Laminin, Fibronectin, and Collagen in Synaptic and Extrasynaptic Portions of Muscle Fiber Basement Membrane

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ABSTRACT   Light and electron microscope immunohistochemical methods were used to study the distribution of several proteins in rat skeletal muscle. The aims were to identify components of muscle fiber basement membrane and to compare the small fraction (0.1%) of the basement membrane that extends through the synaptic cleft at the neuromuscular junction with the remaining, extrasynaptic portion. Synaptic basement membrane is functionally specialized and plays important roles in neuromuscular function and regeneration. Laminin, fibronectin, collagen IV, collagen V, and a collagenous protein (high-salt-soluble protein [HSP]) are all present in muscle fiber basement membrane. Laminin and collagen IV are concentrated in basal lamina (the feltlike, inner layer of the basement membrane) and are shared by synaptic and extrasynaptic regions. Fibronectin, also present synaptically and extrasynaptically, is present in basal lamina and in the overlying reticular lamina. Collagen V and HSP are present throughout extrasynaptic basement membrane but are absent from synaptic sites; HSP is concentrated in the reticular lamina and on the outer surface of the basal lamina. These results, together with experiments reported previously (Sanes and Hall, 1979, *J. Cell Biol.* 83:357–370), provide examples of three classes of components in muscle fiber basement membrane—synaptic, extrasynaptic, and shared.

Each muscle fiber in vertebrate skeletal muscles is ensheathed by a basement membrane. A small fraction of the basement membrane, ~0.1% of the total, occupies the synaptic cleft between nerve and muscle at the neuromuscular junction. Recent experiments have demonstrated that this synaptic portion of the basement membrane is specialized and plays important roles in neuromuscular function and regeneration (1, 2, 5, 19, 28, 36). These results motivate molecular analysis of muscle-fiber basement membrane and comparison of its synaptic and extrasynaptic portions. In a previous study, we used immunohistochemical methods to show that several antigens are present in synaptic but not extrasynaptic basement membrane (35). Here, to extend this analysis to extrasynaptic areas, we asked two questions: (a) Are any proteins that have been purified and characterized from other sources present in rat muscle fiber basement membrane? And (b) are proteins which are present in extrasynaptic basement membrane also present at the synapse or are there antigens specific to extrasynaptic regions? We found that three proteins recently shown to be components of basement membranes in several tissues—laminin (9, 13, 25, 32, 37, 46), collagen IV (4, 33, 38, 45, 51), and fibronectin (10, 26, 29, 42, 50)—are present in synaptic as well as extrasynaptic muscle fiber basement membrane. Two other

proteins—collagen V (4, 6, 11, 15, 16, 31, 33) and a collagenous protein (HSP) isolated from skeletal muscle connective tissue—are present in extrasynaptic basement membrane but are excluded from synaptic sites.

MATERIALS AND METHODS

Antibodies

LAMININ: Antibodies to laminin were provided by Dr. Rupert Timpl (Max-Planck Institut fur Biochemie) and by Dr. Albert Chung (University of Pittsburgh). Dr. Timpl and colleagues purified laminin from the mouse EHS sarcoma (46), produced antisera in rabbits, and purified the antibody by affinity chromatography (32). Dr. Chung provided both whole rabbit antiserum and affinity-purified antibody to a protein, GP2, isolated from extracellular matrix secreted by the mouse embryonal carcinoma-derived endodermal cell line M1536-B3 (9); GP-2 has been shown to be a subunit of laminin (34, 46). All three preparations were studied by immunofluorescence (Fig. 2a shows anti-EHS laminin and Figs. 3a and 6 show anti-GP2); anti-EHS-laminin antibody (Fig. 4a) and anti-GP2 serum were localized with peroxidase-conjugated second antibody; and anti-EHS laminin was used for experiments with ferritin-conjugated second antibody (Fig. 5c).

COLLAGEN IV: Three highly purified antibodies to collagen IV were used. Two, provided by Dr. Rupert Timpl, were prepared against collagen IV isolated from EHS sarcoma (45); one antiserum was produced in a goat and one in a guinea pig, and both were purified by affinity chromatography. Antibody to the C-chain of collagen IV (23) from human placenta was produced in a rabbit, purified by affinity chromatography (15, 39), and provided by Dr. Steffen Gay

(University of Alabama Medical Center). All three antibodies were studied by immunofluorescence (Fig. 3 e shows goat anti-EHS collagen IV); goat anti-tumor collagen IV was used for immunoperoxidase experiments; and rabbit anti-human collagen IV (C) was used with ferritin-labeled second antibody.

FIBRONECTIN: Affinity-purified rabbit antibody (47) to human plasma fibronectin (27) was the gift of Dr. John McDonald, Department of Medicine, Washington University Medical Center. This antibody was used for light and electron microscope immunohistochemistry (Figs. 2 b, 3 g, 4 c, and 5 e). Two other rabbit antisera to human plasma fibronectin were studied by the immunofluorescence method only: one was provided by Dr. Lan Bo Chen (Harvard Medical School) (8), and the other was purchased from N. L. Cappel Laboratories (Cochranville, PA).

COLLAGEN V: Affinity-purified antibody to collagen V (A and B chains) was provided by Dr. Steffen Gay. The collagen was purified from pepsin digests of human placenta (31), antiserum was produced in rabbits, and specific antibodies were purified by affinity chromatography (39).

HIGH-SALT-SOLUBLE PROTEINS (HSP) FROM MUSCLE: Antisera to a collagenous complex from rat muscle, HSP, were prepared in rabbits. Preparation and characterization of HSP are detailed below. Each of three rabbits was immunized with two aliquots of HSP. The first aliquot was emulsified in complete Freund's adjuvant (Cappel Laboratories) to which 5 mg of tubercle bacillus (Difco Laboratories, Detroit, MI) had been added; the second dose, given 1-3 wk later, was emulsified in incomplete Freund's adjuvant (35). Two rabbits received 2 mg of HSP per dose, and one received 0.4 mg per dose. The rabbits were bled 2 wk after the second injection and at 2- to 4-wk intervals thereafter. Three experiments showed that the sera contained antibodies to HSP. First, immunofluorescent staining of muscle by anti-HSP was markedly reduced by preincubation of the antiserum with electrophoretically pure (Fig. 1 a) HSP (5 μl anti-HSP and 10 μg HSP; Fig. 1 b and c). Second, antibody binding was detected in a solid-state radioimmunoassay, using HSP as antigen (Fig. 1 d). Briefly, wells in a microtiter plate were coated with HSP (50 μg/ml), incubated overnight at 4°C with serial dilutions of antiserum, reincubated for 8 h at 20°C with ^{125}I-goat anti-rabbit IgG, washed, cut out, and counted in a gamma counter (see reference 20 for details). Third, HSP was precipitated from solution by anti-HSP but not by preimmune serum. The immune complex was precipitated with Protein A-Sepharose (Sigma Chemical, St. Louis, MO) and HSP was detected in the precipitate by SDS PAGE (24). One antiserum (from a rabbit injected with 2-mg aliquots) was used for light- and electron-microscope studies; sera from the other two rabbits were studied by immunofluorescence only and gave similar results.

ACETYLCHOLINESTERASE: Rabbit antiserum (17) to acetylcholinesterase purified from bovine brain (7) was a gift of Drs. Anthony Trevor and Alvin Greenberg (University of California, San Francisco). Because brain contains globular acetylcholinesterase but little or none of the collagen-tailed form of the enzyme (3), it is likely that this antiserum recognizes the globular subunit that is shared by tailed and nontailed acetylcholinesterase.

CONTROL SERA: Sera collected from rabbits before they were immunized served as controls.

SECOND ANTIBODIES: Fluorescein-, horseradish peroxidase-, and ferritin-conjugated goat anti-rabbit IgG, fluorescein-conjugated goat anti-guinea pig IgG, and peroxidase-conjugated rabbit anti-goat IgG were obtained from N. L. Cappel Laboratories; fluorescein-conjugated rabbit anti-goat IgG was purchased from Miles Laboratories (Elkhart, IN) and ferritin-conjugated goat anti-rabbit IgG was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands).

Preparation and Properties of HSP

A collagenous complex (HSP) was isolated from rat muscle. A connective tissue-rich fraction was prepared from leg muscle, suspended in 0.5 N acetic acid, and digested with pepsin as previously described (35). After 4 d, solid NaCl was added to 1 M, the extract was centrifuged, and the precipitate was redissolved in 0.5 M NaCl adjusted to pH 7 with Tris. Most of the interstitial and basement membrane collagen was precipitated by dialysis overnight against 4.5 M NaCl, 20 mM Tris, pH 7.4, and removed by centrifugation. Glacial acetic acid was then added to the supernatant to a final concentration of 1 M. After 2 h at 4°C, the precipitate was collected by centrifugation and redissolved in a small volume of water neutralized with Tris. This solution was dialyzed overnight against 5 mM Tris, pH 7.2 to remove residual salt and then centrifuged. The yellowish supernatant was discarded, the precipitate was redissolved in 1 M NaCl and the final dialysis step was repeated. The resulting precipitate, called HSP, was used to immunize rabbits as described above. About 0.1 g HSP was obtained per kilogram of muscle.

HSP consists of high molecular weight complexes of collagenous proteins joined by disulfide bonds. No protein entered 7% SDS polyacrylamide gels (24) in the absence of a reducing agent, while reduction with 2% β-mercaptoethanol revealed three bands with apparent molecular weights of 49,000 ± 500, 57,000 ± 800 and 62,000 ± 900 (mean ± SD of six determinations, using the noncollag-

enous proteins bovine serum albumin, pyruvate kinase, fumarase, ovalbumin, aldolase, and glyceraldehyde-6-phosphate dehydrogenase as standards; see Fig. 1 a). No other major bands were visible on Coomassie-Blue-stained gels. At low concentrations of β-mercaptoethanol (0.001-0.01%), several intermediate bands, presumably representing partially reduced HSP, were visible on gels. The 49,000 and 57,000 subunits are basic (pI ≥ 8) and the 62,000 subunit is acidic (pI ≤ 5) as determined by two-dimensional gel electrophoresis of reduced HSP with isoelectric focussing in the first dimension (method C in reference 41). HSP is collagenous as judged by amino acid analysis of the native complex (mole percents of hydroxyproline, proline, glycine, and hydroxylysine were 8, 9, 28, and 3 in a single determination). The complex is partially hydrolyzed by highly purified bacterial collagenase (Advance Biofactures, Lynbrook, NY) under conditions where hydrolysis of bovine serum albumin (BSA) is negligible (280 U collagenase/ml, 18-h incubation at 37°C), but HSP is far less collagenase-sensitive than rat muscle collagens I or V. In terms of its subunit structure, molecular weight, pI, amino acid composition, and relative insensitivity to collagenase, HSP resembles the "unique collagenous fraction" isolated from placenta by Furoto and Miller (14; see also reference 21).

Histology

IMMUNOFLUORESCENCE: The binding of antibodies to rat skeletal muscle was detected by an indirect immunofluorescence method that has been described in detail elsewhere (35). Briefly, cryostat sections of unfixed diaphragm were incubated with the appropriate antibody and then with a mixture of fluorescein 'second antibody' (goat anti-rabbit IgG or rabbit anti-goat IgG) and rhodamine-α-bungarotoxin (prepared by the method of Ravdin and Axelrod; see reference 30). Stained sections were examined and photographed with filters appropriate to show either fluorescein or rhodamine. Rhodamine-bungarotoxin binds specifically to acetylcholine receptors and thus marks synaptic sites, which are undetectable in cryostat sections without a specific stain.

To study the binding of antibodies to basement membrane in the absence of the underlying plasma membrane, we used injured muscles; myofibers degenerate and are phagocytized after damage, but their sheaths of basement membrane persist (35, 36, 49). Cuts were made in external intercostal muscles as described previously (35). 3 d after surgery, the damaged external intercostals and the adjacent, intact internal intercostals were cut out together and processed as described above.

IMMUNOPEROXIDASE: Peroxidase-labeled antibodies were used to localize basement membrane antigens in the electron microscope (12, 35). 2-3 mm wide strips of intercostal muscle, attached to rib at both ends, were pinned in Sylgard-coated (Dow Corning Corp., Midland, MI) dishes and stained and fixed according to the following schedule: Ringer's solution for 0.5-1.5 h at 4°C; antibody or normal serum diluted in Ringer's plus 10 mg/ml BSA for 2 h at room temperature; three changes of Ringer's with BSA for a total of 2 h at 4°C; peroxidase-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG diluted 1:100 in Ringer's for 2 h at room temperature; three changes of Ringer's with BSA for a total of 1 h at 4°C; Ringer's without BSA for 1 h at 4°C; 1% glutaraldehyde, 110 mM NaCl, 5 mM CaCl₂, 30 mM HEPES for 45 min at 4°C; 150 mM NaCl, 30 mM HEPES, 10 mM glycine, pH 7.2, overnight at 4°C. The Ringer's solution was bubbled with O_2 before being applied to the muscle, and incubations with Ringer's were done in a sealed bell jar flushed with O_2. The next day, external intercostals were removed from the ribs, sliced into 0.5 mm wide pieces, incubated in a mixture of p-cresol and diamenobenzidine (43) for 1 h at 4°C, rinsed in 0.1 M cacodylate buffer, pH 7.2, refixed in 1% O_sO_4 in cacodylate, dehydrated in ethanol, rinsed in propylene oxide, and embedded in Araldite. Thin sections were examined without further staining or, occasionally, after staining with lead citrate.

IMMUNOFERRITIN: Highest resolution localization of antigens was obtained by an indirect procedure that used ferritin-conjugated second antibody. In most experiments, muscles were treated as described above for immunoperoxidase experiments, except that ferritin-conjugated goat anti-rabbit was substituted for peroxidase-conjugated second antibody, and incubation in the cresol-diamino-benzidine mixture was omitted. Because ferritin penetrated live tissue poorly, muscles were lightly fixed and sectioned before staining, in a few cases. Rat diaphragm was fixed in 0.8% paraformaldehyde, 110 mM NaCl, 30 mM HEPES, pH 7.2 for 30 min at 4°C, frozen and sectioned at 30 μm in a cryostat. Sections were then incubated with antibodies, washed, refixed with glutaraldehyde and OsO₄, dehydrated, and embedded. Thin sections were stained in uranyl acetate and lead citrate.

RESULTS

Synaptic and Extrasynaptic Basement Membrane Share Laminin, Collagen IV, and Fibronectin

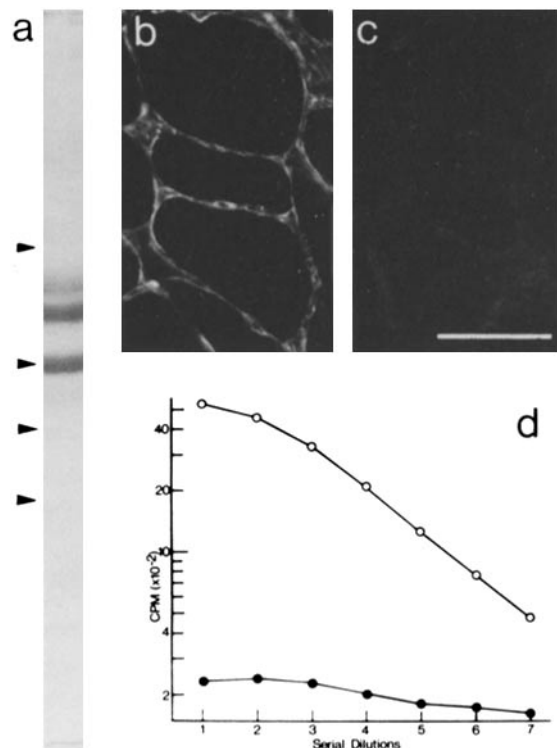LAMININ: The distribution of laminin in rat skeletal mus-

FIGURE 1 Characterization of HSP and its antiserum. (*a*) SDS slab gel of reduced HSP. Three bands of apparent molecular weights 62,000, 57,000, and 49,000 are visible. Arrowheads indicate positions of standards of molecular weights 68,000, 49,000, 43,000, and 36,000 (top to bottom), run in an adjacent lane. (*b* and *c*) Staining by anti-HSP is nearly abolished by adsorption with HSP. Cryostat sections incubated with anti-HSP (*b*) or with anti-HSP that had been preincubated with HSP (*c*) and then stained with fluorescein-second antibody. *b* and *c* were exposed, developed, and enlarged identically. Bar, 50 μm. × 310. (*d*) Solid-phase radioimmunoassay (described in Materials and Methods) shows antibody to HSP in the antiserum. Graph shows the amount of radioactivity bound by wells that had been incubated with HSP, then with serial twofold dilutions of anti-HSP (O) or preimmune serum (●), starting at 1:200, and finally with ¹²⁵I-goat anti-rabbit IgG.

cles was studied by an indirect immunofluorescence method: cryostat sections of unfixed muscle were incubated with anti-laminin and fluorescein-conjugated goat anti-rabbit IgG and examined with epifluorescence optics. As previously reported (9, 32), anti-laminin stained the surface of muscle fibers (Fig. 2*a*). In addition, the surfaces of myelinated axons, perineurial sheaths, and capillaries were stained as were the inner and outer surfaces of large intramuscular blood vessels, the walls of thinner blood vessels, the surface of muscle spindle capsules, and the surface of intrafusal muscle fibers in spindles. However, loose connective tissue surrounding individual muscle fibers (endomysium), fascicles of muscle fibers (perimysium) or whole muscles (epimysium) was not stained, nor was the cytoplasm of any cell stained.

Light and electron microscope immunohistochemical methods were used to determine whether laminin is associated with synaptic as well as extrasynaptic regions of the muscle fiber surface, or whether it is confined to extrasynaptic regions. For light microscopy, synaptic sites were marked with rhodamine-α-bungarotoxin, which binds tightly and specifically to acetylcholine receptors clustered in the postsynaptic membranes at the neuromuscular junction (30, 35). Examination of sections doubly labeled with fluorescein-antibody and rhodamine-toxin showed that anti-laminin stained synaptic as well as extrasynaptic areas (Figs. 3*a* and *b*). Preimmune serum stained neither synaptic nor extrasynaptic areas (Figs. 3*c* and *d*).

Electron microscopy of muscles incubated with anti-laminin and peroxidase-conjugated second antibody confirmed results obtained by immunofluorescence: reaction product covered synaptic and extrasynaptic muscle fiber surfaces, as well as the surface of the overlying Schwann cell (Fig. 4*a*). In contrast, antibodies to synapse-specific components such as acetylcholinesterase stained only the synaptic cleft (Fig. 4*b*). Neither synaptic nor extrasynaptic portions of the muscle fiber surface were stained when preimmune serum was substituted for antibody (not shown). Thus, laminin is present in both synaptic and extrasynaptic portions of the muscle fiber surface.

COLLAGEN IV: Immunofluorescence showed that anti-collagen IV stained both synaptic and extrasynaptic portions of the muscle fiber surface (Figs. 3*e* and *f*) as well as the surfaces of blood vessels, axons, perineurial sheaths, muscle spindles, and capillaries. Electron microscopy of neuromuscular junctions stained with anti-collagen IV and peroxidase-conjugated second antibody revealed reaction product in synaptic and extrasynaptic areas of the muscle fiber surface and on Schwann cell surfaces (not shown). Neither light nor electron microscope immunohistochemical methods revealed any differences between the distribution of laminin and that of collagen IV.

FIBRONECTIN: Like anti-laminin and anti-collagen IV, anti-fibronectin stained both synaptic and extrasynaptic portions of the muscle fiber surface (Figs. 3*g* and *h* and 4*c*) as well as the surfaces of blood vessels, capillaries, muscle spindle capsules, myelinated axons, and perineurium (Fig. 2*b*). In addition, fibronectin was present diffusely throughout endo-, peri-, and epimysial connective tissue (Figs. 2*b* and 4*c*). Since serum contains fibronectin (29, 50), a rat was perfused through the aorta with 1l of Ringer's solution; its diaphragm was carefully examined to be sure it was free of blood and then was prepared for immunofluorescence. Anti-fibronectin staining was not detectably altered by perfusion. Thus, the fibronectin detected in skeletal muscle and on muscle fiber surfaces is not derived from blood trapped in the tissue during its preparation for histological study.

In contrast to its intense staining of the surface of normal skeletal muscle fibers (extrafusal fibers), anti-fibronectin consistently stained intrafusal muscle fibers in muscle spindles relatively poorly (Fig. 2*b*). Anti-fibronectin was the only antibody of those described in this report to distinguish between

FIGURE 2 Components of extracellular matrix are widely distributed in skeletal muscle. Unfixed cross-sections of rat diaphragm were incubated with anti-laminin (*a*), anti-fibronectin (*b*), or anti-HSP (*c*), stained with fluorescein-goat anti-rabbit IgG, and photographed with epifluorescent illumination. Muscle fiber surfaces are stained, as are surfaces of axons and perineural sheaths in intramuscular nerves (*N*), capillaries (*C*), blood vessels (*BV*), and capsules of muscle spindle (*MS*). Anti-laminin and anti-fibronectin stain both inner and outer surfaces of thick-walled vessels (arrowheads), while anti-HSP stains only the outer surface. Anti-laminin and anti-HSP but not anti-fibronectin stain intrafusal muscle fibers in the spindle. Anti-fibronectin and anti-HSP but not anti-laminin stain loose connective tissue of endomysium and perimysium (asterisks). Bar, 100 μm. × 430.
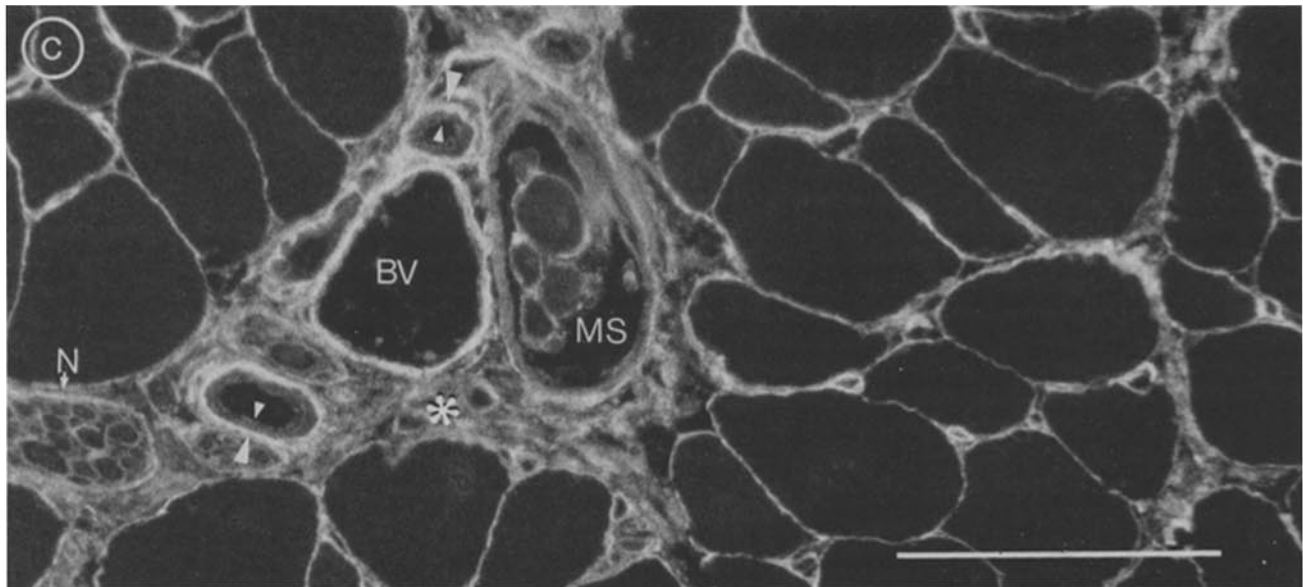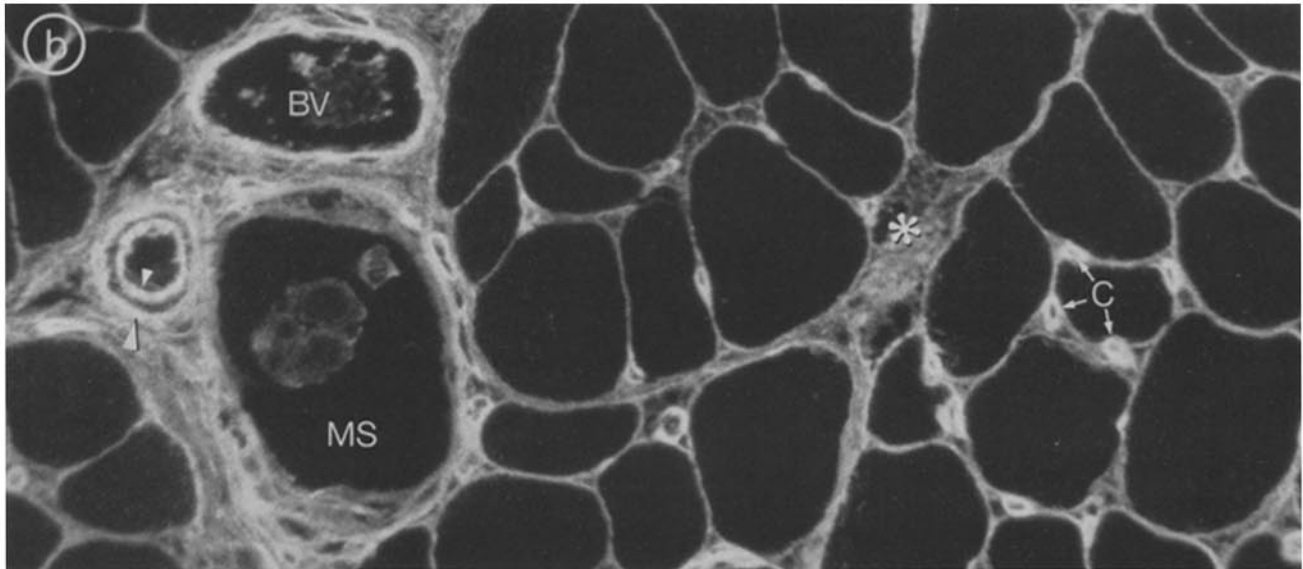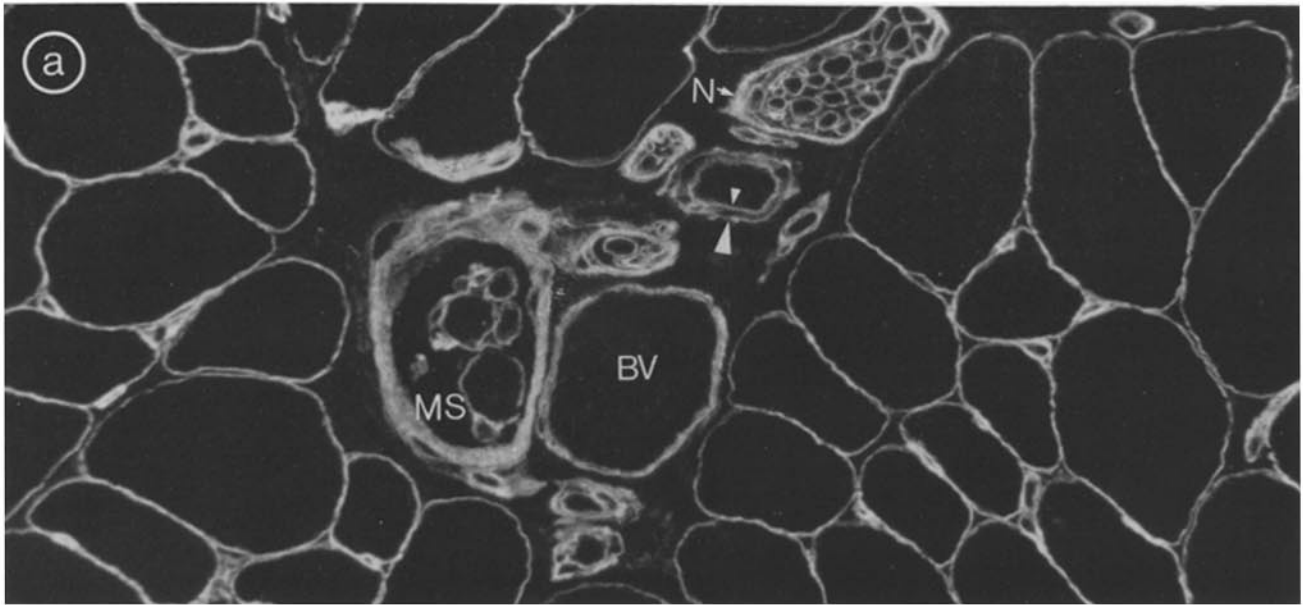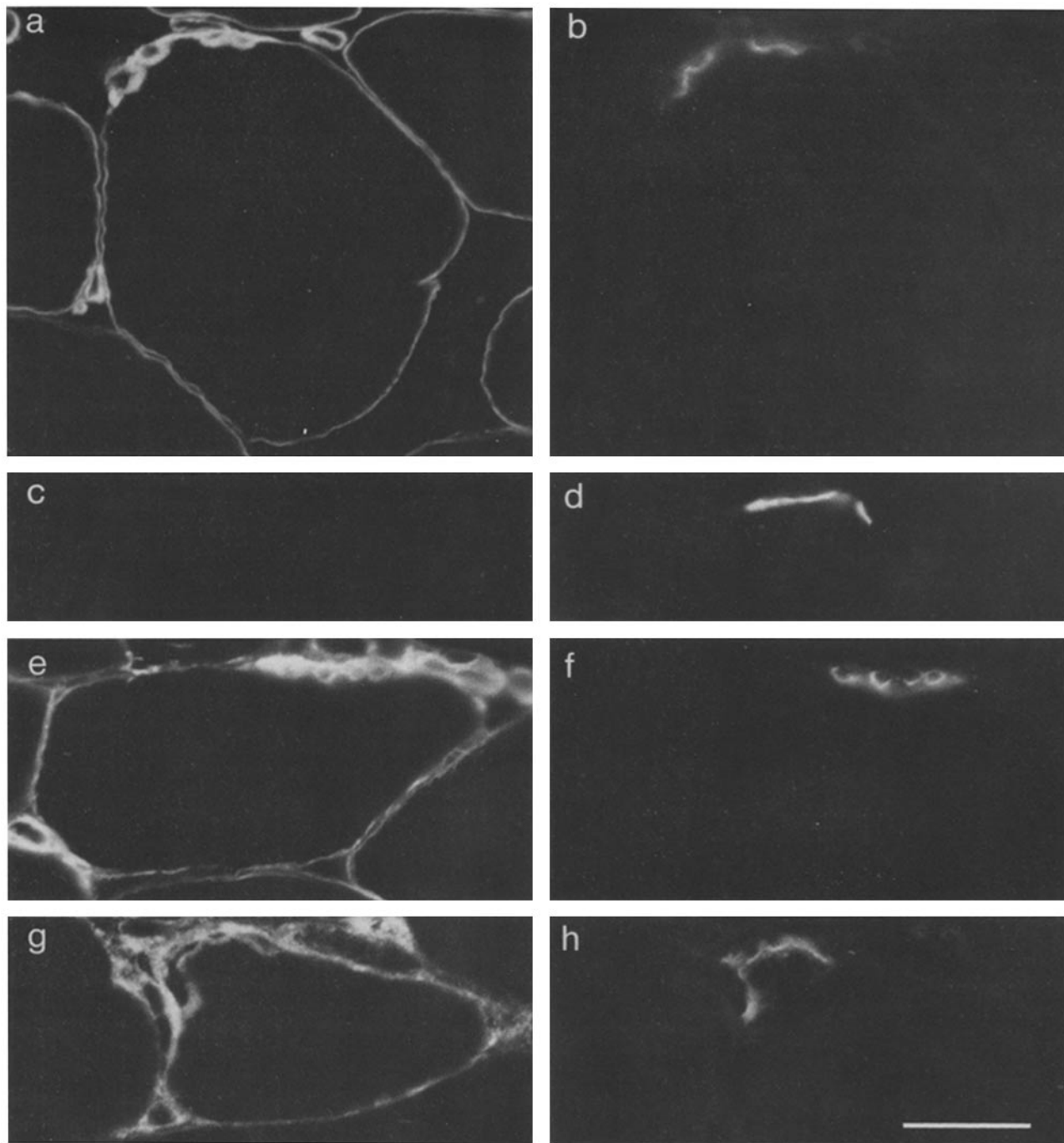
FIGURE 3   Both synaptic and extrasynaptic portions of the muscle fiber surface are stained by anti-laminin (a and b), anti-collagen IV (e and f), and anti-fibronectin (g and h), but not by normal serum (c and d). Cross-sections of rat diaphragm were incubated with antibody, fluorescein-conjugated anti-IgG, and rhodamine-bungarotoxin, and photographed with fluorescein optics to show antibody (a, c, e, and g) or with rhodamine optics to show bungarotoxin and thus synaptic sites (b, d, f, and h). Bar, 20 µm. × 1,000.

extrafusal and intrafusal muscle fiber surfaces.

COMPARISON OF SYNAPTIC AND EXTRASYNAPTIC STAINING: Some antigens are known to be concentrated in either synaptic (35) or extrasynaptic (see below) regions of the muscle fiber's basement membrane. Therefore, it was important to consider the possibility that the uniform staining of the muscle fiber surface observed with anti-laminin, anti-collagen IV, and anti-fibronectin might be due to the presence, in each antibody preparation, of two populations of antibody molecules, one of which recognized a synapse-specific and one an extrasynaptic region-specific antigen. Three lines of evi-

dence suggest that this is not the case but rather that components stained in synaptic and extrasynaptic regions are antigenically closely related if not identical. (a) Two or three independently derived antisera to each basal lamina component (see Materials and Methods) were studied by the indirect immunofluorescence method. In each case, both synaptic and extrasynaptic regions of the muscle fiber surface (as well as other structures specified above) were stained. (b) At least one antibody to each basal lamina component had been purified by affinity chromatography to remove nonspecific antibodies and characterized by radioimmunoassay (references in Mate-
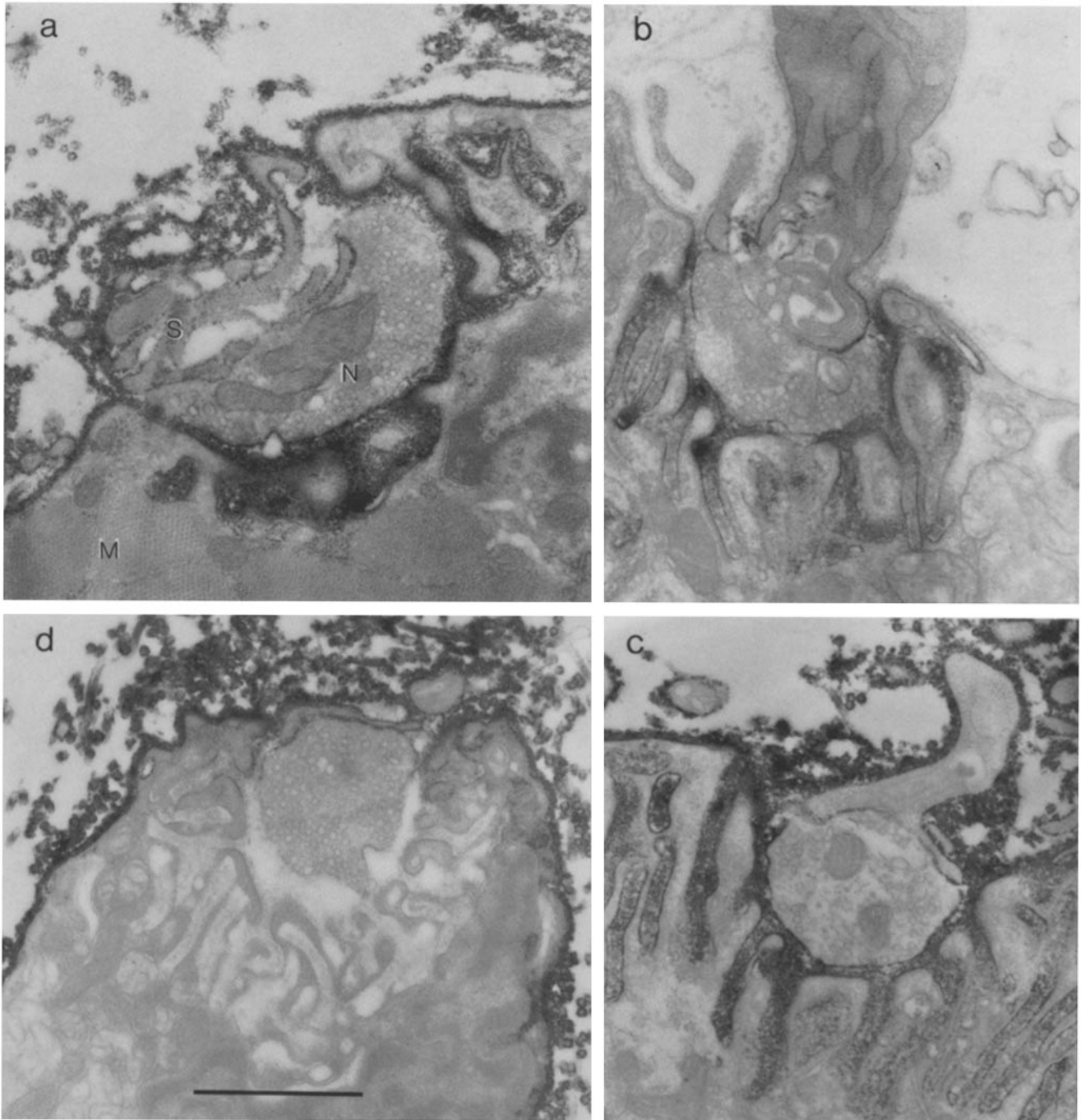
FIGURE 4 Electron microscopy shows differential staining of extrasynaptic, synaptic, and Schwann cell surfaces at the neuromuscular junction. Muscles were incubated with antibody and peroxidase-goat anti-rabbit IgG, then fixed, stained for peroxidase, embedded, and sectioned. Anti-laminin (a) and anti-fibronectin (c) stain synaptic and extrasynaptic surfaces and Schwann cell surface. Anti-acetylcholinesterase (b) stains only synaptic sites. (d) Anti-HSP stains extrasynaptic and Schwann cell surfaces but spares synaptic sites. N, nerve terminal. M, muscle fiber. S, Schwann cell. Bar, 1 μm. × 27,000.

rials and Methods). The purified antibodies, like the unfractionated antisera, bound to synaptic and extrasynaptic sites. (c) One antiserum to each basal lamina component was adsorbed overnight with connective tissue from endplate-free regions of rat diaphragm muscle (35) and then tested by immunofluorescence. In each case, the adsorption abolished staining at synaptic sites as well as in extrasynaptic regions. Taken together, these results provide evidence that laminin-like, collagen IV-like, and fibronectin-like molecules are present throughout the muscle fiber's surface and are not restricted to either synaptic or extrasynaptic domains.

ASSOCIATION WITH BASAL LAMINA: The surface complex or sarcolemma of muscle fibers consists of at least three layers that can be distinguished by electron microscopy (Fig. 5 a; see also reference 36). The innermost layer is the lipid-rich plasma membrane, which is seen as an electron-dense (osmiophilic) bilayer in electron micrographs. A 10–15 nm thick, feltlike basal lamina is separated from the plasma membrane by a narrow electron-lucent gap. A reticular lamina, composed of collagen and other fibrils embedded in a matrix of unknown composition, lies just external to the basal lamina and extends an indeterminate distance into the extracellular space. The
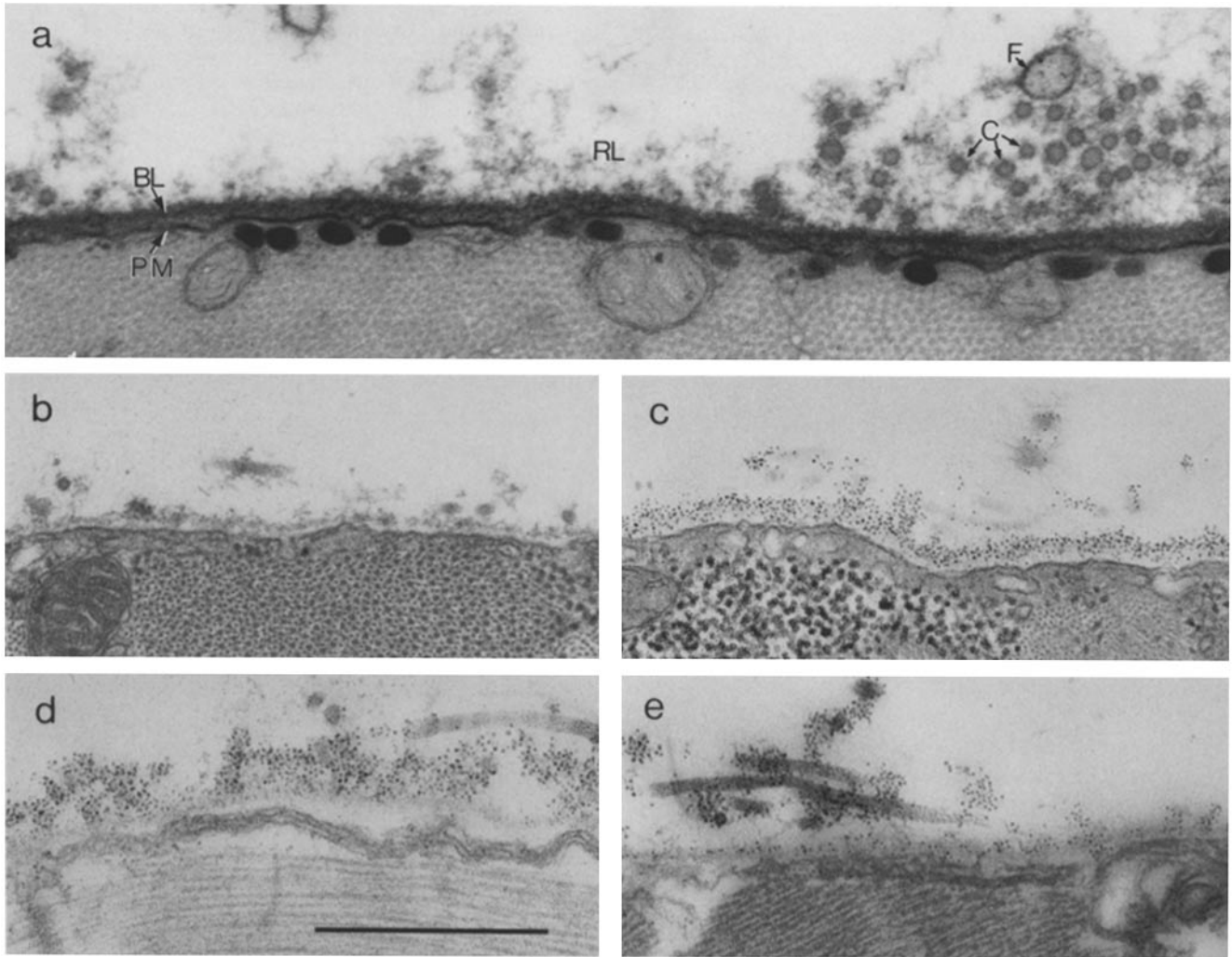
FIGURE 5   Immunohistochemistry with ferritin-conjugated antibody shows that laminin, fibronectin, and HSP are associated with muscle fiber basement membrane. Living muscles (b and c) or lightly fixed sections (d and e) were incubated with normal serum (b), anti-laminin (c), anti-HSP (d) or anti-fibronectin (e), and ferritin-goat anti-rabbit IgG, then prepared for electron microscopy. Laminin is concentrated in the basal lamina (BL), HSP in reticular lamina (RL), and fibronectin in both portions of the basement membrane. The muscle in a was stained with tannic acid (40) but not antibody to show more clearly the division of the muscle fiber surface into plasma membrane (PM), BL, and RL. C, collagen fibril. F, fibroblast process. Bar, 0.5 μm. × 66,000.

basal lamina and reticular lamina together comprise the basement membrane. The tripartite division of sarcolemma into plasma membrane, basal lamina, and reticular lamina is clearly an oversimplification (see reference 18 for example); nevertheless, it provides a convenient framework for considering the fine-structural localization of components of the muscle fiber surface. Three observations revealed that laminin, collagen IV, and fibronectin are all associated, at least in part, with basal lamina.

First, muscles were damaged to remove plasma membrane but not basement membrane and then studied by immunofluorescence. When muscles are injured, the cytoplasm and plasma membrane of muscle fibers degenerate and are phagocytized, but their sheaths of basement membrane survive (35, 36, 49). Antibodies to laminin (Fig. 6; compare with Fig. 2a), collagen IV, and fibronectin stained basement membrane sheaths as intensely in the absence of muscle fibers as in their presence; thus, a major fraction of each of these antigens is associated with the basement membrane.

Second, muscles stained with antibody and ferritin-conjugated second antibody were examined in the electron micro-

scope. The use of ferritin instead of peroxidase as a marker circumvents a limitation of the latter technique: peroxidase reaction product may diffuse before it precipitates and may artifactually stain structures near to but distinguishable from the site to which the enzyme is bound. Basal lamina was heavily labeled with ferritin in muscles incubated with anti-laminin (Fig. 5c) or anti-collagen IV; some ferritin was also bound to collagen and other fibrils in the reticular lamina. In muscles stained with anti-fibronectin, both basal lamina and reticular lamina were labeled (Fig. 5e). Some ferritin was occasionally associated with collagen fibrils but little or none with basal lamina when normal serum was used in place of antibody (Fig. 5b). Thus, laminin, collagen IV, and fibronectin are all associated with muscle fiber basal lamina. Fibronectin is also present in reticular lamina, a localization that might have been expected from the diffuse immunofluorescent staining of spaces between muscle fibers. Laminin and collagen IV may coat collagen fibrils in reticular lamina, although nonspecific staining of these fibrils (with normal serum) renders this observation less reliable.

Finally, the observation that laminin, collagen IV, and fibro-

nectin are all present in synaptic as well as extrasynaptic areas argues that they are not confined to reticular lamina: the basal lamina extends through the synaptic cleft between nerve and muscle, but the reticular lamina does not (36).

Together, these results demonstrate that laminin, collagen IV, and fibronectin are all components of muscle fiber basal lamina. In addition, some laminin and collagen IV may be, and some fibronectin certainly is, associated with the reticular lamina.

Collagen V and HSP Are Present in Extrasynaptic But Not Synaptic Basement Membrane

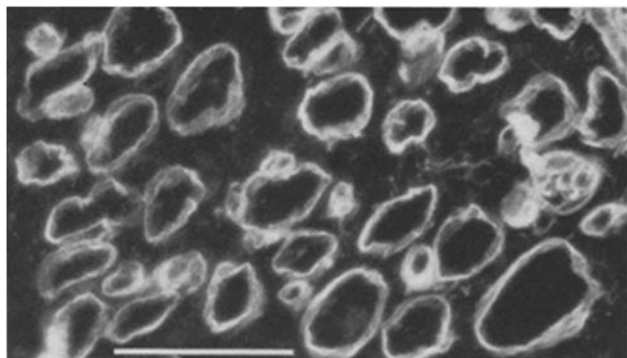The antibodies described so far recognized antigens shared

FIGURE 6 Anti-laminin stains basement membrane sheaths in the absence of muscle fiber cytoplasm and plasma membrane. Muscle was injured (as described in Materials and Methods) to induce degeneration and removal of muscle cells, then frozen, sectioned, and stained with anti-laminin and fluorescein-goat anti-rabbit IgG. Basement membrane sheaths have collapsed but are clearly recognizable. Bar, 50 μm. × 480.

by synaptic and extrasynaptic portions of the muscle fiber basement membrane. In contrast, antibodies to collagen V (AB) and to a high-salt-soluble protein from muscle (HSP) stained extrasynaptic but not synaptic basement membrane. In cryostat sections double-stained with fluorescein-antibody and rhodamine-bungarotoxin, the muscle fiber surface was fluorescent everywhere except at the small patches identified as synaptic sites by bungarotoxin-binding (Fig. 7). Electron microscopy with peroxidase-conjugated second antibody confirmed that anti-HSP (Fig. 4d) and anti-collagen V bound to extrasynaptic and Schwann cell surfaces near the neuromuscular junction but were excluded from the basement membrane in the synaptic cleft and junctional folds. In some electron micrographs, extrasynaptic surface directly adjacent to neuromuscular junctions was only lightly labeled, while in others some reaction product was seen within the margins of the synaptic cleft (Fig. 4d). Poor penetration of immunoreagents could account for the former observation and diffusion of peroxidase reaction product for the latter. However, it is also possible that there is a gradient of antigen concentration at the edges of synaptic sites; further experiments will be required to decide this point. Nevertheless, it is clear that anti-HSP and anti-collagen V stain nearly all (>99%) of the muscle fiber surface but selectively spare synaptic sites.

The antigens revealed by anti-HSP and anti-collagen V are associated with the muscle fiber basement membrane: by immunofluorescence, both antibodies stained basement membrane sheaths from which muscle fibers had been removed by damage-induced degeneration. When anti-HSP and ferritin-conjugated second antibody were used for electron microscopy, the heaviest labeling was seen on the outer surface of the basal lamina and in the portion of the reticular lamina just external to the basal lamina; the basal lamina was labeled lightly and often not at all (Fig. 5d). Thus, HSP may not be a *bona fide*
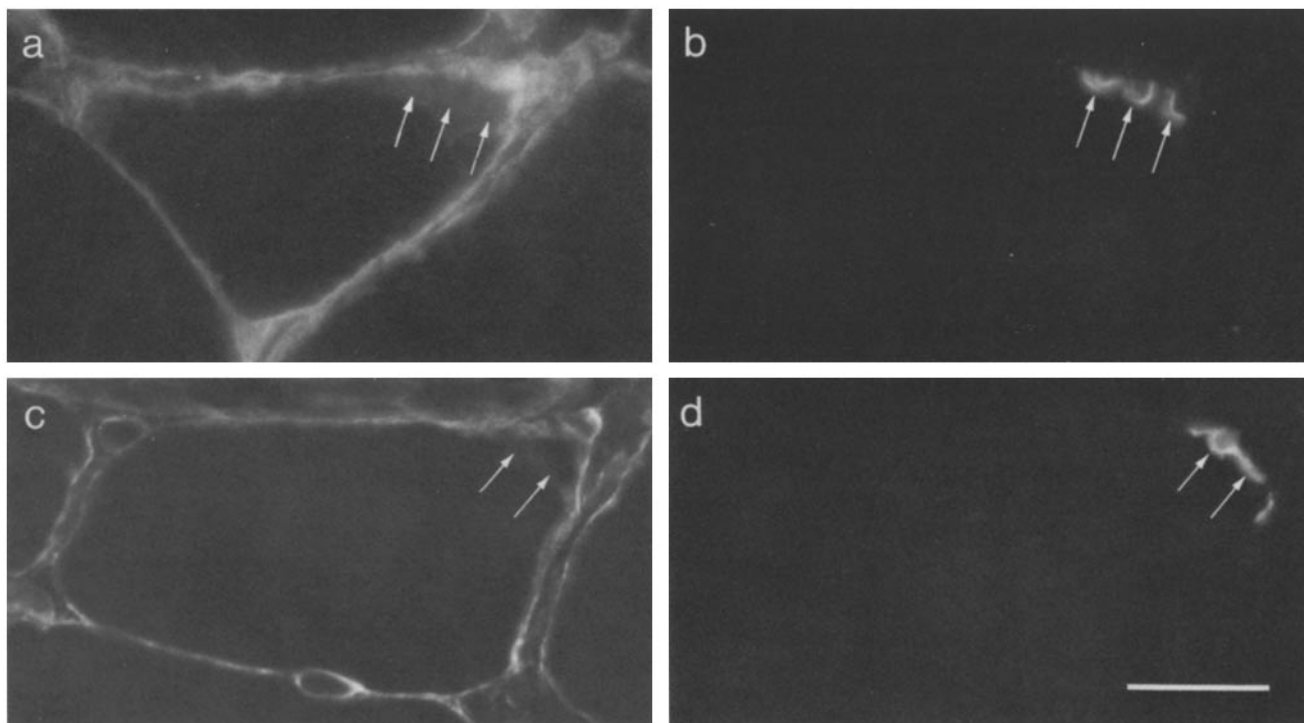

FIGURE 7 Anti-HSP (a and b) and anti-collagen V (c and d) stain extrasynaptic but not synaptic portions of muscle fiber surface. Cross-sections stained with antibody, fluorescein-second antibody, and rhodamine-bungarotoxin were photographed with fluorescein (a and c) or rhodamine (b and d) optics. Arrows mark synaptic sites, defined by toxin-binding. Bar, 20 μm. × 980.

component of the basal lamina, but it is closely associated with basal lamina. Collagen V has not yet been successfully localized by the immunoferritin procedure.

In addition to staining extrasynaptic muscle fiber basement membrane, anti-HSP and anti-collagen V stained the surfaces of myelinated axons, perineurial sheaths, capillaries, blood vessels, muscle spindle capsules, intrafusal muscle fibers, and loose connective tissue in the endo-, peri-, and epimysia (Fig. 2c). Two sites were stained by anti-laminin, anti-collagen and anti-fibronectin but not by anti-HSP or anti-collagen V: synaptic portions of the muscle's basement membrane (Figs. 3, 4, and 7), and the luminal surface of intramuscular arteries (Fig. 2).

DISCUSSION

Three main results emerge from the immunocytochemical analysis of muscle fiber basement membrane reported here: (a) Four proteins that have previously been shown to be associated with basement membranes of other tissues—laminin, fibronectin, collagen IV, and collagen V (references in Introduction)—are components of muscle fiber basement membrane. (b) Three proteins—laminin, fibronectin, and collagen IV (or immunologically closely related molecules)—are shared by the basement membrane's synaptic and extrasynaptic portions. (c) Collagen V and the still-uncharacterized antigen(s) recognized by anti-HSP are present in extrasynaptic but not synaptic basement membrane. These experiments, together with results reported previously (28, 35), provide examples of three classes of components of muscle fiber basement membrane—synaptic, extrasynaptic, and shared (summarized in Table I).

Experiments with ferritin as a marker revealed subtle differences among the antibodies in their distribution across the basement membrane. Laminin and collagen IV are concentrated in the basal lamina, HSP is concentrated on the outer surface of the basal lamina and in the reticular lamina, and fibronectin is present in both basal and reticular laminae. These differences were not detected in immunoperoxidase experiments, presumably because peroxidase reaction product can

migrate for short distances before it precipitates. However, Mayer et al. (26) have found that anti-fibronectin stains embryonic basal laminae in experiments with peroxidase- or rhodamine-conjugated second antibody, but not when ferritin-conjugated second antibody is used, and it may be that some molecules are poorly labeled in some locations by the large (11-nm diameter) ferritin molecule. Ferritin clearly labeled muscle basal lamina in experiments with anti-laminin or anti-collagen IV, but differences in orientation or accessibility amongst antigens could explain the relatively sparse labeling of fibronectin in basal lamina and the apparent absence of HSP from this portion of the basement membrane. Another point of uncertainty is our inability to distinguish the locations of collagen IV and laminin; in skin and glomerular basement membranes, collagen IV is in the basal lamina itself (also called lamina densa), while laminin is concentrated in the electron-lucent gap between basal lamina and plasma membrane (the lamina rara) (13, 25, 33, 51). Muscle basement membrane may be poorly suited for demonstrating this distinction, since it is considerably thinner than the basement membranes of skin or glomerulus; alternatively, muscle basement membrane may resemble the basement membrane of the kidney tubule, in which collagen IV and laminin are apparently codistributed (25). Despite these uncertainties, however, the firm conclusion from our experiments is that all five proteins studied are components of muscle fiber basement membrane and are closely associated with the basal lamina.

Our interest in comparing synaptic and extrasynaptic basement membrane stems from the finding that the synaptic region is functionally specialized: When new neuromuscular junctions form following injury, regenerating axons preferentially contact original synaptic sites on the basement membrane and differentiate into nerve terminals at these sites in the absence of the muscle fiber (36); acetylcholine receptors on regenerating myotubes cluster at original synaptic sites on the basement membrane in the absence of the nerve (1, 5). In addition, acetylcholinesterase, which terminates neurotransmitter action at the neuromuscular junction, is connected to or contained in synaptic basement membrane (19, 28), and adhesion of nerve to muscle at the neuromuscular junction may be mediated by components of synaptic basement membrane (2, 36). One obvious possibility is that components confined to synaptic basement membrane interact with regenerating axons and myotubes to trigger differentiative processes. The discovery that several antigens are present in synaptic but not extrasynaptic basement membrane (35) is consistent with this idea. Another possibility is that components confined to extrasynaptic regions could prevent the formation of nerve-muscle contacts or the appearance of pre- or postsynaptic specializations, either by interacting with nerve or muscle cells or by blocking, everywhere except at the synapse, functionally important sites on molecules that are present throughout the basement membrane. In this regard, it is interesting to note that collagen IV, laminin, and fibronectin, all of which are shared by synaptic and extrasynaptic basement membrane, have been shown to be involved in cellular adhesion and differentiative processes in other tissues (22, 44, 48). The discovery that some antigens are excluded from synaptic sites requires one to consider these latter possibilities.

Table I

Staining of Synaptic and Extrasynaptic Regions by Antibodies that Bind to Muscle Fiber Basement Membrane

	Region of muscle fiber basement membrane stained	
Antibody	Synaptic	Extrasynaptic
Lens capsule	+	−
Acetylcholinesterase	+	−
Muscle basement membrane collagen (adsorbed*)	+	−
Lens-capsule collagen (adsorbed*)	+	−
Laminin	+	+
Collagen IV	+	+
Fibronectin	+	+
Collagen V	−	+
HSP proteins	−	+

The table sumarizes results reported here and in Sanes and Hall (35).
* These sera were synapse-specific only after antibodies to extrasynaptic portions of the muscle fiber surface were removed by adsorption with connective tissue from endplate-free regions of muscle (see Reference 35).

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