

Cytoplasmic Components of Acetylcholine Receptor Clusters of Cultured Rat Myotubes: The 58-kD Protein

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Abstract. A 58-kD protein, identified in extracts of postsynaptic membrane from *Torpedo* electric organ, is enriched at sites where acetylcholine receptors (AChR) are concentrated in vertebrate muscle (Froehner, S. C., A. A. Murnane, M. Tobler, H. B. Peng, and R. Sealock. 1987. *J. Cell Biol.* 104:1633–1646). We have studied the 58-kD protein in AChR clusters isolated from cultured rat myotubes. Using immunofluorescence microscopy we show that the 58-kD protein is highly enriched at AChR clusters, but is also present in regions of the myotube membrane lacking AChR. Within clusters, the 58-kD protein codistributes with AChR, and is absent from adjacent membrane domains involved in myotube–substrate contact. Semiquantitative fluorescence measurements suggest that molecules of the 58-kD protein and AChR are present in approximately equal numbers. Differential extraction of peripheral membrane proteins from isolated AChR

clusters suggests that the 58-kD protein is more tightly bound to cluster membrane than is actin or spectrin, but less tightly bound than the receptor-associated 43-kD protein. When AChR clusters are disrupted either in intact cells or after isolation, the 58-kD protein still codistributes with AChR. Clusters visualized by electron microscopy after immunogold labeling and quick-freeze, deep-etch replication show that, within AChR clusters, the 58-kD protein is sharply confined to AChR-rich domains, where it is present in a network of filaments lying on the cytoplasmic surface of the membrane. Additional actin filaments overlie, and are attached to, this network. Our results suggest that within AChR domains of clusters, the 58-kD protein lies between AChR and the receptor-associated 43-kD protein, and the membrane-skeletal proteins, β -spectrin, and actin.

CLUSTERING of acetylcholine receptors (AChR)¹ in the membrane of embryonic skeletal muscle is one of the earliest events in the formation of the vertebrate neuromuscular junction (reviewed in Schuetze and Role, 1987; Bloch and Pumplin, 1988). Cytoskeletal proteins are believed to participate in the clustering of AChR, but the way they do so is still unknown. Considerable efforts have been made to identify the cytoskeletal proteins at the postsynaptic membrane, and to study their relationship to the AChR (reviewed in Froehner, 1986; Bloch and Pumplin, 1988).

The AChR clusters of cultured rat myotubes have proved to be very useful for such studies. Although they form in aneural muscle cells, these structures are essentially identical to the postsynaptic aggregates of AChR that form at the earliest stage of synaptogenesis (Bloch and Pumplin, 1988). After isolation from intact myotubes by a simple detergent extraction, clusters remain attached to the tissue culture substrate, and retain their original morphology (Bloch, 1984).

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; FGAM, fluorescein goat anti-mouse IgG; LIS, lithium diiodosalicylate; QFDERR, quick-freeze, deep-etch, rotary-replication; R-BT, monotetramethylrhodamine- α -bungarotoxin.

Isolated clusters can be treated to remove peripheral membrane proteins and to observe the consequent changes in AChR organization. Furthermore, these clusters can be studied by both semiquantitative fluorescence techniques, to reveal the approximate stoichiometry of their constituent proteins (Bloch, 1986; Bloch and Froehner, 1987), and by quick-freeze, deep-etch, rotary replication (QFDERR), to reveal their ultrastructural organization (Pumplin, 1989).

Such studies have suggested that the AChR and associated intracellular, peripheral membrane proteins are organized like the membrane skeleton of the human erythrocyte. Clusters contain actin (Bloch, 1986), and an unusual isoform of β -spectrin (Bloch and Morrow, 1989) which may be organized as a network closely applied to the cytoplasmic face of the membrane (Pumplin, 1989; Pumplin, D. W., J. C. Strong, J. G. Krikorian, G. A. Porter, and J. C. Winkelman. 1990. *J. Cell Biol.* 111 [No. 5, Pt. 2]:165a [Abstr.]). Both proteins can be removed from cluster membrane by mild digestion with chymotrypsin (Bloch, 1986; Bloch and Morrow, 1989). After such treatment, AChRs are retained quantitatively but redistribute in the plane of the membrane (Bloch and Froehner, 1987). We have postulated that, like the band 3 protein (anion transporter) in the human erythrocyte (for reviews see Marchesi, 1985; Bennett, 1990), the

AChR itself functions as a binding site on the membrane for a network of actin and spectrin. Unlike the erythrocyte, however, no ankyrin-like protein capable of linking the membrane skeleton to the AChR has been identified in AChR-rich membrane (Flucher and Daniels, 1989).

Two proteins believed to associate with the AChR have been identified in extracts of postsynaptic membrane from *Torpedo* electric organ, with polypeptide chain molecular weights of 43,000 and 58,000 (Sobel et al., 1978; Neubig et al., 1979; Elliott et al., 1980; Porter and Froehner, 1983; Froehner et al., 1987). The 43-kD protein is very closely associated with the AChR in *Torpedo* electric organ, at the neuromuscular junction, and in cultured muscle cells (Sealock, 1982a,b; Nghiem et al., 1983; Burden et al., 1983; Peng and Froehner, 1985; Burden, 1985; Bloch and Froehner, 1987; Toyoshima and Unwin, 1988; Mitra et al., 1989). In *Xenopus* oocytes and in fibroblasts that express the AChR, coexpression of the 43-kD protein causes receptor aggregation (Froehner et al., 1990; Phillips et al., 1991). Less is known about the 58-kD protein. Although it codistributes with AChR under some circumstances, it is more widely distributed than either the 43-kD protein or the AChR (Froehner et al., 1987; Carr et al., 1989; Daniels et al., 1990; Daniels, 1990). In *Xenopus* muscle, a 48-kD protein homologous to the 58-kD protein is also found at the myotendinous junction, and, in culture, at structures resembling focal contacts (Chen et al., 1990). With the exception of actin (Lubit, 1984; Bloch, 1986), no other protein that codistributes with AChR has also been reported to be enriched at focal contacts. Thus, the 58-kD protein may have an unusual association with AChR-rich membrane.

We have used AChR clusters isolated from cultured rat myotubes to study this association. These clusters form at sites of adhesion of the myotube to the culture substrate, and are organized into three distinct types of membrane domains. In two of these domains, one resembling focal contacts of mononucleate cells (Bloch and Geiger, 1980; Bloch et al., 1989; Pumplin, 1989), the other enriched in clathrin (Pumplin, 1989; Pumplin and Bloch, 1990), the membrane lies close to the tissue culture substrate, and so we call them "contact domains" (Pumplin and Bloch, 1983). Membrane in the third type of domain is further from the substrate and is enriched in AChR; we call these "AChR domains" (Pumplin and Bloch, 1983). We show here that the 58-kD protein is highly enriched in the AChR domains, and, contrary to results with *Xenopus* myocytes (Chen et al., 1990), is absent from focal contact domains. We have compared the 58-kD protein to other peripheral membrane proteins at AChR clusters using semiquantitative immunofluorescence measurements and differential extraction protocols, and have localized it in the cytoskeleton by immunogold labeling. Our results suggest that the 58-kD protein forms part of an ordered cytoskeleton on the cytoplasmic surface of AChR domains.

Materials and Methods

Cell Culture

Primary cultures of rat myotubes were prepared by established procedures (Bloch, 1979; Bloch and Geiger, 1980). Briefly, hind limb muscle from neonatal rats were dissociated in collagenase, and suspended at 10^6 cells/ml in

Dulbecco-Vogt modified Eagle's medium containing 10% fetal calf serum (medium). Aliquots of 0.4 ml were applied to glass coverslips (Van Labs, Oxnard, CA), supplemented the next day with 1.5 ml medium, and changed into medium containing 2×10^{-5} cytosine arabinoside 3 d later, when myotubes first start to form. Cells were used on days 6–8 after plating, after large myotubes had formed, but before highly branching syncytia or significant spontaneous electrical activity developed.

Labeling and Isolation of AChR Clusters

Cultures were labeled with monoteletramethyl- α -bungarotoxin (R-BT; 5 μ g/ml), prepared as described (Ravdin and Axelrod, 1977) in Hepes-buffered DMEM containing 5% fetal calf serum, and washed free of unbound toxin. In some experiments, cultures were fixed and permeabilized, either by treatment with 95% ethanol at -20°C , or by exposure for 1–2 min to 0.5% Triton X-100 in 50 mM MES, pH 6.0, followed by fixation in fresh, cold 2% paraformaldehyde in PBS. In most experiments, however, AChR clusters were isolated by subjecting samples to extraction with saponin (Bloch, 1984), or to shearing (Pumplin, 1989). For extraction with saponin, cells were washed sequentially with PBS, and with PBS supplemented with 10 mM MgCl_2 , 1 mM EGTA, 1 mg/ml BSA. Cultures were then placed in the same solution containing 0.2% saponin, and rocked gently for 4–10 min at room temperature (Bloch, 1984). Samples were then either fixed in paraformaldehyde, or treated further and then fixed. For isolation by shearing, cultures were treated for 2 min with a solution containing ZnCl_2 (1 mM ZnCl_2 , 3 mM EGTA, 5 mM MgCl_2 , 100 mM Pipes, pH 6.0; Avnur and Geiger, 1981), then sheared with a stream of buffer containing a high concentration of K^+ (100 mM KCl, 5 mM MgCl_2 , 3 mM EGTA, 20 mM Hepes, pH 7.0; Aggeler and Werb, 1982), and fixed in paraformaldehyde, or treated further and then fixed.

Treatment of Isolated Clusters

Some samples extracted with saponin or prepared by shearing were treated before fixation to remove one or more of the peripheral membrane proteins known to be associated with clustered AChR. These treatments were: buffer A (1 mM Tris HCl, 0.1 mM ATP, pH 8.0; Spudich and Watt, 1971) for 10 min; PBS for 5 min, 10 min, or for longer periods; 10 μ g/ml chymotrypsin for 5 min; 50 mM ethylamine HCl, pH 11, for 5 min (alkaline pH); 20 mM lithium diiodosalicylate (LIS) in 10 mM NaP, 1 mM EDTA, 1 mM EGTA, pH 7.2, for 5 min; 6 M urea in PBS for 5 min. The proteins known to be extracted or degraded by these treatments are summarized with appropriate references in Table I.

Labeling with Antibodies

After fixation, samples were incubated for 15 min in 0.1 M glycine in PBS to inactivate remaining aldehydes, and then washed briefly with PBS containing bovine serum albumin at 1 mg/ml (PBS-BSA). The latter solution was used for all subsequent steps. Samples were labeled with monoclonal antibodies to the 58-kD protein (mAb 1351; see Froehner et al., 1987), kindly provided by Dr. S. Froehner (Department of Biochemistry, Dartmouth Medical School, Hanover, NH), or with the following monoclonal antibodies to other proteins present in AChR clusters: VIII7 anti- β -spectrin (Harris et al., 1986; Bloch and Morrow, 1989), from Dr. J. Morrow (Department of Pathology, Yale University School of Medicine, New Haven, CT); 1579A, anti-43-kD protein (Bloch and Froehner, 1987), and 88B anti-AChR (Froehner et al., 1983), both from Dr. Froehner. Labeling was usually for 30–60 min at room temperature. Samples were then washed in PBS-BSA and incubated further with fluoresceinated goat anti-mouse IgG (FGAM).

In some experiments, we compared the distribution of the 58-kD protein to that of vinculin, as follows. Cultures, unlabeled with R-BT, were subjected to shearing as described above. After fixation, samples were incubated with mAb 1351 and the affinity-purified rabbit anti-vinculin (Bloch and Hall, 1983; Bloch et al., 1989), followed by FGAM and rhodaminylated goat anti-rabbit IgG. Appropriate controls showed that the secondary antibodies were species specific.

After labeling, all samples were washed further in PBS-BSA and mounted in nine parts glycerol, one part Tris-HCl, pH 8, supplemented with 1 mg/ml *p*-phenylenediamine, to reduce photobleaching (Johnson et al., 1982).

Microscopy and Photomicrography

Samples were observed with a Zeiss IM-35 microscope equipped for epifluorescence, and for semiquantitative measurements. For the latter, the

Table I. Removal of Proteins from Isolated AChR Clusters by Different Treatments*

Protein	Low ionic strength	PBS, room temperature, 10 min	Chymotrypsin	pH 11	LIS	Urea
Actin	Removed	— ‡	Removed	Removed	Removed	— ‡
β -spectrin	Bound	Removed§	Removed	Removed	Removed	Removed
43 kD	Bound	Bound	Bound	Removed	Removed	Removed
AChR	Bound	Bound	Bound	Bound	Bound	Bound

* References are as follows: actin, Bloch, 1986; β -spectrin, Bloch and Morrow, 1989; 43-kD protein and AChR, Bloch and Froehner, 1987; effects of extraction with 6 M urea, Krikorian, J. G., and R. J. Bloch, manuscript submitted for publication.

‡ Not determined.

§ Removal is partial, not complete.

incident illumination was limited to a region of the sample 5 μ m in diameter, and the fluorescence from this region was measured with a photomultiplier connected to an I-to-V converter (see Bloch, 1986, for details). Measurements of fluorescein fluorescence, due to bound antibody and FGAM, and of tetramethylrhodamine fluorescence, due to bound R-BT, were always made from the same area, and the ratio, F/R, was calculated after background was subtracted.

Photomicrographs were exposed for 15–30 s. Ilford HP5 film (Ilford Ltd., Basildon, Essex, UK) was processed with Ilford Microphen developer to an ASA of 1,600. Times for exposure and printing were kept constant to allow reliable comparison of different samples.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed on 5–15% gradient acrylamide gels, following the methods of Laemmli (1970). Transfer of material from gels to nitrocellulose was as described (Burnette, 1981). Nonspecific binding of antibodies to nitrocellulose was reduced by applying 3% milk solids, 3% BSA to the blot before exposing it to antibodies, and continuing all incubations in the presence of 0.1% BSA. The buffer used in these incubations was PBS supplemented with 0.5% Tween-20 and 10 mM sodium azide. Blots were incubated further in affinity-purified rabbit anti-mouse IgG. Bound antibodies were visualized with radioiodinated protein A (Amersham Corp., Arlington Heights, IL). Kodak X-Omat film was used for autoradiography and was processed to an ASA of 1600.

ELISA

Plastic 96-well dishes (Immulon II; Dynatech Laboratories, Inc., Alexandria, VA) were coated with 50 μ l aliquots of FGAM (10 ng/ml in buffered saline). Additional protein binding sites were blocked with 1% normal goat serum diluted in buffered saline. Wells were incubated with increasing concentrations of mAb 1351, or with mAb 88B, an IgG₁ anti-AChR (Froehner et al., 1983). Binding of the mouse antibodies to the wells was detected with saturating concentrations of donkey anti-mouse antibodies conjugated to alkaline phosphatase.

Ultrastructural Methods

Immunogold labeling and rapid-freeze, deep-etch, rotary-replication of clusters were performed with the methods and precautions described previously (Pumplin et al., 1990). In brief, clusters isolated by shearing and fixed in paraformaldehyde were labeled with mAb 1351 (50 nM, 1 h, room temperature) and fluoresceinated rabbit antimouse IgG (1:100, 1 h, room temperature). After washing, the samples were incubated overnight at 4°C with goat anti-rabbit IgG adsorbed to 10-nm colloidal gold (Janssen Pharmaceuticals, Beerse, Belgium), diluted 1:10 in BANT (0.1% bovine serum albumin, 0.5 M NaCl, 10 mM MgCl₂, 20 mM Tris, 20 mM NaN₃, pH 7.4; Luther and Bloch, 1989). Well-formed clusters were chosen by rhodamine and fluorescein fluorescence, photographed, and their positions circled with a diamond marking objective. Concentric circles around the marked cluster were cut from the coverslips. Samples were further fixed in 2% glutaraldehyde, then rinsed three times in PBS followed by a rinse in D₂O. After removal of nearly all the overlying liquid, the samples were rapidly frozen by being pressed against a metal mirror at -196°C. Frozen samples were freeze-dried at -95°C under high vacuum until D₂O emission ceased, then rotary replicated with platinum applied at an angle of 20°, followed by a backing layer of carbon. Replicated clusters were readily relocated by reference

to the previously scribed circle. They were floated off the coverslips in 5% HF, rinsed in water, and picked up on formvar-coated slot grids. Electron micrographs were taken at low magnification to compare with corresponding fluorescence images and at high magnification in stereo pairs with $\pm 6^\circ$ of tilt.

Materials

Fragments of postsynaptic membrane were isolated from electric organ of *Torpedo nobiliana* (BioFish, Georgetown, MA), following the method of Porter and Froehner (1983). All fluorescent antibodies were from Jackson ImmunoResearch (West Grove, PA). Unless otherwise noted, all other materials were from Sigma Chemical Co. (St. Louis, MO), and were of the highest grade available. Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Richmond, CA) was used to determine protein concentrations.

Results

Our aim in the following experiments was to learn if a homologue of the 58-kD protein of *Torpedo* electric organ is present in the AChR clusters of cultured rat myotubes, and, if so, to determine its relationship to AChR and to other peripheral membrane proteins concentrated at clusters. We used a monoclonal antibody, mAb 1351, made against the *Torpedo* antigen, as a probe for mammalian protein (Froehner et al., 1987). Immunoblotting of extracts of rat myotubes showed that, as in extracts of C2 mouse muscle cells and of *Torpedo* electric organ (Froehner et al., 1987), 1351 labels a protein in rat myotubes with an apparent molecular mass of 58,000 D (Fig. 1 B). We refer to this protein as the 58-kD protein.

Localization

To localize the 58-kD protein in immunofluorescence studies, we first labeled cultures of rat myotubes with R-BT to visualize AChR, and then permeabilized them to expose the 58-kD protein, using either cold ethanol fixation or brief extraction with Triton X-100 followed by paraformaldehyde. In both preparations, the 58-kD protein, visualized with 1351 and FGAM was highly enriched at AChR clusters (Fig. 2). No concentrations of immunofluorescent label were seen in fibroblasts labeled with mAb 1351, or in myotubes labeled with a control mouse IgG, mAb 21. Some myotubes had membrane regions that were labeled with 1351 and FGAM, but not with R-BT (Fig. 2, A and B, and E and F, brackets). These regions were usually close to an AChR cluster, and the pattern of the immunolabel seemed to extend the pattern of labeling by R-BT. This suggests that the 58-kD protein might be present at "extensions" of AChR-rich membrane. We cannot rule out the possibility that AChRs are present in

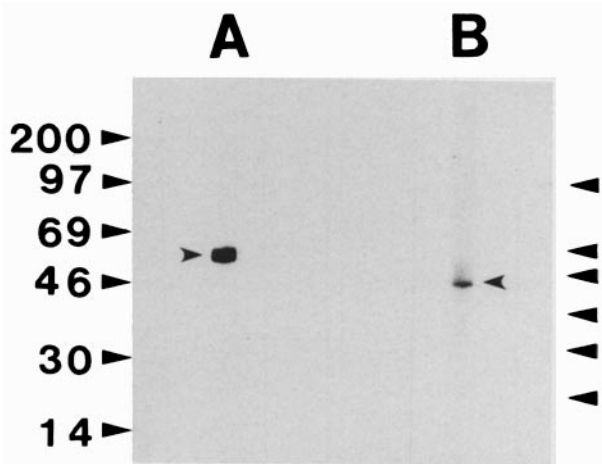


Figure 1. Immunoblotting of the 58-kD protein. Extracts of post-synaptic membrane purified from *Torpedo* electric organ (A) or extracts of rat myotubes (B) were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose. Strips of nitrocellulose were cut, blocked with 3% milk solids, 3% BSA, and incubated

these regions but remain unlabeled by R-BT; ultrastructural studies are now in progress to address this possibility. We saw no other areas of enrichment of labeling for the 58-kD protein in myotubes.

We isolated AChR clusters either by extracting with saponin, or by shearing samples with a stream of buffer (see Materials and Methods), to obtain clearer immunofluorescence images of AChR clusters and associated 58-kD protein. In saponin-extracted clusters, all of the immunofluorescence la-

with mAb 1351, against the 58-kD protein. Binding of mAb was detected by sequential incubations in rabbit anti-mouse IgG and 125 I-protein A, followed by autoradiography. The large arrowheads on the left of lane A indicate positions of several standard proteins with molecular masses (from top to bottom) 200,000, 97,400, 69,000, 46,000, 30,000, and 14,500 D. Large arrowheads to the right of lane B indicate the same bands, but these appear at different positions because different acrylamide concentrations were used in A and B. The results show that mAb 1351 specifically recognizes a band in myotube extracts with an apparent polypeptide chain molecular mass of 58,000 D (small arrowheads).

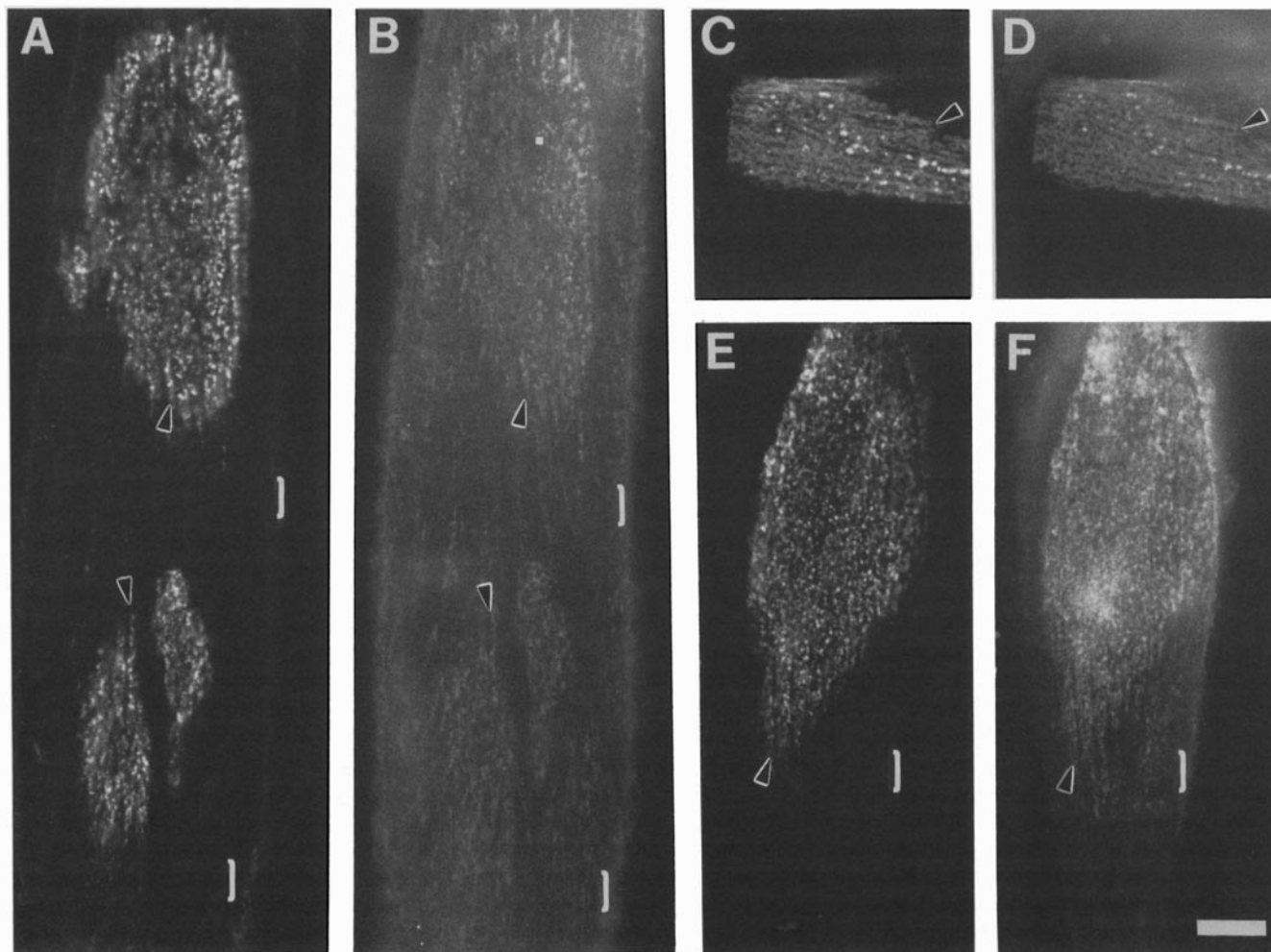


Figure 2. Localization of the 58-kD protein in myotubes by immunofluorescence. Cultures of rat myotubes were labeled with R-BT and either fixed with 95% ethanol at -20°C (C-E), or treated with 0.5% Triton X-100 for 1-2 min and then fixed with 2% paraformaldehyde (A and B). Samples were then labeled with mAb 1351 followed by FGAM. (A, C, and E show fluorescence due to bound R-BT; B, D, and F show fluorescence due to bound mAb 1351 and FGAM. Note that, in each pair of images, R-BT and antibody fluorescence are almost identical (e.g., arrowheads). In some samples, however, fluorescence due to bound antibody is detected in areas apparently lacking significant concentrations of AChR (brackets). Bar, 10 μm .

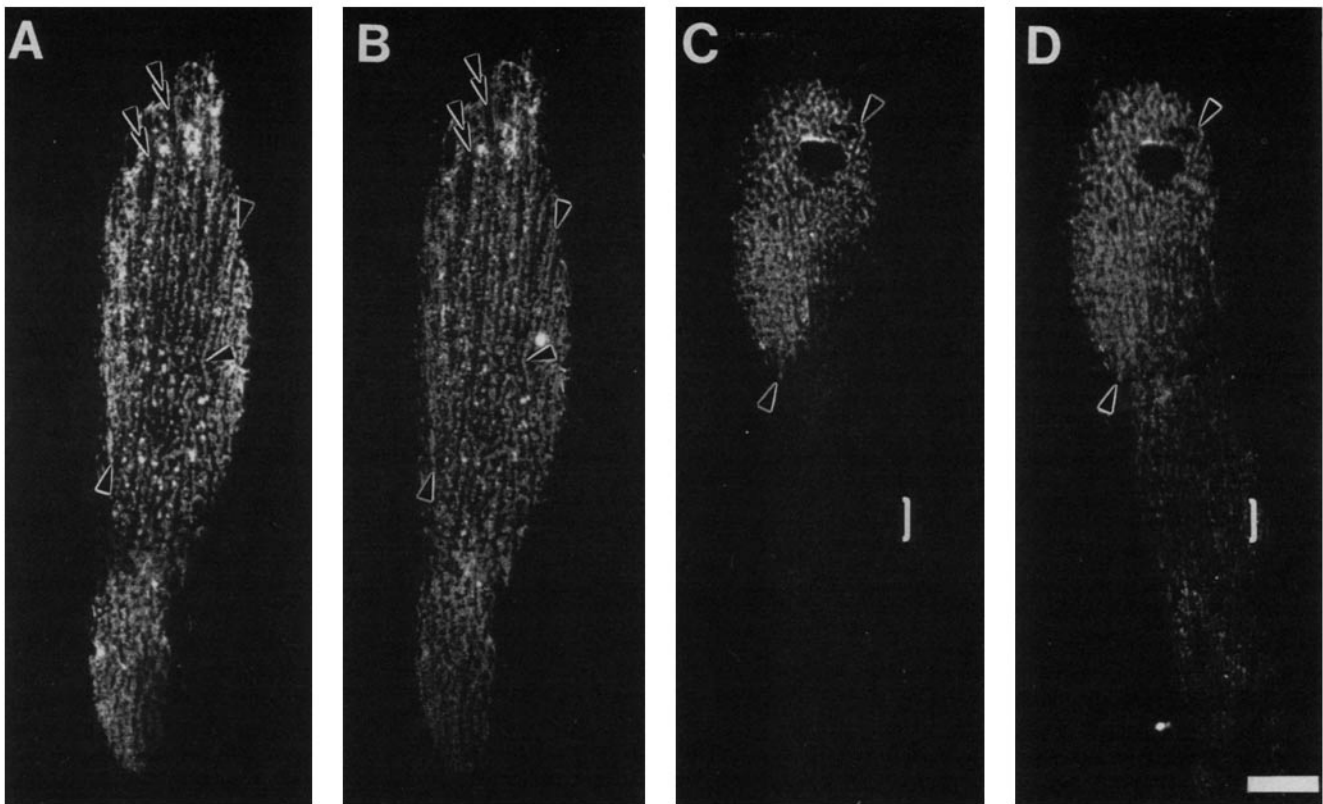


Figure 3. Distribution of 58-kD protein and AChR in isolated AChR clusters. Myotubes were labeled with R-BT, and then subjected to extraction with saponin (*A* and *B*) or to shearing (*C* and *D*) to isolate AChR clusters (see Materials and Methods). After fixation with paraformaldehyde, samples were labeled with mAb 1351 and FGAM. *A* and *C* show fluorescence due to bound R-BT; *B* and *D* show fluorescence due to bound antibody. Results show that the 58-kD protein is present at all sites within AChR clusters that are enriched in AChR (arrowheads), and not at sites, termed contact domains, that are poor in AChR (*A* and *B*, double arrowheads). In some clusters prepared by shearing, bound antibody is detected in areas that appear to lack significant concentrations of AChR (*C* and *D*, brackets). Bar, 10 μm .

bel colocalized with AChR (Fig. 3, *A* and *B*, arrowheads) and was absent from AChR-free regions of the membrane, including the domains of the cluster dedicated to substrate adhesion (Fig. 3, *A* and *B*, double arrowheads). Controls using the mouse IgG, mopc 21, showed no labeling of AChR clusters (not shown). These results suggest that a protein resembling the 58-kD protein of *Torpedo* electric organ codistributes with AChR in the AChR clusters of rat myotubes.

We observed the same enrichment of immunolabel in sheared clusters (Fig. 3, *C* and *D*), but in this case, less intense labeling was sometimes apparent in other, AChR-poor, regions of the membranes (Fig. 3, *C* and *D*, brackets), in agreement with our observations on intact cells, and with previous reports (Froehner et al., 1987). Sheared clusters contain intact "focal contact domains" that are enriched in vinculin and talin (Bloch and Geiger, 1980; Bloch et al., 1989). The fact that mAb 1351 labels only AChR domains within AChR clusters indicates that the 58-kD protein is not a component of focal contacts. This has been confirmed by double immunofluorescence experiments that compared the 58-kD protein to vinculin (not shown). Such comparisons revealed no enrichment of the 58-kD protein at focal contacts of rat myotubes (Pumplin, D. W., and R. J. Bloch, manuscript in preparation). These results contrast with those obtained with cultured *Xenopus* cells, in which a 48-kD ana-

logue of the 58-kD protein is present at focal contacts (Chen et al., 1990).

Quantitation

We performed semiquantitative immunofluorescence measurements to determine the approximate amounts of the 58-kD protein present in clusters. We used saponin-extracted clusters for this purpose, as they contain little 58-kD protein that is not localized with AChR (see above). We added increasing amounts of mAb 1351 to isolated, fixed clusters, and then counterstained with saturating concentrations of FGAM. We then measured the intensity of fluorescein fluorescence (*F*) from clusters and compared it to the intensity of tetramethylrhodamine fluorescence (*R*), due to the R-BT bound in the same region of the cluster. We found that *F* varied linearly with *R* (Fig. 4 *A*). When we plotted the ratio, *F/R*, against the concentration of 1351, we observed saturation at a value of ~ 1 (0.98 ± 0.10 , $n = 3$). mAb 1351 at ~ 3 nM gave half-maximal saturation. We obtained similar results with clusters isolated by shearing (not shown).

The F/R_{max} value of 1 that we obtained for the 58-kD protein with mAb 1351 can be compared to values of ~ 1 obtained in similar experiments with monoclonal antibodies to the AChR itself (Bloch and Froehner, 1987). Experiments using ELISA methods show that FGAM reacts equally well

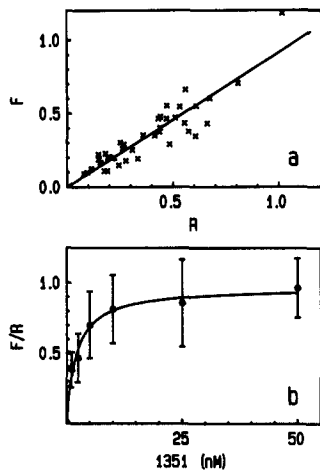


Figure 4. Semiquantitative measurements of immunofluorescence. Samples were labeled with R-BT, isolated by extraction with saponin, fixed, and labeled with different concentrations of mAb 1351, followed by FGAM. Fluorescein fluorescence (F), due to bound antibodies, and rhodamine fluorescence (R), due to bound R-BT, were measured from the same area of AChR clusters, with a photomultiplier. Background readings for F and R , measured away from AChR clusters, were subtracted before plotting. (a) Linear relationship of F and

R , determined at saturating concentrations of 1351 (slope = 0.92 ± 0.07 , $r = 0.915$). (b) Plots of F/R vs. concentration of 1351 indicate that binding of mAb is saturating (rectangular hyperbola fit with $F/R_{\max} = 0.98$ and half-maximal binding at 2.2 nM mAb 1351).

with 1351 and with the anti-AChR mAb, 88B (Fig. 5). If we assume that both antibodies bind to cluster membrane with the same valency, then their respective antigens should be present in clusters in roughly equal amounts.

Extraction

We performed differential extraction experiments to determine how easily the 58-kD protein could be removed from clusters prepared with saponin. Table II summarizes our results. Labeling by mAb 1351 was reduced by treating isolated clusters at alkaline pH, and completely eliminated by treatments with urea, diiodosalicylate, or chymotrypsin.

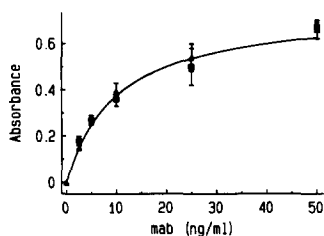


Figure 5. ELISA analysis of FGAM binding to mAb 1351 and anti-AChR mAb 88B. Plates were coated with FGAM and used to capture 1351 or 88B, added at increasing concentrations. Bound mAb was detected with species-specific donkey anti-mouse conjugated to alkaline phosphatase. (Squares) 1351; (triangles) 88B.

Values plotted are means \pm SD ($n = 3$). When both symbols cannot be seen, they overlap. The rectangular hyperbola describing both sets of binding data was fit with a maximum absorbance of 0.75 and a half-maximal concentration of binding of 10 ng/ml. Absorbance recorded from wells lacking FGAM was $<10\%$ of the values obtained here (not shown). The results show that FGAM binds equal amounts of 1351 and 88B.

Table II. Extraction of the 58-kD Protein from Isolated AChR Clusters

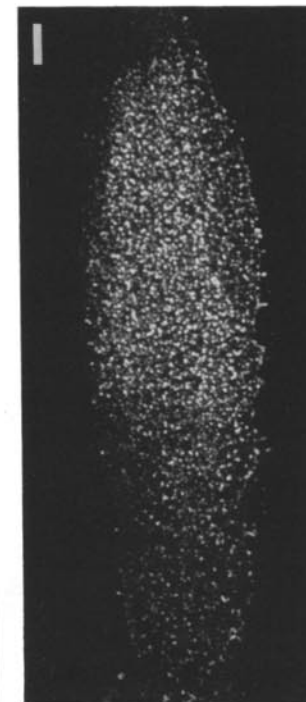
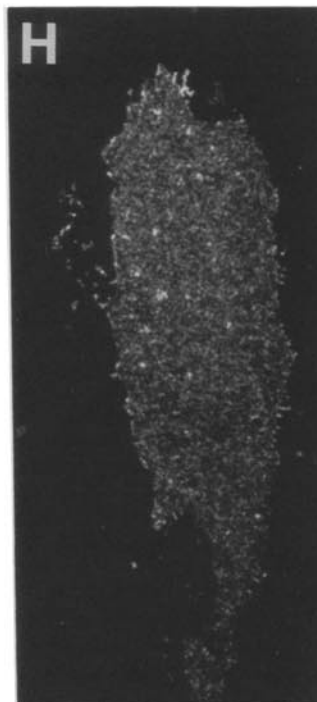
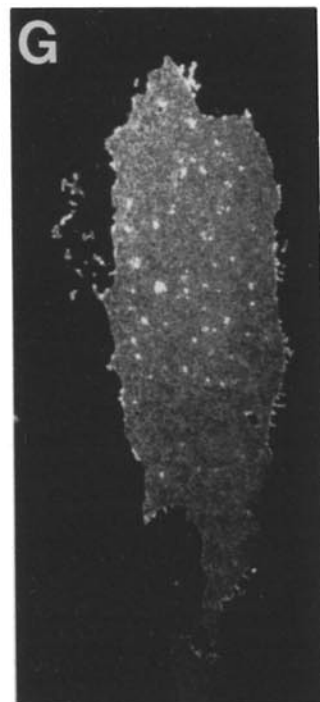
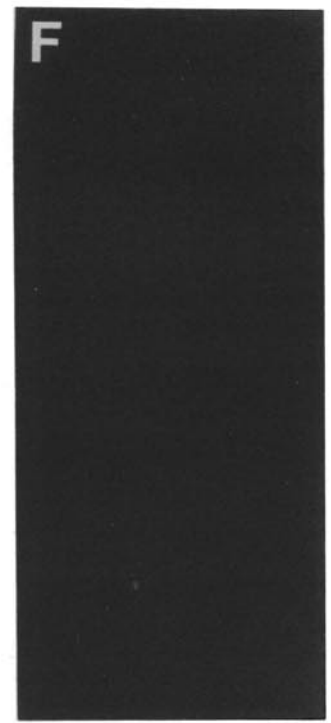
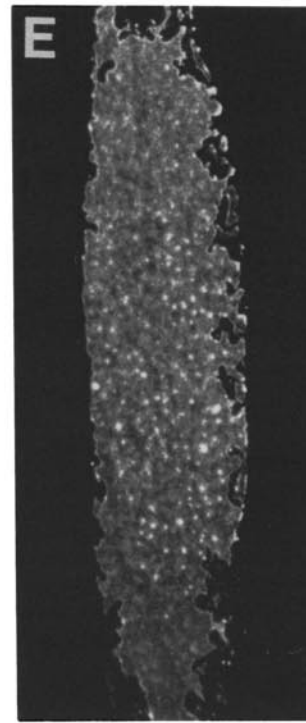
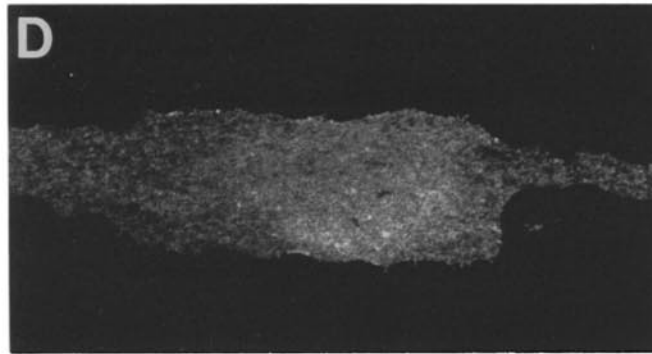
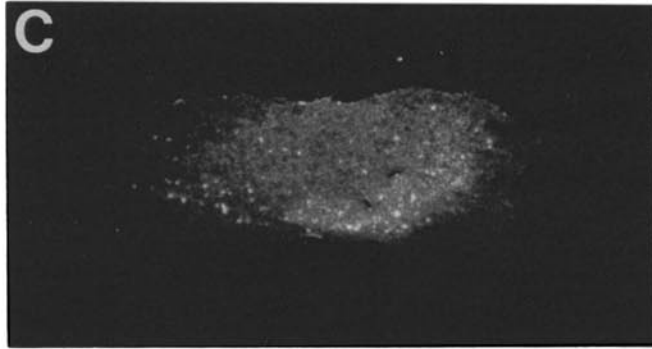
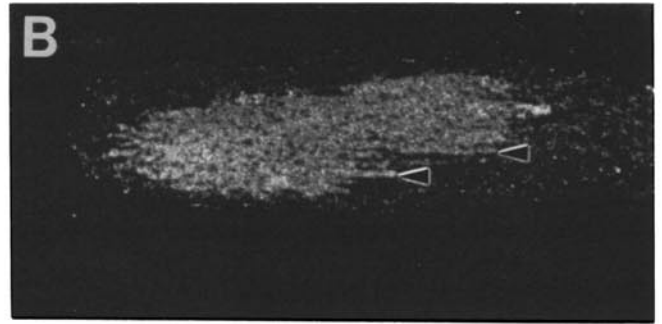
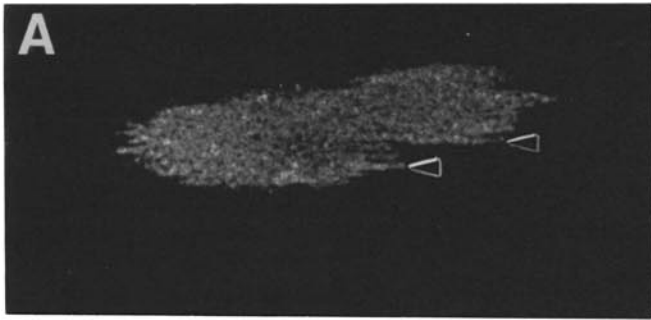
Treatments		
First	Second	F/R
PBS control	—	0.95 ± 0.22
Buffer A	—	0.91 ± 0.21
Chymotrypsin	—	0.04 ± 0.02
Urea, 6 M	—	0.05 ± 0.03
LIS, 20 mM	—	0.06 ± 0.02
Ethylamine HCl, pH 11	—	0.31 ± 0.09
Ethylamine HCl, pH 11	PBS	0.32 ± 0.10
Ethylamine HCl, pH 11	Chymotrypsin	0.01 ± 0.02
Ethylamine HCl, pH 11	Urea, 6 M	0.05 ± 0.03
Ethylamine HCl, pH 11	LIS, 20 mM	0.01 ± 0.03

Myotubes were labeled with R-BT, extracted with saponin, and subjected to additional exposure to PBS, buffer A, chymotrypsin ($10 \mu\text{g/ml}$ in PBS), 6 M urea, a buffered solution of 20 mM LIS, or ethylamine HCl buffer at pH 11. Most samples were fixed immediately; some samples exposed to ethylamine HCl, pH 11, were treated for an additional 5 min before fixation. After fixation and labeling with a saturating concentration of mAb 1351 and FGAM, F/R values were determined with the photomultiplier. Each value represents the mean \pm SD ($n = 10$).

It was not affected by incubation in a buffer of low ionic strength. Comparison of these results to those obtained with other proteins (Table I) shows that the association of the 58-kD protein with AChR clusters resembles that of β -spectrin (Bloch and Morrow, 1989): it is more tightly associated with cluster membrane than actin, but less tightly associated than the 43-kD protein. Unlike spectrin, however, some 58-kD protein remains associated with isolated cluster membrane even after extraction at alkaline pH. The 58-kD protein that remains after extraction at pH 11 can be removed by further treatment with chymotrypsin, urea, or LIS (Table II). Fig. 5 shows the effects of these same treatments on the distribution of AChR and the 58-kD protein. Like β -spectrin (Bloch and Morrow, 1989), labeling for 58-kD redistributes after treatment at low ionic strength (Fig. 6, C and D), is lost upon treatment with chymotrypsin (Fig. 6, E and F) or LIS (Fig. 6, I and J), and is depleted by extraction at pH 11 (Fig. 6, G and H). Chymotrypsin, LIS, and alkaline pH also cause AChR to redistribute.

We attempted to differentiate further between β -spectrin and the 58-kD protein by studying the abilities of these two proteins to remain associated with AChR clusters as a function of time after isolation. Shortly after isolation, cluster membrane starts to lose β -spectrin, and this loss continues for at least 1 h (Bloch and Morrow, 1989). Under the same conditions, the 58-kD protein appears to remain associated with cluster membrane for 10 min, and is lost only slowly thereafter (Fig. 7, solid line). This is true for samples prepared by extraction with saponin, or by shearing. The 43-kD receptor-associated protein is not depleted from AChR

Figure 6. Fluorescence micrographs of extracted AChR clusters. Samples treated as in Table II were photographed under rhodamine (A, C, E, G, and I) and fluorescein (B, D, F, H, and J) optics. (A and B) Sample treated in PBS shows codistribution of label by R-BT and mAb 1351 in typical AChR clusters (arrowheads; c.f. Figs. 2 and 3); (C and D) Extraction in buffer A redistributes both labels in a similar fashion; (E and F) Digestion with chymotrypsin redistributes R-BT label and eliminates subsequent labeling by mAb 1351; (G and H) Treatment at pH 11 redistributes R-BT label, and diminishes subsequent labeling by mAb 1351; (I and J) Extraction with LIS causes AChR to microaggregate, and eliminates labeling by mAb 1351. Bar, 10 μm .



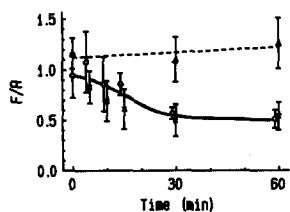


Figure 7. Shedding of the 58-kD protein after isolation of AChR clusters. Myotubes were labeled with R-BT, and AChR clusters were isolated by extraction with saponin (\times , Δ) or by shearing (\circ). Samples were incubated for increasing periods of time in buffered saline, then fixed and

labeled either with mAb 1351 (\times , \circ), or with mAb 1579 (anti-43kD protein; Δ), followed by FGAM. The results show that the 43-kD protein remains stably associated with AChR clusters, and that the 58-kD protein slowly dissociates from clusters after a 5–10-min lag period.

clusters at all under these conditions (Fig. 7, *dashed line*). Thus, the 58-kD protein seems to be more stably bound at AChR clusters than β -spectrin, but less stably associated than the 43-kD protein.

During incubation of isolated AChR clusters for 1 h at room temperature, AChRs redistribute into small aggregates in the muscle membrane (Bloch and Morrow, 1989). If the 58-kD protein is closely linked to the AChR, then it should redistribute into the same microaggregates. Similarly, as clusters are disrupted in intact cells by inhibitors of energy metabolism (Bloch, 1979), the organization of the 58-kD protein should also be disrupted, and the microaggregates of AChR that remain after these treatments should contain the

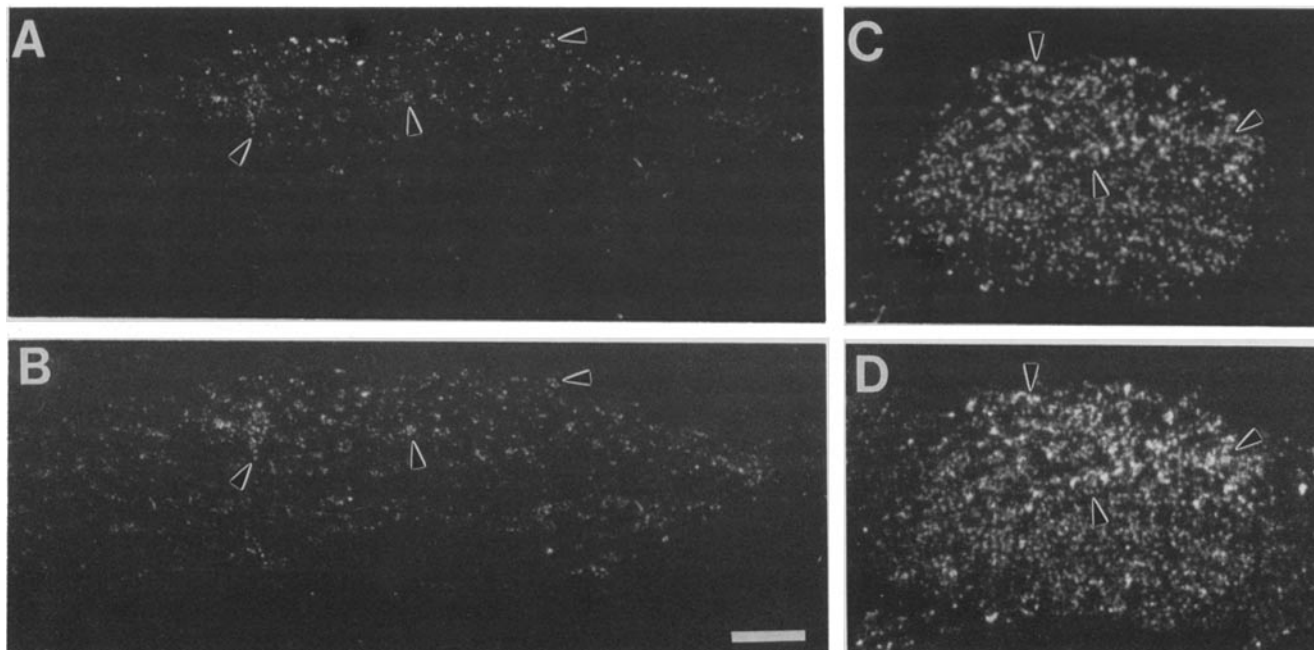
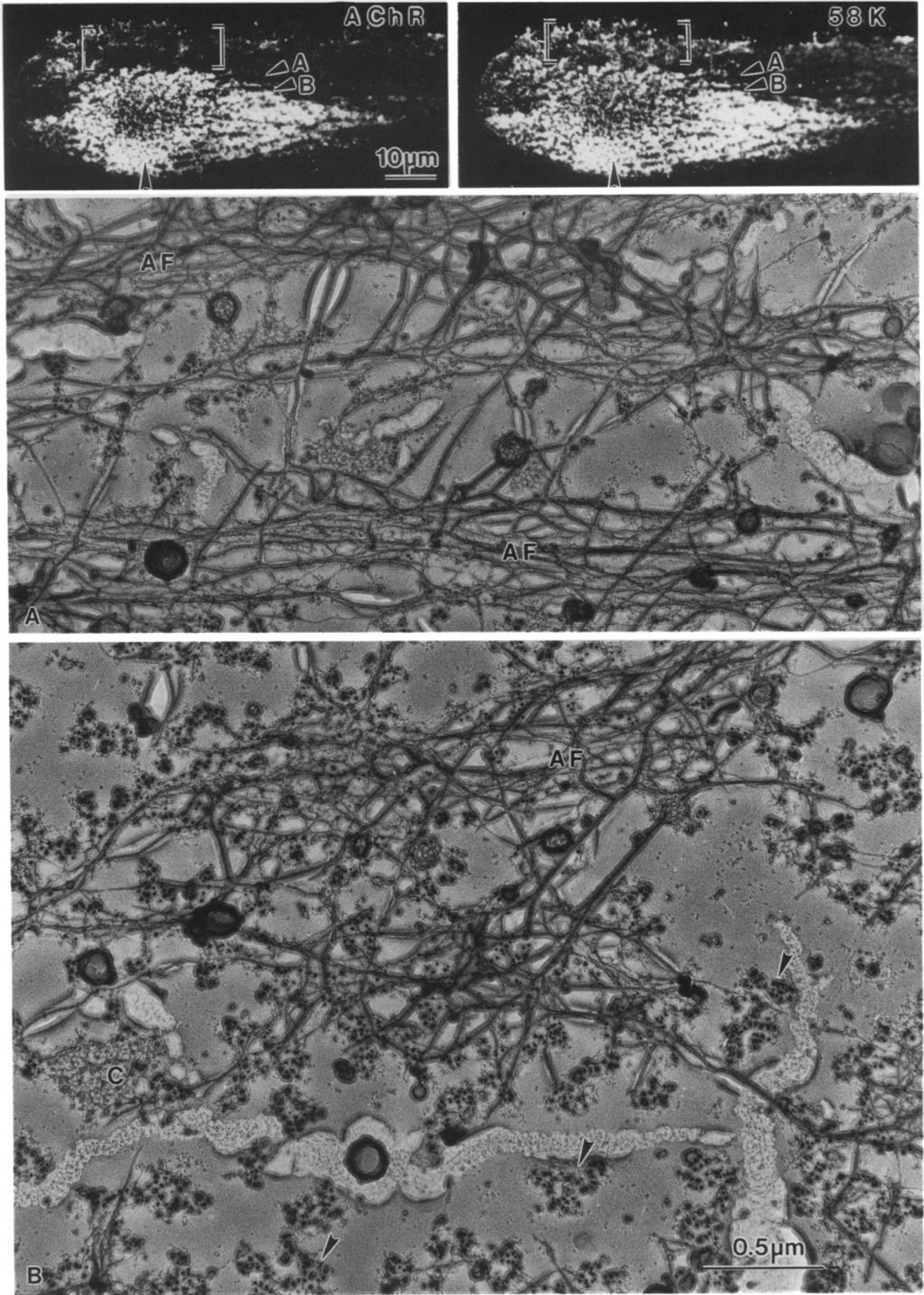


Figure 8. Comicroaggregation of AChR and the 58-kD protein. Myotubes were treated as in the legend to Fig. 6, with saponin extraction followed by incubation in PBS for 60 min (*C* and *D*). Additional cultures were incubated in 5 mM sodium azide for 4.5 h at 37°C, then labeled with R-BT, and extracted with saponin (*A* and *B*). After fixation, samples were labeled with mAb 1351 and FGAM. *A* and *C* show fluorescence due to bound R-BT; *B* and *D* show fluorescence due to bound mAb 1351. The results show that small aggregates of AChR, left after either of these treatments (e.g., *arrowheads*, *A* and *C*) are enriched in the 58-kD protein (*arrowheads*, *B* and *D*). Bar: (*A* and *B*) 10 μ m; (*C* and *D*) 6.6 μ m.

Figure 9. Antibodies to the 58-kD protein localize to AChR domains at the ultrastructural level. A cluster was labeled by R-BT, isolated by shearing, fixed, and labeled with mAb 1351, fluoresceinated rabbit anti-mouse IgG, and goat anti-rabbit IgG adsorbed to 10 nm colloidal gold. After observations of fluorescence, the cluster was visualized further by QFDERR. AChR domains were labeled by R-BT (*upper left*) and also by mAb 1351 (*upper right*). Two portions of the QFDERR replica are shown, corresponding to areas at the tips of *arrowheads A* and *B* in the fluorescence micrographs. Area *A*, having little or no labeling by R-BT or mAb 1351, contains bundles of long, cross-striated actin filaments (*AF*) lying in parallel and oriented with the long axis of the myotube; few gold particles are present. Area *B* is at the edge of an extensive region labeled with both R-BT and mAb 1351. In the corresponding replica, sites of bound mAb 1351 are marked by gold particles forming small groups (*arrowheads*) on the cytoplasmic surface of the membrane. The gold particles decorate a meshwork of filaments closely applied to the inner membrane surface (shown at higher magnification in Fig. 10). Overlying this meshwork is a second group of longer filaments having diameters and cross-striations characteristic of actin filaments. However, the actin filaments overlying regions rich in AChR and the 58-kD protein do not lie in parallel and with each other, and seem to have no preferred orientation with respect to the myotube. These filaments are not labeled with gold. Domains of coated membrane (*C* in lower panel) have their characteristic coat of clathrin and are not labeled by antibodies to the 58-kD protein. For clarity, gold particles are shown as black dots in these non-reversed micrographs. As shown in Figs. 2 and 3, some regions (between *brackets* in top panels) contain the 58-kD protein but little or no AChR.



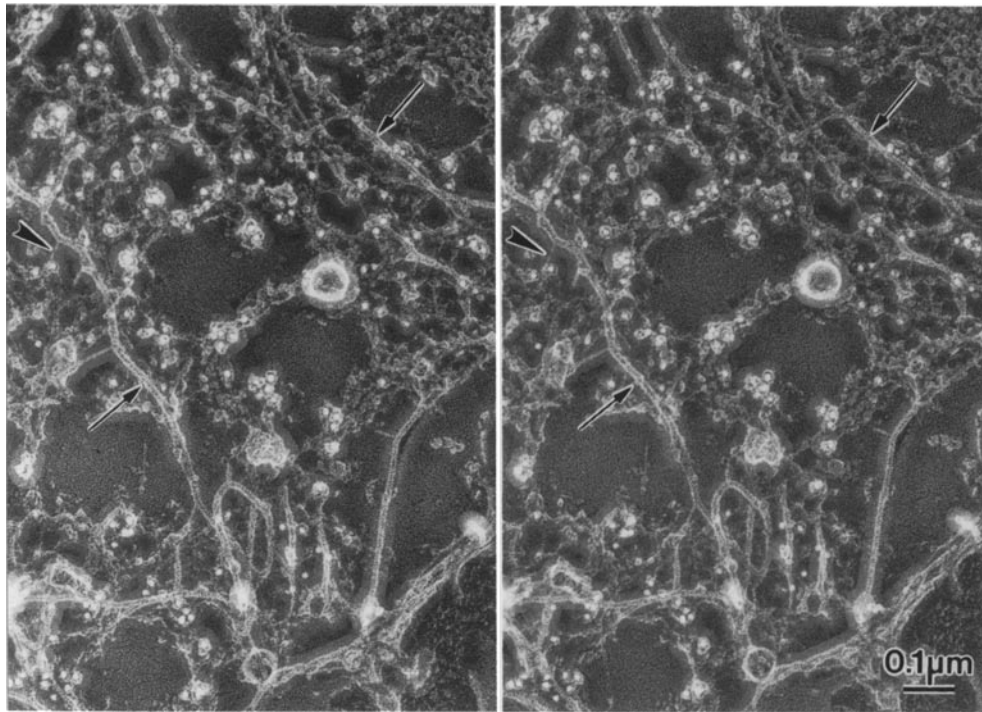


Figure 10. Antibodies to the 58-kD protein bind to the membrane-associated network rather than to overlying actin filaments. This stereo micrograph shows an area rich in both AChR and the 58-kD protein, and is taken from the same QFDERR replica shown in the previous figure (area indicated by vertical arrowheads in upper panels of Fig. 8). Gold particles (shown as white in this photographically reversed micrograph) decorate a meshwork of short filaments and particles lying on the inner membrane face. Actin filaments (*arrows*) pass over this meshwork and are occasionally attached to it by smaller filaments (*arrowheads*). These actin filaments are not labeled by the anti-58-kD antibody.

58-kD protein. These predictions were supported by our experimental observations (Fig. 8).

Fig. 8, *A* and *B* shows small aggregates of AChR and 58-kD protein remaining after treatment with azide. Cultures were treated with sodium azide, labeled with R-BT, and extracted with saponin. The substrate-attached material was fixed and labeled with mAb 1351 followed by FGAM. The small aggregates that remain after this treatment label with both markers. We obtained results similar to those shown for azide when we examined the effect of withdrawing Ca^{2+} from myotubes (not shown), which also disrupts AChR clusters (Bloch, 1983; Bursztajn et al., 1983). AChR clusters that reform after reversal of azide poisoning (Bloch, 1979) also contain the 58-kD protein (not shown).

Fig. 8, *C* and *D* shows punctate fluorescence corresponding to microaggregates of AChR that form spontaneously when isolated AChR clusters are incubated in buffered saline. Microaggregates may be more difficult to discern in these panels because of the higher overall level of labeling. Nevertheless, close examination shows that each small spot of label is discrete and is labeled by both R-BT and mAb 1351. In controls (e.g., Fig. 3), labeling is usually not as punctate, but, instead, is found in more continuous bands.

These results suggest that, even under conditions in which the organization of AChR clusters is greatly altered, the 58-kD protein still codistributes with AChR.

Ultrastructure

The results presented above suggest that the 58-kD protein is closely associated with clustered AChR. The two proteins should therefore lie close to one another at the ultrastructural level. We visualized the cytoplasmic face of AChR clusters using QFDERR electron microscopy (Heuser and Kirchner, 1980; Pumplin et al., 1990), and localized the 58-kD protein

using immunogold cytochemistry. AChR clusters consist of three distinct membrane-skeletal domains, only one of which is enriched in AChR (Pumplin, 1989). A characteristic network of filaments is closely applied to the cytoplasmic surface of this AChR domain, while longer actin filaments overlie and are attached to this network (Figs. 9 and 10; Pumplin, 1989). Immunogold cytochemistry with antibodies against cytoplasmic epitopes of the AChR results in labeling of this domain only (Pumplin, D. W., unpublished results). mAb 1351 also labels this domain preferentially (Figs. 9 and 10). Labeling with mAb 1351 followed by secondary and tertiary antibodies of inappropriate species specificity, or labeling with appropriate secondary and tertiary antibodies but without preincubation with 1351, gave replicas with few or no gold particles (not shown). The images show at the ultrastructural level that the 58-kD protein is concentrated at AChR-rich membrane domains, and is present in the network of filaments on the cytoplasmic surface of these domains. We see little or no immunogold labeling of the overlying actin filaments. Occasionally, however, we detect some immunogold in areas (brackets in upper panels of Fig. 9) that are not apparently recognizable as AChR domains (Pumplin, D. W., manuscript in preparation), in agreement with our observations using immunofluorescence techniques (e.g., Figs. 2 and 3).

Discussion

We have used a morphological approach to study the organization of AChR clusters. This approach has been facilitated by the fact that AChR clusters made by primary cultures of rat myotubes form at sites of myotube-substrate attachment, and can be isolated intact by shearing the cultures or by extracting them with saponin (Bloch and Geiger, 1980; Bloch,

1984; Bloch et al., 1989; Pumplin, 1989). These treatments leave the clusters essentially free of all cytoplasmic constituents, and render the proteins on their intracellular surface accessible to antibodies. We use these isolated cluster preparations for several purposes. The absence of cytoplasmic background makes it easy to determine, at the fluorescence and ultrastructural levels, if a particular protein codistributes with AChR in cluster membrane. The availability of monoclonal antibodies to several components of AChR clusters allows us to use semiquantitative fluorescence techniques to estimate how much of each is present. The use of different extraction protocols reveals the relative tightness of binding of these proteins to the cluster membrane. Finally, the fact that cluster components redistribute together when clusters are disrupted suggests that they are somehow linked to each other in a stable fashion. Using this approach, we have shown that AChRs are associated with at least three peripheral membrane proteins on the cytoplasmic face of AChR clusters: actin, β -spectrin, and the receptor-associated 43-kD protein (Bloch, 1986; Bloch and Morrow, 1989; Bloch and Froehner, 1987). We confirmed the close association of AChR with the 43-kD protein and showed that these two proteins are present in approximately equal amounts in cluster membrane (Bloch and Froehner, 1987). Actin and β -spectrin, in contrast, are less closely associated with clustered AChR (Bloch, 1986; Bloch and Morrow, 1989) and β -spectrin, at least, is probably present at clusters in significantly higher amounts than either the 43-kD protein or the AChR itself (Bloch and Morrow, 1989). The weakness of our approach is that it does not reveal if these proteins bind to each other, or if they interact indirectly, for example, through other protein components of AChR clusters.

We have applied our morphological approach to the study of the M_r 58,000 protein. A homologue of this protein, which was first identified in extracts of *Torpedo* postsynaptic membrane, is present postsynaptically at the neuromuscular and myotendinous junctions of vertebrates and at AChR aggregates that form in vitro (Froehner et al., 1987; Carr et al., 1989; Chen et al., 1990; Daniels, 1990; Daniels et al., 1990). Using the AChR clusters of cultured rat myotubes, we confirm that the 58-kD protein is present, and that its distribution at clusters resembles that of the AChR. Little is known about the closeness of association of the 58-kD protein with clustered AChR. At microaggregates of AChR induced by antibodies (Bloch, R. J., R. Sealock, P. W. Luther, D. W. Pumplin, and S. C. Froehner, manuscript in preparation), or at aggregates of AChR induced to form by extracts from embryonic brain (Daniels, et al., 1990), the 58-kD protein is as likely to be associated with AChR as is the 43-kD protein but, as in adult muscle, the 58-kD protein is also present in regions lacking AChR clusters. Thus, the distribution of the 58-kD protein is similar to that of β -spectrin, which, although enriched at AChR clusters, is present in other regions of rat muscle cells (Bloch and Morrow, 1989).

Despite this similarity, our results suggest that the association of the 58-kD protein with AChR is distinct from that of other peripheral membrane proteins identified at AChR clusters. (a) Semiquantitative immunofluorescence measurements indicate that cluster membrane binds the same amount of anti-58-kD mAb as it binds mAbs to the 43-kD protein and AChR. We have demonstrated here (Fig. 5) that mAb 1351 and anti-AChR mAb 88B bind equally well to

FGAM. If we assume the same for anti-43K mAb, and if we assume that all mAbs bind to cluster membrane with the same valency, our results suggest that clusters contain approximately equal amounts of AChR and the 43- and 58-kD proteins. In contrast, β -spectrin is present in amounts several fold higher (Bloch and Morrow, 1989). (b) Selective extractions distinguish the 58-kD protein from other known proteins at clusters. Like β -spectrin and unlike the 43-kD protein, the 58-kD protein is stably associated with clusters extracted at low ionic strength, but is removed quantitatively by brief treatment with chymotrypsin (Table II). However, some 58-kD protein remains associated with cluster membrane after extraction at alkaline pH, which completely extracts both β -spectrin and the 43-kD protein. Also, the 58-kD protein appears to dissociate more slowly from isolated clusters incubated in buffered saline than does β -spectrin (compare Fig. 7 with Fig. 5 of Bloch and Morrow, 1989). (c) Microaggregation experiments suggest that the 58-kD protein is closely linked to AChR (Fig. 8). Thus, the 58-kD protein may occupy a unique position in AChR clusters, between the 43-kD protein and AChR, on the one hand, and β -spectrin on the other.

Our ultrastructural observations support these results. They show that the 58-kD protein lies close to the inner membrane surface and is part of a network of membrane-associated filaments that are immunolabeled by antibodies to β -spectrin (Pumplin, D. W., J. C. Strong, J. C. Krikoran, G. A. Porter, and J. C. Winkelman. 1990. *J. Cell Biol.* 111 [No. 5, Pt. 2]:165a). Thus, the 58-kD protein is appropriately placed to mediate interactions between AChR in the membrane and a cytoskeleton containing spectrin.

The specific association of the 58-kD protein with AChR and receptor-associated proteins that we have seen differs from a recent report that a homologue of this protein in *Xenopus* myocytes codistributes with talin, implying that it is a component of focal contacts (Chen et al., 1990). Our results suggest that the 58-kD protein is absent from focal contacts. The homologue of the 58-kD protein present in *Xenopus* tissue differs in molecular mass from that in *Torpedo* and in mammalian muscle by 10 kD (Chen et al., 1990). This difference may be accompanied by a change in ligand specificity.

A recent report suggests that the 58-kD protein can associate with a form of dystrophin (Butler, M. H., A. A. Murnane, and S. C. Froehner. 1990. *Soc. Neurosci. Abst.* 16:1004). It seems possible to us that, within AChR clusters, the 58-kD protein can interact either with spectrin or with dystrophin, which is present in AChR clusters in small amounts (Dmytrenko, G. M., D. W. Pumplin, and R. J. Bloch, manuscript in preparation). It could thus form a link between AChR and its associated 43-kD protein, and a membrane skeleton, composed in part of spectrin, dystrophin, and actin. We are currently pursuing experiments to test this possibility.

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