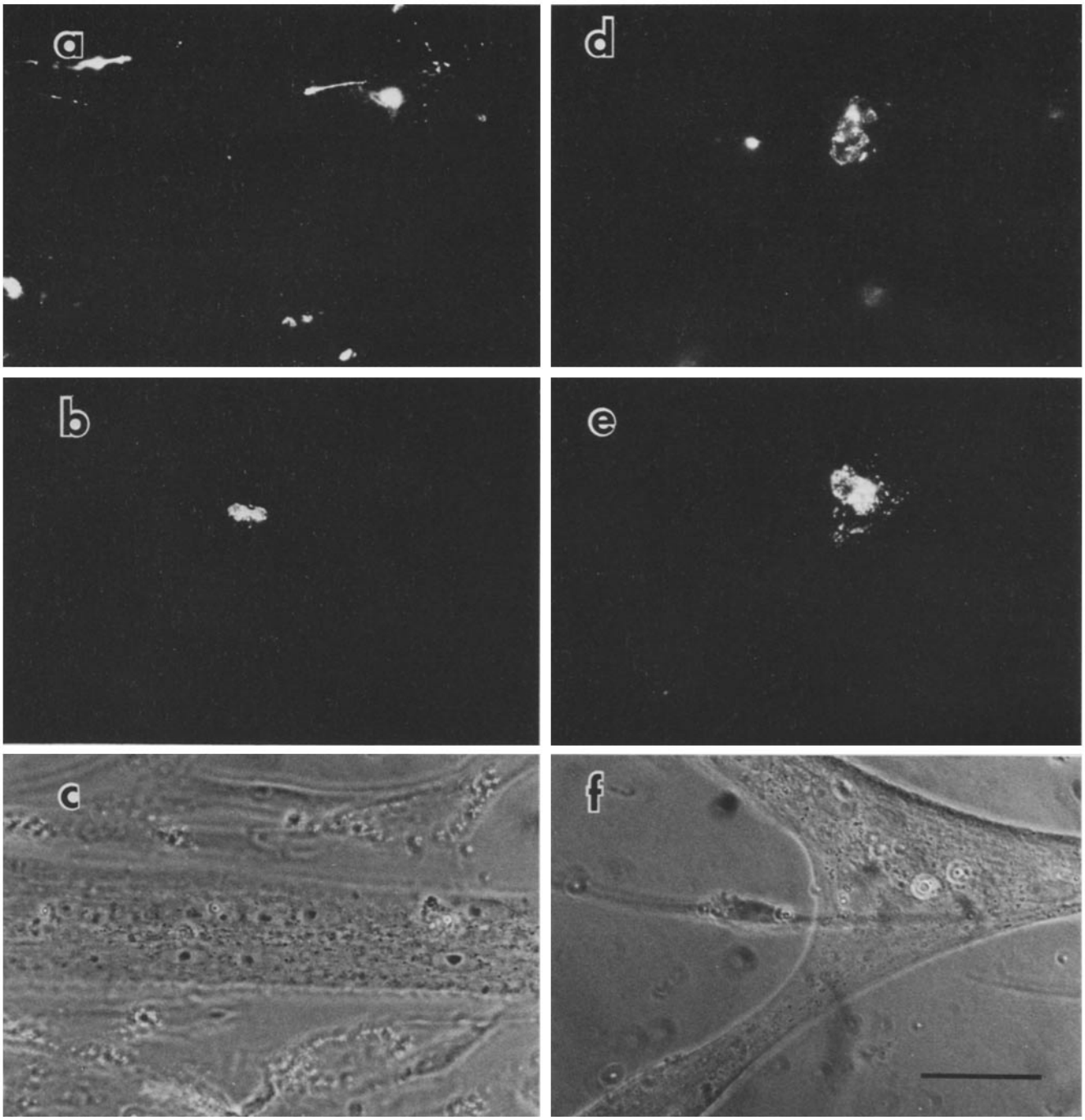


FIGURE 8 Distribution of antigen-39 in chick muscle cultures. Unfixed cultures were treated with TRITC-αBTX followed by monoclonal antibody and fluorescein-conjugated second antibody. *a* and *d*, staining with antibody-39; *b* and *e*, same fields as *a* and *b* but viewed with rhodamine optics to show fluorescent staining of AChR clusters; *c* and *f*, phase-contrast micrographs of the same fields. Often antigen-39 did not co-localize with receptor clusters. When it did, little congruence between the antigen and receptors was noted. Bar, 30 μm. × 617.

that the different antigens co-distributed with AChR clusters to various extents. To rigorously compare the relationship between the organization of each antigen and that of AChR, we scored many AChR clusters in sister cultures for the presence or absence of detectable antigen. Four different sets of cultures, all exhibiting high levels of fusion, were analyzed. Each set contained duplicate cultures stained with each of the five monoclonal antibodies, as well as control cultures stained with antibody from the MOPC P3 × 63 myeloma line. More than 100 receptor clusters were examined in each culture. A receptor cluster was considered to be antigen positive if a localized concentration of antigen showed any overlap with it. The results of this analysis are presented in Fig. 10. As expected from our initial observations, the heparan sulfate proteoglycan recognized by antibody-33 was detected at almost every receptor cluster and little variation was seen among the different sets of cultures. Of 1,324 receptor clusters examined, only 33 were scored as negative. These clusters

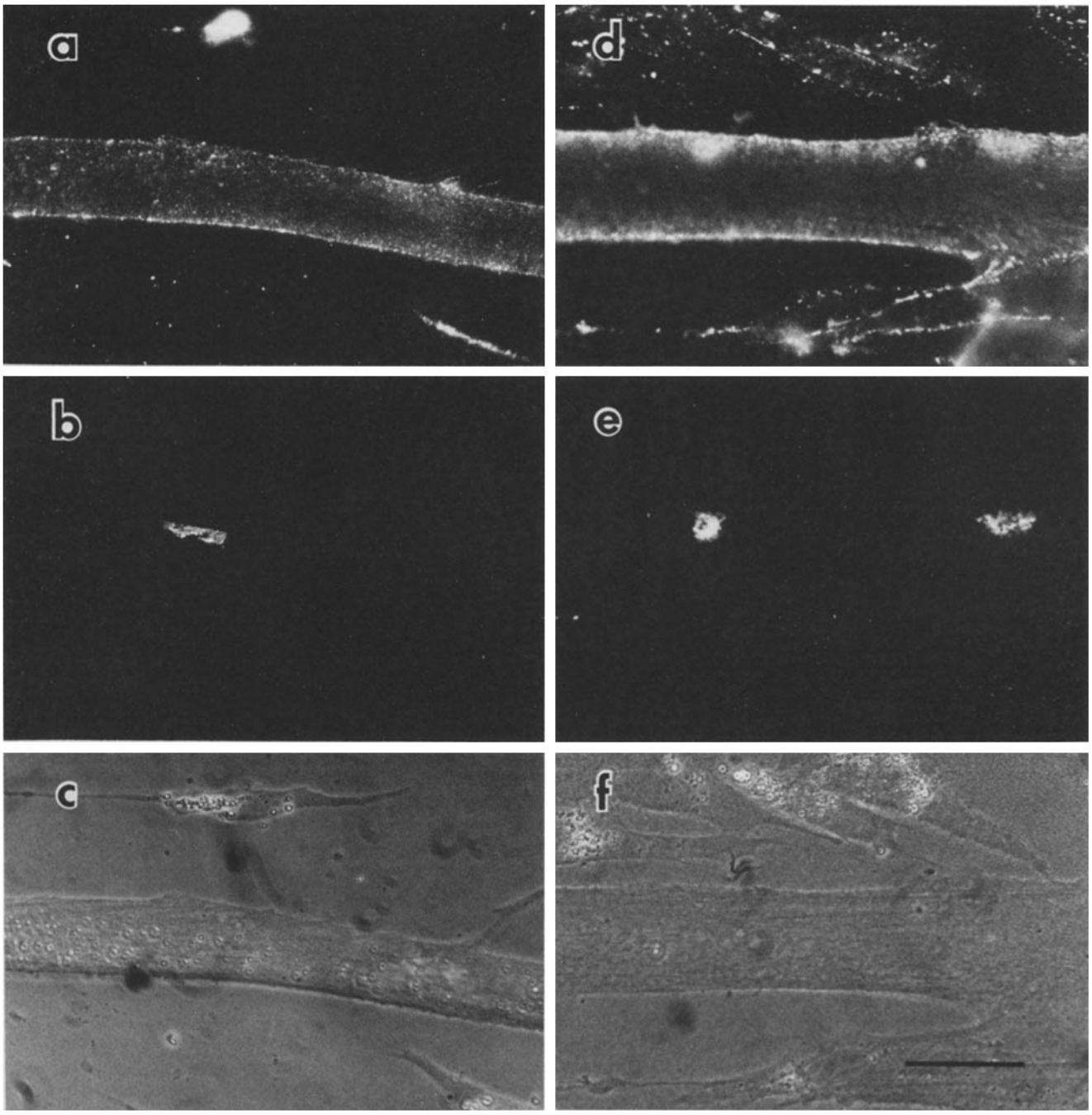


FIGURE 9 Localization of antigen-35 in chick muscle cultures. Unfixed cultures were incubated with TRITC-αBTX, monoclonal antibody, and a fluorescein-conjugated second antibody. *a* and *d* staining with antibody-35; *b* and *e*, same fields, showing rhodamine staining of AChR clusters; *c* and *f*, phase-contrast micrographs of the same fields. In the majority of cases no concentration of antigen (relative to surrounding areas) was associated with AChR clusters (*a–c*). Occasionally, however, co-distribution of antigen and receptor was observed (*d–f*). Bar, 30 μm. × 617.

were invariably on the surface of cells adjacent to the substratum and thus may have been relatively inaccessible to antibody under our staining conditions.

Laminin was also present at most receptor clusters. However, even with this generous scoring method (see above), the degree of co-localization between laminin and AChRs was quite variable, with values ranging from 70 to 90% in different cultures. Despite this variability, the scores obtained for sister cultures within a set were very similar. In contrast to the observations with these basal lamina antigens, fibronectin, which extends through reticular connective tissue in vivo, was detected at a minority of AChR clusters. The proportion of positive receptor clusters in different cultures ranged from 15 to 23%, with an average of 20%. Accumulations of antigens-39 and -35 were likewise found at only 8 and 2% of receptor clusters, respectively.

Accumulation of Heparan Sulfate Proteoglycan during Myogenesis

In view of its striking correspondence in distribution with AChR, the accumulation of the heparan sulfate proteoglycan

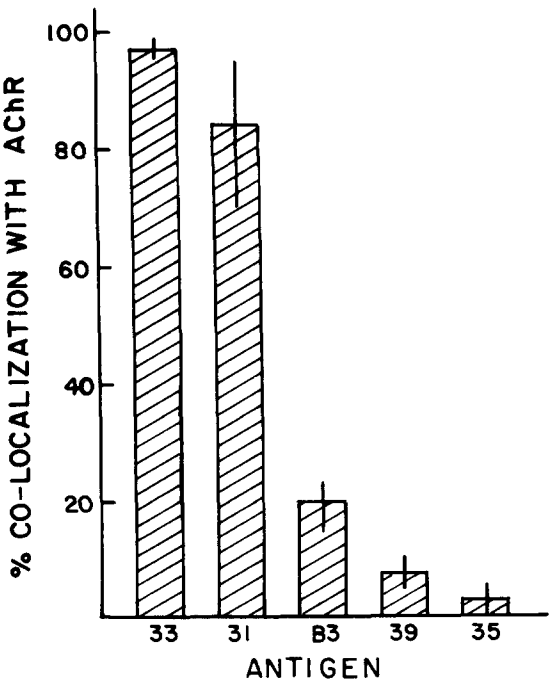


FIGURE 10 Co-localization of matrix antigens and AChR clusters. Muscle cultures were stained with TRITC-αBTX, then monoclonal antibody and a fluorescein-labeled second antibody. By selectively viewing rhodamine fluorescence we were able to identify AChR clusters. Antigen distribution was then analyzed via selective viewing of fluorescein fluorescence. Receptor clusters were scored as positive for co-localization if a concentration of a given antigen (relative to surrounding areas) overlapped the cluster. Bars represent the average percentages of positive AChR clusters in cultures scored for each antigen. Lines represent the range of values obtained from different cultures.

was examined in greater detail primarily to determine whether proteoglycan plaques appeared on the muscle surface before AChR clusters. Primary muscle cultures were thus examined at 24-h intervals following the plating of embryonic myoblasts. After only 1 d in culture, stained material was present on some, but not all, bipolar cells (Fig. 11). Occasional cells with flattened fibroblastic morphology also exhibited staining at this early stage, but no staining of the culture substratum was evident. After 2 d, when bipolar myoblasts were starting to align and fuse, strands containing proteoglycan also became visible on some of the young myotubes. In some cases, this stained material was organized into discrete patches. At this stage, however, AChR clusters revealed by counter-staining with TRITC-αBTX were not yet apparent. Large, branching myotubes had developed by 3 d. Some of these now had AChR clusters, each with a corresponding plaque of antigen. As in the older cultures described earlier, other antigen patches were devoid of AChR clusters. While these observations do not reveal the chronology of appearance of AChR and proteoglycan at individual sites, they do indicate (*a*) that specializations containing the antigen are visible before AChR clustering becomes detectable with these methods, and (*b*) that even at the earliest stages where AChR clusters were detected, a co-distribution between receptors and proteoglycan is evident.

Antigen Organization in Relation to Cultured Fibroblasts

Our findings thus far have demonstrated that each extracellular matrix component shows a different surface organization on cultured myotubes, and that the surface organizations of two basal lamina components correlate closely with adjacent chemical specialization of the myotube plasma-

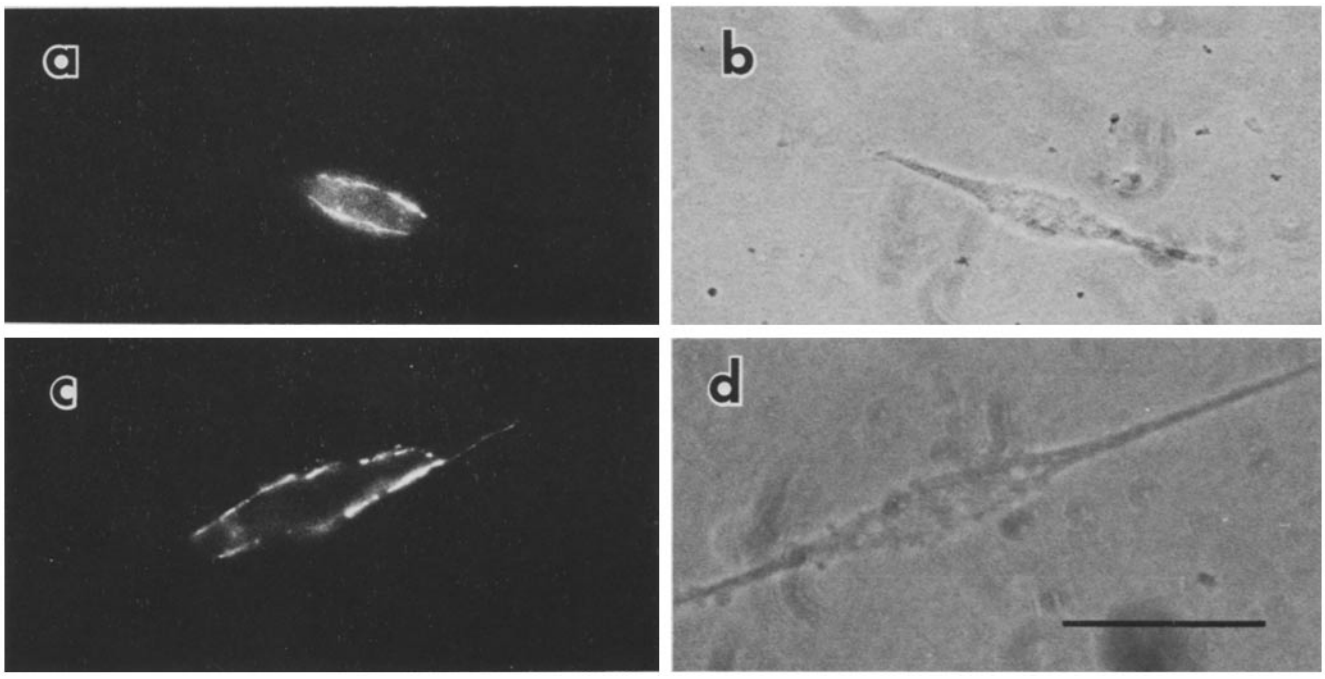
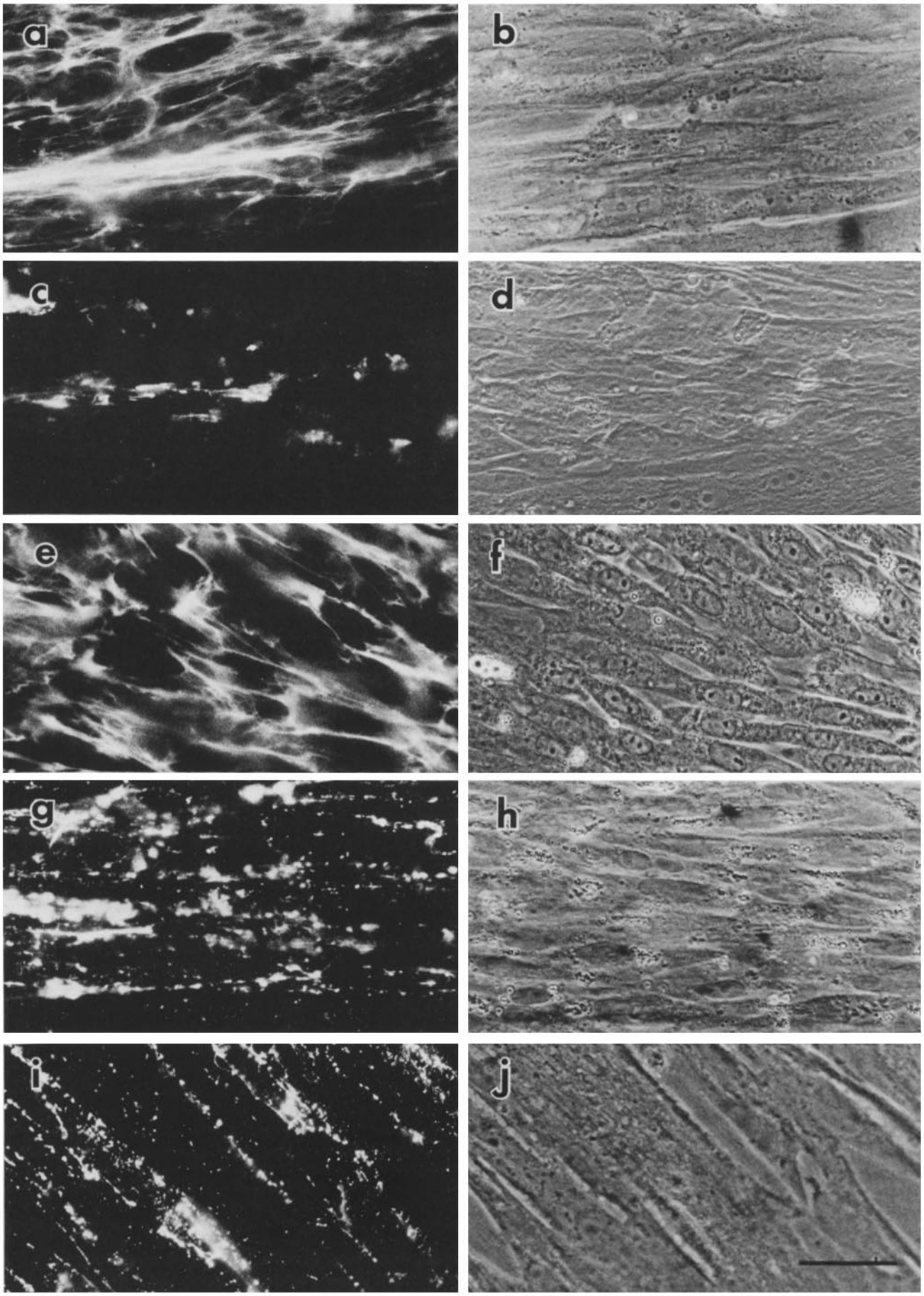


FIGURE 11 Heparan sulfate proteoglycan is associated with myoblasts in 24-h chick muscle cultures. Unfixed cultures were incubated with monoclonal antibody-33 and fluorescein-conjugated second antibody. *a* and *c*, fluorescein fluorescence; *b* and *d*, phase-contrast micrographs of the same fields. Bar, 30 μm. × 882.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lemma. It thus appears likely that specific interactions occur between extracellular matrix components and the muscle plasma membrane. Therefore, it became of interest to examine the arrangement of these same matrix components in relation to fibroblasts. These cells normally reside within muscle connective tissue and are present in conventional muscle cultures. Consequently fibroblasts in muscle cultures, as well as cultures consisting entirely of cells with fibroblastic morphology, were examined (see Materials and Methods).

While little heparan sulfate proteoglycan accumulation was associated with fibroblasts in low density cultures, once confluence was attained a complex fibrillar matrix formed (Fig. 12*a*). This extended over the cell layer showing little obvious correspondence with any individual cell outline and resembled the heparan sulfate proteoglycan-containing matrix that has been described for human embryonic skin fibroblasts (21). Similar matrix accumulations were visible in tertiary fibroblast cultures, and in muscle cultures containing confluent fibroblasts.

Much less staining was seen when cultures containing confluent fibroblasts were exposed to antilaminin antibody-31 (Fig. 12*c*). Morphologically, this matrix was readily distinguishable from that revealed by proteoglycan staining and consisted primarily of scattered patches of stained spots and fibrils. For comparison, Fig. 12*e* shows the elaborate fibronectin-containing network which also forms around confluent fibroblasts, and *g* and *i* (Fig. 12) show antigen-39 and -35 matrices, respectively. Thus, while all of these antigens were found in fibroblast-rich regions, each accumulated with characteristic patterns of distribution. Furthermore, the organization of each antigen was noticeably different in regions of confluent fibroblasts and on myotube surfaces. This observation suggests that each cell type uses different means to control the assembly of extracellular matrix components in its domain.

DISCUSSION

In the present study we have used a number of biochemically characterized monoclonal antibodies to examine the structure and assembly of the extracellular matrix of skeletal muscle. These homogenous immunochemical probes, unlike conventional antisera, can be expected to bind in stoichiometric amounts to individual matrix species, and to be free of contamination with antibodies directed against other matrix substances. These characteristics are obviously of particular value in immunocytochemical studies of the adhesive structural proteins which occupy the extracellular matrix.

In spite of its notable morphological and chemical complexity, the existence of discrete chemical specialization was evident within muscle matrix, even upon analysis at the level of the light microscope. Thus, the organization of basal lamina antigens such as laminin and a heparan sulfate proteoglycan was readily distinguishable from that of other antigens distributed through the reticular lamina and remaining connective tissues of skeletal muscle (see also reference 39). Furthermore, this morphological distinction between the organization of basal lamina and other matrix components was also maintained by embryonic cells developing in the artificial, relative isolation of monolayer cell culture. This could readily be determined by inspection of muscle cultures stained with fluorescent antibody and is demonstrated objectively by the range of correlation that exists between the organization of AChR clusters and the various matrix antigens examined in this study (Fig. 10). This finding is of particular importance since it permits the mechanisms that regulate the assembly of extracellular matrix to be analyzed under controlled conditions in vitro.

While any profound understanding of the assembly of muscle extracellular matrix must obviously await extensive further analysis, several observations of the present study appear significant. The most immediate generalization that can be drawn from these observations of five distinct matrix constituents is that quite different forms of matrix organization develop over myotubes and their adjacent fibroblasts. The extracellular matrix generated by these fibroblasts, isolated from embryonic muscle tissue, showed a complex fibrillar organization which eventually extended over the culture dish without conspicuous relationship to the morphology of individual members of its adjacent cell layer. This, in fact, greatly resembled the extracellular matrix observed with conventional antibody-staining of other fibroblast cells (20, 21). In contrast, the matrix associated with myotubes assumed an obviously different pattern (compare Figs. 6–9 and 12), and showed focal regions of matrix deposition associated with cell surfaces. Indeed, one of these matrix constituents, a heparan sulfate proteoglycan, consistently showed a surface organization very nearly congruent to that of plasma membrane AChR (see also reference 2). These distinctions in matrix organization were apparent even though both cell types make and secrete a variety of these matrix elements (16, 25) and developed together in the presence of the same secreted products. It is reasonable to suppose, from these observations, that the mechanisms regulating the assembly and growth of insoluble matrix structures must depend upon poorly understood characteristics that differ between myogenic and fibroblastic cells, as well as upon passive adhesive interactions that occur between individual matrix components.

Ultrastructural analysis of developing muscle in cell culture has revealed that young myotubes are devoid of the extensive basal lamina which surrounds adult muscle fibers (9, 31). Instead, myotubes in culture initially have only occasional, discrete patches of amorphous extracellular material, some of which appear to coincide with regions of high AChR density (9). Our observations indicate that individual deposits of this amorphous material are likely to vary considerably in chemical composition, and may contain a number of different matrix constituents. Those matrix deposits at AChR clusters are likely to consist primarily of basal lamina components, with less frequent contributions by elements of more distant matrix structures (see also references 2, 11, 42).

The present work demonstrates that AChR clusters are in

FIGURE 12 Antigen distribution in fibroblast-rich regions of chick muscle cultures. Unfixed muscle cultures containing confluent fibroblasts were incubated with monoclonal antibody and fluorescein-labeled second antibody. *a*, Immunofluorescent staining with anti-heparan sulfate proteoglycan antibody-33; *c*, staining with antilaminin antibody-31; (*e*) staining with antifibronectin antibody-B3; (*g*) staining with antibody-39; and *i*, staining with antibody-35. *b*, *d*, *f*, *h*, and *j*, phase-contrast micrographs corresponding to each field. Bar, 30 μm. × 617.

fact almost invariably associated with congruent plaques containing a heparan sulfate proteoglycan. Some, but not all, of these plaques also contain high apparent concentrations of laminin, and occasionally other matrix components. It is thus evident that at these early stages in the development of the myotube basal lamina, the two basal lamina components examined in this study actually showed notably less co-distribution with each other than did proteoglycan and AChR. This observation is of some significance, since it implies that proteoglycan and laminin become deposited on the muscle surface in separate, sequential steps, rather than as a molecular complex with an established stoichiometry. A similar conclusion can be drawn from the even lesser correspondence observed between AChR clusters and extracellular matrix deposits containing a high density of fibronectin.

Our observations likewise indicate that the deposition of laminin within the fibroblast-associated extracellular matrix follows a different time course from that of this proteoglycan (compare Fig. 12, *a* and *c*). Thus an extensive, fibrillar matrix containing the heparan sulfate proteoglycan was evident over fibroblasts at times when laminin deposits could be detected only in discrete, well-separated clumps of short fibrils and spots. This observation differs from the almost complete co-distribution observed for laminin and basal lamina proteoglycan in fibroblast matrices after staining with conventional antisera (20). It remains to be determined whether this apparent contradiction reflects differences in the cellular preparations examined, or in the specificity of the immunochemical reagents.

The particularly close correspondence we have observed between the surface organization of heparan sulfate proteoglycan and AChR aggregates suggests a number of interesting implications. Recently, several independent investigations have reported that heparan sulfate proteoglycans are concentrated in the basal lamina at the neuromuscular junction (2), are present in cholinergic synaptic vesicles (7, 10, 43), show an unusually high avidity for the synaptic form of acetylcholinesterase (47), and may function as substrate-attached nerve growth factors, even for neurons that do not respond to conventional nerve growth factor (27).

In addition to these results, it may also be possible to explain other, more puzzling observations by postulating an intermediary role for proteoglycan. There is evidence that interactions between cultured embryonic muscle cells and either strong electric fields (34) or polycation-coated latex beads (35) can induce a reorganization of the muscle surface, including its distribution of AChR clusters. Since their sulfated glycosaminoglycan side-chains render polyanionic heparan sulfate proteoglycans one of the most highly charged cell surface species, it would not be surprising if important causal events in each of these experiments involved perturbations of proteoglycan organization.

Taken together, these observations suggest that heparan sulfate proteoglycans of the muscle surface are of particular regulatory significance, perhaps contributing to both the immobilization of synaptic elements within a locally specialized region of the cell surface and to the transmission of inductive or "trophic" signals between nerve and muscle. It would, in fact, be particularly satisfying if both of these hypothetical roles were mediated by the same molecular species of heparan sulfate proteoglycan, since this would imply that embryonic motor neurons induce the development of a high junctional concentration of their own survival-promoting proteoglycan during synaptogenesis. Such a reward would be of obvious value for ensuring the survival of neurons with appropriate synaptic connections.

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REFERENCES

1. Anderson, M. J., and M. W. Cohen. 1974. Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. *J. Physiol. (Lond.).* 237:385–400.
2. Anderson, M. J., and D. M. Fambrough. 1983. Aggregates of acetylcholine receptors are associated with plaques of a basal lamina heparan sulfate proteoglycan on the surface of skeletal muscle fibers. *J. Cell Biol.* 97:1396–1411.
3. Bader, D. 1981. Density and distribution of α-bungarotoxin-binding sites in postsynaptic structures of regenerating rat skeletal muscle. *J. Cell Biol.* 88:338–345.
4. Bayne, E. K., J. Gardner, and D. M. Fambrough. 1981. Monoclonal antibodies to extracellular matrix antigens in chicken skeletal muscle. *Cold Spring Harbor Rep. Neurosci.* 2:259–270.
5. Betz, W., and B. Sakmann. 1973. Effects of proteolytic enzymes on function and structure of frog neuromuscular junctions. *J. Physiol. (Lond.).* 230:673–688.
6. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
7. Buckley, K. M., E. S. Schweitzer, G. P. Miljanich, L. Clift-O'Grady, P. D. Kushner, L. F. Reichardt, and R. B. Kelly. 1983. A synaptic vesicle antigen is restricted to the junctional region of the presynaptic plasma membrane. *Proc. Natl. Acad. Sci. USA* 80:7342–7346.
8. Burden, S. J., P. B. Sargent, and U. J. McMahan. 1979. Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of nerve. *J. Cell Biol.* 82:412–425.
9. Burrage, T. G., and T. L. Lentz. 1981. Ultrastructural characterization of surface specializations containing high-density acetylcholine receptors on embryonic chick myotubes *in vivo* and *in vitro*. *Dev. Biol.* 85:267–286.
10. Carlson, S. S., and R. B. Kelly. 1983. A highly antigenic proteoglycan-like component of cholinergic synaptic vesicles. *J. Biol. Chem.* 258:11082–11091.
11. Daniels, M. P., M. Vigny, P. Sonderegger, H. -C. Bauer, and Z. Vogel. 1984. Association of laminin and other basement membrane components with regions of high acetylcholine receptor density on cultured myotubes. *International Journal of Developmental Neuroscience.* 2:87–99.
12. Duance, V. C., H. R. Stephens, M. Dunn, A. J. Bailey, and V. Dubowitz. 1980. A role for collagen in the pathogenesis of muscular dystrophy? *Nature (Lond.).* 284:470–472.
13. Fambrough, D. M., and E. K. Bayne. 1983. Multiple forms of (Na^+ + K^+)-ATPase in the chicken: selective detection of the major nerve, skeletal muscle, and kidney form by a monoclonal antibody. *J. Biol. Chem.* 258:3926–3935.
14. Fambrough, D. M., E. K. Bayne, J. M. Gardner, M. J. Anderson, E. Wakshull, and R. L. Rotundo. 1982. Monoclonal antibodies to skeletal muscle cell surface. *In* Neuroimmunology. J. Brockes, editor. Plenum Press, New York. 49–89.
15. Fischbach, G. D., and S. A. Cohen. 1973. The distribution of acetylcholine sensitivity over uninnervated and innervated muscle fibers grown in cell culture. *Dev. Biol.* 31:147–162.
16. Gardner, J. M., and D. M. Fambrough. 1983. Fibronectin expression during myogenesis. *J. Cell Biol.* 96:474–485.
17. Hall, Z. W., and R. B. Kelly. 1971. Enzymatic detachment of endplate acetylcholinesterase from muscle. *Nat. New Biol.* 232:62–63.
18. Hartzell, H. C., and D. M. Fambrough. 1973. Acetylcholine receptor production and incorporation into membranes of developing muscle fibers. *Dev. Biol.* 30:153–165.
19. Hassell, J. R., D. A. Newsome, and G. R. Martin. 1981. Isolation and characterization of the proteoglycans and collagens synthesized by cells in culture. *Vision Res.* 21:49–53.
20. Hayman, E. G., A. Oldberg, G. R. Martin, and E. Ruoslahti. 1982. Co-distribution of heparan sulfate proteoglycan, laminin, and fibronectin in the extracellular matrix of normal rat kidney cells and their coordinate absence in transformed cells. *J. Cell Biol.* 94:28–35.
21. Hedman, K., S. Johansson, T. Vartio, L. Kjellén, A. Vaheri, and M. Höök. 1982. Structure of the pericellular matrix: association of heparan and chondroitin sulfates with fibronectin-procollagen fibers. *Cell.* 28:663–671.
22. Hogan, B. L. M., A. Taylor, M. Kurkinen, and J. R. Couchman. 1982. Synthesis and localization of two sulphated glycoproteins associated with basement membranes and the extracellular matrix. *J. Cell Biol.* 95:197–204.
23. Kefalides, N. A., R. Alper, and C. C. Clark. 1979. Biochemistry and metabolism of basement membranes. *Int. Rev. Cytol.* 61:167–228.
24. Kennett, R. H., K. A. Denis, A. S. Tung, and N. R. Klinman. 1978. Hybrid plasmacytoma production: fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. *Curr. Top. Microbiol. Immunol.* 81:77–93.
25. Kühl, U., R. Timpl, and K. Von Der Mark. 1982. Synthesis of type IV collagen and laminin in cultures of skeletal muscle cells and their assembly on the surface of myotubes. *Dev. Biol.* 93:344–354.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
27. Lander, A. D., D. K. Fujii, D. Gospodarowicz, and L. F. Reichardt. 1982. Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. *J. Cell Biol.* 94:574–585.
28. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^3H and ^{14}C in

polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
29. Laterra, J., R. Ansbacher, and L. A. Culp. 1980. Glycosaminoglycans that bind cold-insoluble globulin in cell-substratum adhesion sites of murine fibroblasts. *Proc. Natl. Acad. Sci. USA.* 77:6662–6666.
30. Linker, A., and P. Hovingh. 1972. Heparinase and heparitinase from flavobacteria. *Methods Enzymol.* 28:903–911.
31. Lipton, B. H. 1977. Collagen synthesis by normal and bromodeoxyuridine-modulated cells in myogenic culture. *Dev. Biol.* 61:153–165.
32. McClean, I. W., and P. K. Nakane. 1974. Periodate-lysine paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077–1083.
33. McMahan, U. J., J. R. Sanes, and L. M. Marshall. 1978. Cholinesterase is associated with the basal lamina at the neuromuscular junction. *Nature (Lond.).* 271:172–174.
34. Orida, N., and M.-M. Poo. 1978. Electrophoretic movement and localization of acetylcholine receptors in the embryonic muscle cell membrane. *Nature (Lond.).* 275:31–35.
35. Peng, H. B., P.-C. Cheng, and P. W. Luther. 1981. Formation of ACh receptor clusters induced by positively charged latex beads. *Nature (Lond.).* 292:831–834.
36. Rennard, S. I., R. Berg, G. R. Martin, J.-M. Foidart, and P. Gehron-Robey. 1980. Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Biochem.* 104:205–214.
37. Rodén, L. 1980. Structure and metabolism of connective tissue proteoglycans. *In* The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Press, New York. 267–371.
38. Ruoslahti, E., and E. Engvall. 1980. Complexing of fibronectin, glycosaminoglycans and collagen. *Biochim. Biophys. Acta* 631:350–358.
39. Sanes, J. R. 1982. Laminin, fibronectin, and collagen in synaptic and extrasynaptic portions of muscle fiber basement membrane. *J. Cell Biol.* 93:442–451.
40. Sanes, J. R., L. M. Marshall, and U. J. McMahan. 1978. Reinnervation of muscle fiber basal lamina after removal of myofibers. Differentiation of regenerating axons at original synaptic sites. *J. Cell Biol.* 78:176–198.
41. Sanes, J. R., and Z. W. Hall. 1979. Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. *J. Cell Biol.* 83:357–370.
42. Silberstein, L., N. C. Inestrosa, and Z. W. Hall. 1982. Aneural muscle cell cultures make synaptic basal lamina components. *Nature (Lond.).* 295:143–145.
43. Stadler, H., and G. H. C. Dowe. 1982. Identification of a heparan sulfate-containing proteoglycan as a specific core component of cholinergic synaptic vesicles from *Torpedo marmorata. EMBO J. (Eur. Mol. Biol. Organ.)* 1:1381–1384.
44. Suzuki, S. 1972. Chondroitinases from *Proteus vulgarus* and *Flavobacterium heparinum. Methods Enzymol.* 28:911–917.
45. Sytkowski, A. J., Z. Vogel, and M. W. Nirenberg. 1973. Development of acetylcholine receptor clusters on cultured muscle cells. *Proc. Natl. Acad. Sci. USA.* 70:270–274.
46. Timpl, R., H. Rohde, P. G. Robey, S. I. Rennard, J.-M. Foidart, and G. R. Martin. 1979. Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* 254:9933–9937.
47. Vigny, M., G. R. Martin, and G. R. Grotendorst. 1983. Interactions of asymmetric forms of acetylcholinesterase with basement membrane components. *J. Biol. Chem.* 258:8794–8798.
48. Wakshull, E., E. K. Bayne, M. Chiquet, and D. M. Fambrough. 1983. Characterization of a plasma membrane glycoprotein common to myoblasts, skeletal muscle satellite cells, and glia. *Dev. Biol.* 100:464–477.
49. Yamada, K. M., D. W. Kennedy, K. Kimata, and R. M. Pratt. 1980. Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteolytic fragments. *J. Biol. Chem.* 255:6055–6063.