Identification of an Intracellular Postsynaptic Antigen at the Frog Neuromuscular Junction

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ABSTRACT   A layer of amorphous, electron-dense material is situated at the cytoplasmic surface of the postsynaptic membrane of vertebrate neuromuscular synapses. The function of this structure is not clear, but its location suggests that it may have an important role in the formation and/or maintenance of the synapse. This paper demonstrates that a monoclonal antibody raised against antigens from *Torpedo* electric organ binds to an intracellular, postsynaptic protein at the frog neuromuscular synapse. Indirect immunofluorescence on frozen sections of frog muscle was used to demonstrate that the antigen is concentrated at synaptic sites in normal muscle. In denervated muscle, the antigen remains concentrated at synaptic sites, but is also present at extrasynaptic regions of denervated myofibers. The antigen cannot be labeled in intact, whole muscle, but only in whole muscle that has been permeabilized with nonionic detergents. The antibody staining pattern in Triton X-100–permeabilized whole-mounts of the frog neuromuscular synapse is arranged in elongate, arborized areas which are characteristic of the frog neuromuscular synapse. The stained areas are striated and the striations occur with a periodicity that corresponds to the regular folding of the postsynaptic membrane. Immunoferritin labeling of fixed, saponin-permeabilized muscle demonstrates that the antigen is associated with amorphous material that is situated between the postsynaptic membrane and an underlying layer of intermediate filaments. The antigen, solubilized from *Torpedo* electric organ by high ionic strength, was identified by antibody binding to nitrocellulose replicas of SDS gels of *Torpedo* tissue. In *Torpedo* tissue, the antibody binds to a single protein band at 51,000 daltons (51 kd). The 51-kd protein shares an antigenic determinant with intermediate filament proteins, since a monoclonal antibody to all intermediate filaments reacts with the same 51-kd protein. The monoclonal antibody also reacts with a 55-kd protein in frog skin which is localized to the perinuclear region of the epithelial cells.

More is known about the neuromuscular synapse than any other synapse. Its relative simplicity and accessibility have provided us with a detailed account of what a synapse looks like and how a synapse functions. We remain, however, rather poorly informed about the biochemical events involved in the formation and maintenance of this highly specialized and organized region of cell-to-cell contact.

Although there is considerable evidence that interaction between nerve and muscle during development is necessary for the complete structural and functional differentiation of the synapse (2, 19, 43), little information is available concerning how nerve and muscle interact and how the differentiated structures of the synapse are assembled. One hindrance to a detailed examination of the sequence of steps in synapse formation is our rather limited knowledge of the macromolecules present at synapses; acetylcholine receptors and acetylcholinesterase are the only synaptic macromolecules which have been

presently characterized in detail (8, 15, 31). To gain further insight into the mechanisms involved in the formation and maintenance of synapses, it is important to identify and characterize macromolecules that are present at synapses. As a first step to identify synaptic macromolecules, I have produced monoclonal antibodies that are directed against components of the neuromuscular synapse. In this paper a synaptic macromolecule is identified by a monoclonal antibody and shown to be an intracellular postsynaptic protein which has a distribution similar to acetylcholine receptors in both normal and denervated muscle.

MATERIALS AND METHODS

Animals

and *Rana temporaria*) were obtained from commercial suppliers, and *Torpedo* electric organ was obtained frozen from Pacific Biomarine (Venice, CA).

Operation

Frogs were anaesthetized in 0.1% tricaine methanesulfonate (Finquel, Ayerst Laboratories, NY) and a 0.5-cm incision was made in the skin at the lateral edge of the *cutaneous pectoris* muscle (29). The nerve to the muscle was cut within 1 mm of the muscle's edge and 5 mm of the central nerve stump was removed. The skin incision was closed with 7-0 sutures.

Cell Line

The nonsecreting variant cell line P3/NSI/1-AG4-1 (NS-1) derived from the BALB/c myeloma P3 (25) was used for fusion. This cell line and hybridoma cell lines were maintained in tissue culture medium (see below) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cell density was maintained at 1×10^4 to 4×10^5 cells/ml by passaging the cells every few days.

Media

The parent myeloma and hybridomas were grown in RPMI 1640 (Flow Labs, McClean, VA) with the appropriate supplements and fetal calf serum (FCS). The myeloma was grown in 7.5% FCS; the hybridomas were initially grown in 15% FCS and gradually adapted to 7.5% FCS. Hypoxanthine-thymidine (HT) or selective hypoxanthine-aminopterin-thymidine (HAT) medium (30) were prepared as described by others.

Frog Ringer was 2 mM potassium chloride, 1.8 mM calcium chloride, 115 mM sodium chloride, and 1 mM sodium phosphate, pH 7.3. Phosphate buffered saline (PBS) was 150 mM sodium chloride, 10 mM sodium phosphate, pH 7.3.

Immunogen

Torpedo electric organ was chosen as the source of synaptic antigens because the organ is enriched in cholinergic synapses and synaptic material is available in large quantities (7, 12). There is good evidence that components of both the extracellular matrix and the intracellular cytoskeleton have a role in the differentiation of the neuromuscular synapse (2, 6, 44). The electric organ was fractionated to enrich for extracellular matrix and cytoskeletal antigens. The electric organ was homogenized with a motor driven Potter-Elvehjem pestle and insoluble material was collected after centrifugation. The tissue was homogenized in 10 mM Tris, 1 mM EDTA, 0.3 mM phenylmethylsulfonylfluoride (PMSF), pH 7.4 (1 vol tissue/2.5 vol solution; solution A), the pellet was collected after centrifugation (30,000 g for 30 min) and washed twice by centrifugation. The pellet was then resuspended and homogenized in solution A plus 2% Triton X-100 (solution B). The pellet was collected and washed with solution B as described above. The pellet was finally resuspended, homogenized and washed in distilled water and collected. All solutions were maintained on ice during the entire procedure. The yield from 10 g (wet wt) of electric organ was ~220 mg (dry wt) which was stored at −80°C.

Immunization

Mice (BALB/c) were primed subcutaneously with 200 μg of protein (insoluble material described above) emulsified in complete Freund's adjuvant. After 10 d, the mice were boosted intraperitoneally with an additional 200 μg of immunogen emulsified in incomplete Freund's adjuvant and boosted again intraperitoneally after another 3 wk. The serum titer was monitored 6–10 d after a boost.

Cell Fusion

Fusion of myeloma cells and spleen cells and subsequent selection were performed essentially as described by others (27, 38, 51). 3–4 d after the last boost, the spleen was removed from the immunized mouse and a single cell suspension was prepared. The washed spleen cell suspension was mixed with washed NS-1 cells and centrifuged in a conical tube. The ratio of spleen cells to NS-1 cells varied between 4:1 and 10:1. The supernatant was carefully removed and 1 ml of 50% polyethylene glycol (PEG 1500, BDH Chemicals Ltd., Poole, England; 50% wt/vol in RPMI at pH 7.4) at 37°C was gradually added to the gently stirred pellet over 1 min. The pellet was stirred for an additional minute. Subsequently, 2 ml of RPMI were added over 2 min and finally 8 ml of RPMI were added over 3 min with stirring. The cells were centrifuged and gently resuspended in 20 ml of RPMI-HAT-15% FCS. The cells were plated in 24-well tissue culture plates (Linbro, McClean, VA) at a density of 5×10^5 large, viable cells (determined by Trypan Blue exclusion) per well in 1 ml. The next day, 0.8 ml of RPMI-HAT-15% FCS was added to each well. Half of the medium was

replaced every fourth day with fresh medium; on the twelfth day, aminopterin was omitted from the HAT medium; after 1 wk in RPMI-HT-15% FCS, the HT was omitted.

Hybridoma supernatants were screened on frozen sections of frog muscle (see below) as early as 1 wk after fusion. Cells from positive wells were cloned by limiting dilution in 96-well microculture plates with peritoneal macrophages as feeder cells. Positive microcultures were expanded and twice recloned by limiting dilution.

Cells ($1-2 \times 10^6$ cells/vial in 0.3 ml of 90% FCS, 10% dimethylsulfoxide [DMSO]) were frozen for 10 min at −30°C and then maintained in liquid nitrogen.

Tumor Transplantation

Hybridomas were injected intraperitoneally into nu/nu mice which had been primed with pristane (2,6,10,14-tetramethyl pentadecane) 2–7 d previously. Usually 10^6 cells were injected in 0.2 ml of RPMI. Ascites fluid was collected from the tumor-bearing mice, clotted and frozen or immediately purified.

Subclass Identification and Purification

The subclass of monoclonal antibodies was determined by immunodiffusion against subclass specific reagents (Miles Laboratories, Inc., Elkhart, IN) and with fluorochrome conjugated subclass specific antibodies (kindly supplied by Dr. M. C. Raff) on frozen sections of muscle (see below). The monoclonal antibody described in this report is an IgG1. Monoclonal antibodies were purified from supernatant and ascites fluid by Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography as described by others (14).

Immunofluorescence

FROZEN SECTIONS: The *cutaneous pectoris* muscle of the frog was pinned in a Sylgard (Dow Corning Corp., Midland, MI) coated petri dish. A small vaseline chamber was made around the muscle and the chamber was filled with O.C.T. embedding medium (Lab-Tek, Naperville, IL). The muscle was frozen in liquid N_2 and bisected with a transverse cut through the center of the muscle. 4-μm frozen sections were cut in a Bright cryostat and the sections were collected on multispot slides (PTFE slides; Hendley-Essex, Essex, England).

Frozen sections were incubated in 20 μl of antibody (undiluted supernatant during screening; ascites fluid or purified antibody diluted 1:10,000 in Ringer) for 30 min, rinsed in Ringer for 30 s, incubated in 20 μl of fluorescein coupled-goat-anti-mouse IgG (F1-G-α-MIgG; Nordic, Tillburg, The Netherlands; 1:150 in Ringer) for 30 min, washed for 1 min in Ringer and mounted in 20% glycerol, 80 mM sodium bicarbonate, pH 9.5 with 10^{-4} M o-phenylenediamine). Synaptic sites in the muscle were marked by including tetramethylrhodamine-α-bungarotoxin (40) (TMR-α-BGT; 4×10^{-8} M) in the secondary antibody incubation. All incubations were done at room temperature.

The sections were viewed with a Zeiss microscope equipped with epifluorescence optics. Fluorescein fluorescence was visualized with Zeiss filters selective for fluorescein (Zeiss 487709, BP 450-490 excitation filter, LP 520 barrier filter and FT 510 dichromatic reflector) and tetramethylrhodamine fluorescence was visualized with Zeiss filters selective for rhodamine (Zeiss 487714, BP 515-560 excitation filter, LP 590 barrier filter, and FT 580 dichromatic reflector). In these experiments, tetramethylrhodamine fluorescence was not visible when viewed with filters selective for fluorescein, and fluorescein fluorescence was not visible when viewed with filters selective for rhodamine.

Whole-mounts

ANTIBODY LABELING: The *cutaneous pectoris* muscle of the frog was fixed in 1% formaldehyde (in 110 mM sodium chloride, 10 mM sodium phosphate, pH 7.2) for 45 min at room temperature. The muscle was washed in Ringer for 5 min, permeabilized with 0.5% Triton X-100 (in Ringer) for 10 min, incubated in monoclonal antibody (0.5 μg/ml in 0.5% Triton-Ringer) for 1 h, washed (in 0.5% Triton-Ringer) for 30 min, incubated in tetramethylrhodamine coupled-goat-anti-mouse IgG (TMR-G-α-MIgG; Nordic; 1:150 in 0.5% Triton-Ringer) for 1 h, washed for 1 h and fixed in 1% formaldehyde as described above. All incubations were done at room temperature. The muscle was mounted whole (in 20% glycerol, 50 mM sodium phosphate, pH 7.0) and the fluorescent staining was viewed with filters selective for rhodamine as described above.

Horseradish Peroxidase Coupled-α-Bungarotoxin Labeling of Acetylcholine Receptors

Horseradish peroxidase (HRP) was coupled to α-bungarotoxin (α-BGT) with glutaraldehyde and the conjugate was separated from free α-BGT as previously

described (6). Acetylcholine receptors were labeled by incubating live, or 1% formaldehyde fixed, muscles with 10^{-7} M HRP-α-BGT for 1 h at room temperature. The muscle was washed for 15 min in several changes of Ringer's, fixed in 1% glutaraldehyde (in 60 mM sodium phosphate, pH 7.3) for 30 min, washed (in 0.1 M Tris, pH 7.3) for 10 min, treated with 1% (wt/vol) cobalt chloride (in 0.1 M Tris, pH 7.3) for 10 min, rinsed twice (in 0.1 M Tris, pH 7.3) for 5 min and then incubated for ½ h–1 h at room temperature in 0.05% 3,3'diaminobenzidine, 0.01% H_2O_2 (in 0.1 M Tris, pH 7.3). The muscle was dehydrated, cleared in xylene, mounted whole in Permount (Fisher Scientific Company, Fair Lawn, NJ) and viewed with bright-field optics. The cobalt intensification (1) of the HRP reaction produces a blacker product and provides better contrast.

Photography

Immunofluorescent and tetramethylrhodamine-α-bungarotoxin staining were photographed on Kodak Ektachrome 400 ASA film which was processed at 400 ASA. The color transparencies were duplicated on Kodak Plus X film.

Immunoelectron Microscopy

The *cutaneous pectoris* muscle of the frog was fixed in 1% formaldehyde as described above (low concentrations of glutaraldehyde eliminate antibody binding). The muscle was washed with Ringer's for 15 min and permeabilized with 0.1% saponin (Sigma Chemical Co., St. Louis, MO; wt/vol, in 115 mM ammonium chloride, 10 mM sodium phosphate pH 7.2) for 15 min (37, 52). The muscle was incubated in monoclonal antibody (0.5 μg/ml in 0.1% saponin-Ringer's) for 3 h, washed (in 0.1% saponin-Ringer's) for 30 min, incubated in ferritin coupled-goat-anti-mouse IgG (Cappel Laboratories, Cochranville, PA, Lot #12624, further purified on a mouse Ig affinity column; 1:25 in 0.1% saponin-Ringer's) for 3 h, washed for 1 h and fixed with 1% glutaraldehyde (in 60 mM sodium phosphate, pH 7.2) for 45 min. All incubations were performed at room temperature. The muscle was washed (in 90 mM sodium phosphate, 24 mM sucrose, pH 7.3) for 15 min, treated with OsO_4 (1% in 90 mM sodium phosphate, pH 7.0) for 1 h, stained en bloc with uranyl acetate (0.5% in 100 mM sodium acetate, pH 5.2) and embedded in Epon. Thin sections were stained with lead citrate (49) and viewed in a Jeol 100 CX electron microscope.

Identification of Antigen after SDS PAGE

To identify the antigen for the monoclonal antibody, proteins from *Torpedo* electric organ were resolved in SDS gels and labeled with the antibody. Small pieces (1g wet wt) of frozen *Torpedo* electric organ were pulverized under liquid nitrogen with a mortar and pestle, lyophilized and the powder was dissolved in boiling SDS sample buffer (3% SDS, 300 mM mercaptoethanol). Proteins in extracts of the electric organ were also resolved in SDS polyacrylamide gels and labeled with antibody. *Torpedo* electric organ was sequentially homogenized and extracted as described previously for preparation of immunogen. After extraction with Triton X-100, however, the pellet was washed in solution A and then further extracted with high ionic strength (0.6 M potassium iodide, 6 mM sodium thiosulfate, 1 mM EDTA, 0.3 mM PMSF, 10 mM Tris, pH 8.0; 1 vol original tissue/1 vol of solution) for 20 min. The tissue extracts were assayed for the presence of antigen by ELISA assays (50). Only the high ionic strength extract contained detectable quantities of antigen. Proteins in the tissue extracts were resolved in SDS polyacrylamide gels (8.75%; 26) and electrophoretically transferred from the gel to nitrocellulose (48). The paper was incubated with 5% normal goat serum (NGS, in PBS) for 15 min, incubated with monoclonal antibody (0.2 μg/ml in PBS with 2% NGS) for 1 h, washed for 5 min (in PBS), incubated in horseradish peroxidase coupled-rabbit-anti-mouse IgG (Nordic; 1:1,000 in PBS with 2% NGS) for 1 h, washed for 5 min (in PBS), and incubated in 0.05% 3,3' diaminobenzidine (wt/vol) and 0.01% hydrogen peroxide (in 50 mM Tris, pH 7.3). All incubations were done at room temperature.

To identify the antigen for the monoclonal antibody in skin, small pieces of skin from the abdomen of frogs (*R. pipiens*) were frozen, pulverized, and dissolved in SDS sample buffer as described for *Torpedo* electric organ. The proteins were resolved in SDS polyacrylamide gels and labeled with antibodies as described above.

RESULTS

Production of Monoclonal Antibodies

Using the procedures described in Materials and Methods several different hybridoma cell lines which secrete antibodies that stain structures in frog muscle were produced. Different antibodies could often be distinguished by their staining pattern in frozen sections of muscle: some antibodies stain only syn-

aptic sites, others stain synaptic sites and structures within intramuscular nerves and still others stain circumferentially around myofibers and within intramuscular nerves. In a typical fusion, all wells contained growing hybridomas, 25–45% of the wells contained antibodies that stain structures in frozen sections of frog muscle, and 10–15% of the wells contained antibodies that stain only the synaptic site.

Monoclonal Antibody Binds to Synaptic Sites in Frog Skeletal Muscle

The distribution of both synaptic sites and antibody binding sites can be determined in a single frozen section. Frozen sections of muscle were incubated with monoclonal antibody followed by a mixture of TMR-α-BGT and F1-G-α-MIgG and the distribution of each was determined by fluorescence microscopy. Fig. 1 demonstrates that the distribution of α-BGT binding sites and antibody binding sites is strikingly similar. All of the α-BGT sites stain with antibody and all antibody-stained sites stain with α-BGT ($>$100 synaptic sites in five muscles). Thus, the antigen is highly concentrated at synaptic sites in normal frog muscle.

This antibody does not react with synaptic sites in frozen sections of either rat or mouse skeletal muscle, nor does the antibody label synaptic sites in parasympathetic neurons of the frog cardiac ganglion (33).

Distribution of the Antigen Changes after Denervation

The antibody staining which is observed in normal muscle could be associated with the nerve terminal, the synaptic portion of the myofiber, or the synaptic cleft material. To determine whether the antigen was associated with the nerve terminal, the *cutaneous pectoris* muscle of the frog was denervated and stained with antibody 1 wk later. In the frog, nerve terminals degenerate within 3 d after cutting the nerve at the edge of the muscle and the cellular debris is removed shortly thereafter (5, 29). The antibody staining at denervated synaptic sites remains as intense as at normal synaptic sites (Fig. 2); this suggests that little, if any, antibody staining is present within nerve terminals. Antibody staining, however, is now readily apparent at extrasynaptic regions of denervated myofibers; this suggests that the antigen is synthesized by and associated with the myofibers. Moreover, antibody staining is also apparent within denervated myofibers. Thus, most, if not all, of the antigen is associated with the myofiber; furthermore, the distribution of the antigen is strikingly altered after denervation.

Distribution of Antibody Staining in Whole-mounts of Muscle

The *cutaneous pectoris* muscle of the frog is sufficiently thin (2–4 myofibers thick) that synapses can be visualized in whole-mounts of the muscle (34). The distribution of surface acetylcholine receptors in a whole-mount preparation stained with HRP-α-BGT is illustrated in Fig. 3 a. The acetylcholine receptors are arranged in elongate, arborized areas; within the area of stain are striations which occur with a periodicity of ~0.5 μm and correspond to the rather regular longitudinal folding of the postjunctional membrane (4). When antibody incubations were performed in whole, live muscle or in whole, fixed muscle no antibody staining was observed. When whole, fixed muscle was permeabilized with Triton X-100 and subsequently
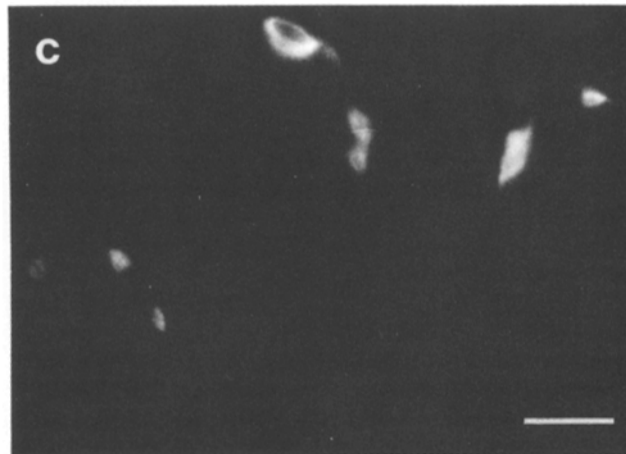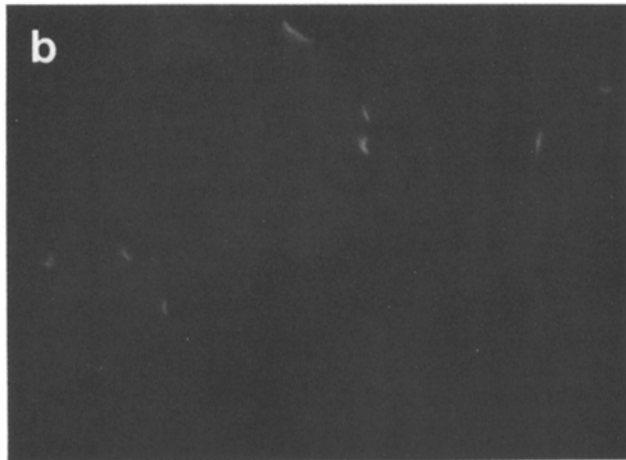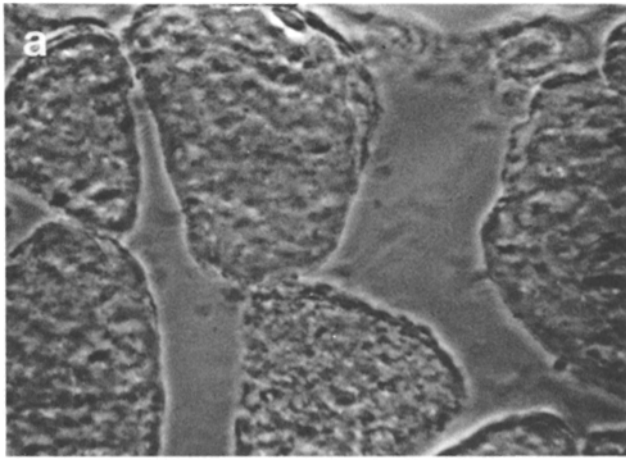
FIGURE 1 The monoclonal antibody stains synaptic sites in frozen sections of frog muscle. (a) Phase optics illustrates the myofibers, (b) tetramethylrhodamine α-bungarotoxin binding is visualized with fluorescence optics which are selective for rhodamine, and (c) monoclonal antibody binding is visualized with fluorescence optics which are selective for fluorescein. Bar, 15 μm.

the antigen is only accessible to antibody in whole muscle whose membrane structure has been perturbed by extraction with nonionic detergents.

Antibody Binds to a Postsynaptic Intracellular Structure

The ability to label the antigen with antibody in whole muscle that has been permeabilized with nonionic detergent suggests that the antigen is intracellular. It is possible, however, that the detergent alters the membrane structure so as to expose determinants within the membrane. To determine whether the antibody binds to an antigen within the membrane or to an intracellular antigen, the antibody binding was visualized with ferritin-labeled antibodies and electron microscopy.

The *cutaneous pectoris* muscle was fixed with 1% formaldehyde and treated with 0.1% saponin to disrupt the membrane. The muscle was incubated with monoclonal antibody followed by ferritin-labeled G-α-MIgG. Fig. 4a demonstrates that the immunoferritin is concentrated at the intracellular surface of the postsynaptic membrane. Furthermore, the immunoferritin is concentrated in an ~50 nm zone of amorphous material that is situated between the postsynaptic membrane and a deeper layer of intermediate filaments (Fig. 4b). This amorphous material is not well resolved, but it is clearly distinguished from the underlying filaments. This pattern of staining was observed at all synaptic sites (31 synaptic sites in 3 muscles) in normal muscle. Thus, the antigen is associated with an intracellular, postsynaptic structure that is situated between the postsynaptic membrane and a layer of intermediate filaments that lie ~50 nm beneath the membrane.

Antibody Binds to a 51,000 Dalton Protein

To identify the antigen for the monoclonal antibody, proteins from *Torpedo* electric organ were resolved in SDS gels and
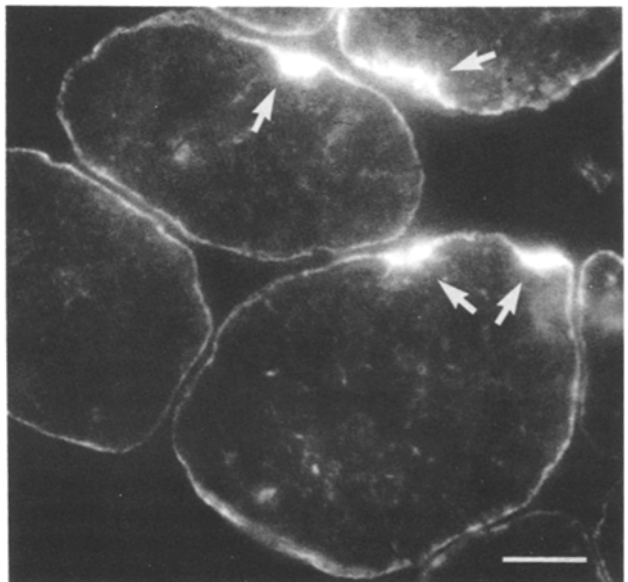
FIGURE 2 After denervation the antigen remains concentrated at synaptic sites but also appears at extrasynaptic regions of myofibers. The antibody binding is visualized with fluorescence optics which are selective for fluorescein. The arrows indicate denervated synaptic sties which were identified by tetramethyl rhodamine α-bungarotoxin. The increase in density of acetylcholine receptors in denervated muscle is not detected with TMR-α-BGT. The muscle was denervated for 6 d. Bar, 10 μm.

incubated with antibodies, the antibody staining pattern was essentially identical to the pattern of α-BGT staining in whole, nonpermeabilized muscle (Fig. 3b). The antibody staining is arranged in elongate, arborized areas that correspond in size and shape to α-BGT stained areas; the striations within the antibody stained area occur at ~0.5 μm intervals and are likely to correspond to the position of the postjunctional folds. Thus,
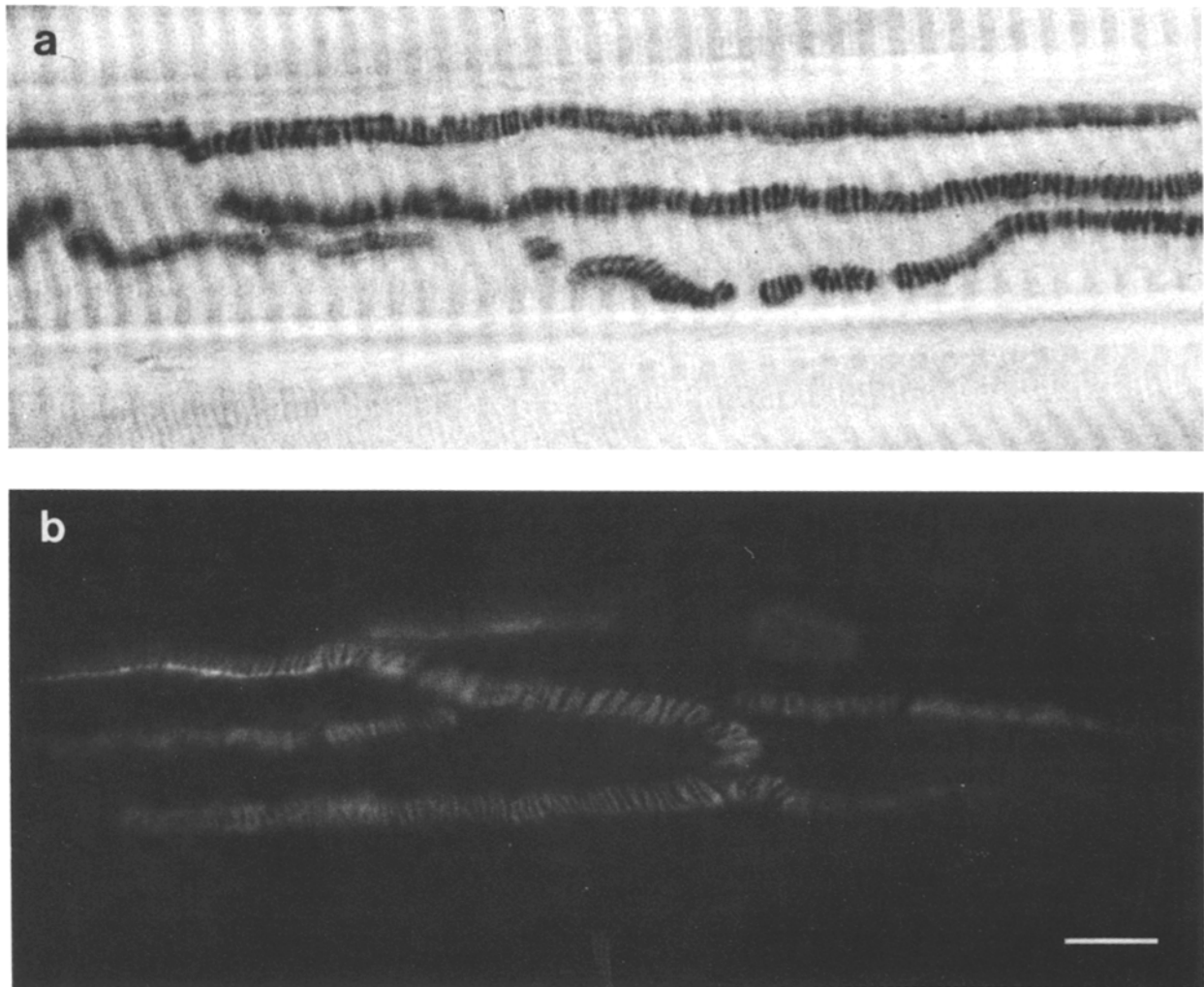
FIGURE 3   The antibody staining pattern in Triton X-100–permeabilized whole-mounts of the *cutaneous pectoris* muscle is arranged in elongate arborized areas which are characteristic of the frog neuromuscular synapse. (a) HRP-α-bungarotoxin staining in a whole-mount of intact muscle illustrates the characteristic arborization of the synaptic site in frog muscle. (b) Immunofluorescence staining of monoclonal antibody binding in a whole-mount of muscle permeabilized with Triton X-100. The pattern of antibody staining is essentially identical to the pattern of α-bungarotoxin staining. The striations within the stained area occur with a periodicity of 0.5 μm and correspond to the regular folding of the postsynaptic membrane. Antibody staining is not observed when detergent is omitted from the incubations. Bar, 8 μm.

labeled with the antibody. Fig. 5 b demonstrates that a protein of 51,000 daltons (51 kd) is labeled by the antibody; in freshly boiled tissue no other protein bands are labeled. Thus, the antibody is termed anti-51 kd (α-51kd).

It has not yet been possible to isolate the 51-kd protein without significant proteolysis. The 51-kd protein remains insoluble after extraction of the electric organ with low ionic strength buffer and low ionic strength buffer with 2% Triton X-100 (see Materials and Methods); the protein can be solubilized, however, by further extraction of the detergent-insoluble residue with high ionic strength (0.6 M potassium iodide, see Materials and Methods). The 51-kd protein which is solubilized by high ionic strength is, however, severely proteolyzed. Antibody labeling of gel transfers of the high ionic strength extract shows that as many as 10 bands are labeled by α-51 kd (Fig. 5 d). The smallest polypeptide fragment that is labeled, is 33,000 daltons. Inclusion of a variety of protease inhibitors (0.3 mM PMSF, 10 mM DFP, 10 mM EGTA, 1 mM EDTA, 10 mM N-ethyl maleimide [NEM]) in all buffers has not yet simplified the proteolysis.

A Monoclonal Antibody to All Intermediate Filaments Reacts with the 51-kd Protein

The close association of the 51-kd protein with intermediate-sized filaments at the neuromuscular junction raised the possibility that α-51 kd reacted with a cytoskeletal component. Fig. 5 c demonstrates that a monoclonal antibody directed against all classes of intermediate filaments (α-IFA; 39) labels a 51-kd protein from *Torpedo* electric organ. Autolysis of the 51-kd protein occurs during its isolation and the proteolytic fragments which are generated are labeled by both α-51 kd and α-IFA (Fig. 5d and e). Since α-IFA and α-51 kd label the same proteolytic fragments (Fig. 5 d and e), it is likely that the two different monoclonal antibodies react with the same 51-kd protein. Based on its reactivity with α-IFA, the 51-kd protein must share an antigenic determinant with intermediate filament proteins. Since proteins of higher molecular weight are labeled by α-IFA and not by α-51 kd (Fig. 5 b and c), the two antibodies must react with different determinants and hence different sites on the 51-kd protein. Furthermore, α-51 kd stains only synaptic
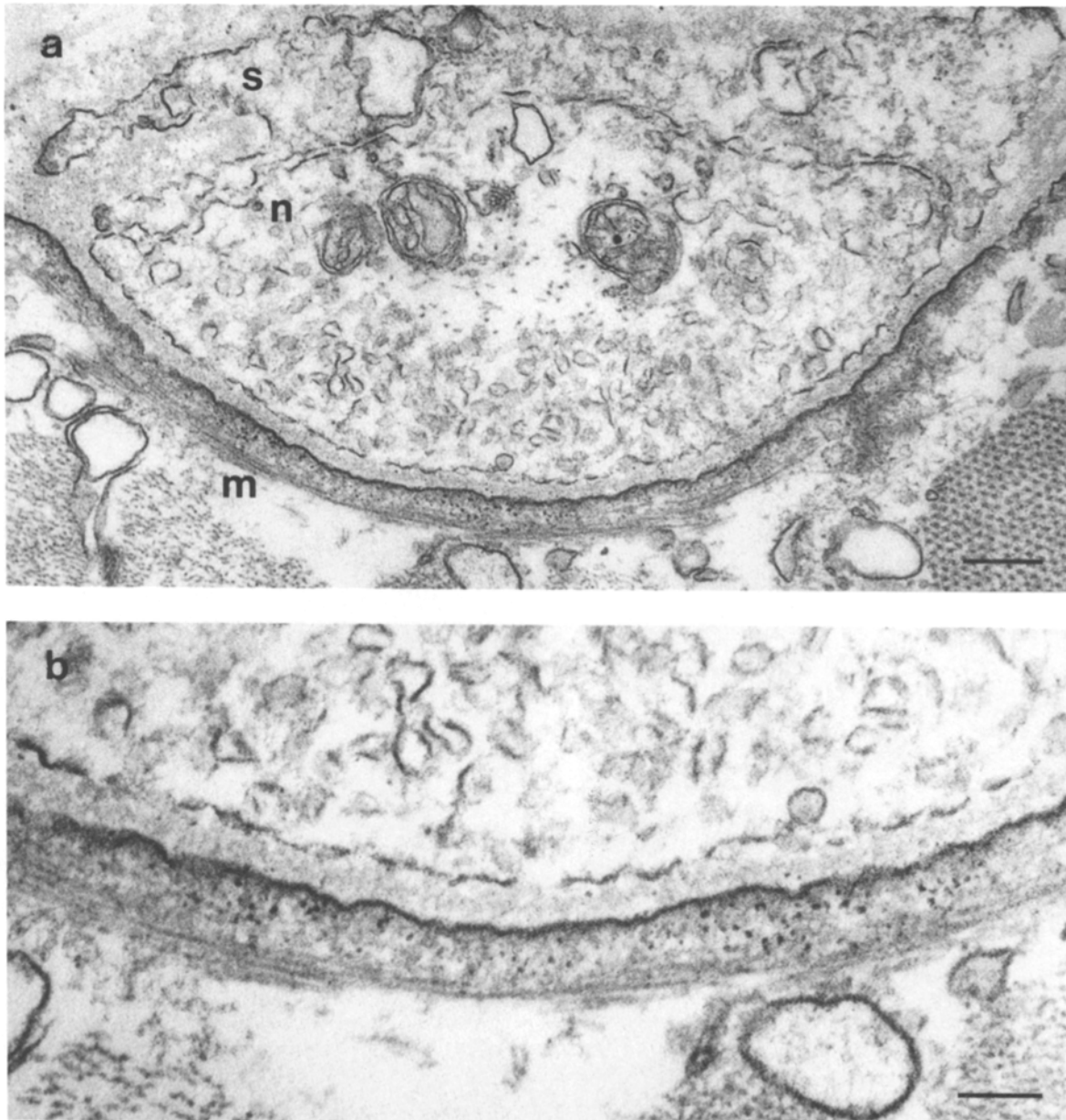
FIGURE 4 The monoclonal antibody binds to an antigen which is associated with amorphous material that is situated between the postsynaptic membrane and an underlying layer of intermediate filaments. The *cutaneous pectoris* muscle was fixed with 1% formaldehyde, permeabilized with 0.1% saponin, incubated with monoclonal antibody followed by ferritin-conjugated goat-anti-mouse IgG, and prepared for electron microscopy. (a) The immunoferritin is concentrated at the synapse on the cytoplasmic side of the postsynaptic membrane. (b) The immunoferritin is concentrated in a 50-nm zone of amorphous material that is interposed between the postsynaptic membrane and a layer of intermediate filaments. Postsynaptic intermediate filaments are aligned parallel to the junctional folds and are oriented parallel to the postsynaptic membrane in transverse sections (23). s, Schwann cell; n, nerve terminal; m, myofiber. Bar, (a) 200 nm, (b) 100 nm.

sites in frozen sections of frog muscle, whereas α-IFA stains axons, Schwann cells, structures within myofibers, and synaptic sites (Fig. 6, and reference 39).

α-51 kd Stains the Perinuclear Region in Epithelial Cells of Frog Skin

Since the 51-kd protein shares an antigenic determinant with intermediate filament proteins, it seemed possible that the 51-kd protein was a previously characterized intermediate filament protein. Intermediate filament proteins are those which can assemble into 7–11 nm filaments. These include: neurofilaments, glial filaments, desmin, vimentin, and tonofilaments

(28). The different filaments are distinguished from each other by their polypeptide composition and by antisera to the individual polypeptides. For example: in mammals, neurofilaments are composed of polypeptides 210 kd, 150 kd, and 70 kd, and vimentin is a single polypeptide of 58 kd. Neurofilaments and glial filaments are found in neurons and astrocytes respectively. Desmin is present in muscle cells and concentrated at the Z-band. Vimentin is present in mesenchymal cells and concentrated at the Z-band in muscle cells. Tonofilaments (cytokeratin filaments) are present in epithelial cells where they are concentrated and organized at desmosomes. The function of these filaments in any cell is not clear.
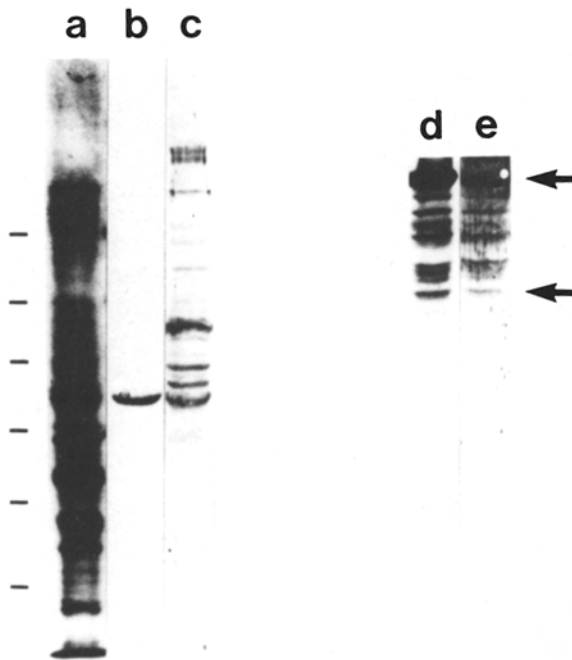
FIGURE 5   The monoclonal antibody reacts with a protein of 51 kd that shares an antigenic determinant with intermediate filament proteins. Proteins from *Torpedo* electric organ were resolved in SDS polyacrylamide gels (8.75%), transferred to nitrocellulose and the paper replicas were stained with antibodies. In (a, b, and c) the sample was a homogenate of the electric organ and in (d and e) the sample was a high ionic strength extract of the electric organ (see Materials and Methods). (a) the gel is stained for protein with Coomassie Brilliant Blue; (b and d) the paper replicas were incubated with monoclonal antibody to the postsynaptic, intracellular antigen (anti-51 kd); (c and e) the paper replicas were incubated with a monoclonal antibody to intermediate filament protein (anti-IFA; 39) (see Materials and Methods for details). (b) a single protein band at 51 kd is labeled. (c) a protein band at 51 kd and several proteins of higher molecular weight are labeled. The position of standard proteins is indicated to the left of a: (phosphorylase A, 95 kd; bovine serum albumin, 68 kd; catalase, 60 kd; actin, 42 kd; aldolase, 40 kd; carbonic anhydrase, 29 kd). (d and e) the same proteolytic fragments are labeled by anti-51-kd and anti-IFA. In (d and e) only the region below 51 kd is shown. The arrows indicate the position of 51 kd and 33 kd.

In frozen sections of frog muscle α-IFA stains axons, Schwann cells, cytoplasmic structures within myofibers, and synaptic sites (Fig. 6, and reference 39). Since α-51 kd stains only synaptic sites in frozen sections of frog muscle (Fig. 1), α-51 kd does not react with vimentin, desmin, or neurofilaments in unfixed frozen sections.

α-51 kd does, however, stain epithelial cells in frog skin. Fig. 7 d demonstrates that α-IFA staining is present throughout the cytoplasm of the differentiated epithelial cells in frog skin. α-51 kd staining, however, is restricted to the perinuclear region in all layers of frog skin (Fig. 7 b). Thus, most of the tonofilaments that are stained by α-IFA are not stained by α-51 kd. Furthermore, gel transfers of proteins from frog skin demonstrates that α-IFA reacts predominantly with proteins of 65 kd, 63 kd, 60 kd, and 48 kd; several protein bands between 60 kd and 48 kd are stained less intensely (Fig. 8 c). α-51 kd reacts predominantly with a protein of ~55 kd in frog skin; a protein band at 65 kd is stained much less intensely (Fig. 8 b). Thus, a protein of ~55 kd is restricted to the perinuclear region of epithelial cells in frog skin and is not the major intermediate

filament protein in these cells. It is not yet clear whether the 55-kd protein in frog skin has homology with intermediate filament proteins nor whether this protein is similar to the 51-kd protein present in *Torpedo* electric organ. It is also not clear whether the 55-kd protein is associated with the nuclear membrane-matrix or with structures in the perinuclear cytoplasm (16). Nevertheless, it is clear that α-51 kd does not react with any previously characterized intermediate filament proteins (neurofilaments, glial filaments, vimentin, desmin, and cytokeratin).

DISCUSSION

Using monoclonal antibodies to identify synaptic macromolecules, I have identified a protein of 51 kd that is associated with postsynaptic intracellular material at the frog neuromus-
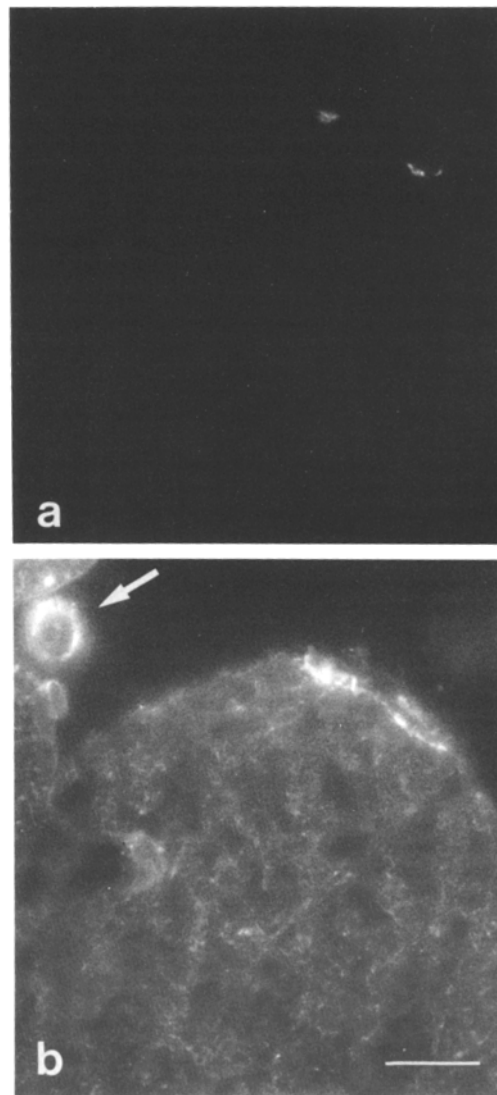

FIGURE 6   Anti-IFA stains synaptic sites and structures within myofibers. Frozen sections of frog muscle were stained with α-IFA and TMR-α-bungarotoxin as described in Materials and Methods. (a) TMR-α-bungarotoxin binding is visualized with fluorescence optics which are selective for rhodamine, (b) α-IFA binding is visualized with fluorescence optics which are selective for fluorescein. α-IFA staining is present throughout myofibers and is more intense at synaptic sites; the intense synaptic staining extends laterally beyond the TMR-α-bungarotoxin stained site. α-IFA also stains Schwann cells and axons (arrow, 39). Bar, 10 μm.
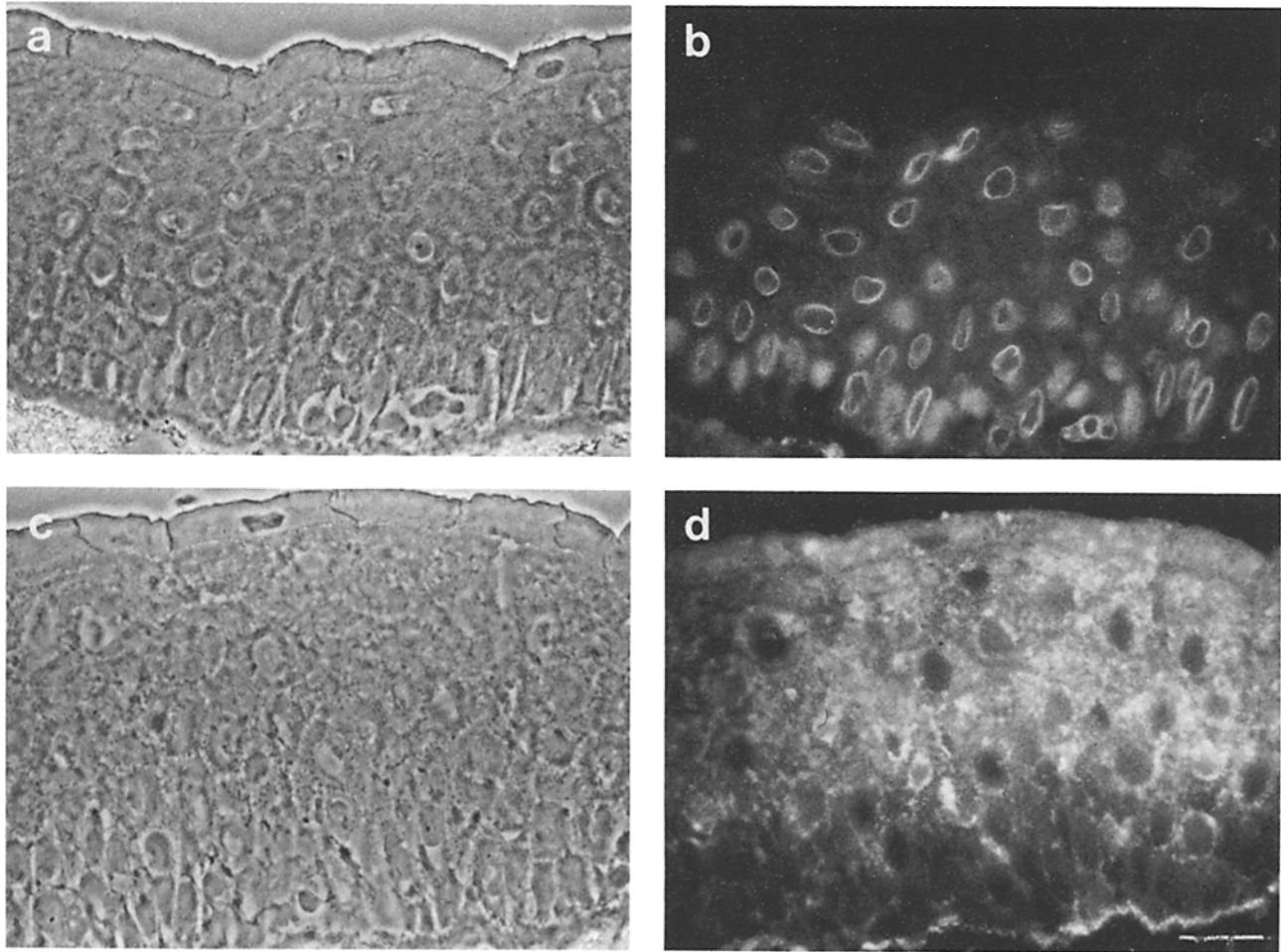
FIGURE 7   Anti-51 kd stains the perinuclear region of epithelial cells in frog skin. (a and b) Paired phase and immunofluorescence micrographs of α-51 kd staining on a frozen section of frog skin. (c and d) paired phase and immunofluorescence micrographs of α-IFA-staining in a frozen section of frog skin. α-51-kd stains the perinuclear nuclear region of epithelial cells in frog skin. α-IFA stains throughout the cytoplasm of epithelial cells in frog skin; more intense staining is present in the more differentiated layers of the skin. Bar, 15 μm.

cular junction. In normal muscle the protein is highly concentrated at synaptic sites. In denervated muscle the antigen remains concentrated at synaptic sites but also appears at lower density at extrasynaptic regions of myofibers.

Many physiological and biochemical changes are known to occur in denervated muscle. The most striking of the denervation changes is the dramatic increase in the number of acetylcholine receptors at extrasynaptic areas of denervated myofibers (3, 35). The increase in the density of extrasynaptic acetylcholine receptors in denervated frog muscle is well apparent within 1 wk after denervation and attains its peak value after 2 wk (11). The time course of the change in antigen number after denervation has not yet been determined, but extrasynaptic staining is apparent within 1 wk after denervation and persists for at least 1 mo. α-51 kd staining is also apparent within denervated myofibers. The fine-structural localization of the antigen in denervated muscle has not yet been determined; it is likely, however, that some of this internal staining reflects synthesis of antigen that is destined for the extrasynaptic surface.

The pattern of α-51 kd staining in Triton X-100 permeabilized whole muscle is essentially identical to the pattern of α-bungarotoxin staining in intact whole muscle. The striations

within the HRP-α-BGT stain results from the high concentration of acetylcholine receptors along the sides of the postjunctional folds (6, 17). The similar pattern of α-51 kd staining in whole-mounts suggests that the 51-kd protein is likewise concentrated along the sides of the postjunctional folds and not restricted to the crests of the folds or evenly distributed between the folds. Confirmation of this hypothesis will require immunoelectron microscopy of muscle that has been longitudinally sectioned to reveal the regular postsynaptic folds.

Immunoelectron microscopy in saponin-permeabilized muscle demonstrates that the 51-kd protein is associated with amorphous material that is interposed between the postsynaptic membrane and an underlying layer of intermediate filaments. A monoclonal antibody to intermediate filament proteins (α-IFA) reacts with the same 51-kd protein. It is interesting that although the 51-kd protein shares an antigenic determinant with intermediate filament proteins, the 10-nm filaments at the neuromuscular junction are apparently not labeled by α-51 kd. It is possible that the 51-kd protein is associated with the assembled 10-nm filaments, but that the determinant recognized by α-51 kd is sterically hindered or altered when the 51-kd protein is associated with assembled filaments. Further experiments will be necessary to determine whether the 51-kd
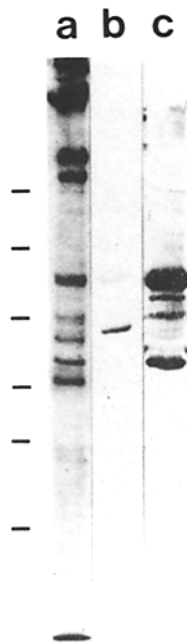
FIGURE 8 Anti-51-kdalton does not label the major cytokeratin proteins in frog skin. Proteins from frog skin were resolved in SDS polyacrylamide gels (8.75%), transferred to nitrocellulose, and the paper replicas were stained with antibodies. (a) the gel is stained for protein with Coomassie Brilliant Blue; (b) the paper replicas were incubated with anti-51-kdalton; (c) the paper replicas were incubated with anti-IFA. (b) The antibody reacts predominantly with a protein of ~55 kdaltons and less intensely with a protein of 65 kdaltons. (c) The antibody reacts predominantly with proteins of 65 kdaltons, 63 kdaltons, 50 kdaltons, and 48 kdaltons; several proteins between 48 kdaltons and 60 kdaltons are labeled less intensely. The position of standard proteins (see Fig. 5) are indicated to the left of (a).

protein is present only within the amorphous material or whether it is also associated with the 10-nm filaments. Presently, I have not been able to do immunoelectron microscopy with α-IFA; the interaction of α-IFA with its synaptic antigen is extremely sensitive to fixation.

The structure of the subsynaptic sarcoplasm at the neuromuscular junction has been elegantly described by Couteaux and colleagues (9, 10). They were the first to describe the presence of intermediate filaments at neuromuscular synapses in frog skeletal muscle (10). These filaments have also been observed at neuromuscular synapses in rapidly frozen frog muscle (23) and at mammalian neuromuscular synapses (13). In the frog the 10-nm filaments are oriented parallel to the axis of the postjunctional folds (perpendicular to the long axis of the myofiber) and are concentrated as a core between the folds. Poorly resolved material is found between this core of filaments and the postsynaptic membrane. This amorphous material is present at the crests and along the sides of the postjunctional folds, and is sparse, or absent, from the bottoms of the folds (9, 10). Thus, this amorphous material has a distribution similar to that of acetylcholine receptors (18). Since the receptors are thought to extend 50 Å beyond the bilayer on the extracellular surface, but only 15 Å beyond the bilayer on the intracellular surface (42) it seems unlikely that the receptor itself can account for much of this more extensive amorphous material.

In *Torpedo* electric organ, a network of filaments, which are ~10 nm in diameter, is found throughout the cytoplasm of the

electrocyte (24, 41). Most of these filaments are not labeled by α-51 kd, since α-51 kd staining in the electric organ is coincident with TMR-α-BGT staining and not observed within the electrocyte (data not presented). The cytoplasmic filaments may, however, be intermediate filaments, since α-IFA stains structures throughout the electrocyte (data not presented).

Two antigens, in addition to the 51-kd protein, have been identified as intracellular, postsynaptic proteins. A 43-kd protein, which copurifies with acetylcholine receptor-enriched membrane fragments from *Torpedo* electric organ (46), is concentrated at the innervated face of the electrocyte (21). The 43-kd protein can be selectively solubilized from the membrane fragments by alkaline pH without an alteration of acetylcholine receptor function (36). Antisera against the 43-kd protein isolated from *Torpedo* stains synaptic sites in frozen sections of mammalian muscle (21), and does not label synapses in intact, whole muscle (20). The antigen for this antiserum, however, has not been identified in mammalian tissue. Affinity purified antibodies to *Aplysia* body-wall actin have been used to identify a cross-reacting antigen which is concentrated at mammalian neuromuscular junctions (22). The antibodies do not stain myofibrils in mammalian skeletal or smooth muscle, but do stain cytoplasmic filaments within mammalian fibroblasts and myoblasts (32); these results suggest that the antibodies react with nonmuscle, cytoplasmic actin. The antigen for these antibodies, however, has not been biochemically characterized in mammalian tissue. Nevertheless, these antibodies stain synaptic sites in frozen sections of mammalian muscle and the synaptic staining persists after denervation (22).

The 51-kd protein is clearly different than the 43-kd protein and *Torpedo* actin (47): the molecular weights of the proteins are different and α-51 kd does not react with the 43-kd protein or *Torpedo* actin in gel transfers (data not presented).

The structure of the subsynaptic sarcoplasm has received considerable attention (9, 10, 23, 24, 45); the function of this specialization, however, remains one of conjecture. A first step in characterizing the function of this structure is to identify its components. The monoclonal antibody to the 51-kd protein should be a useful probe to study the distribution of the protein during development, its interaction with other synaptic proteins and its role in the formation and maintenance of neuromuscular synapses.

REFERENCES

1. Adams, J. C. 1977. Technical considerations on the use of horseradish peroxidase as a neuronal marker. *Neurosci.* 2:141–145.
2. Anderson, M. J., and M. W. Cohen. 1977. Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.).* 268:757–773.
3. Axelsson, J., and S. Thesleff. 1959. A study of supersensitivity in denervated mammalian muscle. *J. Physiol. (Lond.).* 147:178–193.
4. Birks, R., H. E. Huxley, and B. Katz. 1960. The fine structure of the neuromuscular junction of the frog. *J. Physiol. (Lond.).* 150:134–144.
5. Birks, R., B. Katz, and R. Miledi. 1960. Physiological and structural changes at the

amphibian myoneural junction, in the course of nerve degeneration. *J. Physiol. (Lond.).* 150:145–168.

6. 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.

7. Cohen, J. B., M. Weber, M. Huchet, and J. P. Changeux. 1972. Purification from *Torpedo marmorata* electric tissue of membrane fragments particularly rich in cholinergic receptor protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 26: 43–45.

8. Couteaux, R. 1955. Localization of cholinesterases at neuromuscular junctions. *Int. Rev. Cytol.* 4:335–375.

9. Couteaux, R. 1981. Structure of the subsynaptic sarcoplasm in the interfolds of the frog neuromuscular junction. *J. Neurocytol.* 10:947–962.

10. Couteaux, R., and Pécot-Dechavassine. 1968. Particularities structurales du sarcoplasma sous-neural. *C. R. Acad. Sci. Paris.* 266:8–10.

11. Dreyer, F., and K. Peper. 1974. The spread of acetylcholine sensitivity after denervation of frog skeletal muscle fibers. *Pfleugers Arch.* 348:287–292.

12. Duguid, J. R., and M. A. Raftery. 1973. Fractionation and partial characterization of membrane particles from *Torpedo californica* electroplax. *Biochemistry.* 12:3593–3597.

13. Ellisman, M. H., J. E. Rash, L. A. Staehelin, and K. R. Porter. 1976. Studies of excitable membranes. II. A comparison of specializations at neuromuscular junctions and nonjunctional sarcolemmas of mammalian fast and slow twitch muscle fibers. *J. Cell Biol.* 68:752–774.

14. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG₁, IgG₂ₐ, and IgG₂ᵦ immunoglobulins from mouse serum using protein A-Sepharose. *Immunochemistry.* 15:429–436.

15. Fambrough, D. M. 1979. Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* 59:165–227.

16. Farquhar, M. G., and G. E. Palade. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* 26:263–291.

17. Fertuck, H. C., and M. M. Salpeter. 1974. Localization of acetylcholine receptor by ¹²⁵I-labeled-bungarotoxin binding at mouse motor endplates. *Proc. Natl. Acad. Sci. U. S. A.* 71:1376–1378.

18. Fertuck, H. C., and M. M. Salpeter. 1976. Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after ¹²⁵I-bugarotoxin binding at mouse neuromuscular junctions. *J. Cell Biol.* 69:114–158.

19. Frank, E., and G. D. Fischbach. 1979. Early events in neuromuscular junction formation *in vitro. J. Cell Biol.* 83:143–158.

20. Froehner, S. C., and V. G. Gulbrandsen. 1981. Antiserum to *Torpedo* 43k protein recognizes an intracellular postsynaptic membrane protein of the rat neuromuscular junction. *Neurosci. Abstr.* 227:7.

21. Froehner, S. C., V. Gulbrandsen, C. Hyman, A. Y. Yeng, R. R. Neubig, and J. B. Cohen. 1981. Immunofluorescence localization at the mammalian neuromuscular junction of the Mₑ 43,000 protein of *Torpedo* postsynaptic membranes. *Proc. Natl. Acad. Sci. U. S. A.* 8:5230–5234.

22. Hall, Z. W., B. W. Lubit, and J. H. Schwartz. 1981. Cytoplasmic actin in postsynaptic structures at the neuromuscular junction. *J. Cell Biol.* 90:789–792.

23. Heuser, J. E. 1980. 3-D visualization of membrane and cytoplasmic specializations of the frog neuromuscular junction. *In* Ontogenesis and Functional Mechanisms of Peripheral Synapses J. Taxi, editor. Inserm Symposium no. 13. Elsevier/North Holland, Amsterdam 139–155.

24. Heuser, J. E., and S. R. Salpeter. 1979. Organization of acetylcholine receptors in quick-frozen, deep-etched, and rotary-replicated *Torpedo* postsynaptic membrane. *J. Cell Biol.* 82:150–173.

25. Kohler, G., and C. Milstein. 1976. Derivation of specific antibody producing tissue culture and tumour lines by cell fusion. *Eur. J. Immunol.* 6:511–519.

26. Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* 80:575–599.

27. Lake, P., E. A. Clark, M. Khorshidi, and G. H. Sunshine. 1980. Production and characterization of cytotoxic Thy-1 antibody-secreting hybrid cell lines. Detection of T cell subsets. *Eur. J. Immunol.* 9:875–886.

28. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of space. *Nature (Lond.).* 283:249–256.

29. Letinsky, M. S., K. Fischbeck, and U. J. McMahan. 1976. Precision or re-innervation of original synaptic sites in frog muscle after a nerve crush. *J. Neurocytol.* 5:691–718.

30. Littlefield, J. W. 1964. Selection of hybrids from mating of fibroblasts in vitro and their presumed recombinants. *Science (Wash. D. C.).* 145:709–710.

31. Massoulié, J., and S. Bon. 1982. The molecular forms of cholinesterase and acetylcholin-esterase in vertebrates. *Annu. Rev. of Neurosci.* 5:57–106.

32. Lubit, B. W., and J. H. Schwartz. 1980. An antiactin antibody that distinguishes between cytoplasmic and skeletal muscle actins. *J. Cell Biol.* 86:891–897.

33. McMahan, U. J., and S. W. Kuffler. 1971. Visual identification of synaptic boutons on living ganglion cells and of varicosities in postganglionic axons in the heart of the frog. *Proc. Roy. Soc. Lond. B.* 177:485–508.

34. McMahan, U. J., N. C. Spitzer, and K. Peper. 1972. Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc. Lond. B* 181:421–430.

35. Miledi, R. 1960. Acetylcholine sensitivity of frog muscle fibers after complete or partial denervation. *J. Physiol. (Lond.).* 151:1–23.

36. Neubig, R. R., E. K. Krodel, N. D. Boyd, and J. B. Cohen. 1979. Acetylcholine and local anesthetic binding to *Torpedo* nicotinic postsynaptic membranes after removal of nonreceptor peptides. *Proc. Natl. Acad. Sci. U. S. A.* 76:690–694.

37. Ohtsuki, I., R. M. Manzi, G. E. Palade, and J. D. Jamieson. 1978. Entry of macromolecular tracers into cells fixed with low concentrations of aldehydes. *Biologie Cellulaire.* 31:119–126.

38. Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobin-producing hybrid cell lines. *In* Selected Methods in Cellular Immunology. Vol. 3. B. B. Mishell, and S. M. Shugi, editors. W. H. Freeman and Co. S.F. 51.

39. Pruss, R. M., R. Mirsky, M. C. Raff, R. Thorpe, A. J. Dowding, and B. H. Anderton. 1981. All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell.* 27:419–428.

40. Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of α-bungarotoxin: preparation, separation and characterization. *Anal. Biochem.* 80:585–592.

41. Rosenbluth, J. 1975. Synaptic membrane structure in *Torpedo* electric organ. *J. Neurocytol.* 4:697–712.

42. Ross, M. J., M. W. Klymkowsky, D. A. Agard, and R. M. Stroud. 1977. Structural studies of a membrane-bound acetylcholine receptor from *Torpedo californica. J. Mol. Biol.* 116:635–659.

43. Rubin, L. L., S. M. Schuetze, C. L. Weill, and G. D. Fischbach. 1980. Regulation of acetylcholinesterase appearance at neuromuscular junctions *in vitro. Nature (Lond.).* 283:264–267.

44. Sanes, J. R., L. M. Marshall, and U. J. McMahan. 1978. Re-innervation of muscle fiber basal lamina after removal of myofibres. *J. Cell Biol.* 78:176–198.

45. Sealock, R. 1982. Cytoplasmic surface structure in postsynaptic membranes from electric tissue visualized by tannic-acid-mediated negative contrasting. *J. Cell Biol.* 92:514–522.

46. Sobel, A., T. Heidmann, J. Hofler, and J. P. Changeux. 1978. Distinct protein components from *Torpedo marmorata* membranes carry the acetylcholine receptor site and the binding site for local anesthetics and histrionicotoxin. *Proc. Natl. Acad. Sci. U. S. A.* 75:510–514.

47. Strader, C. D., E. Lazarides, and M. A. Raftery. 1980. The characterization of actin associated with postsynaptic membranes from *Torpedo californica. Biochem. Biophys. Res. Comm.* 92:365–373.

48. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76:4350–4354.

49. Venable, J. H., and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 24:407–408.

50. Voller, A., D. Bidwell, and A. Bartlett. 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections. *In: Manual of Clinical Immunology.* N. R. Rose and H. Friedman. *Am. Soc. Microbiol.* 506–512.

51. Williams, A. F., G. Galfre, and C. Milstein. 1977. Analysis of cell surfaces by xenogeneic myeloma hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell.* 12:663–673.

52. Willingham, M. C., S. Yamada, and I. Pastan. 1978. Ultrastructural antibody localization of α-macroglobulin in membrane-limited vesicles in cultured cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:4359–4363.