Localization of Dystrophin Relative to Acetylcholine Receptor Domains in Electric Tissue and Adult and Cultured Skeletal Muscle

R. Sealock,* M. H. Butler,‡ N. R. Kramarcy,* K.-X. Gao,* A. A. Murnane,‡ K. Douville,‡ and S. C. Froehner‡

*Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27599; ‡Department of Biochemistry and Program in Molecular and Cellular Neurosciences, Dartmouth Medical School, Hanover, New Hampshire 03756

Abstract. Two high-affinity mAbs were prepared against *Torpedo* dystrophin, an electric organ protein that is closely similar to human dystrophin, the gene product of the Duchenne muscular dystrophy locus. The antibodies were used to localize dystrophin relative to acetylcholine receptors (AChR) in electric organ and in skeletal muscle, and to show identity between *Torpedo* dystrophin and the previously described 270/300-kD *Torpedo* postsynaptic protein. Dystrophin was found in both AChR-rich and AChR-poor regions of the innervated face of the electroplaque. Immunogold experiments showed that AChR and dystrophin were closely intermingled in the AChR domains. In contrast, dystrophin appeared to be absent from many or all AChR-rich domains of the rat neuromuscular junction and of AChR clusters in cultured muscle (*Xenopus laevis*). It was present, however, in the immediately surrounding membrane (deep regions of the junctional folds, membrane domains interdigitating with and surrounding AChR domains within clusters). These results suggest that dystrophin may have a role in organization of AChR in electric tissue. Dystrophin is not, however, an obligatory component of AChR domains in muscle and, at the neuromuscular junction, its roles may be more related to organization of the junctional folds.

DUCHENNE and Becker muscular dystrophies are diseases of muscle necrosis and wasting that arise from qualitative and/or quantitative defects in dystrophin, a cytoplasmic protein associated with all regions of the sarcolemma in skeletal, cardiac, and smooth muscle (reviewed in Hoffman and Kunkel, 1989; Witkowski, 1989). Dystrophin was unknown before its discovery by genetic methods, and its cell biological activities have not yet been directly demonstrated. However, the deduced amino acid sequence shows that dystrophin is a member of the spectrin/alpha-actinin family of actin-binding, triple helix rod-containing proteins (Hammonds, 1987; Koenig et al., 1988). The skeletal muscle isoform, with a predicted molecular weight and length of 427,000 D and 150 nm (Koenig et al., 1988), has been isolated after detergent solubilization as a complex of several proteins including at least one integral membrane protein (Ervasti et al., 1990). The role of this protein complex is not yet known, but two hypotheses have been proposed. By analogy to the role of spectrin in the erythrocyte, dystrophin in normal muscle may act to stabilize the plasma membrane against contraction-induced mechanical damage (Mandel, 1989); mechanical damage is an early event in necrosis of Duchenne muscle cells (Rowland, 1980; Engel, 1986). Alternatively, dystrophin may be involved in regulation of Ca^{2+}-permeable leak or stretch-inactivated channels such as that absence of dystrophin entrains the high internal [Ca^{2+}] and poor calcium regulation characteristic of dystrophin-minus muscle (Mongini et al., 1988; Franco and Lansman, 1990; Fong et al., 1990).

In mammalian skeletal muscle, the neuromuscular junction appears to be a site at which dystrophin is particularly concentrated, since junctions were very brightly labeled by anti-dystrophin antibodies in several studies (e.g., Shimizu et al., 1989). In addition, Fardeau et al. (1990) found that antibodies against the NH_2-terminal, central rod, and COOH-terminal portions of dystrophin stained junctions in muscle from Duchenne patients and the mdx mouse, a dystrophin-minus mutant (Hoffman et al., 1987; Sicinski et al., 1989). Since the sarcolemmae were not stained (confirming the absence of bona fide dystrophin), the cross-reactivity must have been due to a distinct but dystrophin-like protein or proteins. Alpha-actinin and an unusual form of beta-spectrin have also been detected at the junction (Bloch and Hall, 1983; Block and Morrow, 1989). Thus, evidence exists for the presence at the junction of at least four members of the dystrophin/

K.-X. Gao's present address is Department of Animal Science, University of Tennessee, Knoxville, TN 37901. M. H. Butler's present address is Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510. A. A. Murnane's present address is Genentech, Inc., South San Francisco, CA 94080.

alpha-actinin/spectrin family. This suggests that there are particular requirements for proteins of this type in molecular organization of the cholinergic postsynaptic membrane. The nature of these requirements is in general unknown, particularly with respect to the mature junction. The beta-spectrin isoform has, however, been directly implicated in clustering of acetylcholine receptors (AChR)[1] in cultured, aneural muscle (Bloch and Morrow, 1989; Pumplin, 1989).

Dystrophin or a very similar protein is also a component of the postsynaptic membrane in electric tissue of electric rays (*Torpedo* sp.; Chang et al., 1989; Jasmin et al., 1990). Electric tissue is a highly simplified, noncontractile tissue that derives embryologically from striated muscle (Fox and Richardson, 1978); it is well known as a source of several proteins including AChR that have homologues in skeletal muscle. The discovery that it also contains dystrophin should be of great importance for biochemical studies, since highly purified postsynaptic membranes are readily prepared from electric tissue. In this study, we have used two high-affinity mAbs to localize dystrophin relative to domains of high AChR density in electric tissue, in mature rat skeletal muscle, and in cultured skeletal muscle from *Xenopus*. The aims were to identify possible roles of dystrophin in postsynaptic structure and molecular organization and to compare electric tissue to muscle with regard to these roles.

Materials and Methods

Materials

Electric rays (*Narcine brasiliensis*, *Torpedo californica*, and *T. nobiliana*) were obtained from Gulf Specimen Co. (Panacea, FL), Pacific BioMarine (Venice, CA), and Bio-Fish, Inc. (Georgetown, MA), respectively. Munich Wistar rats and *Xenopus laevis* were obtained from local breeding colonies. Control mice (C57BL/10SnJ) and mdx mice (C57BL/10ScSn-mdx) were purchased from Jackson Laboratories (Bar Harbor, ME). FITC-alpha-bungarotoxin and FITC-labeled, affinity-purified rabbit anti-bungarotoxin were prepared by conventional procedures (Anderson and Fambrough, 1983; Ravdin and Axelrod, 1977). Other fluorescent antibodies were Affini-Pure antibodies from Jackson Immunoresearch Laboratories (West Grove, PA). Rabbit anti-mouse IgG was prepared locally or was from Fisher Scientific Co. (Springfield, NJ). Goat anti-rabbit IgG complexed to 10 nm colloidal gold was obtained from Energy Beam Sciences (Agawam, MA). mAbs 1234A and 264E have been described previously (Froehner et al., 1983; Sealock et al., 1984; Peng and Froehner, 1985). Gelatin from the skin of cold water fish was from Sigma Chemical Co. (St. Louis, MO; catalog number G-7765).

Production and Characterization of mAbs

Torpedo AChR-enriched membranes and peripheral membrane proteins therefrom were prepared as previously described (Porter and Froehner, 1983). mAbs were IgG_1s purified from ascites fluids on protein A columns. Procedures for their preparation and characterization are described in detail in earlier publications (Froehner et al., 1983; Froehner, 1984).

Western Blotting

Transfer of proteins to nitrocellulose (0.45-μm; Bio-Rad Laboratories, Richmond, CA) was carried out in a cooled Transfor apparatus (Hoefer Scientific Instruments, San Francisco, CA). For *Torpedo* proteins, transfer was carried out in 114 mM glycine, 25 mM Tris, pH 8.3, for 16 h at an initial current of 300 mA or for 2 h at an initial current of 900 mA. In both cases, the current increased to 1,200–1,500 mA during the transfer period. For detection of dystrophin in mouse skeletal muscle, ~125 μg of microsomal membranes were run on a 7.5% acrylamide/0.14% bisacrylamide SDS gel. After electrophoresis, the proteins were transferred to nitrocellulose by electrophoresis in 75 mM glycine, 20 mM Tris, pH 8.4, at 70 V for 6 h in the cold. Other methods have been described in detail previously (Froehner et al., 1983; Morton and Froehner, 1989). Radioiodinated (Froehner et al., 1983) and alkaline phosphatase-conjugated second antibodies were used on blots of *Torpedo* proteins and skeletal muscle proteins, respectively.

Immunopurification of Torpedo Dystrophin

Torpedo nobiliana AChR-rich membranes prepared through the first sucrose gradient in the procedure of Porter and Froehner (1983) were suspended to 2 mg/ml in buffer A (10 mM phosphate, pH 7.4, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 2.5 μg/ml PMSF, 5 mM iodoacetate). Triton X-100 was added to a final concentration of 1%. After 20 min on ice and centrifugation for 30 min at 30,000 *g*, the supernatant was diluted 1:1 with buffer A, then incubated (2°C, 1 h) on a rotating wheel with 2 ml Affigel-10 derivatized with mab 1808 IgG (1 mg IgG/ml of Affigel). The gel was then poured into a column and washed successively with 15 ml buffer A containing 1% Triton, 15 ml buffer A containing 1% Triton and 1 M NaCl, and 15 ml buffer A. Bound protein was eluted with 0.1 M NH_4-acetate, pH 11.5. Fractions (1 ml) were collected into tubes containing 5 μl 1 M DTT and 100 microliters 10 M NH_4-acetate, pH 6.0 to neutralize the samples. Proteins in these fractions were precipitated with TCA before SDS gel electrophoresis.

Competition ELISA Assays

Methods for preparation of biotinylated mAbs and their use in competition assays have been described (LaRochelle and Froehner, 1986).

Cultured Muscle

Cells from the myotomal muscle masses of stage 20–22 *Xenopus laevis* embryos were cultured according to Peng and Nakajima (1978) but on coverslips treated with Alscian Blue (10 mg/ml for 10 min). The cells were grown at 10°C (Anderson, 1986).

Immunoelectron Microscopy

Anesthetized electric rays (Narcine) were fixed by perfusion (Froehner et al., 1987). Fragments of the fixed tissue were prepared and labeled as described previously (Wray and Sealock, 1984; Froehner et al., 1987) except that mAbs and rabbit anti-mouse IgG were followed by 10 nm colloidal gold complexed to goat anti-rabbit IgG. The labeling buffer was Ringer containing 0.8% BSA-1.0% fish gelatin or 0.8% BSA-5% normal goat serum-0.1% fish gelatin.

Open membrane fragments were prepared from frozen electric tissue (*T. californica*) by homogenization in the presence of zinc ion as described previously (Sealock et al., 1984). They were labeled by the microculture well method (Wray and Sealock, 1984) using the immunogold method described above plus alpha-bungarotoxin and affinity-purified rabbit anti-bungarotoxin in double-label experiments, with PBS-0.8% BSA-1.0% fish gelatin as buffer.

All mAbs were used over a concentration range (15–1,000 nM). To control for nonspecific binding, mAbs were replaced by commercial pooled mouse IgG.

Immunofluorescence

Unfixed mouse diaphragms and fixed electric tissue (see above) were prepared for cryostat sections (6 μm) and subjected to double-label immunofluorescence by standard methods. Control experiments established that cross-reactivity among antibodies used was negligible. Sections of electric tissue (Fig. 5, *A*, *A'*) were photographed with Kodak Technical Pan developed in Kodak HC110.

Muscle cell cultures were labeled with FITC-alpha-bungarotoxin, then fixed in ice-cold ethanol. They were then labeled by standard methods with mAb 1958 followed by rhodamine-conjugated goat anti-mouse IgG plus FITC-bungarotoxin, with PBS containing 0.8% BSA and 1% fish gelatin as buffer.

High resolution immunofluorescent localization of mAb binding sites in muscle tissue was done on the flexor digitorum longus of 4–6-wk-old Munich Wistar rats. Dissected muscles were fixed in 1% paraformaldehyde in PBS. During fixation (30–45 min), small bundles of muscle fibers joined

1. *Abbreviation used in this paper*: AChR, acetylcholine receptor.

at one end to their tendon were prepared. Penetration of reagents to the interior of bundles was encouraged by longitudinal cuts through the endplate zones. Even with this precaution, labeling intensity in the interior was always considerably lower than on the bundle surfaces. Groups of bundles were permeabilized with 0.5% Triton X-100 and treated with 0.2 M NH_4Cl in PBS.

For labeling, the bundles were incubated with FITC-alpha-bungarotoxin and test antibody (mAb 1808, mAb 1958, mAb 1234, or pooled mouse IgG) followed by combined FITC-conjugated rabbit anti-toxin and rhodamine-conjugated goat anti-mouse IgG. After a final wash, the labeled samples were fixed in 4% paraformaldehyde and wafer embedded in Epon 812 between sheets of Aclar film (Kingsley and Cole, 1988). Endplates zones (located in the polymerized wafers by fluorescence using a 10× lens) were glued to a blank block for sectioning parallel to the surface of the wafer. Sections were 0.2–0.4 μm thick, the smallest value practicable given the intensity of fluorescein labeling achieved in this procedure. To provide access to the fluorophores for anti-photobleaching agents (essential for photography of such thin sections), the sections were put on slides, treated with Na-methoxide/toluene to remove the Epon (Mayor et al., 1961), passed into water, and mounted in glycerol containing *N*-propyl gallate (Giloh and Sedat, 1982) without being allowed to dry. They were photographed with a 63×, NA 1.3 Planapo lens (Zeiss) on Kodak T-Max film developed in Diafine (exposure index ~3,200). Exposure times were typically 10–30 s for rhodamine and 60–90 s for fluorescein.

Results

Anti-dystrophin mAbs

mAbs 1808 and 1958 were developed from mice immunized with peripheral membrane proteins extracted from *Torpedo* postsynaptic membranes (see Materials and Methods). On Western blot analysis of the intact membranes (seen in Coomassie blue staining in Fig. 1 *A*), both mAbs recognized a single protein having a relative molecular mass of ~300,000 (Fig. 1, *B* and *C*). The mAbs are directed against distinct epitopes, since they did not compete for binding sites in solid phase competition assays (LaRochelle and Froehner, 1986; data not shown). A protein at M_r ~300,000 was also recognized by two sheep antisera against distinct fusion proteins of mouse dystrophin (Hoffman et al., 1987). One of these antisera (anti-60 kD, Fig. 1 *E*) gave a much stronger reaction than the other (anti-30 kD, Fig. 1 *F*), but both appeared to be specific since normal sheep antiserum showed no reactivity (Fig. 1 *G*). The mAbs and the anti-60-kD antiserum also stained Western blots of the material immunopurified from detergent-solubilized membranes using mAb 1808 (Fig. 2, *A*, *B*, *C*, and *E*). All three of these antibodies therefore recognize the same *Torpedo* protein.

Both mAbs also recognize mammalian skeletal muscle dystrophin. Dystrophin was located on Western blots of microsomal membranes from mouse muscle by staining with the anti-60-kD antiserum (Fig. 3 *A*, lane *2*). It consistently ran slightly faster than the *Torpedo* antigen (Fig. 3 *A*, lane *1*). No specific staining by the antiserum was obtained with muscle from mdx mice, a dystrophin-minus mutant (Hoffman et al., 1987; Sicinski et al., 1989). The same results were obtained when the blots were probed with mAbs 1808 and 1958 (Fig. 3 *B*, lanes *1–3*). When applied to cryostat sections of normal mouse diaphragm, each mAb gave strong immunofluorescence staining of the entire sarcolemma, with particularly strong staining of neuromuscular junctions (identified with fluorescent alpha-bungarotoxin, Fig. 4, *E* and *H*). Neither antibody stained sections of mdx mouse muscle (Fig. 4, *F* and *I*). These are the expected results for anti-dystrophin antibodies, confirmed by staining with the anti-60-kD antiserum (Fig. 4, *A–C*). The mAbs similarly labeled normal human muscle, but not Duchenne muscle (data not shown). They also gave strong, exclusively sarcolemmal, labeling of skeletal muscle from other species (rat, *Xenopus laevis*, and chicken), of cardiac muscle (rat and normal, but not mdx, mouse), and of smooth muscle (chicken gizzard) (data not shown). These results demonstrate that mAbs 1808 and 1958 recognize muscle dystrophin, and reinforce the conclusion (Chang et al., 1989) that electric tissue contains a form of dystrophin or a closely related protein. In agreement with Chang et al., we call this protein *Torpedo* dystrophin.

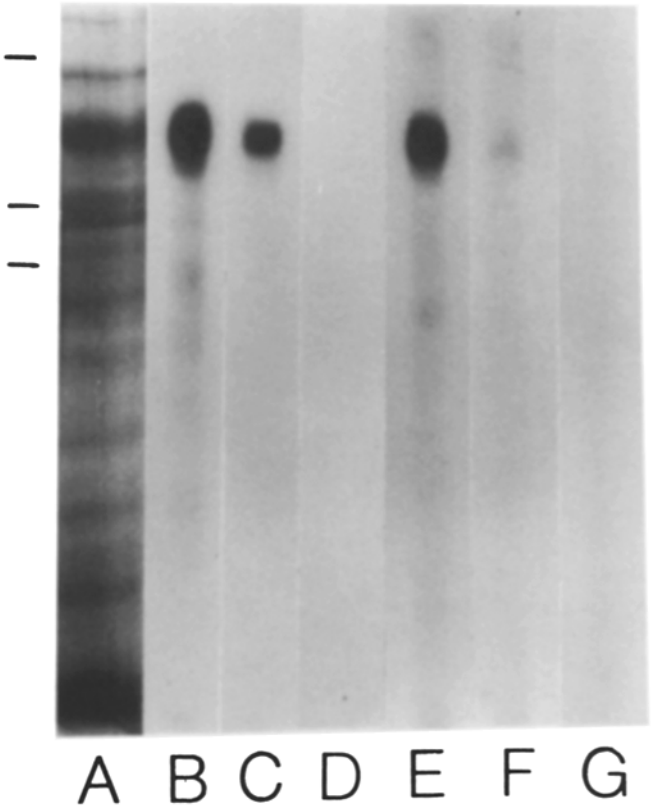

Figure 1. Western blot analysis of AChR-rich membranes. Proteins of electric organ (*T. nobiliana*) postsynaptic membranes were separated by SDS gel electrophoresis and (*A*) stained with Coomassie blue or transferred to nitrocellulose and incubated with (*B*) 50 nM 1808 IgG, (*C*) 50 nM 1958 IgG, (*D*) 130 nM control mouse IgG, (*E*) anti-60-kD dystrophin serum (diluted 1:500), (*F*) anti-30-kD dystrophin serum (1:500), and (*G*) control sheep IgG (20 μg/ml). The markers on the right indicate (from the bottom) the position of human erythrocyte beta-spectrin (known molecular weight, 246 kD; Winkelmann et al., 1990), alpha-spectrin (280 kD; Sahr et al., 1990), and alpha2-macroglobulin (358 kD; Sottrup-Jensen et al., 1984).

mAbs 1808 and 1958 appear to be highly specific for dystrophin. They did not stain the interiors of any muscle fibers, showing that they do not share cross-reactivities (with the C-protein and an isoform of alpha-actinin) that have been identified with other anti-dystrophin antibodies (Yamaguchi et al., 1990; Hoffman et al., 1989). The failure to label Duchenne muscle establishes that they do not cross-react detectably with human muscle spectrin, which has a normal sarcolemmal distribution in Duchenne muscle (Appleyard et al., 1984). They also do not recognize *Torpedo* spectrin,

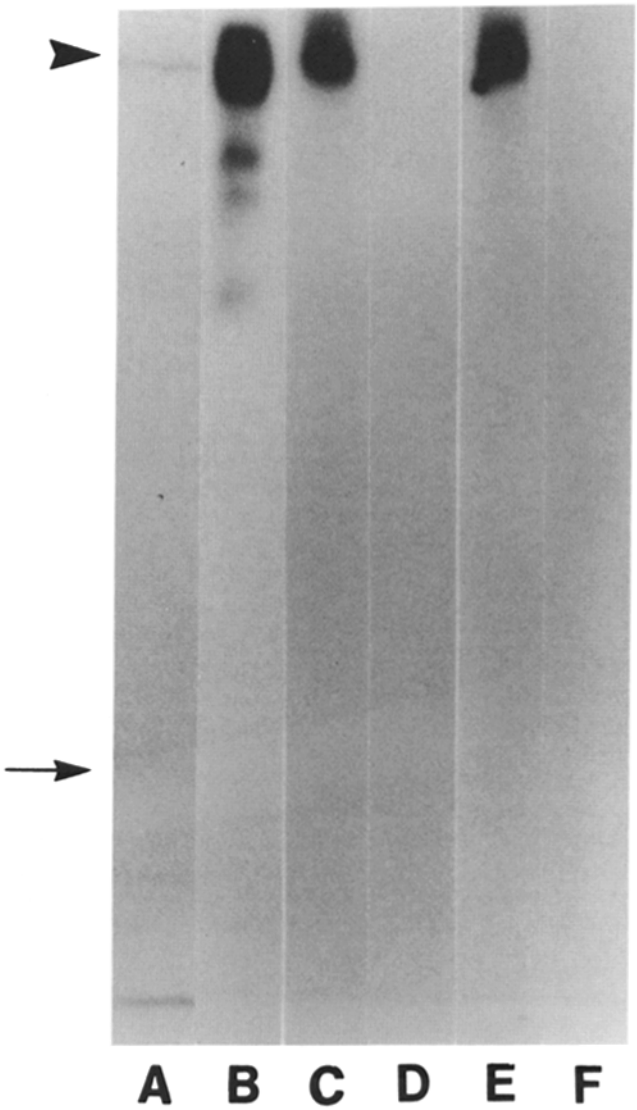

Figure 2. Western blot analysis of affinity-purified *Torpedo* dystrophin. Dystrophin was purified on a mAb 1808 affinity column and analyzed by SDS gel electrophoresis. (*A*) Coomassie blue staining. The arrowhead indicates the M_r 300,000 protein and the arrow points to a protein of ~60-kD present in most preparations. The same sample was subjected to Western blot analysis with (*B*) 50 nM 1808 IgG, (*C*) 50 nM 1958 IgG, (*D*) 130 nM control mouse IgG, (*E*) anti-60-kD dystrophin serum (1:500) and (*F*) control sheep IgG (20 μg/ml).

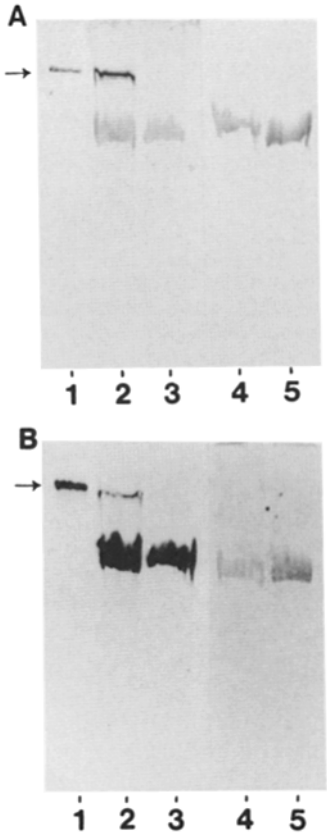

Figure 3. Western blot analysis of normal and mdx mouse muscle. Samples in *A* and *B* were electric organ postsynaptic membranes (lane *1*) and microsomal membranes from skeletal muscle of normal mice (lanes *2* and *4*) or mdx mice (lanes *3* and *5*). Blots in *A* were probed with anti-60-kD dystrophin serum 1:500 (lanes *1–3*) or control sheep serum 20 μg/ml (lanes *4* and *5*). In *B*, a mixture of mAbs 1808 and 1958 (50 nM each) (lanes *1–3*) or control mouse IgG (100 nM) (lanes *4* and *5*). The nonspecifically stained band seen in all muscle samples is probably myosin.

since they do not label the noninnervated face of the electroplaque (see below). Finally, the failure to label the neuromuscular junction in mdx mice establishes that they do not cross-react with the dystrophin-like protein identified by Fardeau et al. (1990) or the chromosome 6-encoded, dystrophin-related protein (Buckle et al., 1990; Khurana et al., 1990), which appears to be selectively concentrated at the junction (Froehner, S. C., and R. Sealock, unpublished results).

Woodruff et al. (1987) and Carr et al. (1989) have described antibodies against *Torpedo* proteins at M_r 300,000 and 270,000, respectively. These proteins are immunologically cross-reactive, and are believed to be the same protein (Carr et al., 1989). Antibodies against these proteins (kindly furnished by S. Burden [Massachusetts Institute of Technology] and J. B. Cohen [Washington University]) also recognized immunoaffinity purified *Torpedo* dystrophin on Western blots (data not shown). The 270/300-kD protein is therefore Torpedo dystrophin.

Localization of Dystrophin in Torpedo Postsynaptic Membranes

Torpedo AChR is not homogeneously distributed within the electrogenic cell (electroplaque). It is present on the innervated (postsynaptic) face of the cell, but absent from the noninnervated face (see Sealock and Kavookjian, 1980). It is most densely concentrated in the postsynaptic membrane closest to nerve terminals (juxtaneural regions), and less densely concentrated in the deep regions of the characteristic postsynaptic invaginations. Within juxtaneural regions, AChR is frequently distributed in distinct rows (Heuser and Salpeter, 1979; Hirokawa, 1983). Identification of any role dystrophin may have in AChR organization thus requires localization of dystrophin relative to AChR at several different levels.

We localized dystrophin at the tissue level by double-label immunofluorescence of cryostat sections from perfusion-fixed electric tissue (*N. brasiliensis*). mAbs 1808 and 1958 labeled only the innervated face (Fig. 5 *A'*). A similar result was obtained by Jasmin et al. (1990) using polyclonal antibodies. These results together suggest that the labeling of both faces by Chang et al. (1989) resulted from a cross-reactivity not revealed by Western blotting. Both juxtaneural regions and the postsynaptic invaginations were clearly visible in our images (*arrowheads* in Fig. 5 *A'*). In contrast,

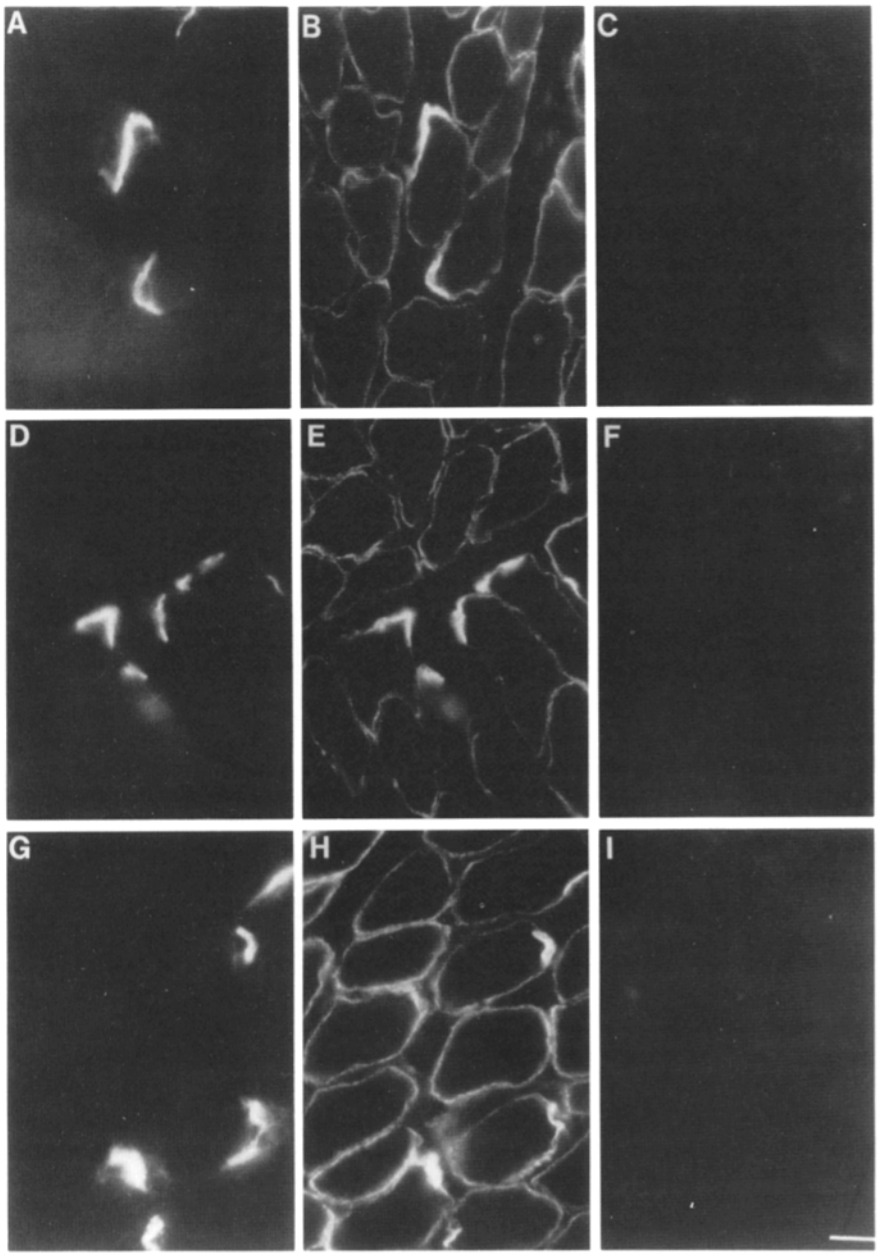

Figure 4. Immunofluorescence staining of control and mdx mouse diaphragm. Cryostat sections of normal mouse diaphragm (*A*, *B*, *D*, *E*, *G*, and *H*) and mdx mouse diaphragm (*C*, *F*, and *I*) were labeled with anti-60-kD dystrophin antiserum 1:500 (*B* and *C*), mAb 1808, 50 nM (*E* and *F*) and mAb 1958, 50 nM (*H* and *I*). Sections from normal muscle were double-labeled with rhodamine-conjugated alpha-bungarotoxin (*A*, *D*, and *G*) to identify AChR. All antibodies gave very similar labeling of normal muscle, but no labeling of mdx muscle. Bar (*I*) 20 μm.

strong labeling of the same sections by alpha-bungarotoxin and anti-toxin antibodies was confined to the juxtaneural regions and the superficial portions of the invaginations (Fig. 5 *A*). This agrees with previous visualizations of bungarotoxin binding in the electroplaque (Sealock and Kavookjian, 1980). In these experiments, we confirmed that this correctly represents the AChR distribution, since an mAb against a cytoplasmic epitope of AChR (mAb 264; Froehner et al., 1983) gave an image indistinguishable from that given by toxin/anti-toxin (data not shown). On the basis of these data, dystrophin appears to be more homogeneously distributed over the innervated face than is AChR.

At the ultrastructural level (immunogold experiments), mAbs 1808 and 1958 labeled only the cytoplasmic surface of the postsynaptic membrane (Fig. 5 *B*). In agreement with the fluorescence data, background levels of gold particles were found on the noninnervated face (not shown), in cytoplasm, and on all structures outside the electroplaque (see Fig. 5 *B*). On the postsynaptic membrane, gold particles occurred in clumps (*arrows* in Fig. 5 *B*) or short continuous stretches (*arrowheads*). This establishes that juxtaneural dystrophin is closely intermingled with AChR, since juxtaneural regions that do not contain AChR at high density are extremely rare (Sealock, 1980). Clusters on the sides of invaginations in the deep regions often seemed paired (Fig. 5 *B*, *arrows*), a feature previously noted in labeling of the 58-kD postsynaptic protein (Froehner et al., 1987). We found little indication of regular patterns of clusters like those reported in skeletal muscle (Watkins et al., 1988; Cullen et al., 1990).

Localization of dystrophin relative to AChR microdomains such as rows of AChR is best done by double-labeling using an extracellular label on AChR. However, the overlying basal lamina in the intact tissue impedes free access of

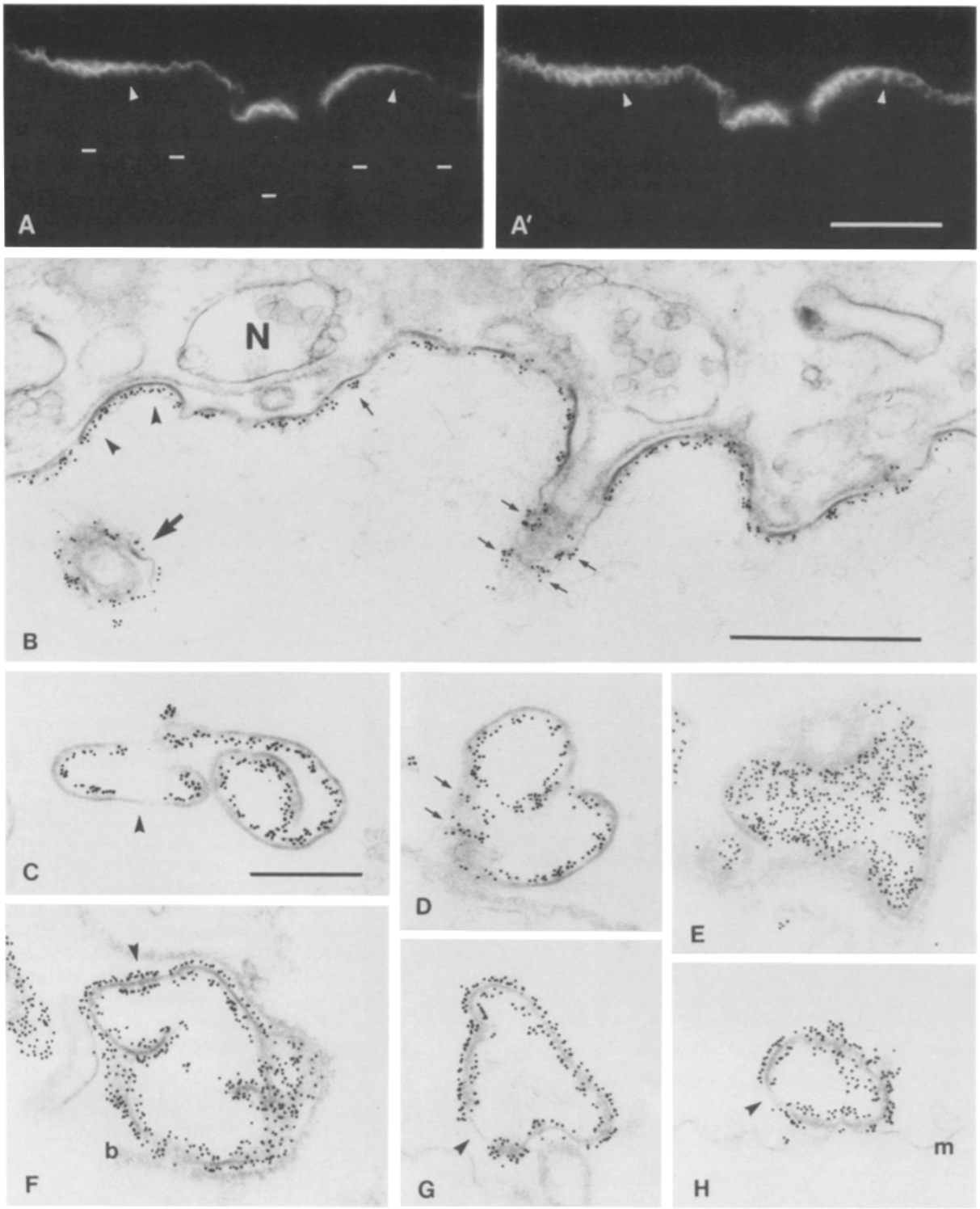

Figure 5. Localization of dystrophin on Torpedo postsynaptic membranes. (*A* and *A'*) Cryostat sections of perfusion-fixed electric tissue (*N. brasiliensis*) were double-labeled for immunofluorescence with bungarotoxin/anti-toxin antibody (*A*) and mAb 1808 (*A'*). Only the innervated face is labeled. Invaginations are evident in *A'* but not at the same positions in *A* (*arrowheads*). The noninnervated face is outlined by the white dashes in *A*. (*B*) Immunogold electron microscopy on fixed electric tissue (*N. brasiliensis*) with mAb 1958. Gold particles on the postsynaptic membrane occurred in clusters (*small arrows*) or short continuous stretches (*arrowheads*). A transversely sectioned invagination (*large arrow*) and a nerve terminal (*N*) are indicated. (*C–H*) Postsynaptic membrane fragments from unfixed tissue (*T. californica*). (*C–E*) mAb 1808 alone. Substructure in the labeling pattern was sometimes apparent (*D, arrows*), but more homogeneous labeling was the rule, even when fragments were viewed en face (*E*). The morphology of the membrane in the gap in *C* (*arrowhead*) strongly suggests a domain free of AChR (criteria in Sealock, 1980). (*F–H*) mAb 1808 plus bungarotoxin/anti-toxin antibody. The two labels were often precisely coextensive (*arrowheads*). *b*, basal lamina; *m*, bottom of the microculture well used for the labeling reaction. Bars; (*A'*) 10 μm; (*B*) 1 μm; (*C*) 0.5 μm.

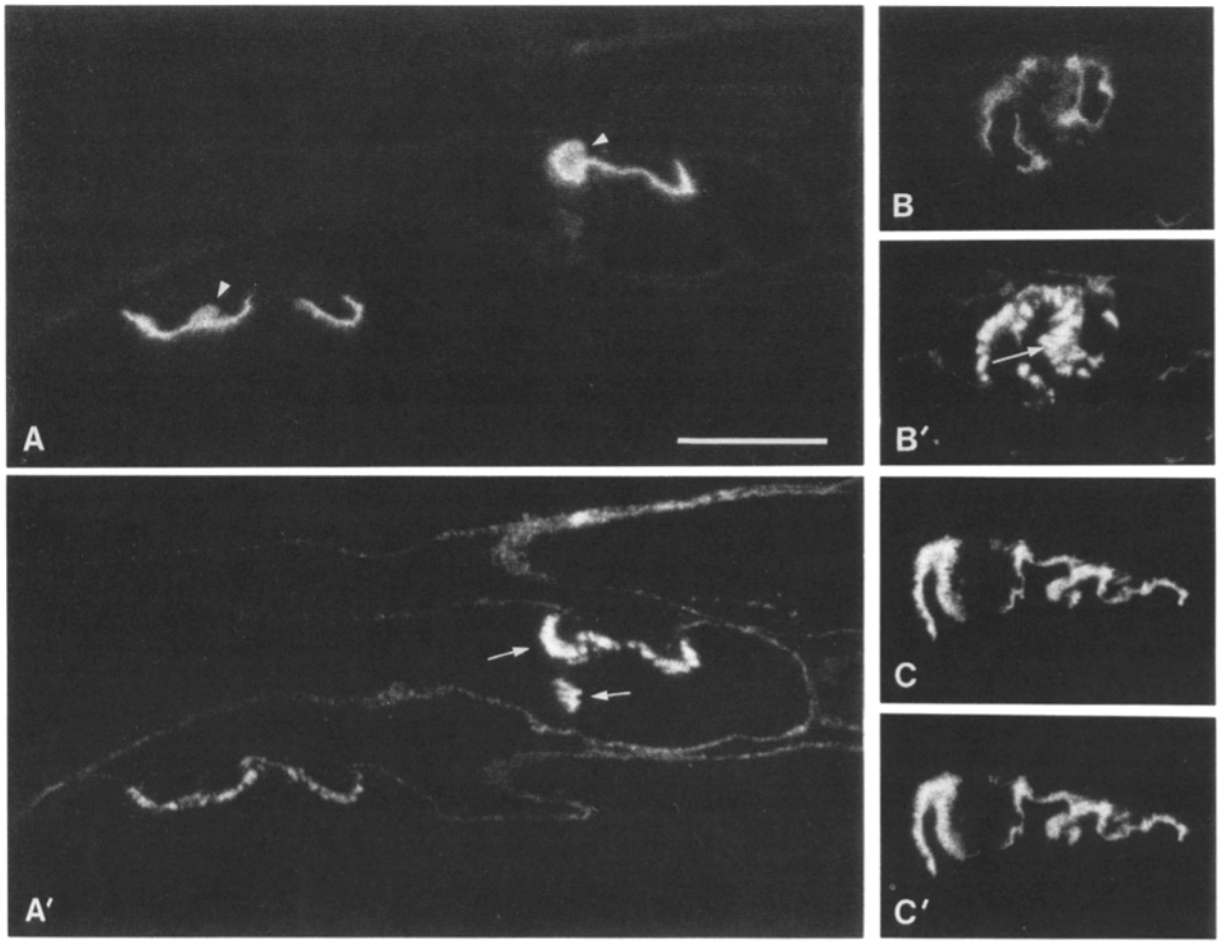

Figure 6. Rat neuromuscular junction. Muscle pieces were double-labeled for immunofluorescence with bungarotoxin/anti-toxin antibody (*A–C*) plus mAb 1808 (*A'* and *B'*) or a mAb (mAb 1234) against the 43-kD protein (*C'*), then embedded in Epon and sectioned (0.2–0.4 μm) for microscopy. Zones labeled by mAb 1808 but not bungarotoxin (*arrows* in *A'*, *B'*) correspond to the junctional folds. Zones labeled by toxin/anti-toxin but not mAb 1808 (*arrowheads* in *A*, smaller zones at various places in *A* and *B*) correspond to the AChR-rich crests of the junctional folds. In contrast, images given by toxin/anti-toxin and mAb 1234 were essentially identical (*C* and *C'*). Bar, 10 μm.

conventional gold particles to the membrane (Sealock et al., 1984). We therefore double-labeled membrane fragments which had been prepared from unfixed electric tissue in the presence of Zn^{2+} to prevent the formation of sealed vesicles (Sealock et al., 1984). The probes were mAb 1808 and alpha-bungarotoxin plus anti-toxin antibodies. Each probe alone labeled only the expected membrane surface, e.g., the intracellular labeling by mAb 1808 shown in Fig. 5, *C* and *D*. The intensity of labeling by mAb 1808 was higher with these fragments than with tissue, presumably reflecting improved access of antibody to dystrophin. Substructure in the labeling pattern could be found (*arrows* in Fig. 5 *D*), but so could zones that were nearly homogeneously labeled even when viewed en face (Fig. 5 *E*). When the membranes were double-labeled, AChR domains were dystrophin-positive (Fig. 5, *F–H*), and domains in which the two labels were coextensive were common (*arrowheads*). These results further establish that dystrophin is intimately intermingled with AChR in the electroplaque postsynaptic membrane, apparently at the level of AChR microdomains.

The labeling intensities given by mAb 1808 and by toxin/anti-toxin were comparable in the experiments of Fig. 5, *C–H*). However, gel electrophoresis of purified postsynaptic membranes shows that dystrophin molecules are far less numerous than AChR; Woodruff et al. (1987) estimated one molecule of the 300-kD protein for every 10 molecules of AChR on this basis. The discrepancy is explained by our observation that with several probes for AChR (toxin/anti-toxin or various mAbs followed by protein A-gold or second antibody gold), gold particle density saturates at or below 2,000 particles/μm^2 for all probes (Sealock, R., unpublished results). This compares to 8,000–10,000 molecules of AChR/μm^2 (hence 16,000–20,000 toxin binding sites/μm^2) in regions of highest AChR density (Heuser and Salpeter, 1979; Bridgman et al., 1987). Because of this limitation in the immunogold method, our results are strictly qualitative.

Localization of Dystrophin in the Neuromuscular Junction

To determine whether dystrophin also occurs on AChR-rich domains at the neuromuscular junction, small pieces of fixed rat skeletal muscle were subjected to immunofluorescent labeling for dystrophin and AChR, embedded in Epon, sectioned at 0.2–0.4 μm thickness, and prepared for fluorescence microscopy (see Materials and Methods). The resulting images could be divided into three zones: (*a*) those that

were strongly labeled by mAb 1808 but not by the toxin and anti-toxin (Fig. 6, *A*, *A'*, *B*, *B'*). On the basis of the appearance of these zones in a large number of sections, they correspond to the deeper regions of the junctional folds, where AChR density is low (Fertuck and Salpeter, 1976). We have previously shown by direct means that the junctional folds can be reliably identified in fluorescent images of this sort (Froehner et al., 1987). (*b*) Smaller zones labeled by toxin/anti-toxin but not by anti-dystrophin. These correspond to the receptor-rich crests of the junctional folds, and were best seen in grazing sections (*arrowheads* in Fig. 6 *A*). (*c*) Zones that contain both probes, either because AChR and dystrophin were intermingled in these regions or because dystrophin and AChR-rich regions were superimposed along the optical axis of the microscope. Corresponding results were obtained with mAb 1958 and *Xenopus* skeletal muscle (data not shown). In contrast, when mAb 1808 was replaced with an mAb against the AChR-associated 43-kD protein (mAb 1234; Peng and Froehner, 1985), the two images were virtually identical down to the finest details, even in the most complicated images (Fig. 6, *C* and *C'*). From these results, dystrophin is most clearly a component of the junctional folds. It may also be present on some AChR-rich regions, but it is not an obligatory component of regions of highest AChR density.

Outside the neuromuscular junction, labeling by mAbs 1808 and 1958 was evenly distributed along the sarcolemma; no periodicity suggestive of a relationship to sarcomeric structures could be detected. No labeling of nerve terminals, axons, or other nonmuscle structures could be detected.

Localization of Dystrophin in Cultured Muscle

Aggregates or clusters of AChR develop on the substrate-attached surfaces of cultured muscle cells by processes believed to be similar to those involved in AChR accumulation at developing neuromuscular junctions (reviewed in Bloch and Pumplin, 1988). To identify the relationship between AChR and dystrophin during the clustering process, we labeled cultured myotomal muscle cells from *Xenopus* tadpoles using bungarotoxin and mAb 1958 (indirect immunofluorescence). At 2 d in culture, these mononucleated, nonfusing cells showed prominent AChR clusters (Fig. 7, *A–C*) similar to clusters described previously (Anderson et al., 1977). Anti-dystrophin staining was detectable in all muscle cells, but in highly variable patterns ranging from weak and diffuse (Fig. 7 *A'*) to somewhat organized (Fig. 7, *B'* and *C'*). Correlations between AChR and dystrophin staining were weak at best (Fig. 7, *C*, and *C'*).

In older cultures (10-d cultures are shown), all muscle cells stained strongly for dystrophin, generally in a rich and highly variable pattern of streaks and patches (Fig. 7, *D'–K'*). Similar patterns of streaks and patches have been observed in labeling of cultured human muscle cells with anti-dystrophin antibodies (Miranda et al., 1988). Virtually every AChR cluster (Fig. 7, *D–K*) was a site of prominent dystrophin staining, with the distribution of dystrophin often bearing an obvious general relationship to the cluster as a whole. Most cells also had dystrophin-positive sites that were not associated with clusters (not shown). On detailed examination at cluster sites, the dystrophin distribution generally appeared to surround or interdigitate with AChR-rich domains (the *arrows* in Fig. 7 indicate corresponding points in pairs of images). Interdigitation is illustrated in Fig. 7, *F* and *F'* and, at higher magnification, in *J* and *J'*. Fig. 7, *I* and *I'* shows a particularly good example of dystrophin surrounding an AChR cluster. Fig. 7, *G* and *G'* (shown at higher magnification in *K*, *K'*) and *H*, *H'* show intermediate situations.

Dystrophin could not be detected in any nonmuscle cell type present in these cultures (fibroblasts and melanocytes), as expected from the very low levels of dystrophin mRNA in fibroblasts (Chelly et al., 1988). Neurons were not present in sufficient quantity to have been examined.

Discussion

In this study, we have described two mAbs against distinct epitopes of *Torpedo* and muscle dystrophins and used them to localize dystrophin relative to AChR domains in electric tissue and skeletal muscle. We have also shown that Torpedo dystrophin is identical to the 270/300-kD protein (Woodruff et al., 1987; Carr et al., 1989), a fact that was originally obscured (Chang et al., 1989; Jasmin et al., 1990) by the low relative molecular mass of the latter compared with the value usually cited for muscle dystrophin (~400,000; known molecular weight, 427 kD). That value was determined by extrapolation from lower molecular weight standards (Hoffman et al., 1987). We have confirmed, however, that both dystrophins migrate on our gels as though their relative molecular masses were ~300,000 when compared to proteins of known, bracketing molecular weights. The possibility that the low value in our experiments is due to proteolytic degradation is very unlikely, since the Torpedo and mouse muscle proteins, which were prepared in the presence of a cocktail of protease inhibitors (see Materials and Methods), would have to have been degraded to the same extent. In addition, Torpedo dystrophin appears as a thin, flexible rod ~175 nm long after immunoaffinity purification and rotary shadowing (Wallace, N. R., and R. Sealock, unpublished results). This agrees well with the predicted shape and length (150 nm) of muscle dystrophin (Koenig et al., 1988). The deduced amino acid sequence of the COOH-terminal domain of the *Torpedo* protein also shows a high degree of homology to human skeletal muscle dystrophin (Yeadon et al., 1991). Thus, the identification of the *Torpedo* protein as an authentic, full-sized dystrophin is supported by a substantial body of evidence.

The discovery of postsynaptic dystrophin at cholinergic synapses (Shimizu et al., 1989; Jasmin et al., 1990) raised the possibility of a role for dystrophin in molecular organization of AChR, an incompletely understood process (reviewed in Bloch and Pumplin, 1988; Pumplin, 1989). The close intermingling of *Torpedo* dystrophin with AChR that we have found in electric tissue is consistent with such a role. However, the presence of the AChR-specific 43-kD protein in both muscle and electric tissue (reviewed in Mitra et al., 1989*a* and *b*) and the unequivocal demonstration that the 43-kD protein can cause AChR clustering (Froehner et al., 1990) make it very likely that AChR is organized by similar mechanisms in the two tissues. It was thus surprising to find that dystrophin is apparently absent in regions of highest AChR density in the neuromuscular junction, and absent or present in low concentrations and poorly organized at young AChR clusters in *Xenopus* muscle. Furthermore, dystrophin's occurrence in domains that surround and interdigitate with AChR domains in older clusters is shared with several pro-

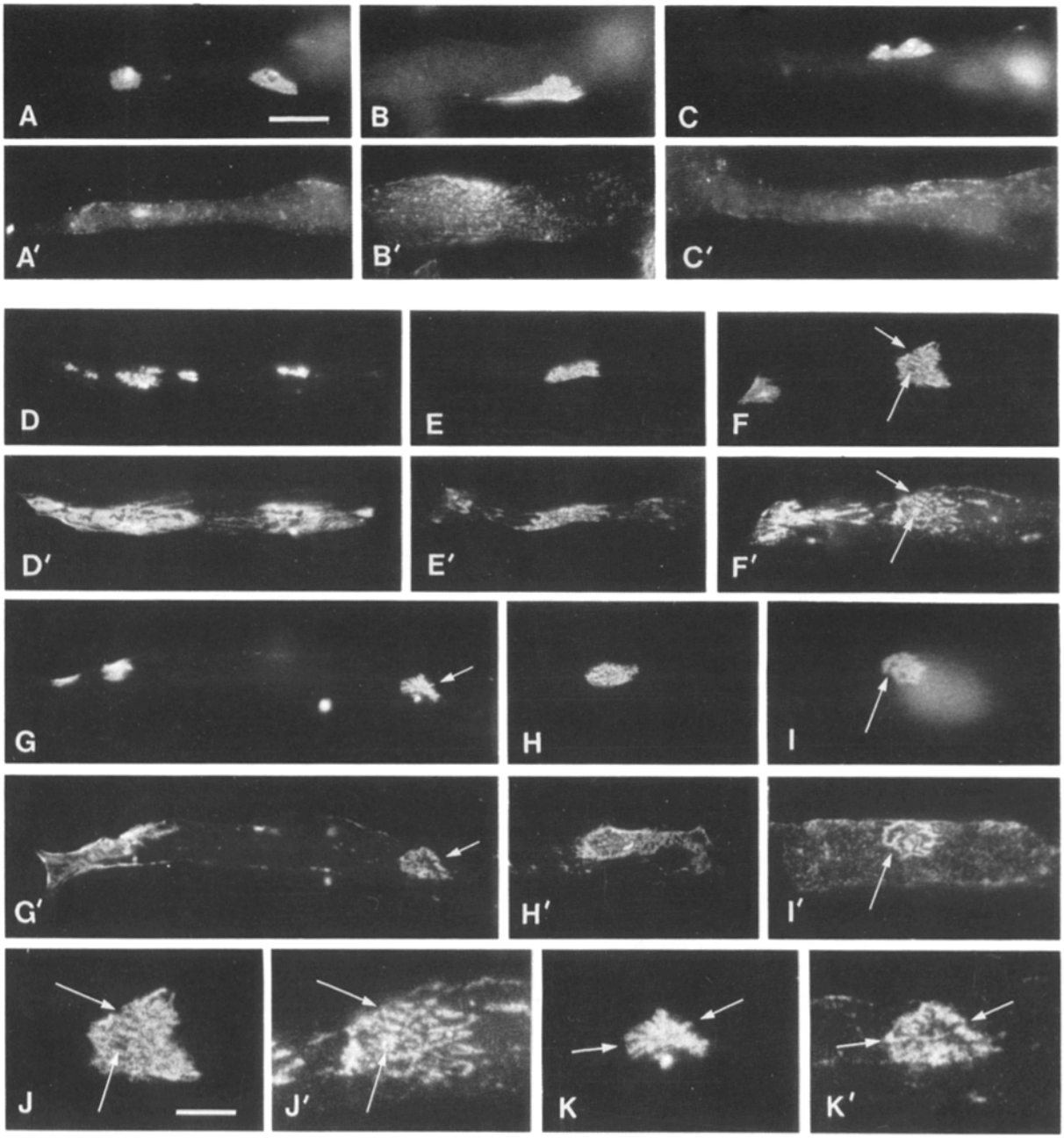

Figure 7. AChR clusters on cultured *Xenopus* muscle. Cultures were double-labeled for fluorescence with bungarotoxin (*unprimed letters*) and mAb 1958 (*primed letters*). In 2-d cultures (*A–C'*), dystrophin was often barely detectable (right hand cluster in *A* and *A'*) or organized with only a vague relationship to clusters (*B* and *B'*). When a relationship was evident, the dystrophin concentration was low (*C* and *C'*). In 10-d cultures (*D–I'*), the dystrophin distribution at clusters was typically broader than the AChR distribution, but usually had an obvious general relationship to it. Striations in the AChR cluster (*F* and *H*) were accompanied by striations in the dystrophin distribution (*F'* and *H'*), with AChR domains lying between dystrophin domains; *arrows* in *F* and *F'* indicate corresponding positions. Some AChR clusters were surrounded by dystrophin (*G–I'*), sometimes strikingly so (*I* and *I'*). Dystrophin patches also occurred on most cells in regions devoid of AChR clusters (not shown). *J,J'*: detail from *F,F'*. *K,K'*: detail from *G,G'*. Arrows indicate corresponding positions. Bars, *A* (10 μm) applies to *A–I'*; J (5 μm) applies to *J–K'*.

teins such as vinculin, which can be extracted from clusters with little alteration of the AChR distribution (Bloch, 1984). Cluster-associated proteins of this type thus appear to have at best an indirect role in AChR organization in muscle. This would be consistent with the normal miniature endplate potentials, AChR distribution and AChR content in junctions from Duchenne patients (Sakakibara et al., 1977).

Since dystrophin occurs in the junctional folds, a partial, trivial explanation for the particularly bright staining of the junction in low resolution samples such as cryostat sections is the increased membrane area in the folds. The important question of whether the density of dystrophin (molecules/squared microns) on the junctional fold membrane may also be increased relative to the extrajunctional sarcolemma has not been addressed directly. Interestingly, the junction also appeared substantially brighter than the surrounding sarco-

lemma in very thin sections (Fig. 6 *A'*). This is consistent with a higher density of dystrophin in the folds, but further interpretation would not be justified without quantitative measures of membrane area, the photographic intensities, and the dose–response characteristics of the film.

Two possible general explanations could account for the apparently different localizations of dystrophin relative to AChR in muscle and electric tissue. One would assume a similar role for postsynaptic dystrophin in the two tissues. In the electroplaque, distinct spaces can occur between rows of AChR (Heuser and Salpeter, 1979; Hirokawa, 1983). If dystrophin is confined to these spaces, its relationship to AChR would be analogous to that in AChR clusters in cultured muscle. Should mixing of dystrophin and AChR domains occur during preparation of membrane fragments (Fig. 5), the two proteins would appear intermingled. While it seems unlikely that such a mechanism would account for images like those in Fig. 5, *G–I*, it cannot be ruled out without further evidence. At the junction, on the other hand, the membrane proteins to which dystrophin is believed to bind (Ervasti et al., 1990) may be excluded from the close two-dimensional arrays of AChR at the crests of the junctional folds (Hirokawa and Heuser, 1982), while remaining in the deep parts of the folds.

Alternatively, dystrophin in the electroplaque could serve a role in AChR organization that is assumed by another protein in muscle. At clusters, AChR domains are overlaid by a unique isoform of beta-spectrin (Bloch and Morrow, 1989; Pumplin, 1989). Several lines of evidence suggest that this spectrin has a key role in AChR anchoring, possibly acting in concert with actin and the 43-kD protein. A corresponding protein has not been identified in the electroplaque; spectrin has thus far been found only on the noninnervated face (Kordeli et al., 1987). However, dystrophin is homologous to beta-spectrin (the actin-binding subunit of spectrin) (Byers et al., 1989; Hoffman and Kunkel, 1989). If a spectrin or spectrin-like protein is essential for AChR organization, dystrophin potentially has the activities and localization for assuming such a role in the electroplaque.

Exploration of the second explanation will require detailed localization of the long dystrophin molecule relative to the characteristic rows in which AChR is organized in electric tissue (Heuser and Salpeter, 1979; Hirokawa, 1983). Our results (Fig. 5) have established that the epitope recognized by mAb 1808 can lie in AChR domains. However, we do not yet know where on the dystrophin molecule that epitope occurs, and if dystrophin should lie partly in and partly outside AChR domains, other mabs could give quite different results. This uncertainty is unlikely to be resolved until epitopes along the full length of the dystrophin molecule can be localized relative to undisturbed AChR domains (rows) or until dystrophin can be directly visualized along with AChR on the membrane surface.

At AChR clusters in rat myotubes, AChR domains interdigitate with domains of closest contact between the muscle cell membrane and the culture substrate (Bloch and Pumplin, 1988). The dystrophin domains in the *Xenopus* cells (Fig. 7) correspond to these contact domains on the basis of their morphology and the fact that they contain talin, a focal adhesion protein (Kramarcy and Sealock, 1990). This agrees with our finding that dystrophin is most reliably concentrated in the junctional fold region of the mature junction (Fig. 6), since the contact domains at clusters are believed to be analogues of the folds (Bloch and Pumplin, 1988). A specific role for dystrophin in elaboration or maintenance of the folds could offer an explanation for the facts that in Duchenne muscle, the folds show focal degeneration with accumulation of excess basal lamina (Engel, 1986), and in the mdx mouse soleus muscle the folds of otherwise normal appearing junctions are on average 50% shorter than in normal muscle (Torres and Duchen, 1987).

Dystrophin is one of four cytoplasmic peripheral membrane proteins identified thus far in *Torpedo* postsynaptic membranes. Of these, only the 43-kD protein has proved to be specific to and associated with AChR. The remaining two proteins, a 58- or 51-kD protein (Froehner et al., 1987; Carr et al., 1989) and an 87-kD protein (Carr et al., 1989), have immunochemically detectable counterparts in muscle which are cytoplasmic sarcolemmal proteins. These proteins share with dystrophin the interesting property of occurring in patches on the surface of immature (cultured) skeletal muscle, but spreading to the entire sarcolemma in mature muscle. In addition, the 58-kD protein and dystrophin have very similar distributions on the electroplaque postsynaptic membrane in immunogold experiments (cf. Fig. 5 and Froehner et al., 1987). Like dystrophin, the muscle 58-kD protein is a prominent component of the myotendinous junction (Chen et al., 1990) and codistributes precisely with talin outside of AChR clusters (Kramarcy and Sealock, 1990). Antibodies against the 58-kD protein also label the sarcolemmae of rat cardiac muscle and chicken gizzard cells. These facts clearly suggest that these proteins could be part of a functional complex with dystrophin. In support of this hypothesis, we report elsewhere that the 58-kD protein copurifies with Torpedo dystrophin and that in the absence of dystrophin (i.e., in mdx mouse muscle) its association with the sarcolemma is greatly reduced (Butler, M. H., K. Douville, A. A. Murnane, R. Sealock, and S. C. Froehner. 1990. *J. Cell. Biol.* 111:165a).

We wish to thank H. B. Peng for assistance with the *Xenopus* cultures and S. J. Burden, J. B. Cohen, L. M. Kunkel, and E. P. Hoffman for generously providing antibodies.

Supported by National Institutes of Health grants NS15293 (R. Sealock) and NS14871 (S. C. Froehner) and by a grant from the Muscular Dystrophy Association (S. C. Froehner). S. C. Froehner is an Established Investigator of the American Heart Association and M. H. Butler is the recipient of a Muscular Dystrophy Association postdoctoral fellowship.

Received for publication 29 November 1990 and in revised form 25 February 1991.

References

Anderson, M. J. 1986. Nerve-induced remodeling of muscle basal lamina during synaptogenesis. *J. Cell Biol.* 102:863–877.

Anderson, M. J., and D. M. Fambrough. 1983. Aggregates of acetylcholine receptors are associated with plaques of a basal lamina heparin sulfate proteoglycan on the surface of skeletal muscle fibers. *J. Cell Biol.* 97:1396–1411.

Anderson, M. J., M. W. Cohen, and E. Zorychta. 1977. Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol. (Lond.).* 268:731–756.

Appleyard, S. T., M. J. Dunn, V. Dubowitz, M. L. Scott, S. J. Pittman, and D. M. Shotton. 1984. Monoclonal antibodies detect a spectrin-like protein in normal and dystrophic human skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 81:776–780.

Bloch, R. J. 1984. Isolation of acetylcholine receptor clusters in substrate-attached material from cultured rat myotubes using saponin. *J. Cell Biol.* 99:984–993.

Bloch, R. J., and Z. W. Hall. 1983. Cytoskeletal components of the vertebrate neuromuscular junction: vinculin, alpha-actinin, and filamin. *J. Cell Biol.* 97:217–223.

Bloch, R. J., and D. W. Pumplin. 1988. Molecular events in synaptogenesis: nerve-muscle adhesion and postsynaptic differentiation. *Am. J. Physiol.* 254:C345–C364.

Bloch, R. J., and J. S. Morrow. 1989. An unusual beta-spectrin associated with clustered acetylcholine receptors. *J. Cell Biol.* 108:481–493.

Bridgman, P. C., C. Carr, S. E. Pedersen, and J. B. Cohen. 1987. Visualization of the cytoplasmic surface of Torpedo postsynaptic membranes by freeze-etch and immunoelectron microscopy. *J. Cell Biol.* 105:1829–1846.

Buckle, V. J., J. L. Guenet, D. Simon-Chazottes, D. R. Love, and K. E. Davies. 1990. Localisation of a dystrophin-related autosomal gene to 6q24 in man, and to mouse chromosome 10 in the region of the dystrophia muscularis (dy) locus. *Hum. Genet.* 85:324–326.

Byers, T. J., A. Husain-Chishti, R. R. Dubreuil, D. Branton, and L. S. B. Goldstein. 1989. Sequence similarity of the amino-terminal domain of *Drosophila* beta spectrin to alpha actinin and dystrophin. *J. Cell Biol.* 109: 1633–1641.

Carr, C., G. D. Fischbach, and J. B. Cohen. 1989. A novel 87,000-M_r protein associated with acetylcholine receptors in Torpedo electric organ and vertebrate skeletal muscle. *J. Cell Biol.* 109:1753–1764.

Chang, H. W., E. Bock, and E. Bonilla. 1989. Dystrophin in electric organ of *Torpedo californica* homologous to that in human muscle. *J. Biol. Chem.* 264:20831–20834.

Chelly, J., J.-C. Kaplan, P. Maire, S. Gautron, and A. Kahn. 1988. Transcription of the dystrophin gene in human muscle and non-muscle tissues. *Nature (Lond.).* 333:858–860.

Chen, Q., R. Sealock, and H. B. Peng. 1990. A protein homologous to the *Torpedo* postsynaptic 58K proteins is present at the myotendinous junction. *J. Cell Biol.* 110:2061–2071.

Cullen, M. J., J. Walsh, L. V. B. Nicholson, and J. B. Harris. 1990. Ultrastructural localization of dystrophin in human muscle by using gold immunolabelling. *Proc. R. Soc. Lond. B. Biol.* 240:197–210.

Engel, A. G. 1986. Duchenne dystrophy. *In* Myology. A. G. Engel and B. Q. Banker, editors. McGraw-Hill Book Co., New York. Vol. 2. 1185–1240.

Ervasti, J. M., K. Ohlendieck, S. D. Kahl, M. G. Gaver, and K. P. Campbell. 1990. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature (Lond.).* 345:315–319.

Fardeau, M., F. M. S. Tome, H. Collin, N. Augier, F. Pons, J. Léger, and J. Léger. 1990. Présence d'une protéine de type dystrophine au niveau de la jonction neuromusculaire dans la dystrophie musculaire de Duchenne et la souris mutante "mdx". *Compt. Rend. Acad. Sci. Paris Serie III.* 311: 197–204.

Fertuck, H. C., and M. M. Salpeter. 1976. Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after ^{125}I-alpha-bungarotoxin binding at mouse neuromuscular junctions. *J. Cell Biol.* 69:144–158.

Fong, P., P. R. Turner, W. F. Denetclaw, and R. A. Steinhardt. 1990. Increased activity of calcium leak channels in myotubes of Duchenne human and mdx mouse origin. *Science (Wash. DC).* 250:673–676.

Fox, G. Q., and G. P. Richardson. 1978. The developmental morphology of *Torpedo marmorata*: electric organ-myogenic phase. *J. Comp. Neurol.* 179:677–698.

Franco, A., and J. B. Lansman. 1990. Calcium entry through stretch-inactivated ion channels in mdx myotubes. *Nature (Lond.).* 344:670–673.

Froehner, S. C. 1984. Peripheral proteins of postsynaptic membranes from *Torpedo* electric organ identified with monoclonal antibodies. *J. Cell Biol.* 99:88–96.

Froehner, S. C., K. Douville, S. Klink, and W. J. Culp. 1983. Monoclonal antibodies to cytoplasmic domains of the acetylcholine receptor. *J. Biol. Chem.* 258:7112–7120.

Froehner, S. C., A. A. Murnane, M. Tobler, H. B. Peng, and R. Sealock. 1987. A postsynaptic M_r 58,000 (58K) protein concentrated at acetylcholine receptor-rich sites in *Torpedo* electroplaques and skeletal muscle. *J. Cell Biol.* 104:1633–1646.

Froehner, S. C., C. W. Luetje, P. B., Scotland, and J. Patrick. 1990. The postsynaptic 43K protein clusters muscle nicotinic acetylcholine receptors in *Xenopus* oocytes. *Neuron.* 5:403–410.

Giloh, H., and J. W. Sedat. 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. *Science (Wash. DC).* 217:1252–1255.

Hammonds, R. G., Jr. 1987. Protein sequence of DMD gene is related to actin-binding domain of alpha-actinin. *Cell.* 51:1.

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

Hirokawa, N. 1983. *In* Structure and Function of Excitable Cells. C. D. Chang, editor. Plenum Publishing Corp., New York. 113–141.

Hirokawa, N., and J. E. Heuser. 1982. Internal and external differentiations of the postsynaptic membrane at the neuromuscular junction. *J. Neurocytol.* 11:487–510.

Hoffman, E. P., and L. M. Kunkel. 1989. Dystrophin abnormalities in Duchenne/Becker muscular dystrophy. *Neuron.* 2:1019–1029.

Hoffman, E. P., R. H. Brown, Jr., and L. M. Kunkel. 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell.* 51:919–928.

Hoffman, E. P., S. C. Watkins, H. S. Slayter, and L. M. Kunkel. 1989. Detection of a specific isoform of alpha-actinin with antisera directed against dystrophin. *J. Cell Biol.* 198:503–510.

Jasmin, B. J., A. Cartaud, M. A. Ludosky, J.-P. Changeux, and J. Cartaud. 1990. Asymmetric distribution of dystrophin in developing and adult *Torpedo marmorata* electrocyte: evidence for its association with the acetylcholine receptor-rich membrane. *Proc. Natl. Acad. Sci. USA.* 87:3938–3941.

Khurana, T. S., E. P. Hoffman, and L. M. Kunkel. 1990. Identification of a chromosome 6-encoded dystrophin-related protein. *J. Biol. Chem.* 265: 16717–16720.

Kingsley, R. E., and N. L. Cole. 1988. Preparation of cultured mammalian cells for transmission and scanning electron microscopy using Aclar film. *J. Electr. Microsc. Techn.* 10:77–85.

Koenig, M., A. P. Monaco, and L. M. Kunkel. 1988. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell.* 53:219–228.

Kordeli, E., J. Cartaud, H.-O. Nghiem, L.-A. Pradel, C. Debreuil, D. Paulin, and J.-P. Changeux. 1987. Evidence for a polarity in the distribution of proteins from the cytoskeleton in *Torpedo marmorata* electrocytes. *J. Cell Biol.* 102:748–761.

Kramarcy, N. R., and R. Sealock. 1990. Dystrophin as a focal adhesion protein: Colocalization with talin and the M_r 48,000 sarcolemmal protein in cultured *Xenopus* muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 272:171–174.

LaRochelle, W. J., and S. C. Froehner. 1986. Determination of the tissue distributions and relative concentrations of the postsynaptic 43-kDa protein and the acetylcholine receptor in Torpedo. *J. Biol. Chem.* 261:5270–5274.

Mandel, J. L. 1989. Dystrophin: the gene and its product. *Nature (Lond.).* 339:584–586.

Mayor, H. D., J. C. Hampton, and B. Rosario. 1961. A simple method for removing the resin from epoxy-embedded tissue. *J. Biophys. Biochem. Cytol.* 9:909–910.

Miranda, A. F., E. Bonilla, G. Martucci, C. T. Moraes, A. P. Hays, and S. Dimauro. 1988. Immunocytochemical study of dystrophin in muscle cultures from patients with Duchenne muscular dystrophy and unaffected control patients. *Am. J. Pathol.* 132:410–416.

Mitra, A. K., M. P. McCarthy, and R. Stroud. 1989*a*. Three-dimensional structure of the nicotinic acetylcholine receptor and location of the major associated 43-kD cytoskeletal protein, determined at 22 A by low dose electron microscopy and X-ray diffraction to 12.5 A. *J. Cell Biol.* 109:755–774.

Mitra, A. K., M. P. McCarthy, and R. Stroud. 1989*b*. Correction. *J. Cell Biol.* 109:1885.

Mongini, T., D. Ghigo, C. Doriguzzi, F. Bussolino, G. Pescarmona, B. Pollo, D. Schiffer, and A. Bosia. 1988. Free cytoplasmic Ca^{++} at rest and after cholinergic stimulus is increased in cultured muscle cells from Duchenne muscular dystrophy patients. *Neurology.* 38:476–480.

Morton, M. E., and S. C. Froehner. 1989. The alpha1 and alpha2 polypeptides of the dihydropyridine-sensitive calcium channel differ in developmental expression and tissue distribution. *Neuron.* 2:1499–1506.

Peng, H. B., and S. C. Froehner. 1985. Association of the postsynaptic 43K protein with newly formed acetylcholine receptor clusters in cultured muscle cells. *J. Cell Biol.* 100:1698–1705.

Peng, H. B., and Y. Nakajima. 1978. Membrane particle aggregates in innervated and noninnervated cultures of *Xenopus* embryonic muscle cells. *Proc. Natl. Acad. Sci. USA.* 75:500–504.

Porter, S., and S. C. Froehner. 1983. Characterization of the M_r=43,000 proteins associated with acetylcholine receptor-rich membranes. *J. Biol. Chem.* 258:10034–10040.

Pumplin, D. W. 1989. Acetylcholine receptor clusters of rat myotubes have at least three domains with distinctive cytoskeletal and membranous components. *J. Cell Biol.* 109:739–753.

Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethylrhodamine derivatives of alpha-bungarotoxin: Preparation, separation and characterization. *Anal. Biochem.* 80:585–592.

Rowland, L. P. 1980. Biochemistry of muscle membranes in Duchenne muscular dystrophy. *Muscle Nerve.* 3:3–20.

Sahr, K. E., P. Laurila, L. Kotula, A. L. Scarpa, E. Coupal, T. L. Leto, A. J. Linnenbach, J. C. Winkelmann, D. W. Speicher, V. T. Marchesi, P. J. Curtis, and B. G. Forget. 1990. The complete cDNA and polypeptide sequences of human erythroid alpha-spectrin. *J. Biol. Chem.* 265:4434–4443.

Sakakibara, H., A. G. Engel, and E. H. Lambert. 1977. Duchenne dystrophy: Ultrastructural localization of the acetylcholine receptor and intracellular microelectrode studies of neuromuscular transmission. *Neurology.* 27:741–745.

Sealock, R. 1980. Identification of regions of high acetylcholine receptor density in tannic acid-fixed postsynaptic membranes from electric tissue. *Brain Res.* 199:267–281.

Sealock, R., and A. Kavookjian. 1980. Postsynaptic distribution of acetylcholine receptors in electroplax of the torpedine ray, *Narcine brasiliensis*. *Brain Res.* 190:81–93.

Sealock, R., B. E. Wray, and S. C. Froehner. 1984. Ultrastructural localization of the M_r 43,000 protein and the acetylcholine receptor in *Torpedo* postsynaptic membranes using monoclonal antibodies. *J. Cell Biol.* 98:2239–2244.

Shimizu, T., K. Matsumura, Y. Sunada, and T. Mannen. 1989. Dense immuno-

staining on both neuromuscular and myotendon junctions with an anti-dystrophin monoclonal antibody. *Biomed. Res.* 10:405–409.

Sicinski, P., Y. Geng, A. S. Ryder-Cook, E. A. Barnard, M. G. Darlison, and P. J. Barnard. 1989. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science (Wash. DC).* 244:1578–1580.

Sottrup-Jensen, L., T. M. Stepanik, T. Kristensen, D. M. Wierzbicki, C. M. Jones, P. B. Lonblad, S. Magnusson, and T. E. Petersen. 1984. Primary structure of human alpha2-macroglobulin. V. The complete structure. *J. Biol. Chem.* 259:8318–8327.

Torres, L. F. B., and L. W. Duchen. 1987. The mutant mdx: inherited myopathy in the mouse. *Brain.* 110:269–299.

Watkins, S. C., E. P. Hoffman, H. S. Slayter, and L. M. Kunkel. 1988. Immunoelectron microscopic localization of dystrophin in myofibres. *Nature (Lond.).* 333:863–866.

Winkelmann, J. C., J. G. Chang, W. T. Tse, A. L. Scarpa, V. T. Marchesi, and B. G. Forget. 1990. Full-length sequence of the cDNA for human erythroid beta-spectrin. *J. Biol. Chem.* 265:11827–11832.

Witkowski, J. A. 1989. Dystrophin-related muscular dystrophies. *J. Child Neurol.* 4:251–271.

Woodruff, M. L., J. Theriot, and S. J. Burden. 1987. 300-kD subsynaptic protein copurifies with acetylcholine receptor-rich membranes and is concentrated at neuromuscular synapses. *J. Cell Biol.* 104:939–946.

Wray, B. E., and R. Sealock. 1984. Ultrastructural immunocytochemistry of particulate fractions using polyvinyl chloride microculture wells. *J. Histochem. Cytochem.* 31:1117–1120.

Yamaguchi, M., S. Ishiura, H. Takano-Ohmuro, T. Tsukahara, K. Arahata, T. Obinata, T. Tamiya, T. Tsuchiya, and H. Sugita. 1990. Detection of a fast isoform of C-protein with an antiserum directed against the N-terminal portion of dystrophin. *Biochem. Biophys. Res. Commun.* 169:57–63.

Yeadon, J. E., H. Lin, S. M. Dyer, and S. J. Burden. 1991. The 300kDa subsynaptic protein and synaptogenesis. *J. Cell Biochem. Suppl.* 15C:84.