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,
Extracellular Matrix Organization on Skeletal Muscle

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 BALB/cJ 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 12- to 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 PMSF, 1 mM PMSF, washed again, resuspended in 50 ml of similar buffer, and extracted with stirred 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, 125I-labeled rabbit antimouse 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.

Materials and Methods

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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.
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* condronitae 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-Cl, pH 6.8, containing 10 mM CaCl2, 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 gels. Samples were boiled for 5 min unless otherwise specified. After electrophoresis, the gels were impregnated with 2,5-diphenyloxazole (6, 28) or Enhance Acufine developer. Bands the gels were visualized with narrow-band selective filter combinations for FITC and TRITC. Immunoprecipitates and conditioned medium were prepared for electrophoresis by addition of concentrated sample buffer, to yield final concentrations of 5% SDS, 0.125 M Tris-Cl, pH 6.8, 0.1 M dithiothreitol. Samples were boiled for 5 min unless otherwise specified. After electrophoresis, the 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 pre flaming) at −70°C. Apparent molecular weights of cell products were estimated as compared with standards including: fibronectin (Mr = 220,000), β-galactosidase (Mr = 116,000), phosphorylase a (Mr = 95,000), BSA (Mr = 68,000), pyruvate kinase (Mr = 57,000), ovalbumin (Mr = 43,000), and deoxyribonuclease (Mr = 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. Cultures were coated with rat-tail collagen. Chick muscle-derived fibroblasts were obtained from similar suspensions by plating the cells for 1 h and centrifugation. After digestion in molecular weight to the unlabeled mouse laminin were observed. 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 [³⁵S]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 immunochemical analysis of antigen distribution in muscle sections (see below), strongly suggest that antibody-31 recognizes chicken laminin. 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.
Antibody-33 immunoprecipitates also contained $[^{35}S]$methionine-labeled polypeptides of apparent molecular weights of 230,000 and 95,000 (Fig. 2a) that were without detectable sulfate label under our experimental conditions (Fig. 2b). 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.)
dase and keratanase exhibited detectable levels of protease contamination, the chondroitinase ABC and two separate with the analogous.

These observations are virtually identical to those obtained 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.

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

As both the immunocytochemical (see below) and immunohistochemical 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. 2a, lane 2, and 2b, 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 (Fig. 4a, 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 Duanne 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. 4b, 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 \(\beta\)-aminopropionitrile and ascorbate) was used in these experiments. Control experiments were carried out using a rabbit antiserum to chicken collagen. As shown in Fig. 4b (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

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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. 4b, 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. Immuno precipitation performed with antibody-35 (from [35S]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 muscular 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. 5c), 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. 5c), 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. 5c) 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. 5d), 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-α-BTX 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-α-BTX (data not shown). It should also be noted that under these experimental conditions, some of the fibronectin occasionally appeared to co-distribute with labeled α-BTX. 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-α-BTX 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

![Image of figure 5 showing the distribution of extracellular matrix antigens in chicken skeletal muscle.](https://example.com/figure5.jpg)

**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.
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 plasmamembrane (1). Under these conditions it was obvious that there were correlations between the organization of extracellular matrix, particularly basal lamina, and the adjacent plasmamembrane. 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 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, 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...
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 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. x 617.
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
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-
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. 12a). 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. 12c). 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. 12e 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](https://example.com/figure12.png) 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-83; (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.

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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 immunological 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), and are present in cholinergic synaptic vesicles (7, 10, 43), show an unusually high avidity for the synaptic form of acetylcholine receptors on embryonic chick myotubes in wvo and in vitro. Beil. Biol. 85:267-286.


