

# Acetylcholine Receptor–aggregating Proteins Are Associated with the Extracellular Matrix of Many Tissues in *Torpedo*

Earl W. Godfrey, Mary E. Dietz, Ann L. Morstad, Peter A. Wallskog, and Donald E. Yorde

Department of Anatomy and Cellular Biology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

**Abstract.** The synaptic basal lamina, a component of extracellular matrix (ECM) in the synaptic cleft at the neuromuscular junction, directs the formation of new postsynaptic specializations, including the aggregation of acetylcholine receptors (AChRs), during muscle regeneration in adult animals. Although the molecular basis of this phenomenon is unknown, it is mimicked by AChR-aggregating proteins in ECM-enriched fractions from muscle and the synapse-rich electric organ of the ray *Torpedo californica*. Molecules immunologically similar to these proteins are concentrated in the synaptic basal lamina at neuromuscular junctions of the ray and frog. Here we demonstrate that immunologically, chemically, and functionally similar AChR-aggregating proteins are also associated with the ECM of several other tissues in *Torpedo*. Monoclonal antibodies against the AChR-aggregating proteins from electric organ intensely stained neuromuscular junctions and the ventral surfaces of electrocytes, structures with a high density of AChRs. However, they

also labeled many other structures which have basal laminae, including the extrajunctional perimeters of skeletal muscle fibers, smooth and cardiac muscle cells, Schwann cell sheaths in peripheral nerves, walls of some blood vessels, and epithelial basement membranes in the gut, skin, and heart. Some structures with basal laminae did not stain with the antibodies; e.g., the dorsal surfaces of electrocytes. Bands of similar molecular weight were detected by the antibodies on Western blots of extracts of ECM-enriched fractions from electric organ and several other tissues. Proteins from all tissues examined, enriched from these extracts by affinity chromatography with the monoclonal antibodies, aggregated AChRs on cultured myotubes. Thus, similar AChR-aggregating proteins are associated with the extracellular matrix of many *Torpedo* tissues. The broad distribution of these proteins suggests they have functions in addition to AChR aggregation.

EXTRACELLULAR material is present in the synaptic clefts of both central and peripheral synapses (28). At the vertebrate neuromuscular junction, this material consists of the synaptic portion of the muscle fiber's basal lamina sheath. The synaptic basal lamina is chemically specialized in that it contains synapse-specific antigens (34, 35) and a high concentration of cholinesterase (22). Functionally, the synaptic basal lamina is also unique. Unlike the extrasynaptic regions of the basal lamina, it triggers the differentiation of both pre- and postsynaptic structures at regenerating neuromuscular junctions (4, 36). Adult muscle fibers regenerating inside the basal lamina sheaths of damaged cells form new junctional folds and aggregates of acetylcholine receptors (AChRs)<sup>1</sup> at the original synaptic sites (4), even in the absence of nerve terminals and Schwann cells (21). The synaptic basal lamina, therefore, can direct the localization of synaptic structures during regeneration and may play a role in the development of these structures as well.

It is likely that postsynaptic differentiation caused by synaptic basal lamina is mediated by specific molecules associated with this structure. In an effort to identify such molecules, extracellular matrix (ECM)-enriched fractions have recently been screened for their ability to aggregate AChRs, using cultured embryonic myotubes as a bioassay (11, 44). The synapse-rich electric organ of the marine ray *Torpedo californica*, a tissue analogous to muscle, is a rich source of synaptic macromolecules, including AChRs and acetylcholinesterase (16, 17). Fractions enriched in ECM from *Torpedo* electric organ and muscle contain AChR-aggregating proteins (12), also termed "agrin" (18, 19, 31). Immunologically similar molecules are concentrated in the synaptic basal lamina of neuromuscular junctions in electric rays (8) and in the frog (19). Here we present evidence that proteins immunologically, chemically, and functionally similar to the AChR-aggregating proteins extracted from electric organ are also associated with the extracellular matrix of several other tissues in *Torpedo*. Monoclonal antibodies against these proteins stain several tissues in a pattern consistent with the localization of the AChR-aggregating proteins in many, but not

1. Abbreviations used in this paper: AChR, acetylcholine receptor; CSAT, cell substrate attachment; ECM, extracellular matrix.

all, basal laminae. This work has been presented in preliminary form (10).

## Materials and Methods

### Preparation of AChR-aggregating Proteins

Insoluble ECM-enriched fractions were prepared as previously described (12) from electric organ of adult *Torpedo californica* (Marinus, Inc., Long Beach, CA). The protease inhibitors leupeptin and pepstatin (0.5 µg/ml; Sigma Chemical Co., St. Louis, MO or Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA were added to buffers used in all steps. Additional protease inhibitors, 5 mM EGTA and 5 mM iodoacetamide, failed to increase biological activity in the preparations or simplify the pattern of immunoreactive bands on Western blots (cf. Fig. 1), so they were omitted. The ECM-enriched fractions were extracted five times with 50 mM sodium citrate buffer, pH 5.5, 50 mM NaCl, 0.01% NaN<sub>3</sub>, using a Polytron homogenizer (Brinkmann Instruments Co., Westbury, NY) at nearly full speed for 1 min, followed by stirring 30 min at 4°C. The insoluble material was removed by centrifugation (15 min, 19,000 g); the supernatants ("pH 5.5 extracts") contained AChR-aggregating activity (12).

The AChR-aggregating activity was purified ~1,000-fold (relative to electric organ homogenates) by a modification of the procedure of Nitkin et al. (26). Solid Tris base (Sigma Chemical Co.) and NaCl were added to the pH 5.5 extracts to final concentrations of 50 and 200 mM, respectively; the pH was adjusted to 9.0 with NaOH. This solution was pumped at 60 ml/h over a column of reactive blue 2-agarose (Sigma Chemical Co.; 40–80 ml of beads for extracts from 5 kg of tissue) equilibrated in 10 mM Tris-Cl, pH 9.0, 300 mM NaCl, 5% (vol/vol) glycerol, 0.01% NaN<sub>3</sub>. The column was washed with 2 column volumes of buffer, and the AChR-aggregating activity was eluted with buffer containing 3 M NaCl. Eluted fractions with the highest concentration of activity (70–100 ml; the "blue column-enriched proteins") were pooled and fractionated by gel filtration on a 2.5 × 120-cm column of Bio-Gel A 1.5m (Bio-Rad Laboratories, Richmond, CA) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, 0.5 M NaCl, 5% glycerol, 0.01% NaN<sub>3</sub>. The active fractions were dialyzed against 10 mM sodium phosphate buffer, pH 8.0, 5% glycerol, 0.01% NaN<sub>3</sub>, and were pumped at ~40 ml/h over a 1–2 ml column of DEAE cellulose (DE-52, Whatman Inc., Clifton, NJ). The column was washed with 40 ml of buffer and AChR-aggregating activity ("DEAE-enriched proteins") was eluted with buffer containing 200 mM NaCl. Protein was estimated by the method of Bradford (3), using BSA (ICN Immunobiologicals, Lisle, IL) in the the appropriate buffer as a standard.

### AChR Aggregation Assays

Aggregation of AChRs was quantitated as previously described, using cultures of chick embryo muscle cells (12). Samples to be assayed were added to the culture medium of the muscle cell cultures for overnight (14–16 h) incubation; AChRs were labeled with rhodamine- $\alpha$ -bungarotoxin (30) and AChR aggregates were counted.

### Monoclonal Antibodies

One male 5-wk-old BALB/c mouse (Jackson Laboratory, Bar Harbor, ME) was immunized intraperitoneally two times, 1 mo apart, with 50 µg of AChR-aggregating proteins (DEAE-enriched) from *Torpedo* emulsified with an equal volume of complete (first injection) or incomplete (second injection) Freund's adjuvant (Gibco, Grand Island, NY); 3 and 13 d after the second injection, DEAE-enriched proteins (10–20 µg) diluted twofold with 10 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS), were injected intravenously into the tail. 4 d after the last injection, the spleen cells were fused with P3x63 Ag 8.653 myeloma cells (2:1 ratio) by a modification of the procedure of Oi and Herzenberg (27).

Supernatant media from hybridoma isolates were initially screened for antibodies against DEAE-enriched proteins by ELISA. Bound antibodies were detected with horseradish peroxidase-conjugated, affinity-purified goat anti-mouse immunoglobulins (Bio-Rad Laboratories) and *O*-phenylenediamine. Media testing positive in this ELISA were screened for ability to precipitate AChR-aggregating activity. Sepharose CL4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) were activated with cyanogen bromide and coupled with immunoglobulins (13 mg protein/ml beads) from goat anti-mouse gamma globulins (Antibodies, Inc., Davis, CA). AChR-

aggregating proteins were incubated with 0.1 ml of hybridoma supernatant for 2 h at 25°C. Goat anti-mouse beads (0.1 ml of a 1:1 suspension in PBS) were added, and the mixture was rotated 2–3 h at 25°C. Beads were removed by centrifugation (1 min, 10,000 g) and the supernatant was assayed in duplicate for AChR-aggregating activity. If supernatant media precipitated 50% or more of the activity, the hybridomas were cloned by limiting dilution and clones were cultured in the presence of mouse peritoneal macrophages. The immunoglobulin type of monoclonal antibodies was determined by immunodiffusion and ELISA using subclass-specific antisera (ICN Immunobiologicals). Ascites fluid containing these antibodies or control monoclonal antibodies (CSAT, reference 25; a gift of Dr. A. F. Horwitz, University of Pennsylvania) was obtained from pristane-primed BALB/c mice injected intraperitoneally with 5 × 10<sup>6</sup> hybridoma cells.

### Mouse Antiserum

To enrich the AChR-aggregating proteins, 84 µg of DEAE-enriched proteins (1,000 U of activity) was passed over a 0.1-ml column of IF9B-Sepharose (made as described below, containing 25 µg of IF9B antibodies). The column was washed with 2 ml of 20 mM Na phosphate, pH 7.4, 300 mM NaCl and 1 ml PBS. The beads with adsorbed antigens were suspended in 0.5 ml PBS and injected intraperitoneally into a male BALB/c mouse. Serum was collected by tail bleeding 20, 44, and 47 d later.

### SDS-PAGE and Western Blotting

Analysis by SDS-PAGE was carried out with 10% acrylamide gels (13 × 18 cm × 1.5 mm; 6 × 8 cm × 1.5 mm for Fig. 2) by the method of Laemmli (15); no reducing agent was used, except for molecular weight standards, which contained 5% 2-mercaptoethanol. Samples were concentrated by precipitation overnight at 4°C in 10% TCA; precipitates were washed three times in H<sub>2</sub>O-saturated ethyl ether and once in 3:1 ethyl ether/acetone and dissolved in SDS sample buffer by heating 1 h at 37°C. Electrophoresis was carried out at 30 mA at 25°C. Proteins were transferred to nitrocellulose (0.45 µm pore size; Schleicher & Schuell, Inc., Keene, NH) overnight at 150 mA at 4°C in the buffer of Burnette (5). Lanes containing molecular weight standards (Bio-Rad Laboratories) were cut from the nitrocellulose and stained with Amido black. Lanes containing samples were stained with antibodies as described (24), except that ascites fluid was used as primary antibody at 1:50, followed by rabbit anti-mouse IgG (Sigma Chemical Co.) at 1:100, then goat anti-rabbit (IgG + IgM) conjugated with 10-nm gold particles (EM grade, Janssen Life Sciences Products, Piscataway, NJ) at 1:50. Normal goat serum (1%; Biologos, Naperville, IL) was included in the first two antibody steps. Antibody-labeled bands were revealed with IntenSE silver enhancement reagent (Janssen Life Sciences Products) applied twice, 10 min each.

### Immunohistochemistry

Tissues were dissected from adult *Torpedo* (Marinus, Inc.) and washed briefly in cold *Torpedo* saline (9). Small pieces were mounted on slices of cork, surrounded with tragacanth gum (10% in H<sub>2</sub>O; Fisher Scientific Co., Fair Lawn, NJ) and quick-frozen in liquid nitrogen-cooled *n*-methyl butane. Frozen sections (10–20 µm thick) were made with a cryostat and thawed onto acid-cleaned, poly-L-lysine-coated glass slides. For immunohistochemistry, slides were placed in PBS-BSA (5 mg/ml) for 1 h at 25°C (and subsequent steps), rinsed in PBS, and fixed 5–10 min in 1% paraformaldehyde in PBS. In some experiments, slides were incubated in fixative first to increase adherence of sections; the order of these steps and the brief fixation did not affect the intensity or pattern of antibody staining. Slides were rinsed in PBS, incubated 1 h in a humid chamber with 0.1 ml per slide of monoclonal antibodies (ascites fluid; 1:1,000 in PBS-BSA), and rinsed (here and after subsequent antibodies) four times in PBS. Next they were incubated 1 h with affinity-purified, FITC-labeled, rabbit anti-mouse immunoglobulins (Organon Teknika-Cappel, Malvern, PA) 1:100 in PBS-BSA, rinsed, incubated 1 h with affinity-purified, FITC-labeled goat anti-rabbit immunoglobulins (Organon Teknika-Cappel) 1:100 in PBS-BSA (with 83 ng/ml rhodamine- $\alpha$ -bungarotoxin in experiment of Fig. 3), rinsed, and mounted in Tris-buffered glycerol, pH 9.0, containing 6.25 g/l *n*-propyl galate (Sigma Chemical Co.) to retard fading of fluorescence (41). Sections were observed and photographed under epifluorescent illumination.

### Immunoaffinity Chromatography

Immunoglobulins were purified from ascites fluid by ion-exchange chromatography on Zeta-Chrom DEAE disks (AMF-Cuno, Meriden, CT) as

specified by the manufacturer, followed by precipitation with 50–60% saturated  $(\text{NH}_4)_2\text{SO}_4$  and dialysis against 0.1 M Na phosphate, pH 8.0, 0.5 M NaCl. Antibodies were covalently coupled to tresyl-activated Sepharose (Pharmacia Fine Chemicals) by incubation in this buffer 24 h at 25°C. Beads were washed and unreacted tresyl groups were hydrolyzed by treatment with 0.2 M Tris-Cl, pH 8.0. The 1F2F- and 1F9B-Sepharose contained 63 and 254  $\mu\text{g}$  protein/ml beads, respectively. For affinity chromatography, antibody-Sepharose columns (0.1–0.2 ml) were washed with 4 M NaCl in 10 mM Na phosphate, pH 7.5, with PBS containing (in this and subsequent steps) 1 mM EDTA, 1 mM PMSF, 0.5 mg/l leupeptin and pepstatin, and with PBS containing 0.1 mg/ml myoglobin (horse heart; Sigma Chemical Co.). Insoluble fractions and pH 5.5 extracts were prepared from frozen *Torpedo* tissues as described above. The pH 5.5 extracts were adjusted to pH 7 with NaOH, 0.1 mg/ml myoglobin was added, and the solutions were clarified by centrifugation 10–20 min at 2,100 g, if necessary. Extracts were passed over the antibody columns at  $\sim 6$  ml/h; unbound fractions had no AChR-aggregating activity. Active fractions were eluted with 2 ml 50 mM formic acid, pH 2.4, 150 mM NaCl, 0.1 mg/ml myoglobin, and neutralized immediately with 0.15 vol 0.5 M Na phosphate dibasic, pH 8.8. Affinity-enriched proteins from brain were eluted for Western blotting (Fig. 6) with 8 M urea instead. AChR-aggregating activity of eluates was determined (in experiment of Table I) and the volume containing 1 U of activity (12) was estimated by comparison with maximal aggregation caused by blue column-enriched proteins from electric organ.

### Immunoprecipitation of AChR-aggregating Activity

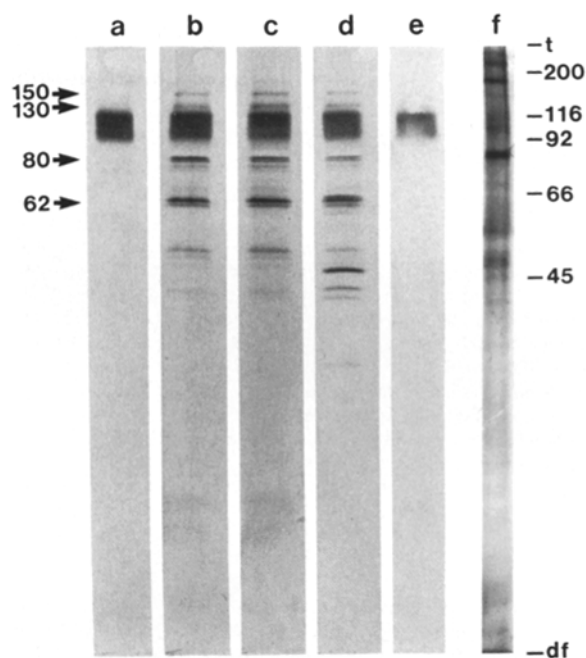
Active molecules were precipitated from affinity-enriched extracts as follows. Sepharose CL4B-*Staphylococcus* protein A beads (Pharmacia Fine Chemicals) were incubated overnight at 4°C with affinity-purified goat anti-mouse immunoglobulins (Organon Teknika-Cappel; 10  $\mu\text{g}$  antibodies per  $\mu\text{l}$  beads) and washed twice for 5 min with PBS. Beads were then coated with monoclonal antibodies (187  $\mu\text{l}$  ascites fluid per 15  $\mu\text{l}$  beads) overnight at 4°C and washed four times, 20 min each, with PBS-BSA (1 mg/ml) to remove unbound antibodies. Antibody-coated beads (15  $\mu\text{l}$ ) were resuspended in an equal volume of PBS-BSA (10 mg/ml) and mixed 4 h at 25°C with immunoaffinity-enriched extracts containing 3–4.5 U of AChR-aggregating activity. Beads were removed by centrifugation (3 min, 10,000 g) and the supernatants were assayed in triplicate for AChR-aggregating activity.

## Results

### Monoclonal Antibodies against AChR-aggregating Proteins

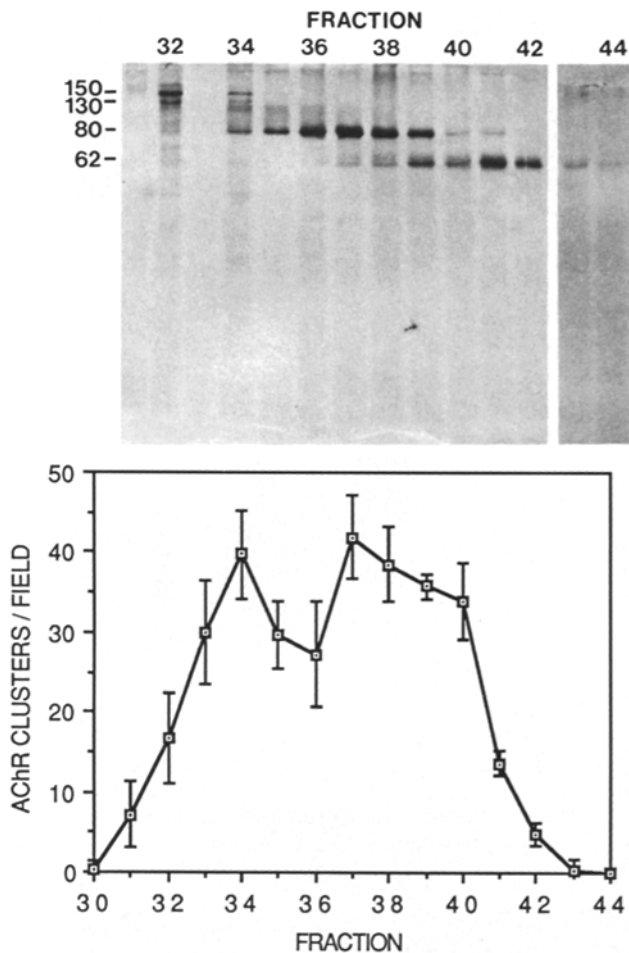
Medium conditioned by two hybridoma isolates, 1F2 and 1F9, consistently immunoprecipitated 80–100% of the biological activity from soluble preparations of AChR-aggregating proteins from *Torpedo* electric organ. We used antibodies made by one clone from each isolate, 1F2F and 1F9B, for the studies reported here. These two antibodies were independently derived, since the immunoglobulin subclasses of 1F2F and 1F9B were IgG<sub>1</sub> and IgG<sub>2b</sub>, respectively. However, these antibodies probably bound to the same or nearby epitopes on the antigenic proteins, since an excess of one antibody blocked 85–100% of the specific binding of the other, <sup>125</sup>I-labeled antibody to AChR-aggregating proteins (blue column-enriched) adsorbed to polystyrene beads (data not shown). Unlike other monoclonal antibodies against these proteins (8), 1F2F and 1F9B did not inhibit biological activity when mixed with the AChR-aggregating proteins. To date, all results with these two antibodies have been identical.

To determine whether the antibodies reacted specifically with AChR-aggregating proteins, we performed two experiments. First, we enriched the proteins by affinity chromatography with 1F9B antibodies covalently bound to agarose beads. The beads removed 94% of the AChR-aggregating activity, but only  $\sim 1\%$  of the protein, from a DEAE-enriched



**Figure 1.** Monoclonal antibodies and mouse antiserum against *Torpedo* AChR-aggregating proteins bind to similar protein bands. Blue column-enriched proteins (not TCA precipitated, see Materials and Methods) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with the monoclonal antibodies CSAT (a), 1F9B (b), 1F2F (c), or with mouse antiserum (d) or normal mouse serum (e), then with rabbit anti-mouse IgG followed by goat anti-rabbit Ig antibodies coupled to colloidal gold, and stained by silver enhancement as described in Materials and Methods. Apparent molecular masses of major stained bands (kD) are shown on left. The broad band of  $\sim 95$ –120 kD (lanes a–e) was nonspecific, since it was seen in lanes a and e treated with control antibodies. Lane f (0.8  $\mu\text{g}$  of protein) was cut from the same gel and silver stained (23). Migration of molecular mass standards (kD), dye front (df), and the top of running gel (t) are shown at right.

preparation. The antibody beads, with adsorbed antigens, were used to immunize a mouse. Antiserum collected from the mouse, unlike the monoclonal antibodies, completely neutralized AChR-aggregating activity when mixed with the proteins before their addition to muscle cell cultures. More importantly, the mouse antiserum and the monoclonal antibodies bound to nearly the same bands on Western blots of AChR-aggregating proteins (Fig. 1). All three antibodies bound specifically to four major bands, with apparent molecular masses of about 62, 80, 130, and 150 kD (arrows, Fig. 1, lanes b–d). The 62- and 80-kD bands were resolved into doublets. These bands were similar in molecular mass to those bound by other monoclonal antibodies against the AChR-aggregating proteins (39). Comparison of Western blots with a silver-stained lane from the same gel (Fig. 1, f) indicates that the four major bands were minor components of this preparation. Several additional bands with molecular masses of 43–52 kD were also detected by these antibodies (lanes b–d). These bands were not usually observed in Western blots of most preparations (see Figs. 2 and 6) and probably represent proteolytic fragments of the four larger bands. The antibodies did not stain any bands if sample proteins were reduced before electrophoresis. Since the antiserum



**Figure 2.** Active AChR-aggregating proteins coelute upon gel filtration with protein bands specifically labeled by 1F9B monoclonal antibodies. *Torpedo* AChR-aggregating proteins (blue column enriched, 78  $\mu$ g, 995 U of activity) were fractionated on a Sephadex G-100 column (1.0  $\times$  120 cm) at a flow rate of 1.5 ml/h in 50 mM Na phosphate, pH 7.4, 2 M NaCl, 0.1% Tween 20, 0.5  $\mu$ g/ml leupeptin and pepstatin, 0.1 mg/ml PMSF, and 1 mM EDTA; 1.2-ml fractions were collected. Western blots (top) of fractions 31–44 were stained as described in Materials and Methods. The sample for fraction 33 was lost during preparation. Apparent molecular masses (kD) of stained bands are shown at left. The AChR-aggregating activity of each fraction was measured in triplicate, and the net number of AChR clusters per field (above untreated cultures; reference 12)  $\pm$  SEM was plotted (bottom).

blocked biological activity of the proteins and bound to a set of bands very similar to those bound by the monoclonal antibodies, we concluded that (a) the monoclonal antibodies bound to all the active AChR-aggregating proteins in soluble preparations; (b) the 62-, 80-, 130-, and 150-kD bands were the major proteins bound by the monoclonal antibodies in most preparations; and (c) the activity was probably associated with these four bands.

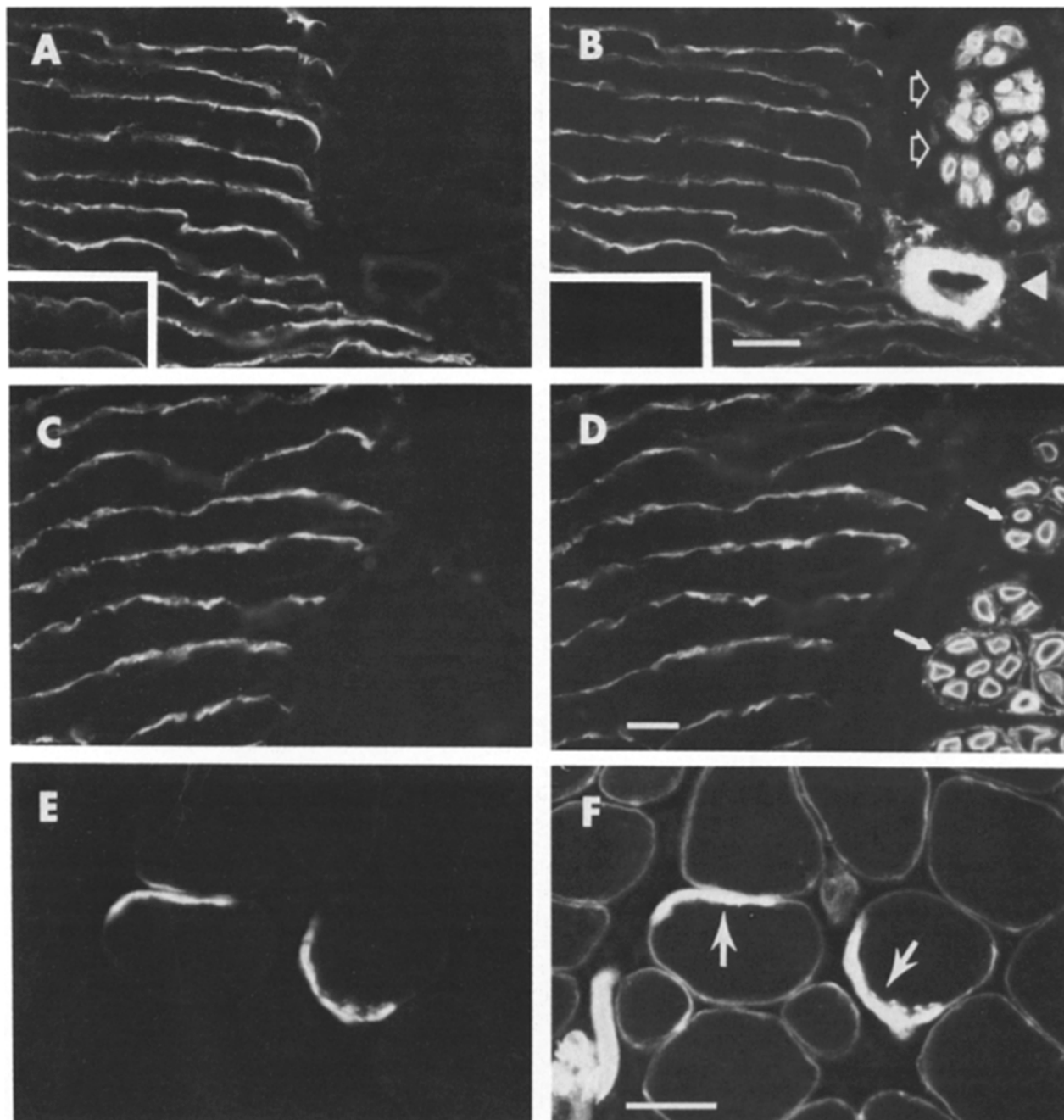
To test this last conclusion, we performed a second experiment. The AChR-aggregating proteins were fractionated by gel filtration on Sephadex G-100 and the elution profiles of biological activity and antibody-stained bands on Western blots were compared (Fig. 2). Two peaks of AChR-aggregating activity were resolved. The first peak (Fig. 2, fractions 32–35) correlated with the 150- and 130-kD bands seen on

the Western blot. These two bands were not separated by the chromatography, so it could not be determined whether the peak of activity correlated with one or both of them. The second peak of activity (Fig. 2, fractions 36–40) correlated with the 80-kD doublet. The 150–130- and 80-kD bands coeluted with peaks of AChR-aggregating activity in six out of seven experiments. In the preparation used in the experiment of Fig. 2, the 62-kD doublet appeared to be inactive; another preparation contained a third peak of activity which eluted at about the same position as this band. The results of these two experiments indicate that the epitope(s) recognized by 1F2F and 1F9B is limited to active AChR-aggregating proteins and closely related molecules.

### Distribution of Antigens in *Torpedo* Tissues

To determine the distribution of AChR-aggregating proteins and related molecules in tissues of *Torpedo*, we stained frozen sections with the monoclonal antibodies. The intensity of antibody staining was increased several fold by the use of two layers of FITC-labeled antibodies (rabbit anti-mouse and goat anti-rabbit). First, we examined electric organ and muscle, tissues with a high density of AChRs at cholinergic synapses. As expected, the AChR-rich ventral surfaces of electrocytes bound the antibodies in a pattern (Fig. 3, B and D) that correlated in detail with the binding of rhodamine- $\alpha$ -bungarotoxin to the AChRs (Fig. 3, A and C). The dorsal surfaces of electrocytes were unstained. In addition, staining was observed around axons (arrows, Fig. 3 B) and in the perineurium (arrows, Fig. 3 D). Surprisingly, antibody staining was most intense in the walls of arteries (arrowhead, Fig. 3 B) in this and other tissues. Control monoclonal antibodies (e.g., CSAT) did not stain any of these structures (inset, Fig. 3 B) or any structures in the other tissues shown in Figs. 2–4, except as noted below. In muscle, neuromuscular junctions stained intensely (Fig. 3 F), in a pattern corresponding closely to the distribution of postsynaptic AChRs (Fig. 3 E). Fainter antibody staining surrounded all muscle fibers (Fig. 3 F). Intramuscular axons (lower left, Fig. 3 F) and arteries (not shown) also stained with the antibodies. Extrajunctional staining surrounded all muscle fibers in both red and white muscle in tail (not shown), as well as in the small white extraocular muscles (Fig. 3 F). This extrajunctional staining was fainter in large-diameter, white muscle fibers than in red or smaller-diameter muscle fibers.

A layer of antibody-stained material surrounded all axons in peripheral nerves. In the electromotor nerve, which innervates the electric organ, this immunofluorescence was external to Schwann cells, as shown by the position of their nuclei (arrows, Fig. 4, A and B). Although staining also appeared to be internal to some of these layers (Fig. 4 B), cross sections of peripheral nerves stained with a honeycomb appearance (not shown), indicating absence of antibody staining in axoplasm. The association of staining with Schwann cells rather than axons was confirmed by examining the sites where electromotor nerves exit the brain (Fig. 4, C and D). Even though axons continue through this region (arrowheads, Fig. 4, C and D), antibody staining appeared abruptly as they exited the electric lobe (left, Fig. 4 D) and entered the peripheral nerve (right, Fig. 4 D). In the electric lobe of the brain, only blood vessels (arrows, Fig. 4, E and F) and meninges were specifically labeled with the antibodies. Small spots of fluorescence were seen in the electromotor neurons (arrowheads, Fig. 4 F), but these were also seen in

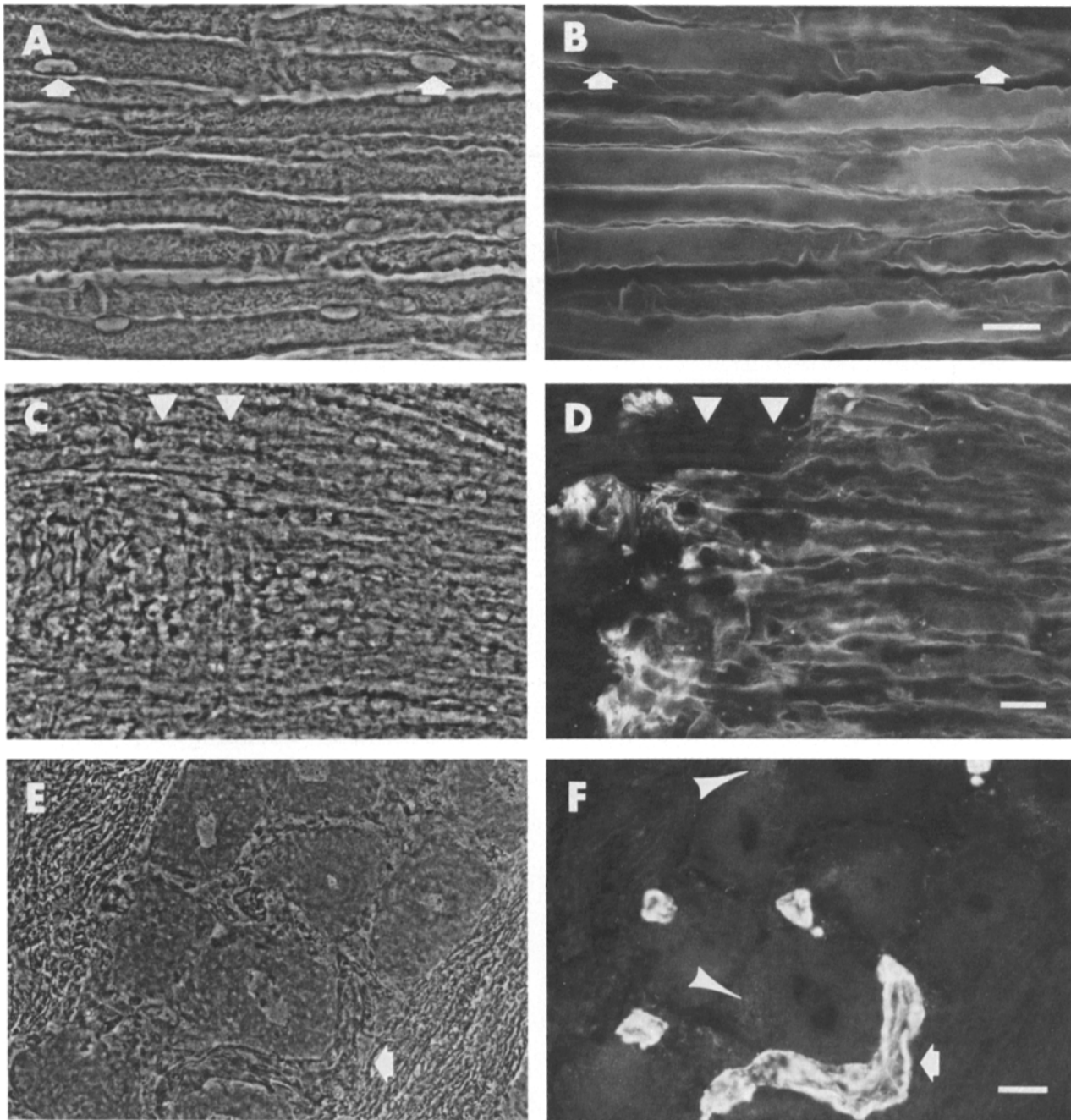


**Figure 3.** Monoclonal antibodies against AChR-aggregating proteins bind to cell surfaces having a high density of AChRs and stain other structures with basal lamina in electric organ and muscle. Frozen sections of adult *Torpedo* tissues were fixed briefly, incubated with monoclonal antibodies (1F2F), fluorescein rabbit anti-mouse Ig, and a mixture of fluorescein goat anti-rabbit Ig and rhodamine- $\alpha$ -bungarotoxin, as described in Materials and Methods. The same microscopic fields were photographed with epifluorescent illumination using the appropriate filters for both rhodamine (A, C, and E) or fluorescein (B, D, and F). In electric organ (A–D), the ventral (AChR-rich; A and C) surfaces of electrocytes stained with the antibodies (B and D). In addition, axons (open arrows, B; D) were surrounded by antibody staining, and the perineurium (arrows, D) and the walls of arteries (arrowhead, B) were labeled. Sections of electric organ were not stained with the control monoclonal antibody, CSAT (inset, B) but were labeled with rhodamine- $\alpha$ -bungarotoxin (inset, A). In extraocular skeletal muscle, neuromuscular junctions were intensely stained with both  $\alpha$ -bungarotoxin (E) and antibodies (arrows, F); antibody staining also surrounded extrajunctional surfaces of muscle fibers (F) and axons (lower left, F). Bars: (A, B, E, and F) 50  $\mu$ m; (C and D) 25  $\mu$ m.

sections treated with a control (or no) monoclonal antibody. Neurons in other regions of the *Torpedo* brain and in dorsal or ventral horns of the spinal cord also failed to stain specifically, although in both dorsal and ventral roots, perineurium and thin sheaths surrounding all axons were stained (data not shown).

Several tissues that do not receive innervation from motor

neurons and do not contain high density clusters of AChRs were also stained with monoclonal antibodies to AChR-aggregating proteins. Basement membranes (arrows, Fig. 5) of the intestinal epithelium (Fig. 5 A), epidermis (Fig. 5 D), and epicardium (Fig. 5 C) all stained intensely with these antibodies. In addition, immunofluorescence surrounded smooth muscle cells in the intestinal wall (Fig. 5 B) and



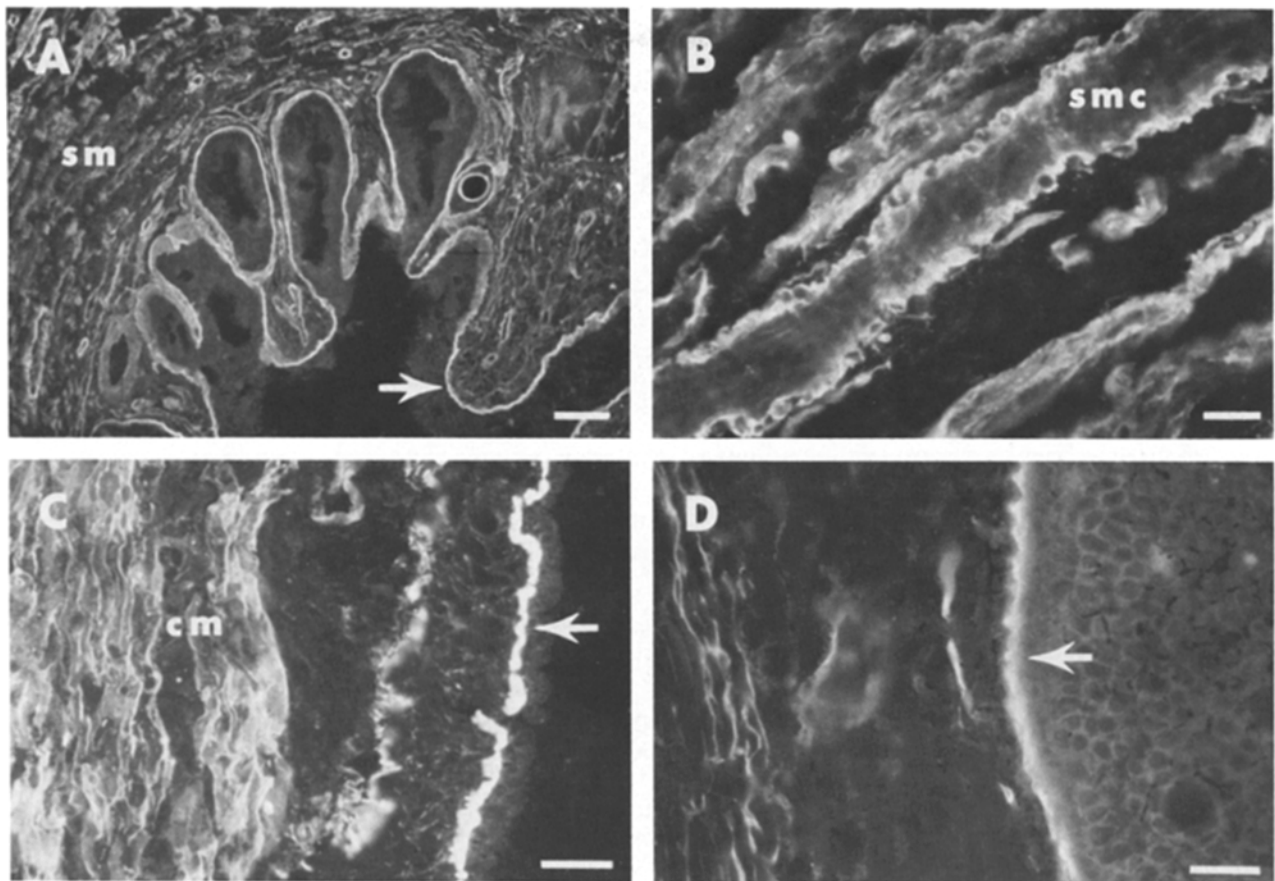
**Figure 4.** Antibodies against AChR-aggregating proteins label peripheral nerves but not neurons or axons in the electric lobe of the brain. Immunohistochemistry was performed with 1F2F antibodies as described in Materials and Methods. The same microscopic fields were photographed using both phase-contrast (*A*, *C*, and *E*) and fluorescence (*B*, *D*, and *F*) optics. Antibody staining ensheathed axons in the electromotor nerve (*B*); these stained sheaths were external to nuclei of Schwann cells (*arrows*, *A* and *B*). Axons within the electric lobe (*arrowheads*, *C* and *D*) were not labeled with antibodies, but staining appeared abruptly as they entered the electromotor nerve (to the right of *arrowheads* in *C* and *D*). Neuronal cell bodies in electric lobe (*E*) did not stain specifically with the antibodies (*F*), while blood vessels (*arrows*, *E* and *F*) stained intensely. *Arrowheads* in *F* indicate punctate staining in neuronal cell bodies; this was also seen in the absence of monoclonal antibodies. Bars: (*A* and *B*) 25  $\mu\text{m}$ ; (*C* and *D*) 50  $\mu\text{m}$ ; (*E* and *F*) 100  $\mu\text{m}$ .

cardiac muscle cells in the conus arteriosus of the heart (Fig. 5 *C*). The coincidence of antibody staining with basement membranes and sites of other basal laminae suggests the antigens are localized in these extracellular structures.

#### **Chemical and Functional Comparison of Antigens**

The broad distribution of structures binding antibodies against AChR-aggregating proteins prompted us to ask whether the

antibody staining in different tissues was due to chemically or functionally similar molecules. To assess the chemical similarity of the cross-reacting antigens in various tissues, we compared the pattern of antibody-labeled bands on Western blots of immunoaffinity-enriched extracts. Bands of similar molecular mass were recognized by 1F9B antibodies in extracts of most tissues examined (Fig. 6). Extracts of electromotor nerve, heart, and gut (lanes *c*, *d*, and *e*) contained immunostained bands that comigrated with doublets of 62



**Figure 5.** Antibodies against AChR-aggregating proteins bind to basement membranes and surfaces of smooth and cardiac muscle cells. Immunohistochemistry was performed as described in Materials and Methods. Antibodies (1F2F in *A*, *C*, and *D*; 1F9B in *B*) bound to epithelial basement membranes (*arrows*) in intestine (*A*), epicardium of heart (*C*), and epidermis in skin (*D*). In addition, smooth muscle cells (individual cells and bundles) in the wall of the intestine (*sm* in *A*, *smc* in *B*) and cardiac muscle fibers (*cm* in *C*) were surrounded by antibody staining. Bars: (*A* and *D*) 100  $\mu\text{m}$ ; (*B*) 25  $\mu\text{m}$ ; (*C*) 50  $\mu\text{m}$ .

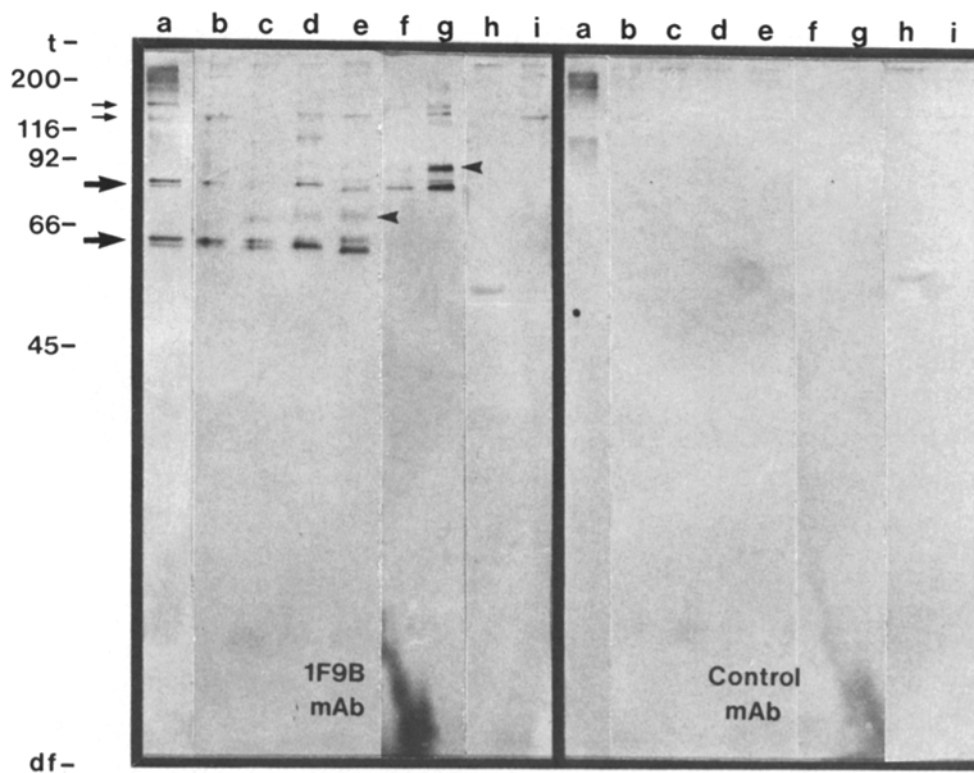
and 84 kD (*large arrows*) in extracts of electric organ (lane *b*) and in AChR-aggregating proteins partially purified from electric organ (lane *a*). Extracts of electric lobe (lane *g*) and other parts of the brain (lane *f*) contained the 84-kD doublet, but not the 62-kD doublet. The 84-kD band stained more intensely in extract of electric lobe (lane *g*) than in the extract from the rest of the brain (lane *f*). Bands of higher molecular mass also bound 1F9B in extracts of heart, gut, and brain (lanes *d*–*g*). Some of these were similar in size to the 135- and 157-kD bands (*small arrows*) in AChR-aggregating proteins enriched from electric organ (lane *a*). Extracts of muscle (lanes *h* and *i*) did not stain specifically with 1F9B under these conditions, possibly due to the sensitivity of the method. Similar results were seen with extracts not enriched by affinity chromatography (data not shown). In summary, the major antibody-stained bands in extracts of most tissues were the two doublets most prominent in AChR-aggregating proteins partially purified from electric organ.

Finally, we determined whether immunoreactive molecules in extracts of these tissues were functionally similar by examining their AChR-aggregating activity. Extracts from ECM-enriched fractions of the tissues (except electric organ) were enriched by immunoaffinity chromatography with 1F2F-Sepharose. All the AChR-aggregating activity in the extracts bound to the antibody columns; activity was eluted from all the columns with formic acid at pH 2.4. The amount of activ-

ity (per gram of tissue) in affinity-enriched extracts of electromotor nerve, heart, and brain was  $\sim 10$ -fold less, and from skeletal muscle and gut  $\sim 100$ -fold less, than in pH 5.5 extracts of electric organ. The AChR-aggregating activity in immunoaffinity-enriched fractions from nerve and brain was due to protein, as it was trypsin sensitive (data not shown). Most (94–100%) of the activity in the affinity-enriched extracts was precipitated with beads coated with 1F2F (Table I), while 16% or less of the activity was precipitated with the control monoclonal antibody, CSAT. Similar results were obtained when activity was precipitated with 1F9B antibodies. Thus, we could extract biologically active AChR-aggregating molecules from ECM-enriched fractions of all tissues examined, and they were recognized by both monoclonal antibodies against AChR-aggregating proteins from electric organ.

### Discussion

Monoclonal antibodies against AChR-aggregating proteins from ECM-enriched fractions of *Torpedo* electric organ were used to study the distribution of similar proteins in a number of *Torpedo* tissues. Immunohistochemical studies showed a distribution of antibody binding sites consistent with their localization of basal laminae in many tissues. While the antibodies intensely stained neuromuscular junc-



**Figure 6.** Antibodies against AChR-aggregating proteins bind to bands of similar apparent molecular mass in extracts of ECM-enriched fractions from several *Torpedo* tissues. AChR-aggregating proteins were enriched from pH 5.5 extracts by affinity chromatography on columns (0.1 ml) of 1F9B-Sepharose and analyzed by SDS-PAGE and Western blotting (Materials and Methods). This figure is a composite of two similar Western blots. Antibodies against AChR-aggregating proteins specifically stained doublet bands of ~62 and 84 kD (large arrows) in blue column-enriched proteins from electric organ (5  $\mu$ g, lane a), and in immunoaffinity-enriched extracts of electric organ (b), electromotor nerve (c), heart (d), and gut (e). The 84-kD band was also seen in extracts of electric lobe of brain (g) and the remainder of the brain (f). No specifically stained

bands were observed in extracts of muscle (h and i). In addition, bands of ~135 and 157 kD (small arrows) were seen in blue column-enriched proteins (a); bands in the same molecular mass range were also seen in extracts of electric organ (b), heart (d), gut (e), and brain (f and g). Bands of ~71 and 95 kD (arrowheads) also appeared in some tissue extracts.

tions and the AChR-rich ventral (but not dorsal) surfaces of electrocytes, they also bound to the extrajunctional perimeters of skeletal muscle fibers, smooth and cardiac muscle cells, Schwann cell sheaths in peripheral nerves, walls of some blood vessels, and basement membranes in the gut, skin, and heart. Proteins recognized by these antibodies were extracted from ECM-enriched (detergent-insoluble) fractions of several tissues; the molecular masses of the major reactive proteins were similar. More importantly, the antibody-binding proteins extracted from all tissues examined had AChR-aggregating activity. These results strongly suggest that similar AChR-aggregating proteins are associated with the ECM of many tissues in *Torpedo*.

The monoclonal antibodies stained four major bands, with molecular masses of ~62, 80, 130, and 150 kD, on Western blots of AChR-aggregating proteins from electric organ. These bands were also the major bands stained by a mouse antiserum made against proteins affinity enriched with the monoclonal antibodies. Since no additional bands were stained by this antiserum in most soluble protein preparations, these four appear to be the major proteins bound by the monoclonal antibodies. In addition, two or three of the four bands coeluted from gel filtration columns with peaks of biological activity; thus, the antibodies appear specific for AChR-aggregating proteins and closely related molecules. Multiple forms of these proteins (reference 39 and this report) may have been generated by proteolysis in vivo rather than during preparation, since neither omitting protease inhibitors from the buffers used in the purification, nor addi-

**Table I.** Immunoprecipitation of AChR-aggregating Activity from Affinity-enriched Extracts of *Torpedo* Tissues

Extract	Monoclonal antibody	Percent activity precipitated
Electric organ	1F2F	94 $\pm$ 2
	CSAT	11 $\pm$ 11
	None	4 $\pm$ 6
Electromotor nerve	1F2F	98 $\pm$ 3
	CSAT	15 $\pm$ 4
Skeletal muscle	1F2F	100 $\pm$ 5
	CSAT	13 $\pm$ 17
Heart	1F2F	100 $\pm$ 5
	CSAT	5 $\pm$ 15
Gut	1F2F	98 $\pm$ 1
	CSAT	16 $\pm$ 6
Brain—electric lobe	1F2F	99 $\pm$ 2
	CSAT	16 $\pm$ 19
Brain—remainder	1F2F	100 $\pm$ 8
	CSAT	13 $\pm$ 9

ECM-enriched fractions were extracted with pH 5.5 buffer and (except for electric organ) extracts were concentrated by affinity chromatography with 1F2F antibodies bound to Sepharose, as described in Materials and Methods. The affinity-enriched extracts were exposed to immobilized 1F2F or CSAT (control) antibodies, the antibodies and proteins bound to them were removed by centrifugation, and supernatants were assayed in triplicate for AChR-aggregating activity, as described in Materials and Methods. The activity of extracts not exposed to immobilized antibodies was also determined; percent precipitation (mean  $\pm$  SD) was calculated in comparison to these values. Skeletal muscle included both red and white muscles from the tail.



tion of the protease inhibitors iodoacetamide and EGTA, affected the antibody staining pattern of the proteins on Western blots (unpublished results).

Our results are similar to those of Smith et al. (39), who found that other monoclonal antibodies against these *Torpedo* proteins ("agrin") bound to four bands of 70, 95, 135, and 150 kD, resolved by SDS-PAGE under reducing conditions, and that biological activity coeluted from gel filtration columns with the 95- and 150-kD bands but not with the 70- and 135-kD bands. The 70- and 95-kD bands shared the same NH<sub>2</sub>-terminal amino acid sequence, while the 135- and 150-kD bands shared yet a different sequence (39), indicating that each pair of proteins is closely related. It is probable that the 70- and 95-kD bands seen by them correspond to the 62- and 80-kD doublets in our Western blots. The differences in apparent molecular mass of these bands may be due to their use of reducing agents; however, we cannot test this hypothesis with our antibodies, since they did not bind to reduced proteins. Barald et al. (1) have extracted an AChR-aggregating protein from an ECM-enriched fraction of innervated regions of rat diaphragms. A monoclonal antibody against this protein stained a doublet of ~75 kD on Western blots of proteins from these regions (1). Further characterization of the rat and *Torpedo* proteins will be required to determine if they are related.

Immunofluorescence due to the monoclonal antibodies was not limited to tissues innervated by motor neurons or sites where AChRs are clustered at high density. Although neuromuscular junctions had a high concentration of molecules reacting with the antibodies, immunologically similar molecules were also found at many nonsynaptic sites. Other monoclonal antibodies against these AChR-aggregating proteins also stain extrajunctional regions of some muscle fibers, walls of arteries and Schwann cell surfaces in muscle, and blood vessels and meninges in brain (8, 18, 19, 31); however, immunohistochemical data for those antibodies in other tissues have not been reported. The staining we observed at nonsynaptic as well as synaptic sites was probably due to AChR-aggregating proteins, since 1F2F and 1F9B appeared to be specific for these proteins and immunoaffinity-enriched proteins from all tissues examined were biologically active.

The localization of antibody staining corresponded to sites of basal laminae, as staining surrounded Schwann cells and all types of muscle cells. Staining in the walls of blood vessels may also have been due to basal laminae of their smooth muscle cells. Confirming that antibody binding sites are in basal laminae will require EM immunocytochemistry in several tissues. Other monoclonal antibodies against these proteins have been shown to label synaptic basal lamina of neuromuscular junctions in *Torpedine* rays (8) and the frog (19) at the ultrastructural level. Although most structures with a basal lamina stained with our antibodies, some did not, most notably the dorsal surfaces of electrocytes (32). It is unclear why the antibodies did not bind to these structures; the highly polarized nature of the electrocytes with regard to membrane proteins (38) may be reflected in the composition of the dorsal and ventral basal laminae.

Extracts of neural tissues (13, 14, 29, 33, 41), including the electric lobe and electromotor nerve of *Torpedo* (7), and media conditioned by neuronal cells (2, 6, 37) aggregate AChRs on cultured myotubes. Therefore, one might expect synaptic molecules that cause differentiation of postsynaptic struc-

tures to be synthesized and secreted by neurons. Our monoclonal antibodies against AChR-aggregating proteins did not, however, specifically stain any neurons in either the electric lobe of the brain, which innervates the electric organ, or the spinal cord. These results are in contrast to those of Magill et al. (18, 20) who, using similar methods, found that other monoclonal antibodies against these proteins specifically stained neuronal cell bodies in both the electric lobe and the ventral (motor) horn of spinal cord in *Torpedo*. Perhaps the antibodies used recognized different epitopes and the proteins in neurons differ enough from those in other tissues that our antibodies did not bind to them. Our results indicate, however, that ECM-enriched fractions from *Torpedo* brain and electromotor nerve contain much less AChR-aggregating activity than fractions from electric organ, raising the possibility that some of the synaptic AChR-aggregating protein is synthesized by postsynaptic cells.

The broad distribution of AChR-aggregating proteins related to those from *Torpedo* electric organ suggests that, in addition to having synapse-specific activities such as AChR clustering, these molecules also play general roles unrelated to the formation or differentiation of neuromuscular synapses. Their primary function could be in ECM assembly, or in linking cell membrane receptors with the basal lamina, as laminin is thought to do (40). The aggregation of AChRs induced in cultured myotubes by both proteins (43) may be secondary to a common mechanism involving cell-matrix interaction. There is good evidence, however, that the AChR-aggregating proteins are not closely related to laminin (8, 12). The AChR-aggregating proteins from *Torpedo* ECM, unlike laminin, cause receptor clustering in culture at concentrations below 10<sup>-12</sup> M (19), suggesting that this response is physiological. Understanding the physiological role of these proteins at the synapse will require further studies, including testing whether antibodies against them interfere with synaptic differentiation, determining which cells make and secrete them into the ECM, and determining their molecular structure and identity.

We thank Patrick Mullen, Richard Moran, Terri Miresse, Deborah Schoenborn, Ruth Siebenlist, and Donna McAllister for valuable technical assistance; Terri Murtha-Wardon for help with cryostat sectioning; Dawn Krokowski-Gorski for excellent photography; Carolyn Snyder for assistance in preparing figures; and Drs. Ed Krug, John Lough, Dan Riley, and Linda Walters for helpful comments on the manuscript.

This work was supported by National Science Foundation grant BNS 84-06790 and program project grant 1P01HD20743 from the National Institutes of Health.

Received for publication 27 July 1987, and in revised form 23 November 1987.

#### References

1. Barald, K. F., G. D. Phillips, J. J. Jay, and I. F. Mizukami. 1987. A component in mammalian muscle synaptic basal lamina induces clustering of acetylcholine receptors. *In Progress in Brain Research*, Vol. 71. F. J. Seil, E. Herbert, and B. M. Carlson, editors. Elsevier/North Holland, Amsterdam. 397-408.
2. Bauer, H. C., M. P. Daniels, P. A. Pudimat, L. Jacques, H. Sugiyama, and C. N. Christian. 1981. Characterization and partial purification of a neuronal factor which increases acetylcholine receptor aggregation on cultured muscle cells. *Brain Res.* 209:395-404.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

4. 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.
5. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
6. Christian, C. M., M. P. Daniels, H. Sugiyama, Z. Vogel, L. Jacques, and P. G. Nelson. 1978. A factor from neurons increases the number of acetylcholine receptor aggregates on cultured muscle cells. *Proc. Natl. Acad. Sci. USA.* 75:4011-4015.
7. Connolly, J. A., P. A. St. John, and G. D. Fischbach. 1982. Extracts of electric lobe and electric organ from *Torpedo californica* increase the total number as well as the number of aggregates of chick myotube acetylcholine receptors. *J. Neurosci.* 2:1207-1213.
8. Fallon, J. R., R. M. Nitkin, N. E. Reist, B. G. Wallace, and U. J. McMahan. 1985. Acetylcholine receptor-aggregating factor is similar to molecules concentrated at neuromuscular junctions. *Nature (Lond.)* 315:571-574.
9. Fox, G. Q., and G. P. Richardson. 1978. The developmental morphology of *Torpedo marmorata*: electric organ-myogenic phase. *J. Comp. Neurol.* 179:677-698.
10. Godfrey, E. W. 1986. Monoclonal antibodies again acetylcholine receptor-aggregating protein from *Torpedo* electric organ bind to cell surfaces in nerve and muscle. *Soc. Neurosci. Abstr.* 12:546.
11. Godfrey, E. W., R. M. Nitkin, B. G. Wallace, and U. J. McMahan. 1983. Identifying components of extracellular matrix that organize ACh receptors at synaptic sites on regenerating muscle cells. In *Developing and Regenerating Vertebrate Nervous Systems*. P. W. Coates, R. R. Markwald, and A. D. Kenny, editors. Alan R. Liss Inc., New York. 61-70.
12. Godfrey, E. W., R. M. Nitkin, B. G. Wallace, L. L. Rubin, and U. J. McMahan. 1984. Components of *Torpedo* electric organ and muscle that cause aggregation of acetylcholine receptors on cultured muscle cells. *J. Cell Biol.* 99:615-627.
13. Jessell, T. M., R. E. Siegel, and G. D. Fischbach. 1979. Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord. *Proc. Natl. Acad. Sci. USA.* 76:5397-5401.
14. Kalcheim, C., Z. Vogel, and D. Duksin. 1982. Embryonic brain extract induces collagen biosynthesis in cultured muscle cells: involvement in acetylcholine receptor aggregation. *Proc. Natl. Acad. Sci. USA.* 79:3007-3081.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
16. Lindstrom, J., R. Anholt, B. Einarson, A. Engel, M. Osame, and M. Montal. 1980. Purification of acetylcholine receptors, reconstitution into lipid vesicles, and study of agonist-induced cation channel regulation. *J. Biol. Chem.* 255:8340-8350.
17. Lwebuga-Mukasa, J. S., S. Lappi, and P. Taylor. 1976. Molecular forms of acetylcholinesterase from *Torpedo californica*: their relationship to synaptic membranes. *Biochemistry.* 15:1425-1434.
18. Magill, C. X., R. M. Nitkin, J. R. Fallon, B. W. Wallace, and U. J. McMahan. 1986. Characterization of agrin and localization of antigenically related molecules in motor neurons. *Soc. Neurosci. Abstr.* 12:190.
19. Magill, C., N. E. Reist, J. R. Fallon, R. M. Nitkin, B. G. Wallace, and U. J. McMahan. 1987. Agrin. In *Progress in Brain Research*, Vol. 71. F. J. Seil, E. Herbert, and B. M. Carlson, editors. Elsevier/North Holland, Amsterdam. 391-396.
20. Magill, C., B. G. Wallace, and U. J. McMahan. 1987. Molecules similar to agrin are concentrated in motor neurons. *Soc. Neurosci. Abstr.* 13:373.
21. McMahan, U. J., and C. R. Slater. 1984. The influence of basal lamina on the accumulation of acetylcholine receptors at synaptic sites in regenerating muscles. *J. Cell Biol.* 98:1453-1473.
22. McMahan, U. J., J. R. Sanes, and L. M. Marshall. 1978. Cholinesterase is associated with the basal lamina at the neuromuscular junction. *Nature (Lond.)* 271:172-174.
23. Merril, C., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science (Wash. DC)* 211:1437-1438.
24. Moeremans, M., G. Daniels, and J. De Mey. 1985. Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. *Anal. Biochem.* 145:315-321.
25. Neff, N., C. Lowrey, A. Tovar, C. Decker, C. Damsky, C. Buck, and A. Horwitz. 1982. A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. *J. Cell Biol.* 95:654-666.
26. Nitkin, R. M., B. G. Wallace, M. E. Spira, E. W. Godfrey, and U. J. McMahan. 1983. Molecular components of the synaptic basal lamina that direct differentiation of regenerating neuromuscular junctions. *Cold Spring Harbor Symp. Quant. Biol.* 48:653-665.
27. Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobulin-producing hybrid cell lines. In *Selected Methods in Cellular Immunology*. B. M. Mishell and S. M. Shiigi, editors. Freeman Cooper and Co., San Francisco. 351-372.
28. Peters, A., S. L. Palay, and H. deF. Webster. 1970. *The Fine Structure of the Nervous System*. Hoeber, New York. 198 pp.
29. Podleski, T. R., D. Axelrod, P. Ravdin, I. Greenberg, M. M. Johnson, and M. M. Salpeter. 1978. Nerve extract induces increase and redistribution of acetylcholine receptor on cloned muscle cells. *Proc. Natl. Acad. Sci. USA.* 75:2035-2039.
30. Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of  $\alpha$ -bungarotoxin: preparation, separation and characterization. *Anal. Biochem.* 80:585-592.
31. Reist, N. E., C. X. Magill, and U. J. McMahan. 1986. Molecules antigenically similar to agrin are highly concentrated in the synaptic basal lamina of skeletal muscles. *Soc. Neurosci. Abstr.* 12:189.
32. Rosenbluth, J. 1975. Synaptic membrane structure in *Torpedo* electric organ. *J. Neurocytol.* 4:697-712.
33. Salpeter, M. M., S. Spanton, K. Holley, and T. R. Podleski. 1982. Brain extract causes acetylcholine receptor redistribution which mimics some early events at developing neuromuscular junctions. *J. Cell Biol.* 93:417-425.
34. Sanes, J. R. 1982. Laminin, fibronectin and collagen in synaptic and extrasynaptic muscle fiber basal lamina. *J. Cell Biol.* 93:442-451.
35. Sanes, J. R., and Z. W. Hall. 1979. Antibodies that bind specifically to synaptic sites on muscle fiber basal lamina. *J. Cell Biol.* 83:357-370.
36. Sanes, J. R., L. M. Marshall, and U. J. McMahan. 1978. Reinnervation of muscle fiber basal lamina after removal of muscle fibers. *J. Cell Biol.* 78:176-198.
37. Schaffner, A. E., and M. P. Daniels. 1982. Conditioned medium from cultures of embryonic neurons contains a high molecular weight factor which induces acetylcholine receptor aggregation on cultured myotubes. *J. Neurosci.* 2:623-632.
38. Sealock, R., B. E. Wray, and S. C. Froehner. 1984. Ultrastructural localization of the  $M_r$  43,000 dalton protein and the acetylcholine receptor in *Torpedo* postsynaptic membranes using monoclonal antibodies. *J. Cell Biol.* 98:2239-2244.
39. Smith, M. A., B. G. Wallace, Y.-M. Yao, J. W. Schilling, P. Snow, and U. J. McMahan. 1987. Purification and characterization of agrin. *Soc. Neurosci. Abstr.* 13:374.
40. Timpl, R., J. Engel, and G. R. Martin. 1983. Laminin—a multifunctional protein of basement membranes. *Trends Biochem. Sci.* 9:207-209.
41. Usdin, T. B., and G. D. Fischbach. 1986. Purification and characterization of a polypeptide from chick brain that promotes the accumulation of acetylcholine receptors in chick myotubes. *J. Cell Biol.* 103:493-507.
42. Valnes, K., and P. Brandtzaeg. 1985. Retardation of immunofluorescence fading during microscopy. *J. Histochem. Cytochem.* 33:755-761.
43. Vogel, Z., C. N. Christian, M. Vigny, H. C. Bauer, P. Sonderegger, and M. P. Daniels. 1983. Laminin induces acetylcholine receptor aggregation on cultured myotubes and enhances the receptor aggregation activity of a neuronal factor. *J. Neurosci.* 3:1058-1068.
44. Wallace, B. G., E. W. Godfrey, R. M. Nitkin, L. L. Rubin, and U. J. McMahan. 1982. An extract of extracellular matrix fraction that organizes acetylcholine receptors. In *Muscle Development: Molecular and Genetic Control*. M. L. Pearson and H. F. Epstein, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 469-479.