A Protein Homologous to the *Torpedo* Postsynaptic 58K Protein Is Present at the Myotendinous Junction

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Abstract. The 58K protein is a peripheral membrane protein enriched in the acetylcholine receptor (AChR)-rich postsynaptic membrane of *Torpedo* electric organ. Because of its coexistence with AChRs in the postsynaptic membrane in both electrocytes and skeletal muscle, it is thought to be involved in the formation and maintenance of AChR clusters. Using an mAb against the 58K protein of *Torpedo* electric organ, we have identified a single protein band in SDS-PAGE analysis of *Xenopus* myotomal muscle with an apparent molecular mass of 48 kD. With this antibody, the distribution of this protein was examined in the myotomal muscle fibers with immunofluorescence techniques. We found that the 48K protein is concentrated at the myotendinous junctions (MTJs) of these muscle fibers. The MTJ is also enriched in talin and vinculin. By double labeling muscle fibers with antibodies against talin and the 48K protein, these two proteins were found to colocalize at the membrane invaginations of the MTJ. In cultured myotomal muscle cells, the 48K protein and talin are also colocalized at sites of membrane-myofibril interaction. The 48K protein is, however, not found at focal adhesion sites in nonmuscle cells, which are enriched in talin. These data suggest that the 48K protein is specifically involved in the interaction of myofibrillar actin filaments with the plasma membrane at the MTJ. In addition to the MTJ localization, 48K protein is also present at AChR clusters both in vivo and in vitro. Thus, this protein is shared by both the MTJ and the neuromuscular junction.

THE myotendinous junction (MTJ)[1] and the neuromuscular junction (NMJ) are two specialized sarcolemmal domains of skeletal muscle. At the MTJ, the force generated by the contraction of myofibrils is transmitted across the plasma membrane to the tendon. At the NMJ, the release of neurotransmitter from the nerve terminal is sensed by the acetylcholine receptors (AChRs) in the postsynaptic membrane. Although these two sarcolemmal domains are distinct in function, they share a number of common features. First, the density of AChRs is elevated above background level at both NMJ and MTJ, although the level at the MTJ never reaches that at the NMJ (12, 13, 17, 23). Second, acetylcholinesterase is concentrated at both the NMJ and the MTJ. Furthermore, among the different molecular forms of this enzyme, the asymmetric form is shared by both junctions (22). Third, the two junctions share certain structural similarities. For example, membrane invagination is a common feature of both the postsynaptic membrane and the MTJ (11, 39, 40). A cytoplasmic density and a filamentous network underlies these specialized membrane domains at both junctions. In addition, they also share a number of peripheral membrane proteins, such as vinculin and talin, which mediate membrane-cytoskeleton interaction (2, 31, 34, 35, 37). Fourth, we have recently shown that positively charged beads induce the formation of AChR clusters as well as a membrane domain that is morphologically analogous to the MTJ (31). Thus, despite their distinct functions, the organization and the development of these two sarcolemmal domains bear much in common.

A great deal of information concerning the structure and biochemistry of the postsynaptic membrane of skeletal muscle has come from the study of AChR-rich synapses in *Torpedo* electric organ. In association with this AChR-rich membrane are several specialized peripheral membrane proteins, such as the postsynaptic 43 and 58K proteins (14). Using mAbs against the *Torpedo* postsynaptic membrane, homologous proteins have also been found in the endplate of skeletal muscle (3, 6, 15, 26). Thus, the postsynaptic membrane in skeletal muscle contains both a set of general cytoskeletal proteins, such as vinculin and talin, and a set of synapse-specific proteins, such as the homologues of the *Torpedo* 43 and 58K proteins. The 43K protein is present in the postsynaptic membrane in a 1-to-1 stoichiometry with the AChR and is probably linked to the receptor (3, 7, 33), and may be directly associated with the lipid bilayer of the postsynaptic membrane (24, 29). Removal of this protein by alkaline treatment results in an increase in both the translational and rotational mobility of AChRs in the membrane (1,

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; MTJ, myotendinous junction; NMJ, neuromuscular junction; R-BTX, tetramethylrhodamine-conjugated α-bungarotoxin.

10, 32). Thus, this protein may be involved in the stabilization of AChRs in the postsynaptic membrane. The function of the 58K protein is unknown. Although it is concentrated in the postsynaptic membrane, it is also present at a reduced level in the extrajunctional region of the muscle cell (15). This suggests its function is not restricted to the maintenance of the AChR clusters.

In view of the structural similarities between the NMJ and MTJ, it is important to know whether these specialized postsynaptic proteins are also present at the MTJ. This knowledge will enable us not only to further understand the function of these proteins, but also to delineate the similarities and differences between NMJ and MTJ. Toward this goal, we have examined the distribution of the postsynaptic 58K protein at the MTJ of *Xenopus* muscle cells both in vivo and in vitro and contrasted that with the distribution of the postsynaptic 43K protein. We report here that a protein homologous to the *Torpedo* 58K is highly enriched at the MTJ, but the 43K protein is totally absent from this sarcolemmal domain. At the MTJ, the 58K protein is colocalized with other proteins involved in membrane-cytoskeleton interaction, such as talin and vinculin. This suggests that the 58K protein is a member of a protein complex mediating the attachment of myofibrils to the plasma membrane.

Materials and Methods

Antibodies

Four different antibodies were used in this study: a mouse mAb against chicken gizzard vinculin (C-19; kindly provided by Dr. K. Burridge, University of North Carolina at Chapel Hill), a rabbit antiserum against chicken gizzard talin (8; also from Dr. K. Burridge) and two mouse mAbs against *Torpedo* electric organ postsynaptic 58K protein (mAb 1351; 15) and 43K protein (mAb 1234; 3, 26). Both of these mAbs against *Torpedo* proteins are gifts of Dr. S. C. Froehner (Dartmouth Medical School).

Immunoblot Analysis

Tails from *Xenopus* tadpoles (stage 52), which are composed primarily of muscle tissue, were homogenized and subjected to electrophoresis on 10% SDS-polyacrylamide gels containing 0.13% bisacrylamide (20). The proteins were transferred onto nitrocellulose paper (38) on a semi-dry electrophoretic transfer device (LKB Instruments, Inc., Gaithersburg, MD) at a current setting of 0.8 mA/cm^2 for 1 h. After blocking unoccupied protein binding sites with 5% horse serum, 5% BSA, and 0.05% Tween-20 in PBS, pH 7.4, for 1 h, the nitrocellulose strips were incubated with mAb 1351 at 20 nM at room temperature for 1 h. After washing with the same buffer, the strips were incubated with biotinylated horse anti-mouse IgG at room temperature for 60 min, and the avidin/biotinylated HRP complex (ABC kit; Vector Laboratories, Inc., Burlingame, CA) at room temperature for 45 min. The strips were developed with the substrate solution (0.4 mg/ml 4-chloronaphthol and 0.2% H_2O_2 in PBS) at room temperature for 5 min.

Dissociation of Myotomal Muscle Fibers and Tissue Culture of Myotomal Muscle Cells

Tails were excised from *Xenopus* tadpoles anesthetized with 0.1% MS-222 (Sigma Chemical Co., St. Louis, MO), skinned and incubated with collagenase (Sigma Type I) at a concentration of 3 mg/ml in frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 5 mM Hepes, pH 7.4) for 2 h at room temperature under gentle agitation on a rotator. At the end of the incubation, the tails were gently vortexed to release the myotomal muscle fibers. They were collected by gentle centrifugation and washed with Ringer solution.

The technique for preparing muscle cell cultures from *Xenopus* embryos followed those described by Peng and Nakajima (27). In short, the dorsal portions of the embryos at stage 20–22 were excised and dissociated in Ca^{2+}, Mg^{2+}-free Steinberg's solution. The resultant single cell suspension was plated onto coverglass and cultured at room temperature. To induce the formation of AChR clusters, the cultures were treated with polyornithine-coated latex beads according to a previously published method (25).

Immunofluorescence

Dissociated myotomal muscle fibers were fixed with Luther's fixative (21; 0.5% paraformaldehyde, 80 mM cyclohexylamine in 10 mM Pipes buffer containing 10 mM $MgCl_2$ and 10 mM EGTA, pH 6.5) for 30 min, and washed with 75 mM Tris-HCl buffer (pH 6.7) containing 10 mM $MgCl_2$, 10 mM NaN_3, and 0.5% Brij 58 (Sigma Chemical Co.) for 1 h. Nonspecific binding sites were blocked with 20 mM Tris-HCl buffer (pH 6.5) containing 0.1% BSA, 10 mM $MgCl_2$, and 10 mM NaN_3 ("blocking buffer") for 1 h. They were then labeled with primary antibodies reconstituted in blocking buffer. The primary antibody staining was carried out overnight at 4°C. The fibers were washed and labeled with rhodamine-conjugated goat anti-rabbit IgG or fluorescein-conjugated goat anti-mouse IgG (Organon Technika, Cochranville, PA) for 1 h at room temperature. They were washed and mounted on slides. Microscopy was performed on a Leitz Orthoplan microscope. Images were recorded on Kodak T-max film.

In some experiments, fibers were double stained with antitalin antiserum and mAb 1351. In this case, a mixture of these two antibodies was used in the primary antibody step and a mixture of appropriate secondary antibodies was used in the secondary antibody step. To label AChR clusters, fibers were treated with 0.3 μM tetramethylrhodamine-conjugated α-bungarotoxin (R-BTX) immediately after fixation. This is followed by permeation with Brij 58 and antibody labeling. To visualize the cell nuclei, fixed, permeabilized fibers were treated with 1 M 4,6-diamidino-2-phenyl-indole (DAPI; Sigma Chemical Co.) for 30 min before mounting.

Cultured muscle cells were fixed with 95% ethanol at −20°C for 4 min, washed with blocking buffer, and labeled with antibodies as described above. To visualize AChR clusters, the cultures were first labeled with R-BTX before fixation.

Immunofluorescence of Cryosections

In addition to dissociated myotomal muscle fibers, we also examined mAb 1351 staining in semi-thin frozen sections of myotomal muscle. The tails of stage 50 *Xenopus* tadpoles were fixed and the myotomes were dissected under fixative. They were infiltrated with 1.6 M sucrose in 0.1 M phosphate buffer at pH 7.2 for 2 h, mounted on metal stubs and frozen in pentane slush. Semi-thin cryosections (estimated thickness, 0.3 μm) were cut and mounted on glass slides. They were treated successively with (1) mAb 1351 or nonspecific mouse IgG, (2) rhodamine-labeled goat anti-mouse IgG containing FITC-conjugated BTX, (3) rhodamine-labeled rabbit anti-goat IgG containing affinity-purified rabbit anti-BTX antibody labeled with fluorescein, and (4) 4% paraformaldehyde in PBS for 30 min. All antibodies were diluted in 10% normal horse serum. Stained sections were mounted in glycerol containing *n*-propylgallate (16) and examined by fluorescence microscopy.

Results

Immunoblots with mAb 1351

Immunoblot analysis of the total tail muscle homogenate with mAb 1351 demonstrated a single protein band of 48 kD (Fig. 1, lane *C*). This is in agreement with previous study of Froehner et al. (15) that mAb 1351 recognized a single molecular weight species in SDS gel analysis of homogenates from *Torpedo* electric organ and C2 mouse muscle cell line. Thus, a protein immunologically related to the *Torpedo* 58K protein is present in the *Xenopus* myotomal muscle. However, the apparent molecular weight of the *Xenopus* antigen on SDS gel is smaller than that reported for *Torpedo* electric organ and C2 muscle culture. Since only a single band was recognized by mAb 1351, this 48K protein is unlikely to be the breakdown product of a larger protein. We conclude that the *Xenopus* homologue of the 58K protein has a lower molecular mass than its *Torpedo* counterpart. For the remainder of this paper, this antigen is referred to as the 48K protein.

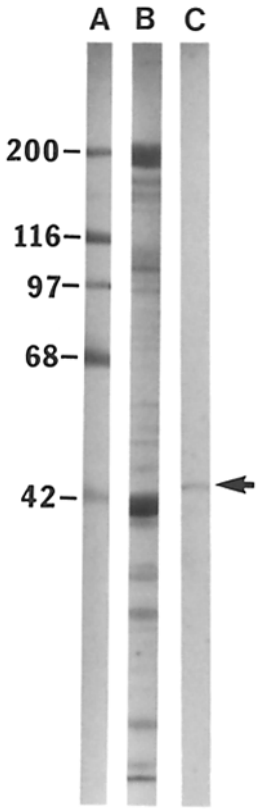

Figure 1. Immunoblot analysis of *Xenopus* myotomal muscle homogenate with mAb 1351. (Lane *A*) Molecular mass standards (in kD); (lane *B*) total muscle homogenate. Lanes *A* and *B* were taken from Coomassie blue-stained gel. (Lane *C*) Immunoblot of total muscle homogenate reacted with mAb 1351 followed by secondary antibody and color reaction. A single band with a molecular mass of 48 kD (*arrowhead*) was recognized by the antibody.

Localization of the 48K Protein at MTJ

Single muscle fibers dissociated from the myotomal muscle were used to examine the distribution of the 48K protein at the MTJ. Examples of these fibers are shown in Fig. 2. They ranged from 20 to 100 μm in diameter and were multiple nucleated. After dissociation, the nerve terminals innervating the ends of these fibers were lost. However, both the MTJ and the postsynaptic apparatus remained intact. The sarcolemma at the ends of the fibers where the MTJs are located is deeply invaginated (19, 31). These invaginations appeared as a series of streaky structures after the fibers were stained with antibodies against vinculin (Fig. 3) or against talin (Fig. 4, *A* and *D*). Previous studies have shown that these two proteins are located at the MTJ (31, 35, 37). Thus, the antitalin staining was used to identify the sites of MTJ.

When dissociated myotomal fibers were double labeled with antitalin antibody and mAb 1351, strong staining by both antibodies was confined to the ends of the cells (Fig. 4, *A–C*). Away from the MTJ, staining was undetectable. At the MTJ, the two staining patterns were very nearly identical (Fig. 4, *A*, *B*, *D*, and *E*). This was true even when the patterns happened to be highly detailed. In Fig. 4 *D*, each invagination revealed by the antitalin staining (Fig. 4 *D*) was also recognized by mAb 1351 in a one-to-one correspondence (Fig. 4 *E*).

To eliminate the possibility that the concentration of mAb 1351 staining at the MTJ was due to an overlapping of the invaginated membrane, we probed semi-thin cryosections of muscle fibers with the antibody. Fig. 5 *B* shows a section that cut several muscle fibers from the tail musculature of a *Xenopus* larva. The labeled profiles appeared as circles when sectioned transversely (the fiber on the left) and as hollow tubes when sectioned longitudinally (on the right). As in mammalian muscle (15), the labeling was confined to the sarcolemma. The center of the tubes, which was occupied by extracellular matrix of the invaginated membrane, was not stained.

In control studies, we labeled the dissociated muscle fibers or cryosections with nonspecific mouse serum followed by usual secondary antibody labeling. An example of the cryosection control is shown in Fig. 5 *D*, which showed no staining of the MTJ and the rest of the muscle fiber in the absence of mAb 1351. Neither was staining observed if the muscle fibers were not permeabilized before mAb 1351 labeling.

Localization in Cultured Cells

To further understand the cellular distribution of the 48K an-

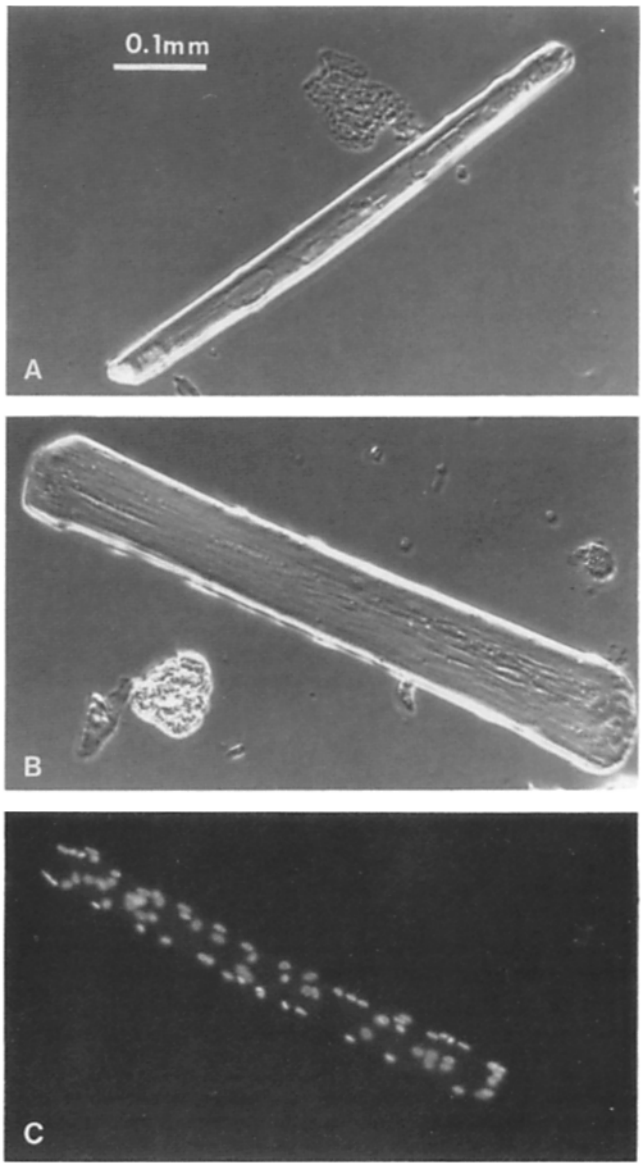

Figure 2. Dissociated muscle fibers from *Xenopus* larvae. *A* and *B* show two live fibers with different diameters in phase contrast. These fibers are multiple nucleated as shown by DAPI staining in *C*.

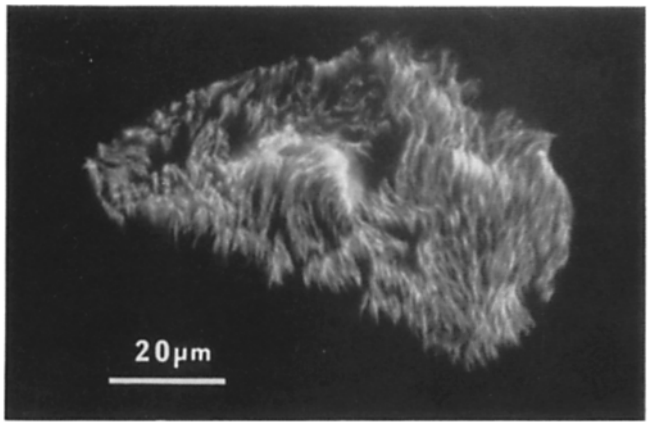

Figure 3. MTJ of the myotomal muscle fiber after labeling with an antibody against vinculin. Membrane invaginations were stained by this antibody and gave a streaky appearance at the MTJ. The rest of the muscle fiber extended downward from this sarcolemmal specialization.

tigen, we studied its localization in cell cultures dissociated from the myotomes of *Xenopus* embryos. These cultures contained predominantly muscle cells, but also included a small number of nonmuscle cells. In muscle, the antigen recognized by mAb 1351 is colocalized with AChR clusters (*arrowhead* in Fig. 6, *A* and *B*; see also reference 15). In addition, this antibody also stained a series of linear, streaky structures that were often located at the periphery or at the ends of the muscle cell (*arrows* in Fig. 6 *A*). By comparing with the phase-contrast image, it can be seen that these fluorescent streaks were colocalized with unstriated fibrils which extended from striated myofibrils (Fig. 6, *A* and *C*). Our previous study on these muscle cells have shown that these unstriated extensions of myofibrils are composed of actin filaments which insert into the plasma membrane (28). Thus, these mAb 1351-labeled streaks are analogous to the MTJ in vivo. To further correlate these streaks with MTJ, we double labeled cultures with both antitalin antibody and mAb 1351. Fig. 7, *A* and *B* show that these two antigens were precisely colocalized at these streaks. Based on these data,

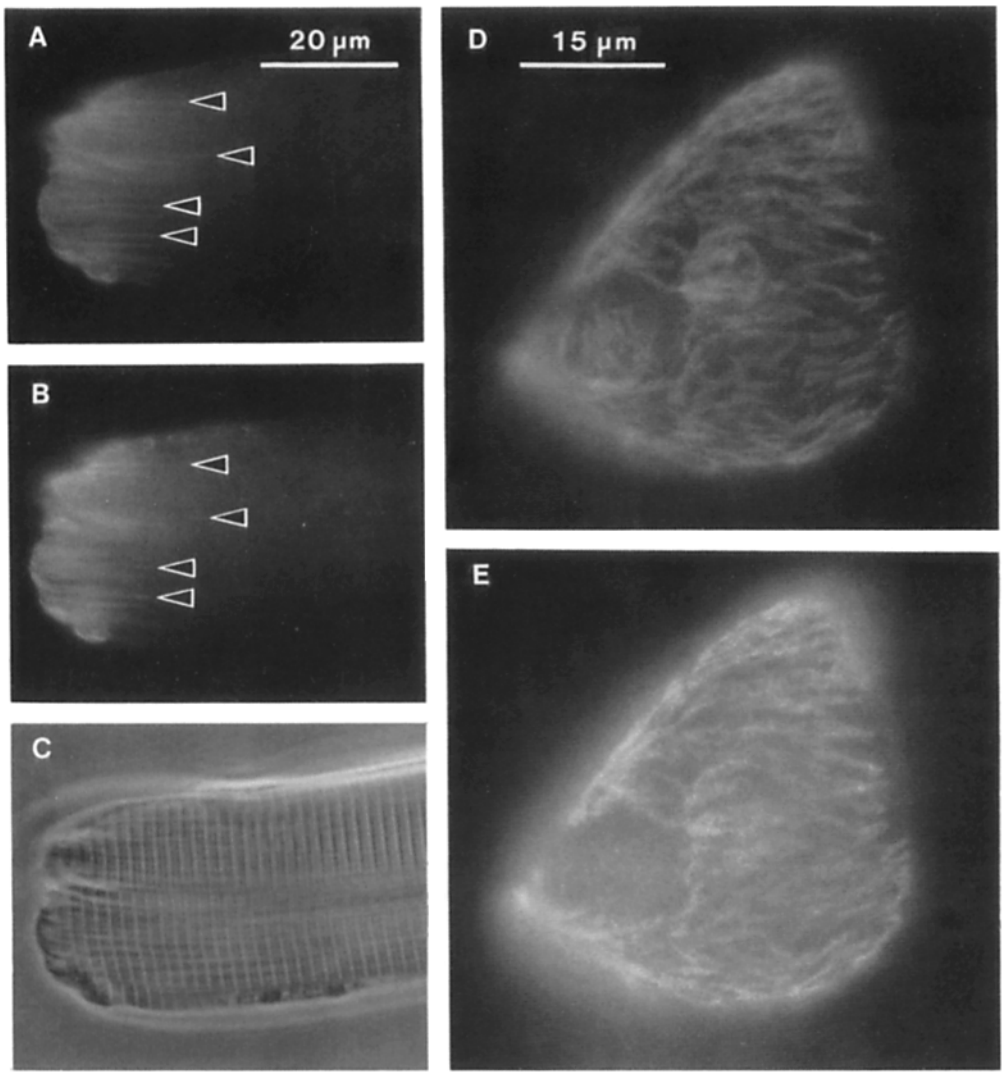

Figure 4. Colocalization of talin and 48K protein at the MTJ. The fibers were double labeled with an antibody against talin (*A* and *D*) and mAb 1351 (*B* and *E*). The talin staining identified the location of the MTJ at the ends of the muscle fiber. The invaginations of the MTJ (*arrowheads* in *A*, *B*) were lined with both talin and the 48K protein. *C* is the phase-contrast view of the area shown in *A* and *B*. *D* and *E* show a more striking example of the correspondence in talin and 48K distribution. Little staining was detected away from the MTJ.

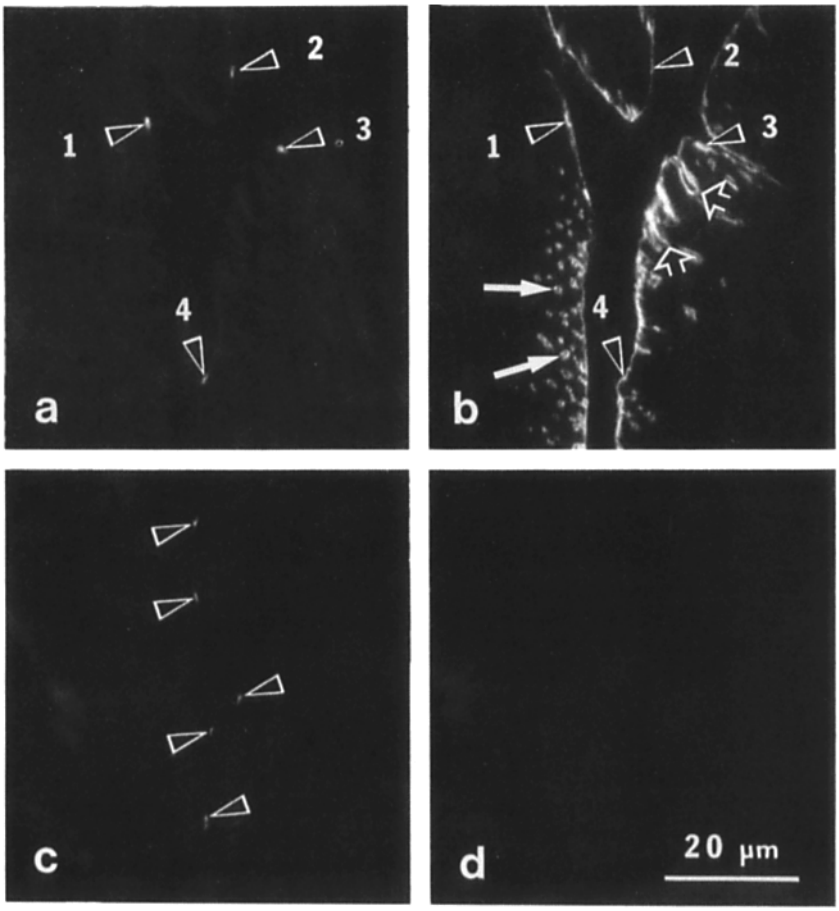

Figure 5. Distribution of the 48K protein and AChRs in the myotomal muscle fibers in semi-thin cryosections. (*a* and *c*) FITC-BTX images; (*b*) rhodamine image of mAb 1351 staining; and (*d*) control image of the same section as in *c*. mAb 1351–labeled membrane invaginations at the MTJ (*white open arrowheads* in *b*). When the fiber was transversely sectioned, these invaginations appeared as circular profiles lined by antibody staining (*white solid arrows* in *b*). Staining was absent from the lumen of these circles, which was the extracellular domain. Outside the MTJ domain, the sarcolemma was not stained. In addition, mAb 1351 staining was also contiguous across the AChR clusters at the NMJ (*black arrowheads* in *a* and *b*) which existed in the midst of the MTJ invaginations. No staining was observed when the primary antibody was a nonspecific mouse serum (*d*), even though the toxin staining was present (*c*).

we conclude that in vitro, the 48K antigen is also localized at sites of myofibril–membrane interaction in a fashion analogous to the MTJ specialization in vivo.

In nonmuscle cells, bundles of actin filaments (stress fibers) interacted with the plasma membrane at sites of focal adhesion. These sites are enriched in talin (9). Since the protein compositions of focal adhesion and MTJ have much in common, and since a 58K protein is detected by mAb 1351 in several nonmuscle tissues in *Torpedo* (our unpublished results; 15), we examined nonmuscle cells, including fibroblasts and epithelial cells, in cultures double stained for talin and 48K. The focal adhesions in these nonmuscle cells were strongly stained by the antitalin antibody, as expected (Fig. 7 *C*), but not by mAb 1351 (Fig. 7 *D*). Thus, the 48K antigen is unique to the site of myofibril–membrane interaction in muscle cells. These data indicate that the molecular interaction between the myofibril and the plasma membrane at MTJ is different from the actin–membrane interaction at focal adhesions.

Relationship of the 48K Protein to AChR Clusters

Previous study has shown that both the postsynaptic membrane at the NMJ and AChR clusters in cultured muscle cells were recognized by mAb 1351. In view of the new information on the MTJ localization of the 48K protein described here, we reexamined the distribution of this antigen at the AChR clusters in *Xenopus* both in vivo and in vitro.

NMJs in *Xenopus* myotomal muscle occur on the ends of the fibers and are essentially surrounded by the MTJ (31). When semi-thin cryosections of the myotomal muscle were double labeled with mAb 1351 and R-BTX, one can see that the postsynaptic membrane exists in the midst of the 48K-positive MTJ specialization (Fig. 5, *A* and *B*). Within the resolution limit of light microscopy, the area occupied by the AChR-rich postsynaptic membrane is also labeled by mAb 1351.

To obtain a panoramic view of the relationship between the 48K protein and AChR clusters, we also examined the dissociated myotomal muscle fibers in whole mount. As shown in Fig. 8, *A-D*, the pattern of 48K protein distribution was distinct from that of the AChR clusters. The AChR clusters occupied a small area in the midst of the 48K-positive membrane span, although these two domains showed considerable overlap with each other. Comparison of these two patterns revealed the following differences: (*a*) the postsynaptic membrane was often composed of punctate AChR patches (Fig. 8 *B*). This fine structure was not seen in the mAb 1351 image. (*b*) Despite the overlap, some AChR patches were located in membrane areas deficient in the 48K antigen (numbered areas in Fig. 8, *A-D*). Since AChR clusters totally devoid of mAb 1351 staining were not observed in semi-thin cryosections, these numbered patches shown in Fig. 8 may have a reduced, but not totally absent, mAb 1351 staining. This reduced staining may have escaped detection due to the intense MTJ staining in their vicinity.

Although the majority of myotomal muscle fibers are in-

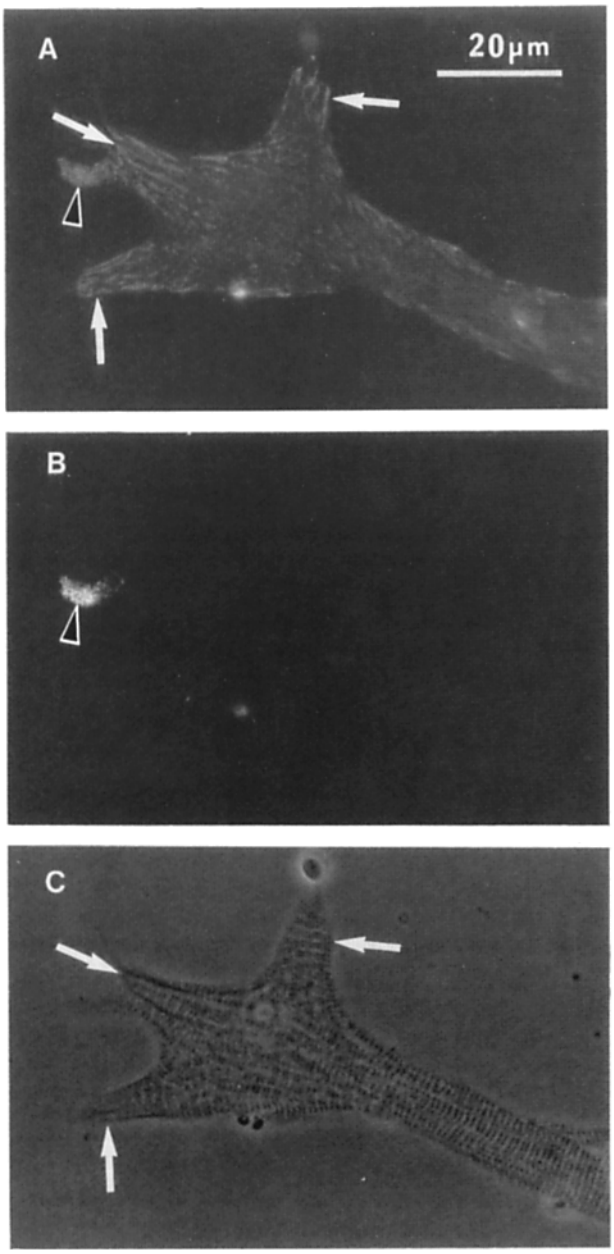

Figure 6. Localization of 48K protein in cultured myotomal muscle cell. (*A*) mAb 1351 staining; (*B*) R-BTX staining; and (*C*) phase contrast. The antibody stained streaky structures (*white arrows* in *A*) located at sites of myofibril–membrane interaction. The myofibrils at these sites were often unstriated. (*white arrows* in *C*). In addition, mAb 1351 also stained the cytoplasmic domain of AChR clusters (*arrowheads* in *A* and *B*).

nervated at both ends, a small number of them are singly innervated as shown by the presence of R-BTX fluorescence at only one end of the fiber. At the noninnervated end, prominent mAb 1351 staining of the MTJ was still observed (Fig. 8, *E* and *F*). This shows that the concentration of the 48K protein at the MTJ is independent of AChR clustering.

AChR clusters can be induced in cultured *Xenopus* muscle cells by polycation-coated latex beads (25). This allows one to examine the relationship between peripheral membrane proteins and developing AChR clusters. As shown in Fig. 9, *a–c*, the bead-induced AChR clusters became positive in mAb 1351 staining as early as 5 h after the beginning of the clustering process set by the addition of beads. As the density of the receptors within the clusters increased with time, evidenced by the increase in R-BTX fluorescence, there was a concomitant increase in the intensity of mAb 1351 staining. In Fig. 9 *g*, several 24-h-old bead–induced clusters are shown. They are accompanied by equally bright mAb 1351 staining (Fig. 9 *h*). Thus, the accumulation of this antigen paralleled the aggregation of AChRs within the clusters. Furthermore, the morphology of the AChR- and 48K-positive membrane domains showed striking similarity (compare Fig. 9, *d* and *e* and *g* and *h*). This suggests that there is a close relationship between the AChRs and the 48K protein at developing AChR clusters in vitro.

Based on these results, we conclude that the 48K protein is shared by both the MTJ and the AChR clusters in *Xenopus* myotomal muscle cells.

Distribution of the 43K Protein in the Myotomal Muscle Fiber

Since both the 58 and 43K proteins are present at the postsynaptic membranes of *Torpedo* and the NMJ of skeletal muscle (14, 15), we wanted to know whether this relationship could also be observed at MTJ. The distribution of the 43K protein in relationship to MTJ and NMJ was examined by double labeling dissociated myotomal muscle fibers with mAb 1234 and R-BTX. As shown in Fig. 10, the 43K protein was colocalized with AChR clusters in the postsynaptic membrane of dissociated myotomal fibers. Although these AChR patches were surrounded by membrane invaginations of the MTJ (Fig. 8, *A-D*), the latter were not labeled by mAb 1234. This is in sharp contrast to the pattern of mAb 1351 staining (Figs. 4 and 8). Thus, the 43K protein was discretely localized at the AChR clusters in the postsynaptic membrane and was absent from the MTJ.

Discussion

Using an mAb against the postsynaptic 58K protein of *Torpedo* electric organ, we have identified a homologous protein in the myotomal muscle of *Xenopus* with an apparent molecular mass of 48 kD. Our previous study showed that the antigen recognized by mAb 1351 is concentrated at the postsynaptic membrane of skeletal muscle and is also present in the extrajunctional membrane (15). This study has shown that 48K protein is, in fact, highly concentrated at the MTJ. At the MTJ, this protein is associated with the cytoplasmic side of the invaginated membrane in a pattern similar to the distribution of two other peripheral membrane proteins, namely talin and vinculin. This suggests that the 48K protein is a member of a protein complex responsible for the integrity of the MTJ specialization. Since the myotomal muscle can be easily dissociated into single muscle fibers small enough for whole-mount immunofluorescence studies and the development of both the NMJ and the MTJ specializations can be studied in vitro, our works were centered on this muscle. However, in addition to the myotomal muscle, we have also examined the distribution of the 48K protein in the submaxillaris muscle of *Xenopus*. This muscle exists both in the larva and in the adult. We found that the 48K protein is

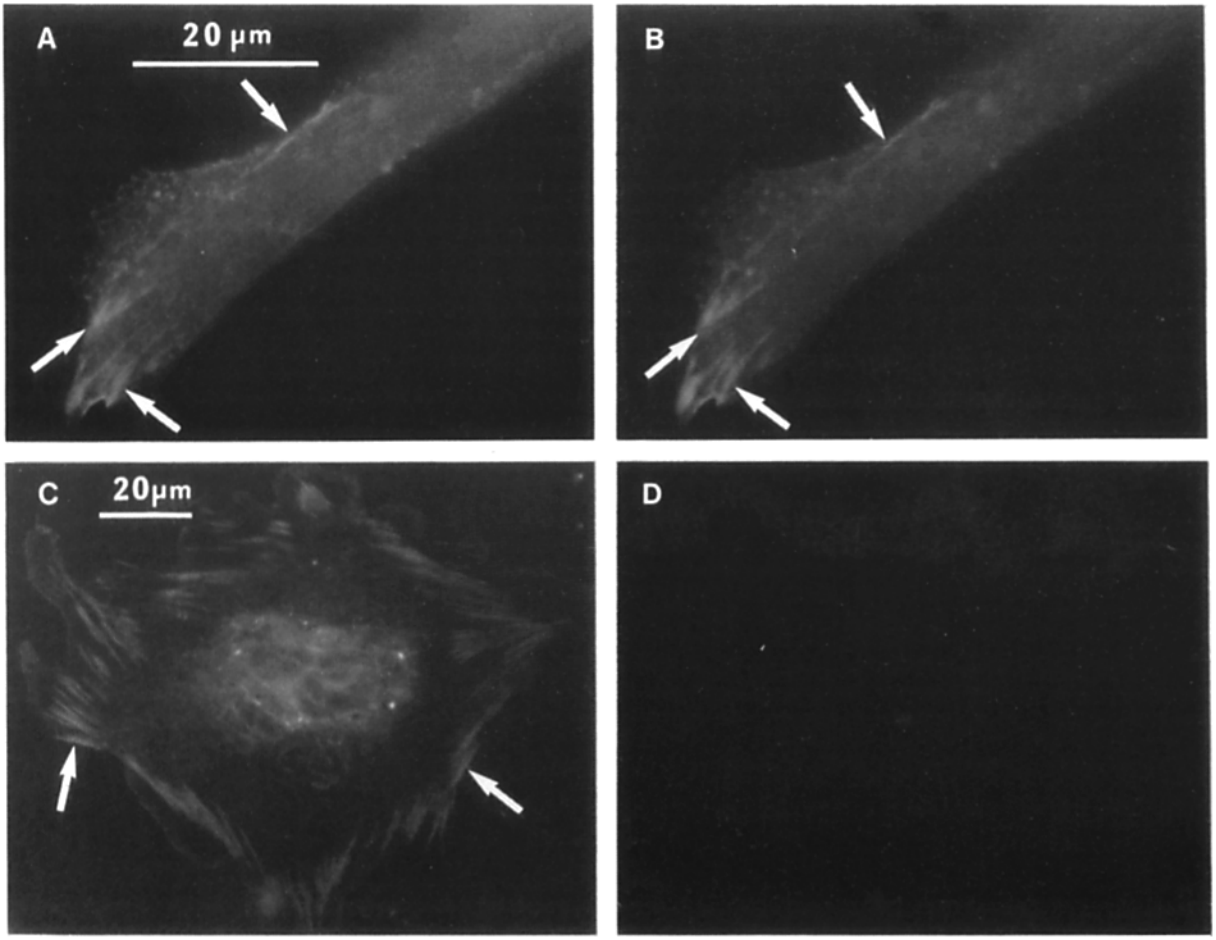

Figure 7. Relationship of talin and the 48K protein in cultured cells. (*A* and *C*) talin image; (*B* and *D*) 48K image. In muscle cell, talin and 48K coexisted at sites of membrane–myofibril interaction (*arrows* in *A* and *B*). Although talin was present at sites of focal adhesion in nonmuscle cell (*arrows* in *C*), such sites were devoid of mAb 1351 staining (*D*).

also concentrated at the MTJ of this muscle (our unpublished results).

The MTJ integrates the contraction of myofibrils and the transmittal of force via the tendon. The membrane invaginations greatly increase the surface area and the strength of this integration (36). Functionally, the MTJ in muscle is analogous to the focal adhesion sites in fibroblasts and other nonmuscle cells. At focal adhesions, the attachment of actin filaments to the plasma membrane is mediated by a complex of proteins including vinculin and talin on the cytoplasmic side and integrin in the membrane (9). Integrin in turn interacts with the extracellular matrix on the substrate. In addition to talin and vinculin, recent evidence suggests that integrin is also localized at the MTJ (5). This suggests that the myofibril–tendon integration at MTJ is also mediated by the same mechanism as that used at the focal adhesion. The colocalization of the 48K protein with talin and vinculin suggests that it may play a similar role in mediating actin–membrane interaction. The fact that the 48K protein is only found at the MTJ, but not at the sites of focal adhesion in nonmuscle cells, suggests a unique role for this protein in mediating the myofibril–membrane interaction. Thus, in addition to general cytoskeletal proteins such as talin and vinculin, the interaction of myofibrils to the plasma membrane may require certain specialized proteins, such as the 48K, which probably are not needed for membrane–actin filament interaction in nonmuscle cells. However, the possibility that a different isoform of the 48K protein that is not recognized by mAb 1351 is present at the focal adhesion sites of nonmuscle cells can not be excluded at present.

Both 43 and 58K proteins are colocalized at the postsynaptic membrane of *Torpedo* electric organ (14). In *Torpedo*, both proteins are localized at the crest of the postsynaptic folds, an area also occupied by AChR clusters (15, 33). This and previous studies have also shown that both proteins are present at developing AChR clusters, such as those induced by polycation-coated beads and electric fields in *Xenopus* cultures (26, 30). Thus, they may play a role in the formation and maintenance of AChR clusters. However, this study has clearly delineated the difference between these two proteins in muscle. While the 43K protein is uniquely associated with the AChR-rich postsynaptic membrane, the 48K protein, a homologue of the *Torpedo* 58K protein, is shared by both the MTJ and the NMJ.

The 43K protein is generally thought to impart stability to AChR clusters, since its removal results in an increase in the mobility of AChRs in isolated *Torpedo* postsynaptic membrane (1, 10, 32). However, recent data have shown that

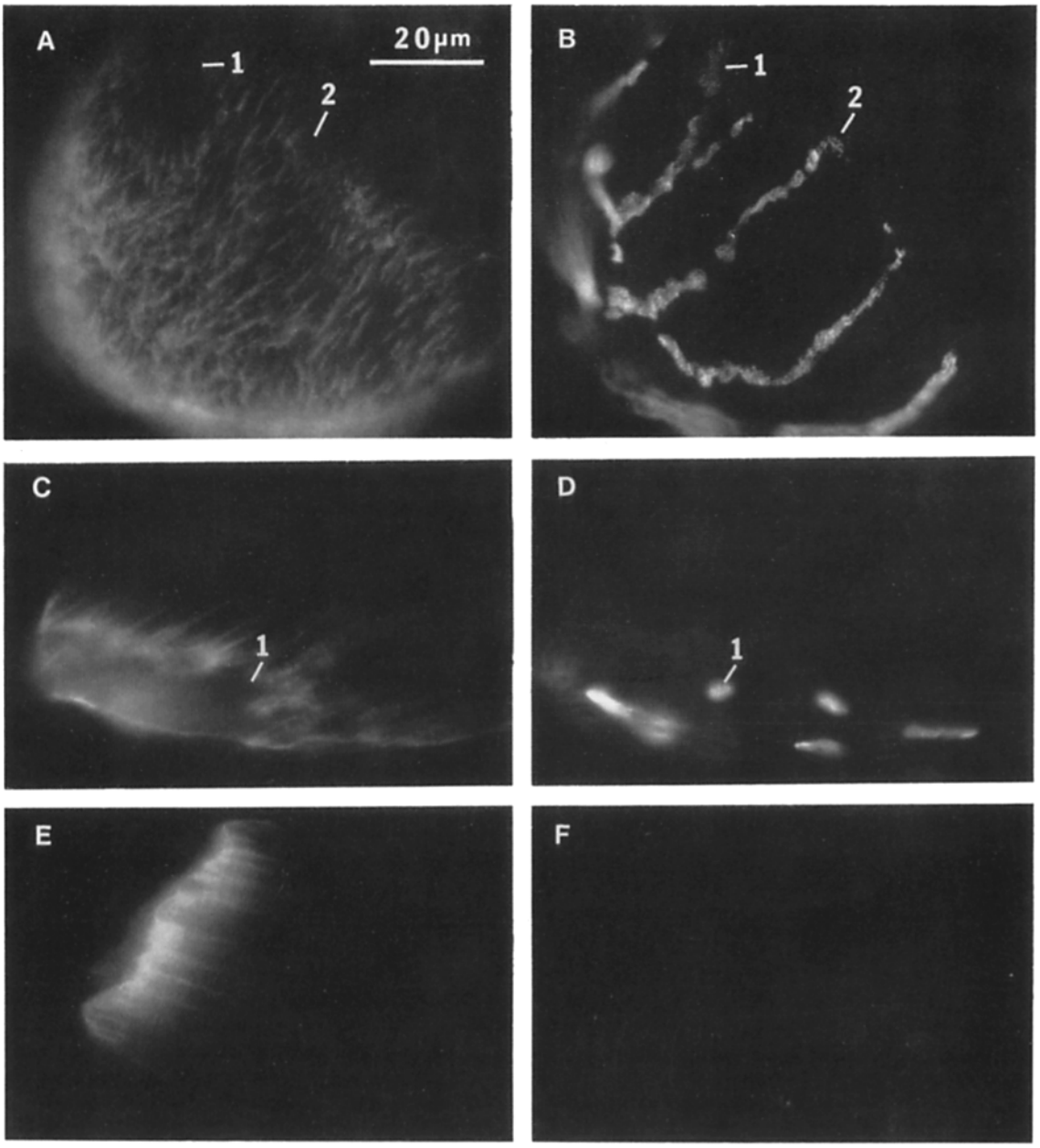

Figure 8. Comparison of the 48K protein distribution and AChR clusters at MTJ and NMJ of myotomal muscle fibers in whole mount. (Left column) 48K localization as shown by mAb 1351 staining; (right column) AChR clusters as shown by R-BTX staining. Each row represents a single fiber examined at the same focal plane. The numbers in *A-D* point to AChR patches located in membrane areas with a much reduced mAb 1351 staining. Note the fine structure of AChR clusters at the NMJ in *B*. Such fine structure was not seen in the 48K image (*A*). In *E* and *F*, a noninnervated end of a fiber is shown. Despite the absence of AChR clustering (*F*), the presence of the 48K protein at the MTJ remained prominent.

43K protein may be absent from early AChR clusters in *Torpedo* embryos (18). This suggests that the earliest assembly of AChRs may comprise of a mechanism independent of the 43K protein. Unlike the 43K protein, the concentration of the 48K antigen is independent of the AChR clustering (Fig. 8 *E* and *F*). This indicates that this protein does not interact directly with AChRs at all sites at which it is concentrated. The MTJ localization of the 48K protein suggests that the 48K protein may mediate the interaction between AChRs and an actin-based cytoskeleton at the postsynaptic membrane. In vitro studies have shown that an actin-based cytoskeleton may be the underlying mechanism for the stabilization of AChR clusters as recent studies by Bloch (2) have shown.

The presence of 48K protein at both MTJ and NMJ demonstrates further homology between these two sarcolemmal specializations. In cultured *Xenopus* myotomal cells, polycation-coated latex beads induce the formation of AChR clusters as well as a membrane domain with features similar to MTJ (25, 31). In this MTJ-like domain, membrane invaginations are formed and they are contacted by myofibrils

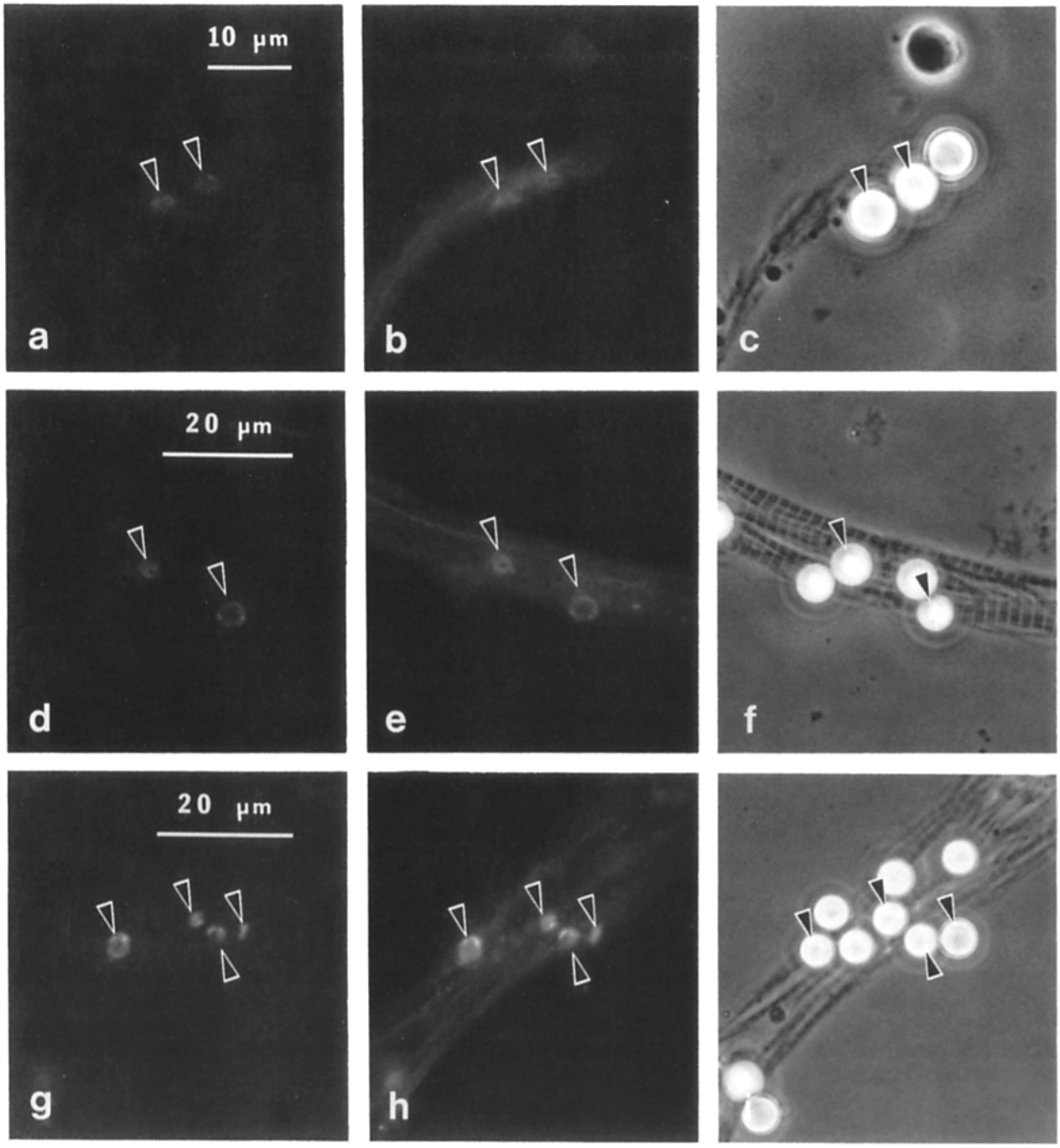

Figure 9. Presence of 48K protein at developing AChR clusters induced by polycation-coated beads. (*a*, *d*, and *g*) R-BTX images; (*b*, *e*, and *h*) FITC images of mAb 1351 staining; and (*c*, *f*, and *i*) phase-contrast images. Presence of 48K protein could be detected as early as 5 h after the onset of AChR clustering process (*a-c*). At 8 h of bead–muscle coculture, the mAb 1351 staining pattern closely resembled that of the AChR clusters (*d-f*). This similarity persisted at well-formed AChR clusters after 24 h of bead–muscle coculture (*g-i*). Arrowheads in *c*, *f*, and *i* point to beads that induced AChR clusters.

in a manner similar to that seen at the MTJ (see Fig. 10 in reference 31). Consistent with this observation is the fact that proteins associated with either the NMJ or MTJ, including AChRs, 43 and 48K proteins, talin and vinculin, are all concentrated at the bead-induced specializations (26, 31). Thus, in vitro, a common signal provided by polycation-coated beads stimulates the muscle cell to form both NMJ and MTJ specializations. In vivo, however, the muscle cell must receive two distinct signals for these two structures, since they develop at distinct sarcolemmal domains. The cell–cell interaction between nerve and muscle may provide the signal for synaptic differentiation, whereas the cell–extracellular matrix interaction may signal the MTJ development. Elucidation of the difference in the cellular response to these two signals may be essential to understanding the biogenesis of the NMJ. In addition, an understanding of the MTJ develop-

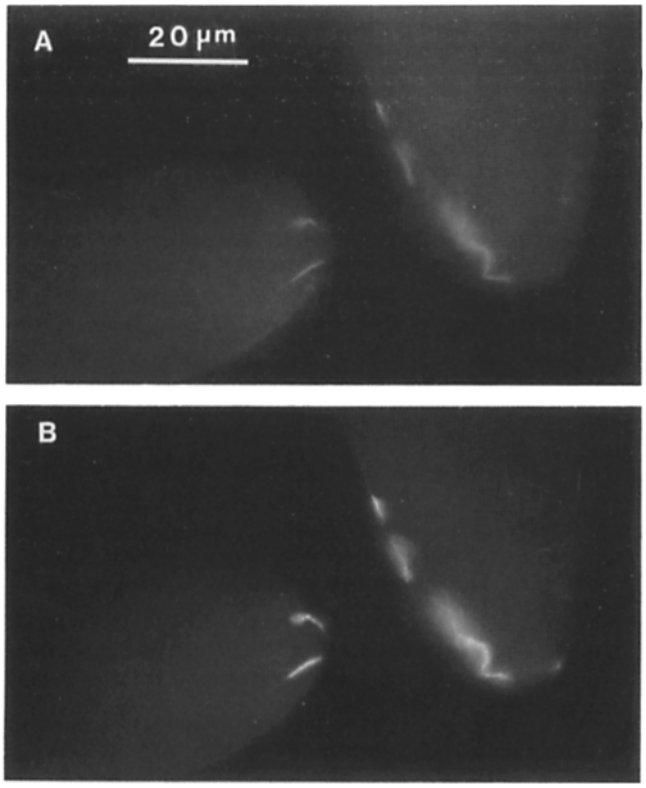

Figure 10. Colocalization of the 43K protein with AChR clusters at the NMJ. (*A*) 43K localization as shown by mAb 1234 staining; (*B*) AChR clusters as shown by R-BTX staining. Although the NMJs were present in the midst of the MTJ, the 43K protein was discretely localized at the NMJ, in sharp contrast to the 48K protein.

ment should provide valuable information on the assembly of the macromolecular complexes involved in the interaction between the cytoskeleton and the plasma membrane.

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