The Subcellular Distribution of Dystrophin in Mouse Skeletal, Cardiac, and Smooth Muscle

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Abstract. We use a highly specific and sensitive antibody to further characterize the distribution of dystrophin in skeletal, cardiac, and smooth muscle. No evidence for localization other than at the cell surface is apparent in skeletal muscle and no 427-kD dystrophin labeling was detected in sciatic nerve. An elevated concentration of dystrophin appears at the myotendinous junction and the neuromuscular junction, labeling in the latter being more intense specifically in the troughs of the synaptic folds. In cardiac muscle the distribution of dystrophin is limited to the surface plasma membrane but is notably absent from the membrane that overlays adherens junctions of the intercalated disks. In smooth muscle, the plasma membrane labeling is considerably less abundant than in cardiac or skeletal muscle and is found in areas of membrane underlain by membranous vesicles. As in cardiac muscle, smooth muscle dystrophin seems to be excluded from membrane above densities that mark adherens junctions. Dystrophin appears as a doublet on Western blots of skeletal and cardiac muscle, and as a single band of lower abundance in smooth muscle that corresponds most closely in molecular weight to the upper band of the striated muscle doublet. The lower band of the doublet in striated muscle appears to lack a portion of the carboxyl terminus and may represent a dystrophin isoform. Isoform differences and the presence of dystrophin on different specialized membrane surfaces imply multiple functional roles for the dystrophin protein.

DYSTROPHIN is a large (427 kD) protein that is absent or at very low levels in individuals suffering from Duchenne muscular dystrophy (Hoffman et al., 1987*a*; Hoffman et al., 1988*a*). It is present in all types of muscle (Hoffman et al., 1988*b*) and immunocytochemical studies using light microscopy have localized the protein in skeletal muscle to the sarcolemma (Arahata et al., 1988; Bonilla et al., 1988; Zubrzycka Gaarn et al., 1988). EM has shown that dystrophin is closely apposed to the cytoplasmic surface of the plasma membrane (Watkins et al., 1988; Carpenter et al., 1990; Cullen et al., 1990) and it has been shown biochemically to associate tightly with a complex of membrane glycoproteins (Campbell and Kahl, 1989). This localization, the fact that membrane defects characterize Duchenne muscular dystrophy (see Rowland, 1980 for review), and sequence similarities with other structural proteins such as alpha-actinin and spectrin have led to speculation that dystrophin is an important structural component of a membrane skeleton that provides resilience to the plasma membrane during cycles of contraction and relaxation (Brown and Hoffman, 1988; Koenig and Kunkel, 1990).

In contrast with most immunocytochemical studies which do not show labeling of structures internal to the surface membrane, biochemical fractionation studies have indicated that dystrophin cofractionates with transverse tubule membranes (Hoffman et al., 1987*b*), and particularly with triads (Knudson et al., 1988; Salviati et al., 1989). These findings are important to confirm since the transverse tubules are not subjected to the same deformations as the sarcolemma, and the presence of dystrophin may suggest a different role for the protein in this location as compared to the proposed strengthening role at the plasma membrane. Such a conclusion is also implied by the recent localization of dystrophin to specific subsets of neuronal cells in the central nervous system (Lidov et al., 1990), another tissue that does not undergo dynamic changes in shape.

Additional evidence indicating a potentially more complex functional role for dystrophin originates from studies of skeletal muscle in which the neuromuscular junction and myotendinous junction have been reported to show increased dystrophin labeling as compared to the rest of the sarcolemma (Miike et al., 1989; Shimizu et al., 1989; Fardeau et al., 1990; Samitt and Bonilla, 1990). However, in two studies some labeling of the same structures was found in the dystrophin-deficient *mdx* mouse (Fardeau et al., 1990; Samitt and Bonilla, 1990), and dystrophin-deficient controls were not utilized in a third (Shimizu et al., 1989). In fact, Fardeau et al. (1990) clearly establish the likely presence of

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a protein that cross reacts with dystrophin antibodies, probably the dystrophin-related protein (Love et al., 1989; Khurana et al., 1990) that has recently been shown to be present at high concentrations in neuromuscular junctions of the *mdx* mouse (Khurana et al., 1991).

The dystrophin gene is transcribed into multiple RNA isoforms, some of which show a distinctive tissue specificity (Chelly et al., 1988; Feener et al., 1989; Nudel et al., 1989). Corresponding isoforms at the protein level have not been shown because of difficulties in resolving small changes in the relative mobility of such a large protein with SDS-PAGE, and because of a lack of isoform-specific probes. Dystrophin separates into two bands on western blots of mouse skeletal and cardiac muscle under conditions of adequate resolution, and a single band is seen in smooth muscle that was reported to correspond to mobility to the lower skeletal muscle band (Hoffman et al., 1988*b*). On this basis it was proposed that the lower band in skeletal muscle might be a smooth muscle isoform due to the presence of vascular tissue in the skeletal muscle sample.

The object of this study was to use a new, highly specific antibody to reevaluate the distribution of dystrophin in skeletal muscle and to define its ultrastructural distribution in other muscle types and peripheral nerve. This is important in light of both cardiac and gastrointestinal involvement in Duchenne muscular dystrophy (see Emery, 1988 for review). In addition, we have optimized the resolution of dystrophin on SDS-PAGE/Western blots and examine it for isoform differences in the same tissues. We find that dystrophin is differentially distributed with respect to membrane specializations in all muscle tissues, and interpret this to imply the likelihood of more than one functional role for the protein.

Materials and Methods

SDS-PAGE, Antibodies, and Immunoblots

Tissues were frozen in liquid nitrogen, homogenized without prior thawing in sample buffer (60 mM Tris-PO_4, pH 6.8, 2.5% [wt/vol] SDS, 10 mM EDTA, 50 mM DTT, 40 μg/ml PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1.5 μg/ml aprotinin, and 10% glycerol), and were boiled for 4 min. Gel samples were equalized according to total protein using a dotblot/amido black staining procedure as described by Nakamura et al. (1985). Electrophoresis was carried out using the Hoefer (San Francisco, CA) Mighty Small slab gel apparatus and the buffer system described by Laemmli (1971) with the addition of 10 mM 2-mercaptoethanol to the running buffer. The separating gel consisted of either 7% acrylamide, 0.08% N,N'-methylene bisacrylamide or a 3.5–12.5% acrylamide (0.1% bis in each) gradient; in each case utilizing a 3% acrylamide, 0.08% bis stacking gel. Gels were transferred to nitrocellulose as described by Burnette (1981).

The production and affinity purification of antibody 6–10 was described in Lidov et al. (1990). Briefly, the antibody was produced in a rabbit immunized with a dystrophin polypeptide expressed in bacteria from dystrophin cDNA residues 6,181–9,544. Antibody D11 was described in Koenig et al. (1990) and was purified by affinity to a polypeptide that was identical in the extent of its dystrophin sequences to the original immunogen (cDNA residues 9,786–11,555). The polypeptide was produced in the pGEX vector as described for the antibody 6–10 immunogen, and as in that preparation, nondystrophin fusion sequences were removed before the coupling of the dystrophin polypeptide to Affi-Gel 10/15 affinity gel (Bio-Rad Laboratories, Richmond, CA).

Western blots were stained with Ponceau red S (Sigma Chemical Co., St. Louis, MO) to check protein loading, rinsed with distilled water, TBS plus 0.1% Tween-20, and then blocked in TBS plus 0.1% Tween-20 and 5% FCS. Blots were incubated with antibodies in TBS plus 0.1% Tween-20 and 5% FCS overnight at 4°C for the primary and 1 h for the secondary antibody (1:1,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG or donkey anti-sheep IgG; Sigma Chemical Co., St. Louis, MO), rinsing between steps in the same buffer without FCS. Blots were developed by incubation with 60 μg/ml nitro blue tetrazolium and 60 μg/ml 5 bromo-4-chloro-3-indolyl phosphate in 0.15 M Tris-HCl, pH 8.8.

Sample Preparation for Immunocytochemistry

Mice were killed by chloroform inhalation and dissected along their ventral midline, and fixed with 2% formaldehyde in 0.1 M PBS (pH 7.4) by ventricular perfusion. After perfusion, the heart, regions of duodenum, and the gastrocnemius, soleus, extensor digitorum longus, and superior rectus muscles were removed. The samples were fixed in 2% formaldehyde, 0.01% glutaraldehyde for an additional hour. Samples for EM were cut into 1-mm cubes and immersed in 2.3 M sucrose overnight. Samples for thick (5 μm) frozen sections were immersed in 30% sucrose overnight.

After overnight infiltration with 30% sucrose, tissue samples were mounted on filter paper and shock-frozen with CryoKwik (Damon) and stored at −80°C for sectioning. Samples for EM were oriented on cutting stubs and shock frozen in liquid nitrogen.

Immunocytochemistry

Frozen sections 5 μm thick were cut for light microscopy using an Ames Cryostat II. Sections were lifted onto poly-L-lysine-coated glass slides and maintained at −30°C until sectioning was complete. Sections were thawed and rehydrated with 0.1 M PBS, blocked using 5% normal goat serum in PBS, and labeled with antibody 6–10 at a dilution of 0.2 μg/ml in PBS. After three washes in PBS, the primary antibody was revealed using a 1:100 dilution of goat antirabbit rhodamine conjugate (Organon Teknika-Cappel, Malvern, PA) in PBS. After a 1-h incubation with the second antibody, the sections were washed three times in PBS, mounted in Gelvatol (Monsanto, St. Louis, MO) and coverslipped. All observations were with a Zeiss Photomicroscope III.

Thin sections (70–100 nm) were cut using a Reichert Ultracut E microtome (Reichert Scientific Instruments, Buffalo, NY) with an FC4D cryoattachment. Sections were lifted onto formvar-coated carbon grids and washed three times in 0.1 M PBS. Nonspecific antibody binding was blocked by subsequent washes in 0.1 M PBS containing 0.5% BSA and 0.15% glycine (buffer 1) followed by a 30 min incubation with 5% normal goat serum in buffer 1. After three further washes in buffer 1, sections were incubated in 1 μg/ml antibody 6–10 in buffer 1 for 30 min, washed three times in buffer 1, then incubated in 5-nm goat antirabbit gold conjugate (Amersham Corp., Arlington Heights, IL) in buffer 1. They were then washed three times in buffer 1, three times in PBS, three times in H_2O and counterstained with neutral 2% uranyl acetate, followed by three washes in H_2O and stained for a further 2 min in acidic 2% uranyl acetate. The grids were mounted in 1.15% methyl cellulose in distilled H_2O and dried. All electron microscopic observations were with a Jeol 100-CX II. Micrographs were taken at a magnification of 29,000×.

Results

Western Blot Analysis

The high specificity of antibody 6–10 for dystrophin in mouse skeletal, cardiac, and smooth muscle is demonstrated in Fig. 1 *a*. No labeling was observed in muscle tissues of the dystrophin-deficient *mdx* mouse. Antibody 6–10 is directed against a portion of dystrophin that is downstream from the stop codon in the *mdx* mutation (Sicinski et al., 1989), and consequently should not recognize even partial products of dystrophin in this mutant. We did not observe a band of similar mobility to dystrophin in sciatic nerve, but a band is seen at ~105 kD in this tissue from both normal and *mdx* mice.

Dystrophin characteristically appears as a doublet in skeletal and cardiac tissue under conditions that are adequate to resolve the two bands (Hoffman et al., 1988*b* and Fig. 1, *a*, *b*, and *d*). The stoichiometry of the two bands is similar from muscle prepared in different ways, using fresh or frozen

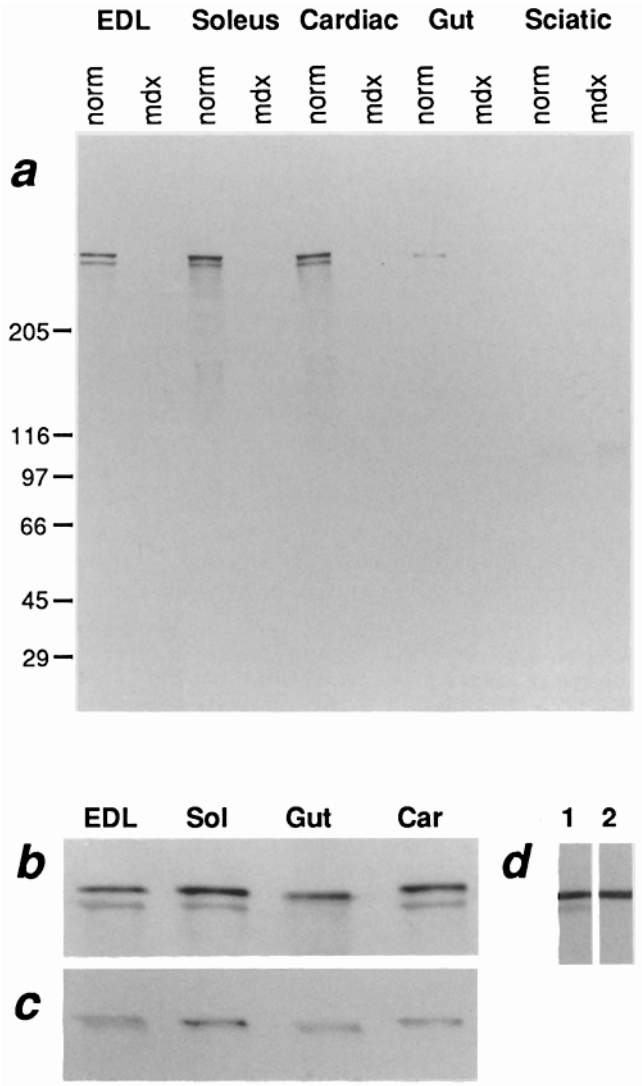


Figure 1. Western blot analysis of dystrophin in mouse tissues. To demonstrate the specificity of antibody 6–10, (*a*) gel samples of muscle and nerve tissue as indicated (*norm*, normal mice; *mdx*, dystrophin deficient mice; *EDL*, extensor digitorum longus) were equalized according to total protein content, electrophoresed on 3.5–12.5% acrylamide gradient gels, and then transferred to nitrocellulose. The blot was probed with 0.3 μg/ml antibody 6–10. *b–d* compare the reactivity of antibody 6–10 and carboxyl-terminal antibody D11 with mouse muscle dystrophin. Gel samples were adjusted for comparable dystrophin staining and run on a 7% acrylamide/0.08% bis gel before transfer to nitrocellulose. *b* and *c* are identical blots of the indicated tissues (*Sol*, soleus; *Car*, cardiac) probed with (*b*) 0.3 μg/ml antibody 6–10 and (*c*) 0.6 μg/ml antibody D11. To show that the upper antibody 6–10 reactive band corresponds to the single D11 reactive band, in *d* adjacent strips from a curtain gel of mouse heart tissue are stained as in *b* and *c* with (lane *1*) antibody 6–10 and (lane *2*) antibody D11.

tissue, with or without denaturing conditions in the initial homogenization, and with different mixes of protease inhibitors (not shown). Thus, we believe that the lower band is probably an isoform of dystrophin. Messenger RNA isoforms that could explain such differences in molecular mass have been described (Feener et al., 1989). Alternatively, if the lower band is a degradation product, the proteolytic cleavage must be very rapid, specific, and must occur in a characteristic subset of molecules (otherwise the stoichiometry might be expected to vary with the preparation). In any case, such a cleavage would probably have physiological significance, as with the limited cleavage of spectrin (fodrin) by calpain (Harris and Morrow, 1990).

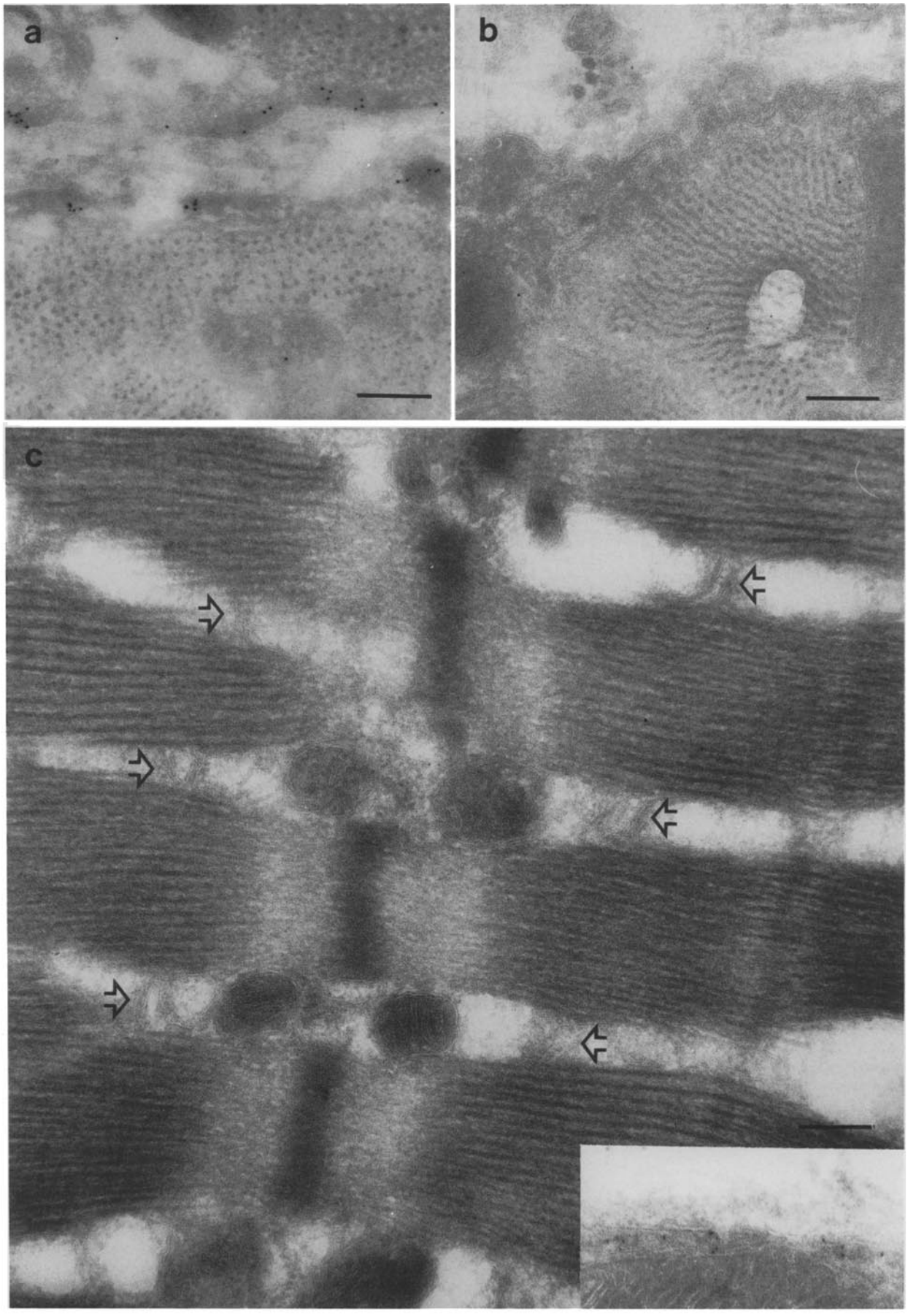
We have used region-specific antibodies to determine which region of dystrophin might be missing from the lower polypeptide band of the dystrophin doublet. The lower band is not recognized by antibody D11 (Koenig and Kunkel, 1990) that was made against the carboxyl-terminal 486 amino acid residues of dystrophin (Fig. 1, *c* and *d*, lane *2*), but is recognized by antibody 6–10 which is directed against the distal portion of the dystrophin repeat domain (Koenig et al., 1988). The anti-30-kD and anti-60-kD antibodies against the middle and proximal regions of the repeat domain also recognize the lower band of the doublet (Hoffman et al., 1987*a* and data not shown). Therefore, based on our inability to detect the lower band with antibody D11, we presume that the difference in mobility between the two bands is accounted for by the absence of carboxyl-terminal sequences in the lower form.

Intestinal smooth muscle displays a single dystrophin band of lower intensity than that of skeletal and cardiac muscle (Fig. 1 *a*) when equalized for total protein. This band runs with a slightly lower apparent molecular mass than the upper band of the skeletal muscle doublet (Fig. 1, *b* and *c*), but the two bands cannot be resolved in mixtures of the two samples (data not shown). One possible explanation for the apparent difference in mobility could be differences in the composition of the two samples. In contrast to the conclusions of Hoffman et al. (1988*b*), it is clear that smooth muscle dystrophin is more similar in molecular mass to the upper rather than the lower band of the striated muscle doublet.

Immunocytochemical Analysis

Skeletal Muscle. Immunofluorescence microscopy of normal skeletal muscle with antibody 6–10 shows a typical membrane localization for dystrophin with no labeling apparent on internal structures of the muscle fibers. Also, no labeling is seen in *mdx* mouse muscle (data not shown). At the electron microscope level (Fig. 2 *a*), labeling is also concentrated at the plasma membrane and does not appear to be associated with other membranous structures near the surface or to extend within the muscle fiber. No labeling is seen in the equivalent *mdx* tissue (Fig. 2 *b*). When muscle is cut at a slightly oblique angle, it is possible to visualize multiple triadic structures between myofibrils (Fig. 2 *c*). In such sections no labeling is seen with antidystrophin at transverse tubule membranes within the muscle (Fig. 2*c*, *open arrows*), though labeling associated with the surface membrane of the same fiber in the same section is strong (Fig. 2 *c*, *inset*).

When subcellular specializations of skeletal muscle are examined, a considerable increase in the concentration of antidystrophin labeling is seen at the myotendinous junction of normal muscle (Fig. 3 *a*). This label appears to extend further into the muscle, away from the plasma membrane, consistent with the increased subplasmalemmal density of the myotendinous junction. When the myotendinous junction in the *mdx* mouse is examined using the same concentration of antibody, no labeling is apparent (Fig. 3 *b*).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The neuromuscular junction in the normal mouse also reacts with antibody 6-10 (Fig. 3 *c*). We used one of the small muscles of the eye, the superior rectus, to maximize the number of end plates examined. We find that the crests of the extensively folded neuromuscular junction membrane reproducibly lack antidystrophin labeling. Labeling is consistently found in the troughs and appears more intense than that of the general plasma membrane. The same results were observed in occasional endplates found in extensor digitorum longus muscle tissue. In the neuromuscular junction of the *mdx* mouse, no labeling is apparent under the same conditions (Fig. 3 *d*).

Peripheral Nerve. No reactivity with antibody 6-10 was found in immunocytochemistry of normal or *mdx* mouse sciatic nerve (Fig. 3 *e*). Western blots also showed an absence of immunoreactivity in the expected region for full-length dystrophin. A faint band was seen, however, at ~105 kD in both normal and *mdx* mouse (Fig. 1 *a*). This band could be a cross-reactive protein, or it could be an alternate product of the dystrophin gene such as that described by Bar et al., (1990). It is possible that this polypeptide is distributed diffusely in the cytoplasm, explaining the lack of labeling in tissue sections.

Cardiac Muscle. Fluorescence microscopy shows that dystrophin labeling clearly extends about the periphery (Fig. 4 *a*) and along the length of the cardiac myocyte (Fig. 4 *c*). No labeling is seen in cardiac tissue of the *mdx* mouse (Fig. 4 *b*), except for occasional positive fibers that are thought to be reversions of the *mdx* mutation (data not shown, see Hoffman et al., 1990). Occasional unlabeled myocytes may be seen within the muscle of normal mice (one or two per section of whole mouse ventricle; Fig. 4, *c* and *d*).

At the electron microscope level, dystrophin is limited to the plasma membrane of the cardiocyte (Fig. 4 *e*). The plasma membrane in the region of the intercalated disk is not labeled, however, and no labeling is seen on any of the membrane systems within heart muscle such as transverse tubules or triads (Fig. 4 *f*). In the *mdx* mouse, no labeling of any kind is apparent within cardiac muscle at the EM level (data not shown).

Smooth Muscle. Light microscopy shows discontinuous labeling of the surface membrane of smooth muscle cells. In transverse section (Fig. 5 *a*, *open arrows*), the labeling appears punctate, and in longitudinal section (Fig. 5 *a*, *closed arrows*), bars are seen that appear to run the length of the cells. No labeling is present in the *mdx* mouse (Fig. 5 *b*).

At the electron microscope level, the labeling for dystrophin in smooth muscle is significantly less than that found for skeletal or cardiac muscle and only occurs near the plasma membrane, but is frequently found at a somewhat greater distance from the membrane than in skeletal or cardiac muscle. An example of what is considered strong labeling of smooth muscle is shown in Fig. 5 *c*. A patchy distribution is seen that is consistent with the immunofluorescence labeling. Labeling seems to be excluded from regions of membrane underlain by dense plaques, and present below the membrane of intervening regions. It also occurs reproducibly on membranous structures below the plasma membrane (Fig. 5 *c*, *inset*). No labeling is apparent in *mdx* mouse smooth muscle under the same conditions (not shown).

Discussion

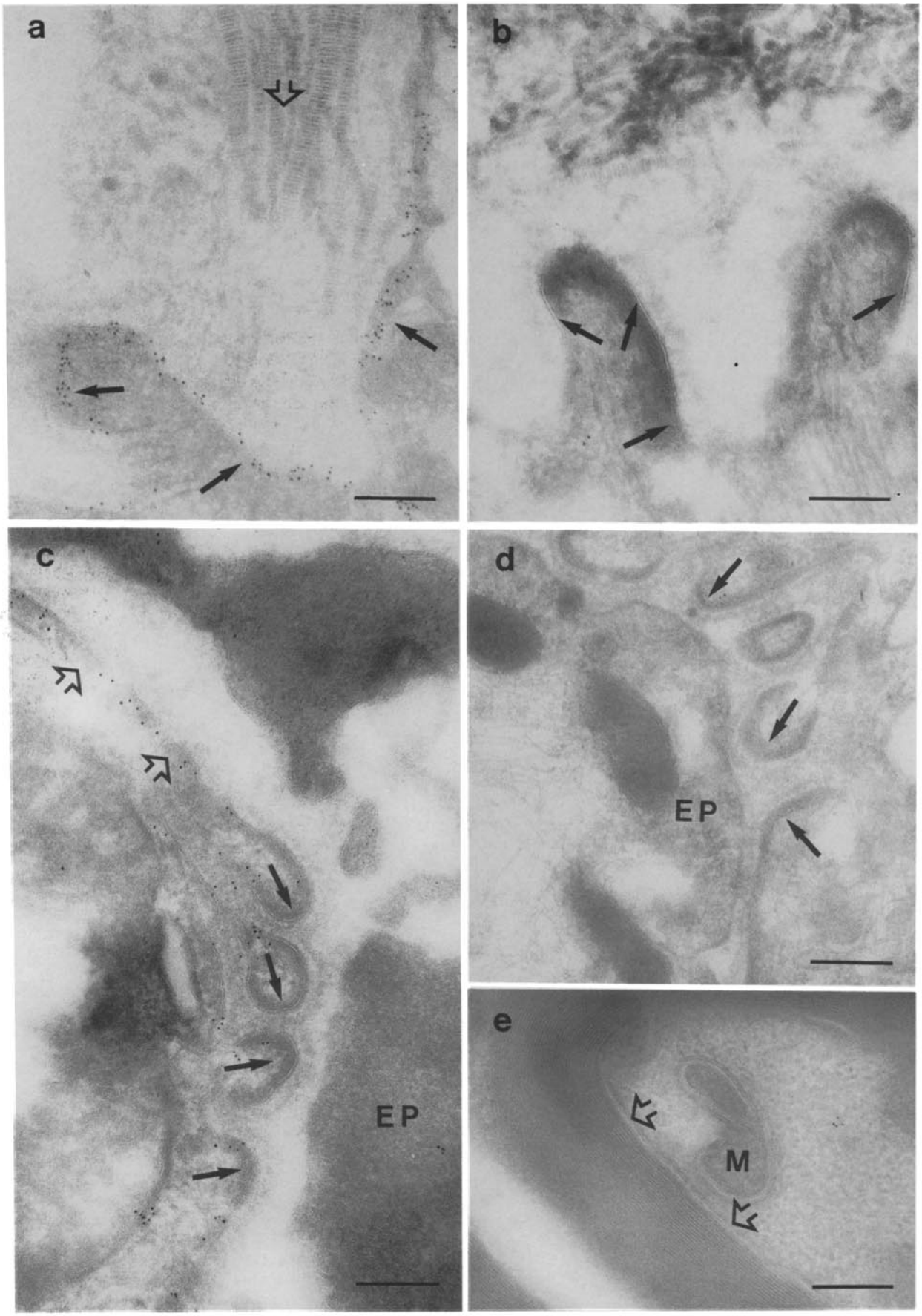
We have used a highly specific and sensitive antibody to reevaluate the subcellular distribution of dystrophin in skeletal muscle and to examine its distribution in other muscle tissues and in peripheral nerve. The presence of dystrophin in regions of muscle and nerve cells that are specialized for very different functions, and probable isoform diversity in the dystrophin protein, argue that dystrophin may fulfill distinct functional roles in different locations.

Skeletal Muscle

Previous immunocytochemical work has shown the bulk of dystrophin to be associated with the sarcolemma of skeletal muscle (Arahata et al., 1988; Bonilla et al., 1988; Zubrzycka Gaarn et al., 1988). Biochemical studies of skeletal muscle membranes have suggested that dystrophin also cofractionates with junctional transverse tubule membranes (Hoffman et al., 1987*b*; Knudson et al., 1988; Salviati et al., 1989), but this observation is not supported by immunocytochemistry. The presence of a membrane cytoskeletal protein such as dystrophin at the triadic membranes could indicate a role in the maintenance of either channel distribution within the triad or the position of the triad structure itself within the infrastructure of the sarcomere. Therefore, it is important in a consideration of the possible functions of dystrophin to confirm its subcellular distribution. In agreement with other immunocytochemical studies (Bonilla et al., 1988; Carpenter et al., 1990; Cullen et al., 1990), we find a strong labeling at the plasma membrane, but no labeling of internal structures or membranes. It is unlikely that dystrophin is being selectively extracted from internal membranes with the techniques that we employ, therefore, we suggest that there is little, if any, dystrophin on internal membranes relative to the plasma membrane. These conclusions are also in agreement with a recent biochemical fractionation study that compared the dystrophin content of cleanly separated sarcolemmal and transverse tubule fractions and verified the content of the fractions using a number of well-defined markers specific to skeletal muscle membrane fractions (Ohlendieck et al., 1991).

At the myotendinous junction, thin filaments of the myofibrils attach and transmit force via the membrane to extracellular structures. Antidystrophin labeling of this struc-

Figure 2. In skeletal muscle, labeling is found only at the plasma membrane. (*a*) This transverse ultrathin frozen section through normal extensor digitorum longus muscle shows labeling under the plasma membrane but not associated with subplasmalemmal membranous structures when incubated with antibody 6-10. (*b*) In the *mdx* mouse, transverse sections of extensor digitorum longus muscle incubated with the same antibody show no evidence of labeling. (*c*) When muscle fibers are cut at a slightly oblique angle, multiple triads may be visualized in a single section. In this micrograph of normal muscle, no labeling of the triads (*open arrows*) is found. Representative labeling at the surface of this fiber is shown in Fig. 2 *c* (inset). Strong subplasmalemmal labeling for dystrophin is apparent. Bars, 0.2 μm.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ture is more intense than that seen at the remainder of the plasma membrane. This finding has been reported previously by Shimizu et al. (1989) and Samitt and Bonilla (1990), but the former authors did not use a dystrophin-deficient control and the latter also reported some labeling in the same location in muscle tissue of the *mdx* mouse. As it is known that the *mdx* mouse lacks dystrophin, Samitt and Bonilla (1990) suggested that they may be detecting another protein besides dystrophin. The cross-reactive protein could possibly be the recently described dystrophin-related protein (Love et al., 1989; Khurana et al., 1990) which shows strong labeling at the *mdx* myotendinous junction by immunofluorescence (Khurana et al., 1991). In this study we have been able to titrate the concentration of antibody 6–10 to a level that is completely negative for *mdx* muscle, but retains a strong reaction in normal tissue and in the occasional revertant fibers of *mdx* muscle (Hoffman et al., 1990). Thus, our results confirm with a high degree of confidence that dystrophin is enriched at the myotendinous junction.

Tidball and Law (1991) have found a deficiency in the attachment of actin filaments to myotendinous junctions of the *mdx* mouse. For this reason, and because of its elevated presence at myotendinous junctions, dystrophin is proposed to be an integral element in the transmission of tension from sarcomeric actin filaments through the plasma membrane to the tendon (Samitt and Bonilla, 1990; Tidball and Law, 1991).

As at the myotendinous junction, dystrophin seems to be present in slightly higher abundance at neuromuscular junctions, confirming the findings of Miike et al. (1989), Shimizu et al. (1989), and Fardeau et al. (1990). Huard et al. (1991) has suggested through inferential reasoning that dystrophin might be present in presynaptic nerve terminals at the neuromuscular junction as well as at the muscle membrane. In this high-resolution study, however, we do not find dystrophin on the neuronal side of the junction.

On the postsynaptic membrane of the neuromuscular junction, dystrophin shows a differential distribution with respect to the junction folds. Other molecular components also segregate into distinct domains in the extensively folded membranes of the neuromuscular junctions. The acetylcholine receptor and 43-kD protein are concentrated at the crests of the junctional folds and Na^+ channels and ankyrin are found predominantly in the troughs (Flucher and Daniels, 1989). Sodium channels are known to interact with ankyrin (Srinivasan et al., 1988) and it is thought that ankyrin may help to restrict the distribution of the channels by linkage to the membrane cytoskeleton. Our localization of dystrophin to the troughs of the junctional folds suggests the possible involvement of dystrophin in such a membrane cytoskeletal structure. The fact that these folds are less pronounced in neuromuscular junctions of DMD patients (Jerusalem et al., 1974) and *mdx* mice (Torres and Duchen, 1987) suggests a structural role for dystrophin in maintaining the folded shape of the membrane in this region. Also, in view of the central importance of acetylcholine receptors to synaptic function, an important corollary to this observation is their apparent lack of co-localization with dystrophin. This finding corroborates the lack of disruption of the distribution and quantity of acetylcholine receptors in DMD patients, despite changes in the folding of postsynaptic membranes (Sakakibara et al., 1977).

Cardiac Muscle

In quality and quantity, the general distribution of dystrophin on plasma membrane surfaces of cardiac muscle is almost indistinguishable from skeletal muscle. This similarity is also true of skeletal versus cardiac dystrophin on Western blots. A major difference, however, is that a specialized portion of the outer plasma membrane of cardiac muscle, the intercalated disk, shows no labeling for this protein whatsoever. The intercalated disk contains adherens junctions that anchor myofibrillar actin filaments and maintain intercellular connections under tension. It has been suggested (see above) that dystrophin plays a role in anchoring actin filaments at the membrane of the myotendinous junctions of skeletal muscle. Thus, it is surprising to find a complete absence of dystrophin at the membrane of the intercalated disks.

We occasionally observed unlabeled cardiocytes in sections of heart tissue. We feel that this is likely to reflect somatic mutations in the dystrophin gene in the nucleus of those particular cells. Such an event would not be as easily observed in skeletal muscle fibers because of their syncytial nature. A reciprocal phenomenon has been described in the *mdx* mouse where occasional positive cardiac myocytes are seen in a field of negatively staining cells. This has been attributed to a somatic reversion of the *mdx* mutation in those cells (Hoffman et al., 1990).

Smooth Muscle

A significantly lower level of dystrophin is observed in smooth muscle as compared with striated muscle, both by Western blot analysis and immunocytochemistry. Also, the distribution at the light microscope level is discontinuous on the surface of the smooth muscle cell, forming a pattern that is stripe-like in longitudinal view and punctate in cross-section. This distribution is very much like the pattern seen

Figure 3. Distribution of dystrophin at membrane specializations of skeletal muscle and in peripheral nerve. (*a*) Ultrathin longitudinal section of normal skeletal muscle at the myotendinous junction shows strong labeling under the plasma membrane (*solid arrows*) with antibody 6–10. The labeling here appears considerably more intense than that seen at the periphery of normal skeletal or cardiac muscle (compare to Figs. 2 *a* and 4 *e*). Collagen fibrils can be seen in close association with the junction (*open arrow*). (*b*) Longitudinal section through *mdx* skeletal muscle at the myotendinous junction labeled under the same conditions as *a*. No labeling is seen under the membrane (*arrows*) or in any other region. (*c*) Ultrathin frozen section through a neuromuscular junction in the superior rectus muscle of normal mouse and labeled as in *a*. Within the folded region of the junction the labeling is predominantly associated with the troughs of the postsynaptic membrane and not with the crests (*closed arrows*). No labeling is seen at the neuronal endplate (*EP*). (*d*) Ultrathin frozen section through a neuromuscular junction in the superior rectus muscle of *mdx* mouse and labeled as in 3 *a*. No labeling of either the crests (*closed arrows*) or folds in this endplate is apparent. (*e*) No labeling is found within the peripheral nerve of normal mouse when labeled with this antibody (*open arrows* delimit the axioplasm; *M*, mitochondrion). Bars, 0.2 μm.

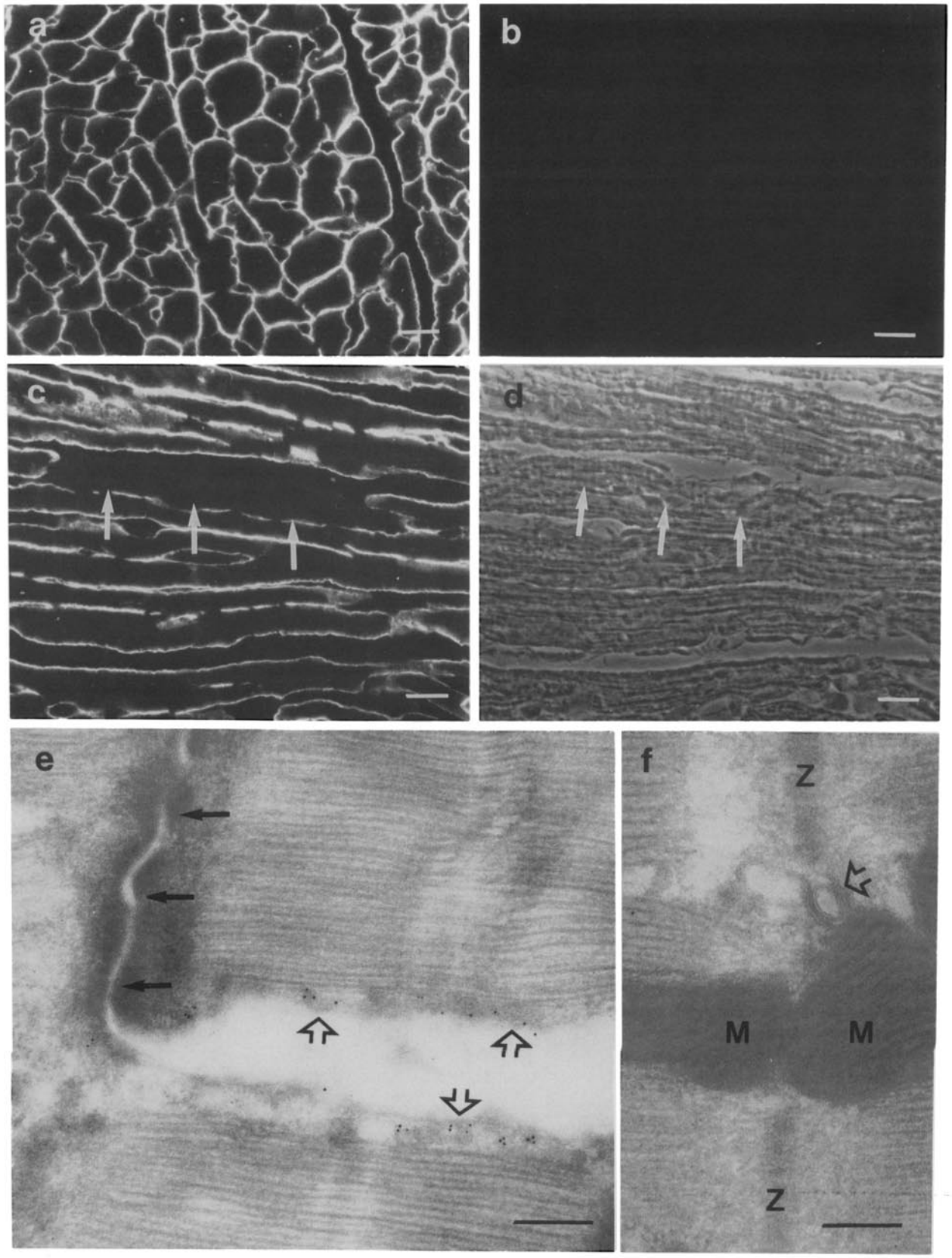

Figure 4. Dystrophin in cardiac muscle. (*a*) Transverse section through normal perfused cardiac ventricle shows uniform immunofluorescent labeling about the periphery of cardiocytes with antibody 6–10. (*b*) In the *mdx* mouse, transverse sections of the cardiac ventricular tissue show no label. (*c*) Longitudinal section through normal cardiac ventricle also shows uniform labeling of cardiocyte membranes. On occasion in normal muscle, totally unlabeled cells are seen (*arrows*). (*d*) Phase-contrast micrograph of *c* showing the cardiocyte that is negative for dystrophin (*arrows*). (*e*) Ultrathin frozen section of normal cardiac muscle labeled with antibody 6–10. Subplasmalemmal labeling is

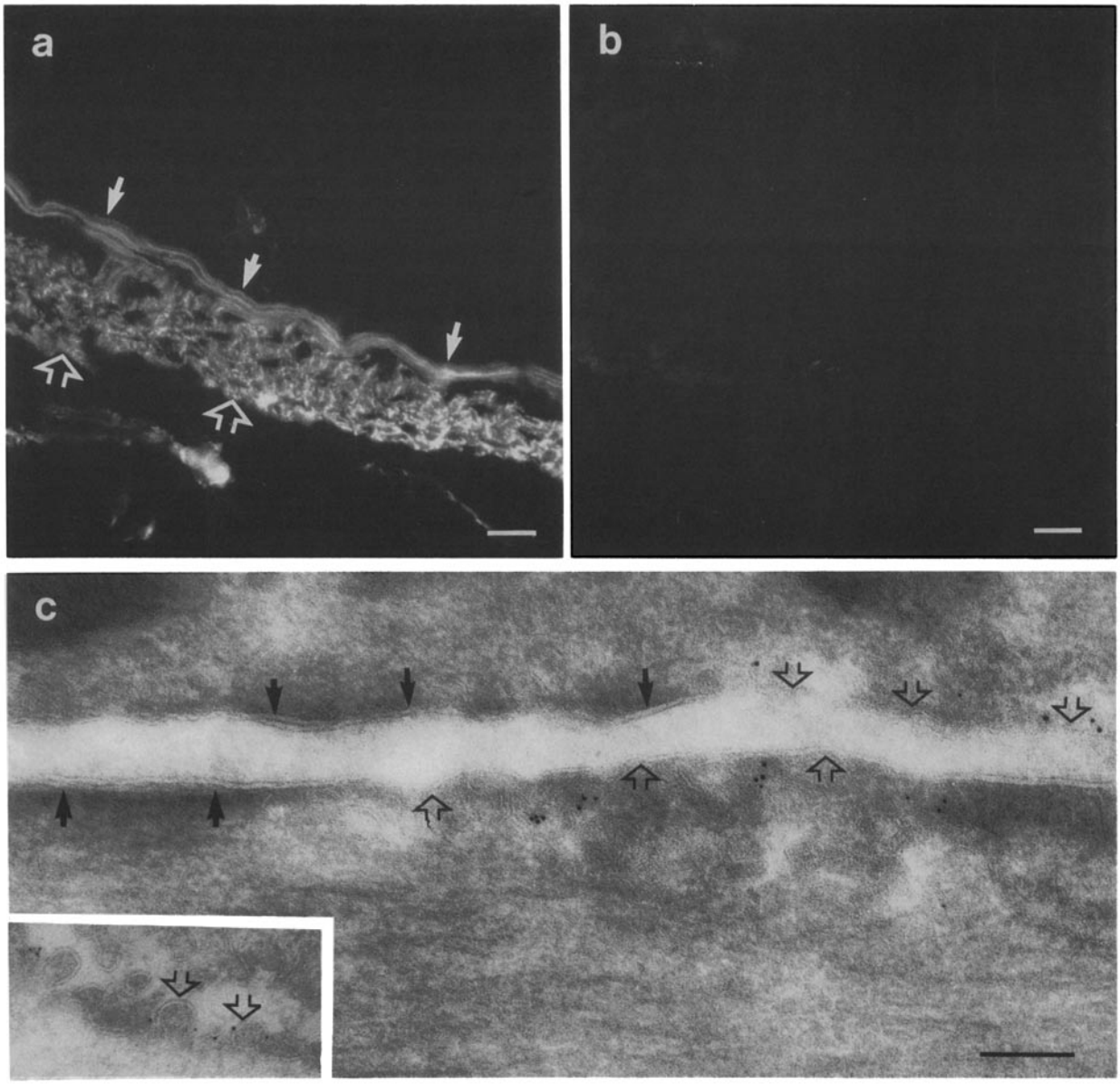

Figure 5. Dystrophin distribution in smooth muscle. (*a*) Immunofluorescent labeling with antibody 6-10 in normal mouse smooth muscle (duodenum) occurs in bars that appear to extend the length of cells cut in longitudinal section (outer layer of cells, *solid arrows*), and in a punctate pattern about the periphery of cells cut in transverse section (inner layer of cells, *open arrows*). (*b*) In the *mdx* mouse no labeling for dystrophin is seen in duodenum under similar conditions. (*c*) Ultrathin longitudinal sections of normal mouse gut smooth muscle labeled with antibody 6-10. The intensity of labeling is less than that seen in skeletal or cardiac muscle. It is seen to be unevenly distributed along the subplasmalemmal surface, being largely excluded from submembranous densities (*closed arrows*) and present in intervening regions (*open arrows*). The labeling does not appear as closely associated with the plasma membrane as in skeletal or cardiac muscle and frequently appears on surface-associated caveolae (*inset, open arrows*). Bars: (*a* and *b*) 20 μm; (*c*) 0.2 μm.

with antibodies to vinculin (Small, 1985), and talin (Drenckhahn et al., 1988). In those studies, examination of isolated cells made it clear that vinculin and talin are present in multiple longitudinal ribs around the perimeter of each cell.

At the TEM level, the smooth muscle membrane displays periodic zones that are underlain by vinculin and talin-containing dense plaques (Geiger et al., 1980; Drenckhahn et al., 1988). In freeze etch EM, the smooth muscle membrane is organized in alternating zones of vesicle-rich surface membrane and smooth membrane that correspond to the dense plaque regions (Devine et al., 1971). We find that dystrophin is predominantly found in vesicle-rich areas, some-

clearly apparent (*open arrows*), though no labeling is seen at the membrane of intercalated disks (*closed arrows*). (*f*) Ultrathin frozen section of normal cardiac muscle labeled as in *e*. A typical triadic structure of cardiac muscle is seen (*open arrow*). No label is associated with the T-tubule or triads (*M*, mitochondrion). Bars: (*a–d*) 20 μm, (*e* and *f*) 0.2 μm.

times associated with vesicles and caveolae near the surface, and not in areas containing dense plaques. These results suggest that dystrophin and vinculin may occur in alternating tracks.

Functional Considerations

Dystrophin is probably capable of bifunctional binding to both membrane elements (Campbell and Kahl, 1989; Ohlendieck et al., 1991) and actin filaments of the cytoskeleton (Hammonds, 1987; Byers et al., 1989). Thus, dystrophin may participate in a membrane skeletal structure that links membrane elements such as channels and receptors to the cytoskeleton, possibly playing a structural and/or regulatory role with respect to these components. The primary functional defect in Duchenne muscular dystrophy is thought to be a destabilization of the muscle plasma membrane due to defects in the dystrophin molecule. Our data, however, along with the data of others, argues that the function of dystrophin is not simply limited to the stabilization of the muscle plasma membrane.

Recently, dystrophin was shown to be present at synaptic densities in neurons of the brain (Lidov et al., 1990). The noncontractile nature of brain tissue implies a potentially different role for dystrophin in this locale than at the muscle plasma membrane, possibly serving to organize synaptic elements such as membrane receptors. It is possible that dystrophin is playing a similar role at the synapse of the neuromuscular junction, distinct from the functions it serves in the nonjunctional plasma membrane of skeletal muscle. Another striking feature of the findings of Lidov et al. (1990) was the apparent absence of detectable dystrophin in nonsynaptic regions of nervous tissue in the central nervous system. We extend this finding to the peripheral nervous system by our observation of a lack of detectable dystrophin in sciatic nerve. Thus dystrophin does not fulfill a general structural role for plasma membranes of the nervous system as it is proposed to in muscle.

Isoform differences might be expected for dystrophin in different specialized regions as has been observed for the ankyrins and spectrins (see Bennett, 1990 for review), and several isoforms have been described in the dystrophin transcript (Chelly et al., 1988; Feener et al., 1989; Nudel et al., 1989). Although further resolution of small differences in this high molecular weight protein will be difficult, we suggest that the lower band of the dystrophin doublet in striated muscle is an isoform that lacks epitopes from the carboxyl terminus. It does not appear to be a smooth muscle isoform as proposed by Hoffman et al. (1988) from comparisons of mouse skeletal muscle and uterus, though, because the single dystrophin band in gut is more similar in mobility to the upper band of the doublet. Instead, our data suggest that the lower band is an isoform that is specialized for a function that is specific to striated muscle.

In smooth and cardiac muscle, adherens junctions (marked by dense plaques under the membrane) anchor actin microfilaments to the membrane and are responsible for maintaining a transmembrane linkage to extracellular structures and adjacent cells under tension (see Geiger et al., 1985 for review). We have found these structures to be the only major regions of the surface membrane of muscle cells that lack dystrophin. This contrasts with dystrophin's proposed role at the myotendinous junction, where it is enriched in submembrane densities and is thought to be involved in the coupling of the myofibrillar apparatus to the membrane, and beyond to the tendon (Samitt and Bonilla, 1990; Tidball and Law, 1991). Clearly this cannot be considered a generalized function of dystrophin in all muscle types.

The absence or very low level of dystrophin in peripheral nerve and structures such as internal membranes of muscle cells and adherens junctions of cardiac and smooth muscle makes these locations unlikely sites of action for dystrophin. On the other hand, probable isoform diversity and the presence of dystrophin in areas that are specialized for very different functions, from membranes of contractile cells to synaptic membranes, support the argument that dystrophin is capable of multiple functional roles in different regions of muscle and neuronal cells. Before the discovery of the dystrophin gene, the multisystemic involvement of muscle and nerve hampered progress in determining the primary cause of Duchenne muscular dystrophy. Further understanding of the multifunctional nature of dystrophin will help to explain the complexity of the Duchenne phenotype.

Thanks to J. V. Small for sharing his expertise on smooth muscle, to H. Slayter for support and useful discussions, and to J. English for technical assistance. Thanks also to the members of the Kunkel laboratory and T. Khurana in particular for comments on the manuscript.

This work was supported by a grant from the Muscular Dystrophy Association and the National Institutes of Health grant NS23740. L. M. Kunkel is an associate investigator of the Howard Hughes Medical Institute.

Received for publication 20 May 1991 and in revised form 11 June 1991.

Note Added in Proof. In a recent report that used different methods (Sealock, R., M. H. Butler, N. R. Kramarcy, K.-X. Gao, A. A. Murnane, K. Douville, and S. C. Froehner. 1990. *J. Cell Biol.* 113:1133-1144), it was also observed that dystrophin is present in deep regions of the folds of neuromuscular junctions and absent from acetylcholine receptor-rich domains.

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