Confocal Laser Microscopy of Dystrophin Localization in Guinea Pig Skeletal Muscle Fibers

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Abstract. A confocal laser microscope was used to analyze the localization pattern of dystrophin along the sarcolemma in guinea pig skeletal muscle fibers. Hind leg muscles of the normal animals were freshly dissected and frozen for cryostat sections, which were then stained with a monoclonal antidystrophin antibody. In confocal laser microscopy, immunofluorescence staining in relatively thick sections could be sharply imaged in thin optical sections. When longitudinal and transverse sections of muscle fibers were examined, the immunostaining of dystrophin was seen as linearly aligned fluorescent dots or intermittent lines along the sarcolemma. In longitudinally cut muscle fibers, many fluorescent dots, but not all, corresponded to the sarcomere pattern, especially the I band. Sections cut tangential to the sarcolemma also showed a lattice-like pattern of longitudinal and transverse striations of fluorescent dots. Double staining for dystrophin and vinculin showed that the two proteins were not exactly colocalized. The end portions of muscle fibers were much more intensely stained with antidystrophin antibody than the central portions, following the contour of elaborate surface specializations at the myo–tendon junction. The staining pattern at the myo–tendon junction was also discontinuous. These confocal microscopic observations suggest that dystrophin may be localized in a nonuniform, discontinuous pattern along the sarcolemma and in some relationship with the underlying myofibrils.

UCHENNE muscular dystrophy (DMD) is an Xp21-linked recessive disease of muscle necrosis and wasting. This disease has recently been demonstrated to be caused by deficiency of a single gene product named dystrophin (Hoffman et al., 1987). Indeed, dystrophin is not detected in muscles of DMD patients (Sugita et al., 1988; Zubrzycka-Gaarn et al., 1988; Hoffman et al., 1988) and the mdx mice (Hoffman et al., 1987; Sugita et al., 1988). Since the cDNA sequence of dystrophin has been completely determined (Koenig et al., 1987), antibodies can be raised against various segments of the dystrophin molecule using its synthetic peptides (Hoffman et al., 1987; Sugita et al., 1988; Zubrzycka-Gaarn et al., 1988). It has been well documented from immunofluorescence studies that dystrophin is localized at the sarcolemma in normal skeletal muscle fibers (Sugita et al., 1988; Zubrzycka-Gaarn et al., 1988; Arahata et al., 1988). Immunoelectron microscopic studies have demonstrated the dystrophin localization on the cytoplasmic face of the sarcolemma (Watkins et al., 1988; Cullen et al., 1990; Wakayama and Shibuya, 1990, 1991), probably constituting the membrane cytoskeleton (Mandel, 1989). In conventional immunofluorescence microscopy, dystrophin is usually stained continuously along the sarcolemma, as described by many investigators. However some preparations with a monoclonal antidystrophin antibody showed a nonuniform and even discontinuous pattern of fluorescence staining along the sarcolemma. Hence, we used a confocal laser microscope to determine more precise pattern of the dystrophin staining.

Materials and Methods

Antibodies

The mAb (4-4C5) used was kindly provided by Ajinomoto Co. and Fujirebio Inc. (Tokyo, Japan). It was raised in a mouse against a synthetic polypeptide corresponding to the COOH-terminal region (amino acid residues from 3495 to 3544) of the human dystrophin sequence. FITC-labeled goat anti–mouse IgG (H+L) was purchased from Cappel Laboratories (Malvern, PA). Texas red–labeled goat anti–mouse IgG+IgM (E-Y Laboratories, Inc., San Mateo, CA) and FITC-labeled phalloidin (Sigma Chemical Co., St. Louis, MO) were used for double staining for dystrophin and F-actin. Polyclonal rabbit anti-vinculin antibody (Transformation Research, Inc., Framingham, MA) and FITC-labeled goat anti–rabbit IgG (H+L) (Wako Chemical Industries, Osaka, Japan) were used for double staining with antidystrophin.

Immunofluorescence Staining

Male guinea pigs of ~200 g body weight were used for this study. They were anesthetized with ether, and their hind leg muscles were dissected out. After cut into small blocks, they were pasted on disks of cork using tragacanth gum (Wako Chemical Industries). They were then frozen in isopentane cooled by liquid nitrogen within 3 min after dissection. Serial 8–10-μm sections from the guinea pig muscle blocks were cut in a cryostat and placed on gelatin-coated slide glasses. After being air dried at room temperature for 30 min, they were fixed with cold acetone for 10 min and

incubated with blocking solution of 0.1% casein and 0.1% gelatin in PBS, pH 7.2, at room temperature for 1 h. Then they were allowed to react with a monoclonal antidystrophin antibody (4-4C5, IgG) in PBS containing 1% BSA for 1 h. They were washed 3 times with PBS for 10 min each, incubated with FITC-labeled goat anti–mouse IgG (H+L) as a second antibody in PBS containing 1% BSA for 1 h, and then washed again in the same way. For double staining of dystrophin and F-actin, specimens were first immunolabeled with monoclonal antidystrophin antibody, treated with Texas red–conjugated goat anti–mouse IgG+IgM as a second antibody, followed by FITC-labeled phalloidin staining. For double staining of dystrophin and vinculin, specimens were immunolabeled with a mixture of monoclonal antidystrophin and polyclonal antivinculin antibodies, washed, treated with a mixture of Texas red–conjugated goat anti–mouse IgG+IgM and FITC-conjugated anti–rabbit IgG (H+L). The sections were mounted with 90% glycerol in PBS containing 5% 1,4-diazabicyclol octane (DABCO) (Aldrich Chemical Co., Milwaukee, WI) as an antiquenching agent.

Epifluorescence and Confocal Laser Microscopy

For conventional epifluorescence microscopy, sections were viewed using a Zeiss Axiophot (Carl Zeiss, Inc., Thornwood, NY) with an epifluorescence attachment and Plan-Apochromat oil immersion objective lens (100× 1.3 NA or 63× 1.4 NA). For confocal laser microscopy, a Bio-Rad Lasersharp MRC-600 (Bio-Rad Laboratories, Richmond, CA) fitted on a Zeiss Axiophot with the same lens was used.

Immunoblot Analysis

Cryostat sections were suspended in 10 vols of SDS solution containing 30 mM Tris-HCl, pH 6.8, 10% SDS, 2.5% β-mercaptoethanol, 5% glycerol, and then boiled for 3 min. SDS-PAGE was performed by the method of Laemmli in 6% acrylamide slab gels (Laemmli, 1975). For immunoblotting, the electrophoresed polypeptides were transferred onto Immobilon-p membrane (Millipore Corp., Bedford, MA) according to the method of Kyhse-Anderson (1984). Proteins on the membrane were allowed to react with the monoclonal antidystrophin antibody (4-4C5). The antibody was detected with an ABC kit (Vectastain; Vecter Laboratories, Inc., Burlingame, CA) and then with Immunostain HRP IS-50B (Konica, Tokyo, Japan). Molecular weight markers were 450 kD laminin A (Cooper et al., 1981), 205 kD myosin, 116 kD β-galactosidase, and 97.4 kD phosphorylase b (Sigma Chemical Co.).

Results

As shown by immunoblot analysis, antidystrophin antibody (4-4C5) used for this study stained a single band of guinea pig skeletal muscle extracts without nonspecific reaction. Its molecular mass was ∼400 kD as judged from its electrophoretic mobility (Fig. 1).

In conventional epifluorescence microscopy, transverse sections of normal skeletal muscle fibers showed a selective immunostaining of dystrophin along the sarcolemma (Fig. 2 A). The staining was usually seen as continuous lines along each muscle fiber. However, when we looked carefully at those fluorescent lines, they were not uniformly stained, showing intermittent or periodic staining patterns. There was no significant immunostaining inside the muscle fiber. The control sections incubated in PBS containing 1% BSA without antidystrophin antibody did not show any staining along the sarcolemma (data not shown).

For further analysis of such a nonuniform appearance of immunostaining, we took advantage of using a confocal laser microscope (Fig. 2 B). In confocal laser microscopy, sharp optical section images were obtained from relatively thick sections. In transverse sections two apposed sarcolemmas of adjacent muscle fibers could be almost always recognized. The staining was discontinuous, showing linearly aligned dots or intermittent lines along the sarcolemma. In some parts the interval of such dots was quite regular. Such discon-
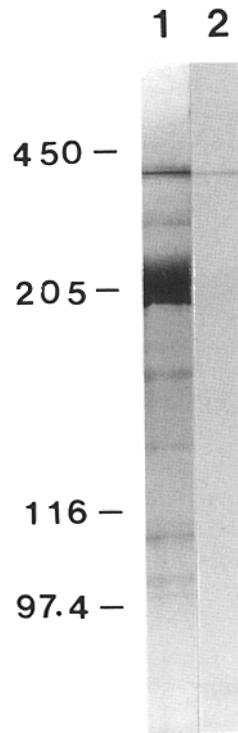

Figure 1. Immunoblot analysis of antidystrophin antibody 4-4C5. Normal guinea pig hind leg muscle extract was electrophoresed. (Lane 1) Coomassie brilliant blue stain. Numbers on the left indicate molecular masses of marker proteins in kD. (Lane 2) Immunoblot using mAb 4-4C5. Only a single protein band of ∼400 kD is stained.
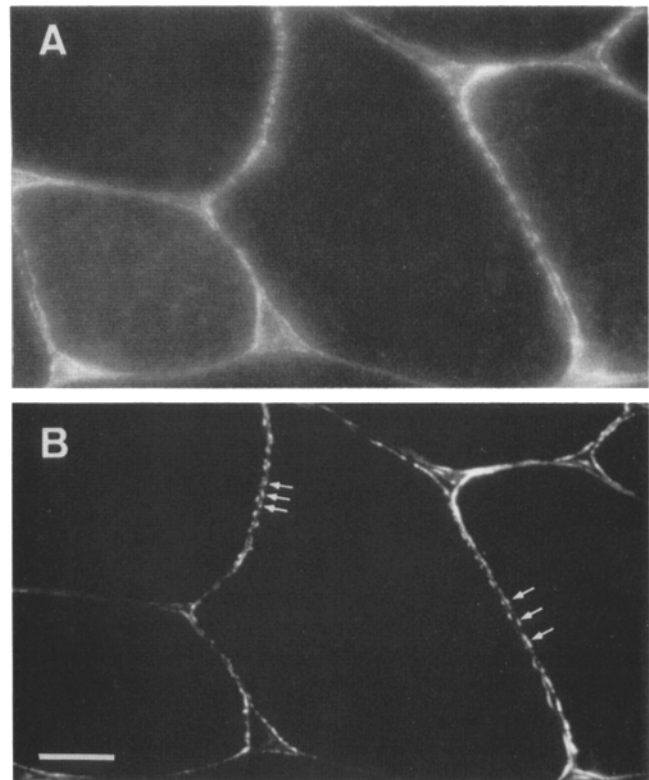

Figure 2. Comparison between a conventional epifluorescence (A) and a confocal laser microscope image (B) of the same transverse section stained with antidystrophin. The nonuniform staining pattern is more prominently recognized in the confocal image, in which the sarcolemmal staining appears as linearly aligned dots or intermittent lines (arrows). In some parts, the dots are regularly spaced. Bar, 10 µm.

tinuous staining could be observed in any optical sections within the whole thickness of a section (data not shown).

In longitudinal sections, dystrophin immunostaining also appeared as intermittent lines along the sarcolemma (Fig. 3). The center-to-center distance between fluorescent dots was 1–3 μm. Regular cross striations were faintly observed inside the fibers, though the significance of such intracellular staining was not clarified. Interestingly, many fluorescent dots, but not all, corresponded to the sarcomere pattern. Double staining with antidystrophin antibody and phalloidin revealed that dystrophin staining along the sarcolemma was situated preferentially at the level of the I band of underlying myofibrils (Fig. 4, A–C). Phalloidin was used to stain the I bands, since it is a cyclic peptide that specifically binds to F-actin (Wulf et al., 1979).

The intermittent or periodic pattern of dystrophin staining was also seen in sections cut tangential to the sarcolemma, showing a lattice-like pattern on the sarcolemma (Fig. 5, A–C). Such staining pattern consisted of transverse and longitudinal striations, along which fluorescent dots were more or less regularly aligned. The transverse striations often appeared to correspond to the sarcomere pattern, while the longitudinal striations ran parallel to each other with nonuniform distances. When muscle fibers were immunostained with antivinculin, vinculin was also seen to be distributed in transverse striations along the sarcolemma. The striations of vinculin were regularly spaced, well corresponding to the sarcomere pattern. Double staining with antivinculin and antidystrophin antibodies clearly showed that both proteins were not exactly colocalized along the sarcolemma, though their fluorescence was partly superimposed (Fig. 6, A and B). Interestingly, dystrophin was less regularly spaced and more densely distributed than vinculin.

At the end portions of muscle fibers, dystrophin was stained much more intensely than at the central portions (Fig. 7 A). The staining was seen as thick lines along the sarcolemma following the contour of elaborate processes and invaginations characteristic of the myo–tendon junction. In confocal laser microscopy, the staining of dystrophin was

also nonuniform, intermittent along the sarcolemma, though the fluorescence was much broader and more continuous in the end portion than in the central one (Fig. 7 B). Cellular profiles in the tendon were weakly fluorescent in the same preparations. However, the control sections without the first antibody also showed weak staining of cellular profiles in the tendon (data not shown), suggesting that this staining is nonspecific.

Discussion

The present study has demonstrated the discontinuous, intermittent pattern of immunostaining for dystrophin localization along the sarcolemma in normal skeletal muscle fibers as revealed by confocal laser microscopy. The confocal laser microscope offers improved rejection of out-of-focus noise, thus, providing a sharper image of any given focus plane with greater resolution than conventional imaging (White et al.,
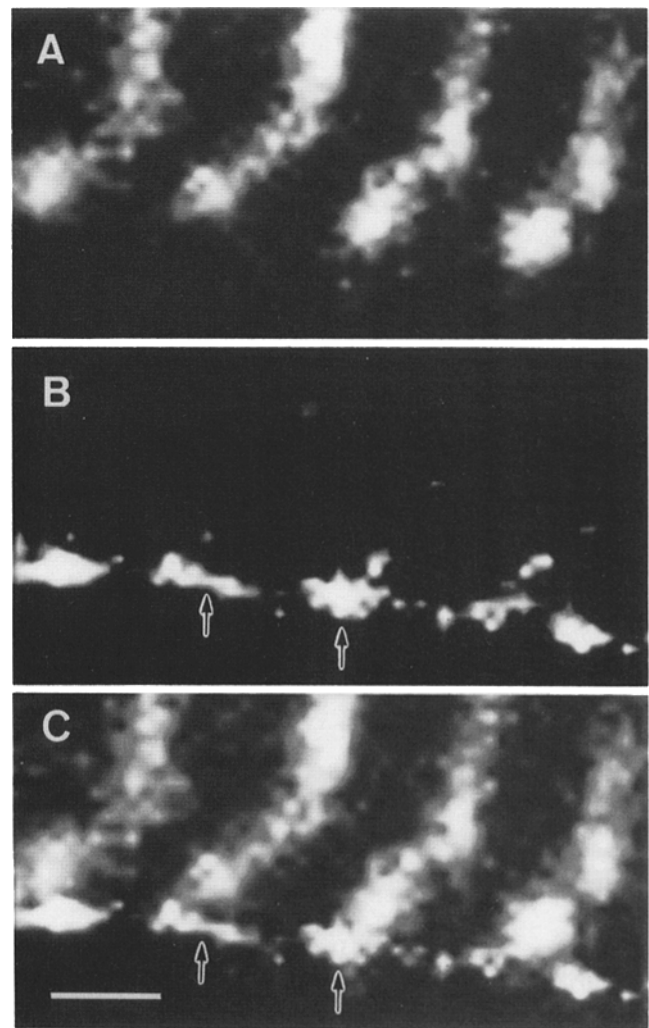
Figure 4. Confocal laser micrographs of the same section double stained with FITC-phalloidin (*A*) and Texas red–labeled antidystrophin (*B*). Dystrophin staining image is superimposed onto the same field of phalloidin staining (*C*). The dot-like staining of dystrophin along the sarcolemma (*arrows*) is found to locate at the same level of the phalloidin-stained I band. Bar, 1 μm.
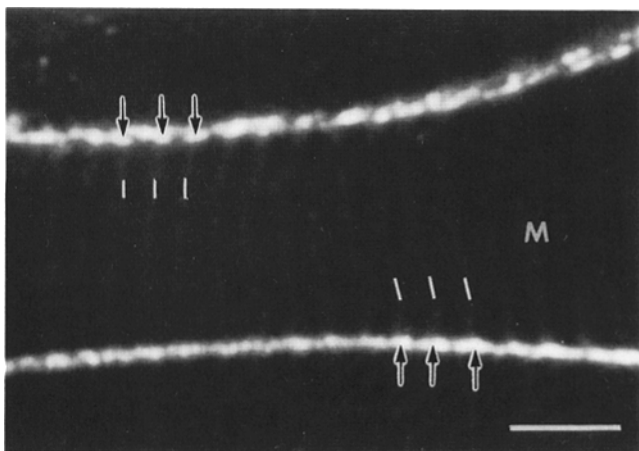
Figure 3. Confocal laser image of a longitudinal section of the muscle fibers stained with antidystrophin. Regular cross striations are faintly observed inside the fibers (*lines*), corresponding to the dystrophin staining along the sarcolemma (*arrows*). *M*, muscle fiber. Bar, 5 μm.
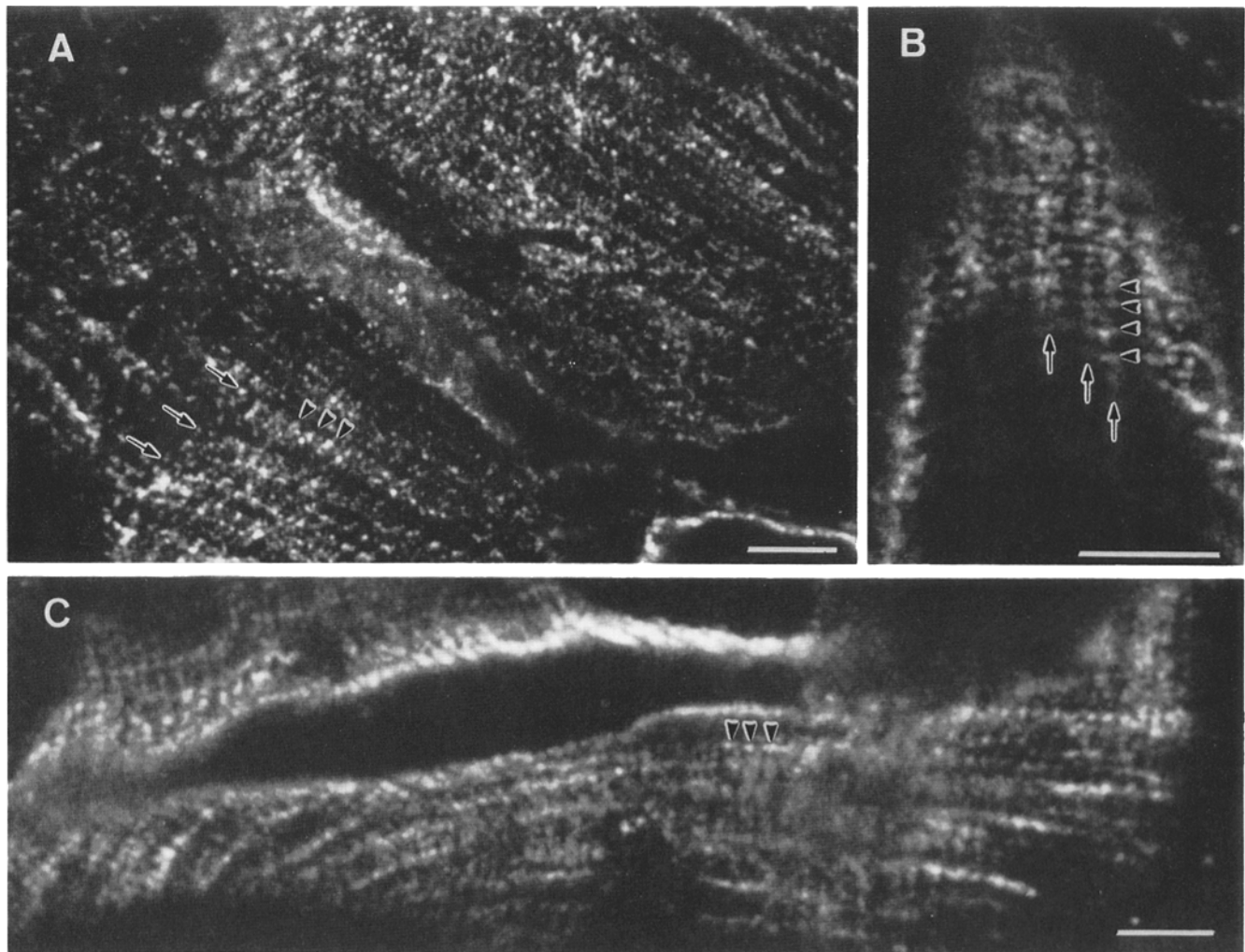
Figure 5. Confocal laser micrographs of sections cut tangential to the surface of muscle fibers stained with antidystrophin (*A–C*). Sarcomere-like cross-striated pattern is seen on the sarcolemma (*arrowheads*), while the longitudinal striations (*arrows*) run parallel to each other. Bars, 5 μm.

1987). Furthermore, such optical sectioning can minimize mechanical damages that might be caused by cutting thinner sections from frozen tissue blocks. The present observation suggests that dystrophin may not be uniformly distributed beneath the sarcolemma and may be spatially associated with the underlying myofibrils.

The dystrophin localization at the sarcolemma has been well documented (Sugita et al., 1988; Zubrzycka-Gaarn et al., 1988; Bonilla et al., 1988; Arahata et al., 1988, 1989; Shimizu et al., 1989; Samitt and Bonilla, 1990; Tanaka et al., 1990). Many investigators described the continuous and uniform immunostaining of dystrophin along the sarcolemma using various antidystrophin antibodies. To our knowledge, no particular attention has been paid to the discontinuous pattern of immunofluorescence along the sarcolemma in normal human and animal skeletal muscle fibers. Interestingly, favorable sections in our preparations showed a discontinuous pattern of immunostaining even under a conventional microscope. Such a discontinuous pattern of staining was much more prominently demonstrated by confocal laser microscopy.

The question naturally arises if such discontinuous stain-ing may be an artifact. Several candidates are conceivable for the possible sources of the artifact. First, the mAb (4-4C5) used may cause a peculiar reaction resulting in the observed staining pattern. It has been reported that a gene is expressed in muscles whose 3' terminal is highly homologous to the 3' terminal of dystrophin gene (Love et al., 1989). The product of the gene was named dystrophin-related protein. On Western blots it binds to a 400-kD protein in DMD patient and mdx mouse muscle as well as normal human and mouse muscle (Tanaka et al., 1991; Ohlendieck et al., 1991; Man et al., 1991). However, 4-4C5 which was raised against the COOH-terminal region of dystrophin stained a 400-kD band in skeletal muscle samples from control human, but not in those from DMD patients (Tanaka and Ozawa, 1990; Tanaka et al., 1991). This data showed that 4-4C5 was specific to dystrophin and did not react to the dystrophin-related protein. Our Western blot analysis for guinea pig skeletal muscle also showed that 4-4C5 only reacted to the 400-kD dystrophin band specifically and did not cross react with any other protein bands, including α-actinin or spectrin. In addition, the same immunofluorescence result was obtained
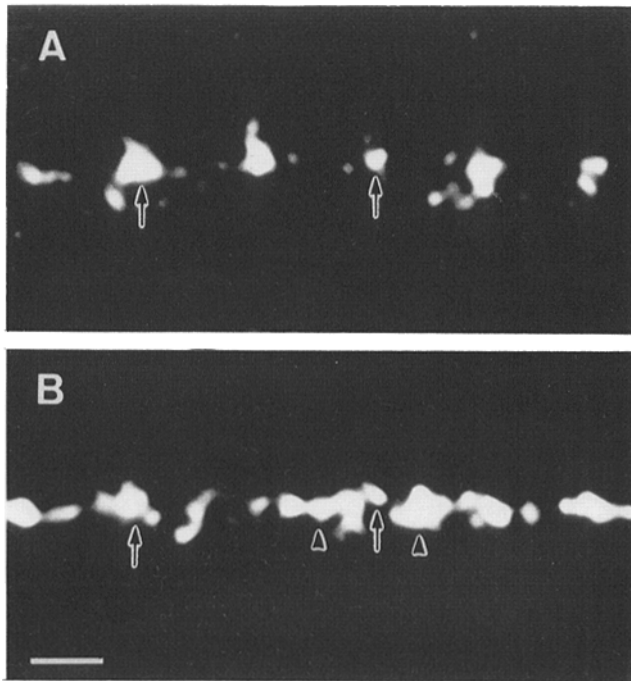
Figure 6. Confocal laser micrographs of the same section double stained with FITC-labeled antivinculin (*A*) and Texas red–labeled antidystrophin (*B*). Dystrophin and vinculin are colocalized at some spots (*arrows*). But there are other spots where they are not colocalized (*arrowheads*). Bar, 1 μm.

using a polyclonal antibody raised in a rabbit against synthetic fusion peptides containing the COOH-terminal region (kindly provided by Dr. Y. Nonomura, University of Tokyo).

Secondly, our samples were prepared from unfixed muscle tissues as in many other studies for conventional immunofluorescence microscopy of dystrophin location. The mAb we used did not stain the formaldehyde-fixed muscle fibers. One might wonder if the sarcolemma in such unfixed muscle fibers have been damaged during the sample preparation, thus resulting in the discontinuous pattern of staining. However, the staining was not random, often showing a lattice-like pattern of transverse and longitudinal striations. Furthermore, without the air-drying step before acetone treatment, we could obtain the same results. This may reasonably exclude the possibility that the discontinuous pattern is caused by reorganization of the membrane cytoskeleton during the air-drying step. Acetone treatment was applied to fix and permeabilize the tissue sections. Even if the sarcolemma has been damaged by the acetone treatment, it is still possible that the sarcolemma or dystrophin-associated structures beneath the sarcolemma might be preferentially preserved in a nonrandom pattern. The membrane preservation often depends on the occurrence of underlying cytoskeletal components, namely the plasmalemmal undercoat (Ishikawa, 1988). The plasmalemmal undercoat may provide the structural support for the membrane and also serve as the link between the plasmalemma and cytoplasmic fibrous structures. Hence, the observed discontinuous staining of dystrophin may be significant, for dystrophin is considered to constitute the membrane skeleton (Mandel, 1989) or the plasmalemmal undercoat. Such cytoskeleton may be unevenly distributed along the sarcolemma.
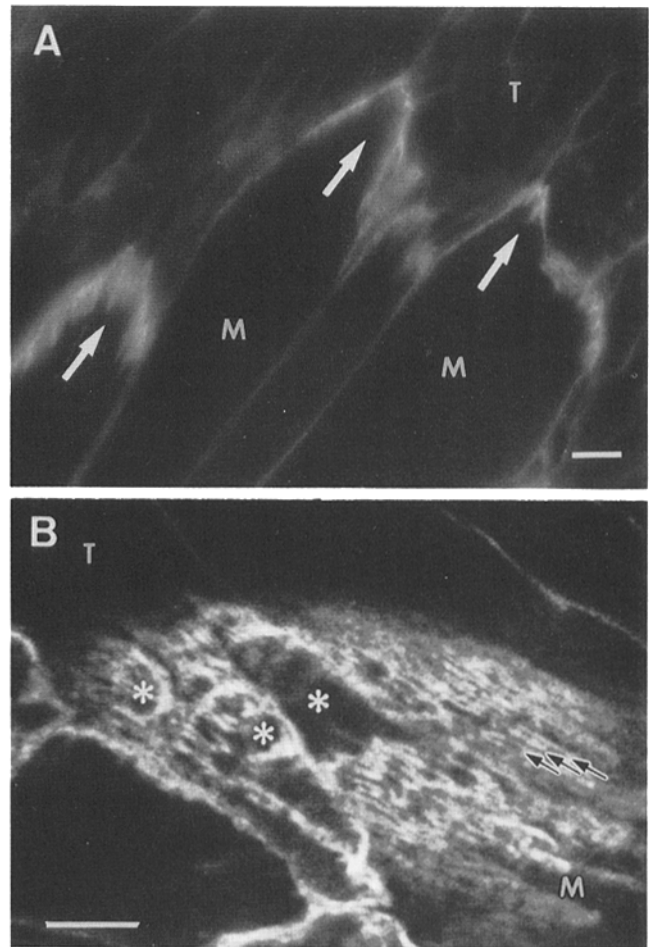

Figure 7. Conventional epifluorescence (*A*) and confocal laser micrograph (*B*) of the myo–tendon junction in a guinea pig hind leg muscle stained with antidystrophin. (*A*) Intense immunostaining is observed at the ends (*arrows*) of muscle fiber (*M*), revealing the elaborate configuration. (*B*) The ends of muscle fibers are intensely stained with a nonuniform pattern. Asterisks represent cut ends of sarcoplasmic processes of the fiber ends. Longitudinal striations (*arrows*) are also prominent on the lateral surface of the muscle fiber (*M*). *T*, tendon tissue. Bars: (*A*) 10 μm; (*B*) 5 μm.

Interestingly, in longitudinal sections the dystrophin staining pattern appeared to correspond to the cross striation of the underlying myofibrils. The center-to-center distance between the fluorescent dots was 1–3 μm. The superimposition analysis of double-stained samples with the confocal laser microscope clearly indicated that the fluorescent dots along the sarcolemma tended to be situated at the same level of the I band which was stained with fluorescent dye-labeled phalloidin. This implies that dystrophin may be located in the structure which is preserved during our specimen preparation and is situated preferentially at the level of the I band or Z disc. In immunoelectron microscopic studies, the occasional periodicity of dystrophin labeling on the sarcolemma has been pointed out (Watkins et al., 1988; Cullen et al., 1990). The periodicity was ~125 nm in both transverse and longitudinal sections. This value corresponds well with the estimated length of a dystrophin molecule (Koenig et al., 1988) but not with the length of the sarcomere or the gap between adjacent myofibrils.

The present study also demonstrated the intense immunostaining of dystrophin along the sarcolemma at the myo–tendon junction, confirming the recent observations (Shimizu et al., 1989; Samitt and Bonilla, 1990). On the other hand, EM revealed that most of the sarcolemma at the myo–tendon junction is lined with a layer of dense material as we call the plasmalemmal undercoat (Ishikawa, 1988). In our correlated confocal laser and thin section EM, the immunofluorescence of dystrophin and the electron-dense undercoat showed the same distribution at the ends of muscle fibers (our own unpublished data). Clearly, dystrophin stain was localized at the dense undercoat at the end portion of the muscle fiber. At the myo–tendon junction the undercoat possesses two domains: a myofibril-anchored domain and an intermediate filament-associated domain (Ishikawa, 1988). It is interesting to ask how dystrophin is associated with such domains at the myo–tendon junction. In this connection, the observation deserves attention to the fact that the sarcolemma in the central portion of the muscle fiber is also lined with the electron-dense undercoats in a scattered manner. Such undercoats appear to serve as the attachment sites for intermediate filaments, which run transversely to be associated with myofibrils at the Z disc level. However, it has not been decided how the intermittent pattern of dystrophin localization is related to the undercoat in the central portion of the muscle fiber.

Vinculin-containing cortical lattice named costamere has been reported to be localized at the sarcolemma. In the longitudinal sections of muscle fibers, the costameres always overlie the I band (Pardo et al., 1983a,b; Shear and Bloch, 1985; Tokuyasu, 1989). In the present study, double immunostaining for dystrophin and vinculin revealed that the two proteins were not exactly colocalized. Further studies are needed to clarify how dystrophin is related to the vinculin-containing costameres.

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