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 it 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

BALB/c and nu/nu mice were provided by the Animal Breeding Unit of the Imperial Cancer Research Fund, Mill Hill, London, England. Frogs (Rana pipiens...
and Rana temporaria) were obtained from commercial suppliers, and Torpedo electric organ was obtained frozen from Pacific Biomarine (Venice, CA).

**Operation**

Frogs were anesthetized in 0.1% tricaine methanesulfonate (Inquil, 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 nomenclature variant cell line P3/NSI-A-4G1 (NS-I) 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% CO₂ and 95% air. The cell density was maintained at 1 x 10⁶ to 4 x 10⁶ cells/ml by passing the cells every few days.

**Media**

The parent myeloma and hybridomas were grown in RPMI 1640 (Flow Labs, McLean, 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 adjusted 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 phenylmethylsulfonyl fluoride (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 5 x 10⁵ large, viable NS-I cells and centrifuged in a conical tube. The ratio of spleen cells to NS-I cells was 100:1. The resulting mixture was homogenized with a motor-driven Potter-Elvehjem pestle and insoluble material was collected after centrifugation. The tissue was 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.

**Subclass Identification and Purification**

The subclass of monoclonal antibodies was determined by immunodiffusion against subclass specific reagents (Miles Laboratories, Inc., Elkhart, IN) and with fluorescein 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 N₂ 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 multistep slides (PFTE slides; Hendley-Essen, 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-a-MIgG; Nordic, Tilburg, 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 μg/ml of o-phenylenediamine). Diamine sites in the muscle were marked by including tetramethylrhodamine-a-bungarotoxin (40 μg/ml; TMR-a-BTX; 4 x 10⁻⁴ 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 487/90, 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 487/74, 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-a-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 assessed with filters selective for rhodamine as described above.

**Horseradish Peroxidase Coupled-a-Bungarotoxin**

**Labeling of Acetylcholine Receptors**

Horseradish peroxidase (HRP) was coupled to a-bungarotoxin (HRP-a-BTX) with glutaraldehyde and the conjugate was separated from free a-BTX as previously described in this report.

522 THE JOURNAL OF CELL BIOLOGY. VOLUME 94, 1982
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 ¼ 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 xylenes, 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-antibody (Nordic) for 1 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 OsmO (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, homogenized 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 thiocyanate, 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 (59). 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.

The muscle was washed for the monoclonal antibody in skin, small pieces of skin from the abdomen of frogs (R. piperis) 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 synaptic 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 FI-G-α-MlgG 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. 3a. 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 postsynaptic 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

Burden Identification of a Postsynaptic Antigen 523

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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 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,
labeled with the antibody. Fig. 5b 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. 5d). 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. 5c 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. 5d 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. 5b 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.
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. 7d 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. 7b). 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 demonstrate 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. 8c). 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. 8b). 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 (neurofilsaments, 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-
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...
A 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 51-kd protein is 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.

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