Dystrophin Colocalizes with β-Spectrin in Distinct Subsarcolemmal Domains in Mammalian Skeletal Muscle

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Abstract. Duchenne's muscular dystrophy (DMD) is caused by the absence or drastic decrease of the structural protein, dystrophin, and is characterized by sarcolemmal lesions in skeletal muscle due to the stress of contraction. Dystrophin has been localized to the sarcolemma, but its organization there is not known. We report immunofluorescence studies which show that dystrophin is concentrated, along with the major muscle isoform of β-spectrin, in three distinct domains at the sarcolemma: in elements overlying both I bands and M lines, and in occasional strands running along the longitudinal axis of the myofiber. Vinculin, which has previously been found at the sarcolemma overlying the I bands and in longitudinal strands, was present in the same three structures as spectrin and dystrophin. Controls demonstrated that the labeling was intracellular. Comparison to labeling of the lipid bilayer and of the extracellular matrix showed that the labeling for spectrin and dystrophin is associated with the intact sarcolemma and is not a result of processing artifacts. Dystrophin is not required for this lattice-like organization, as similar domains containing spectrin but not dystrophin are present in muscle from the *mdx* mouse and from humans with Duchenne's muscular dystrophy. We discuss the possibility that dystrophin and spectrin, along with vinculin, may function to link the contractile apparatus to the sarcolemma of normal skeletal muscle.

DUCHENNE'S muscular dystrophy (DMD)[1] is a relatively common X-linked disorder characterized by progressive loss of muscle tissue due to the degradation of myofibers. DMD is caused by the lack of dystrophin, a high (~400 kD) molecular weight protein encoded by a gene on the X chromosome (Hoffman et al., 1987, 1988). Damaged muscle fibers from patients with DMD show lesions of the sarcolemma and underlying sarcoplasm (Mokri and Engel, 1975; Carpenter and Karpati, 1979) which result from the stress of contraction (Florence et al., 1985; Webster et al., 1988). Dystrophin has been found at the sarcolemma by immunofluorescence and immunoelectron microscopy (Arahata et al., 1988; Bonilla et al., 1988*a*; Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Byers et al., 1991; Ohlendieck et al., 1991; Wakayama and Shibuya, 1991). It has thus been proposed that dystrophin stabilizes the sarcolemma (Beam, 1988), but how it does so has not been determined.

Dystrophin's primary sequence resembles those of spectrin and α-actinin, suggesting that these proteins can be grouped into a super family of cytoskeletal proteins (Davison and Critchley, 1988; Koenig et al., 1988). Spectrins are high molecular weight molecules first discovered in erythrocytes and subsequently found in most mammalian cells (Marchesi, 1985; Morrow, 1989; Bennett, 1990). In the red blood cell, spectrin oligomers are the major component of a sub-membrane network involved in maintaining the distinctive biconcave shape of the cell and in organizing integral membrane proteins in the plasmalemma (Marchesi, 1985; Bennett, 1990). This model of spectrin–membrane organization has been proposed to apply to other cells containing spectrin. For example, this laboratory has previously shown by immunoelectron microscopy that dystrophin (Dmytrenko et al., manuscript submitted for publication) and an unusual isoform of β-spectrin (Bloch and Morrow, 1989; Pumplin, D. W., J. C. Strong, J. G. Krikorian, G. A. Porter, and J. C. Winkelmann, 1990; *J. Cell Biol.* 111:165*a*) are present in a similar sub-membrane network that helps to organize acetylcholine receptors in the membrane of cultured mammalian myotubes. In adult myofibers, dystrophin and spectrin have previously been localized by immunofluorescence to the sarcolemma at the neuromuscular junction and in extrajunctional regions (Repasky et al., 1982; Craig and Pardo, 1983; Nelson and Lazarides, 1983; Appleyard et al., 1984; Arahata et al., 1988; Bonilla et al., 1988*a*; Watkins et al., 1988; Zubrzycka-Gaarn et al., 1988; Bloch and Morrow, 1989; Chang et al., 1989; Shimizu et al., 1989; Byers et al., 1991; Ohlendieck et al., 1991; Pons et al., 1991; Sealock et al., 1991; Yeadon et al., 1991), and dystrophin has been observed in a cytoskeletal network in ultrastructural experi-

1. *Abbreviation used in this paper*: DMD, Duchenne's muscular dystrophy.

ments (Wakayama and Shibuya, 1990, 1991). These proteins may therefore contribute to a sub-sarcolemmal network in the adult myofiber.

We have addressed this possibility by determining if dystrophin and spectrin codistribute under the sarcolemma. Previous reports (Repasky et al., 1982; Nelson and Lazarides, 1983) have demonstrated that spectrin is enriched in skeletal myofibers in a sub-sarcolemmal, two-dimensional lattice containing longitudinal strands, transverse elements over the M lines, and "costameres"—regions of the sarcolemma that overlie the I bands flanking the Z lines (Craig and Pardo, 1983; Pardo et al., 1983*a*). Costameres may be a link between the sarcolemma and the contractile apparatus (Garamvölgyi, 1965; Chiesi et al., 1981; Pierobon-Bormioli, 1981; Craig and Pardo, 1983; Pardo et al., 1983*a*,*b*; Street, 1983; Shear and Bloch, 1985). To determine if both dystrophin and β-spectrin are present in costameres and other sub-sarcolemmal domains, we used antibodies against dystrophin and the major muscle isoform of β-spectrin (Winkelmann et al., 1990*b*) to study the distribution of these proteins in frozen sections of rat skeletal muscle. Fibers sectioned tangentially to reveal large areas of sarcolemma in a single plane of focus showed distinct domains enriched in both spectrin and dystrophin, not only over I bands but also above M lines and in fine longitudinal strands. Controls showed that the labeling was intracellular, was associated with the membrane, and was not due to processing artifacts. These domains are related to costameres, as the costameric protein, vinculin, codistributed with both dystrophin and spectrin. In muscles from the *mdx* mouse and patients with DMD, which lack dystrophin, a sub-sarcolemmal lattice of spectrin resembling that in control muscles was present, suggesting that dystrophin is not required for the lattice to form.

Materials and Methods

Antibodies

Sheep antibodies to a fusion protein containing the amino terminal 60-kD fragment of mouse dystrophin (Hoffman et al., 1987) were obtained from Dr. L. Kunkel (Harvard Medical School, Boston, MA) and Dr. E. Hoffman (University of Pittsburgh, Pittsburgh, PA). A mouse mAb, 1808, to *Torpedo* dystrophin (Sealock et al., 1991), was from Dr. S. Froehner (Dartmouth Medical School, Hanover, NH). Rabbit polyclonal antibody to human fibronectin, AB1940, and mouse mAb against human vinculin, mAb1637, which recognizes vinculin and metavinculin, were obtained from Chemicon (Temecula, CA). Rabbit antibody to vinculin has been described previously (Shear and Bloch, 1985).

The peptide, Gly-Lys-Lys-Asp-Lys-Glu-Lys-Arg-Phe-Ser-Phe-Phe-Pro-Lys-Lys-Lys, representing the carboxy terminal sequence of the major muscle isoform of β-spectrin (Winkelmann et al., 1990*b*), was synthesized (model 430A; Applied Biosystems, Foster City, CA) with Cys-Gly-Gly added to the amino terminus and purified by high performance liquid chromatography on a C-18 reverse phase column (Vydac, Hesperia, CA) at the Biopolymer Laboratory of the Department of Microbiology and Immunology at the University of Maryland School of Medicine (Baltimore, MD). It was coupled (Gentry et al., 1983) to BSA, and 200 μg of the conjugate was injected subcutaneously into rabbits in complete Freund's adjuvant. Rabbits were boosted 1 mo later, and every 2 wk thereafter, with 50 μg of the peptide-BSA conjugate in incomplete adjuvant. Rabbits were bled every week after the first boost. Immunoglobulins were purified from serum by precipitation in 50% ammonium sulfate, followed by dialysis against PBS. The crude immunoglobulin fraction was applied to a column of Affigel 15 (Bio-Rad Laboratories, Richmond, CA) coupled to BSA, and the effluent was then applied to a column of Affigel 15 coupled directly to the peptide, following the manufacturer's instructions. Bound antibody was eluted with 0.1 M glycine-HCl, 0.5 M NaCl, pH 2.7, immediately neutralized, and dialyzed against PBS plus 10 mM sodium azide. Aliquots were stored at −70°C.

Immunoblotting

Rat hindlimb muscle was suspended in a solution containing 1% deoxycholate, 1% NP-40, 10 mM sodium phosphate, pH 6.8, 0.5 M NaCl, and 2 mM EDTA (Hoffman et al., 1989), supplemented with protease inhibitors (0.22 U/ml aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml antipain, 200 μg/ml soybean trypsin inhibitor) and 10 mM sodium azide but containing no SDS. Tissue was homogenized in a Virtis Hi-Speed "45" Homogenizer (Gardiner, NY) at 4°C for a total of 2 min and incubated for 1 h at 4°C. Insoluble material was removed by centrifugation (16,000 rpm, 1 h, 4°C; SS-34 rotor; Sorvall Instruments, Newton, CT), and the supernatant was stored at −50°C.

Proteins (50–60 μg/5-mm lane), separated on 5–15% acrylamide gradient minigels (Hoefer Scientific, San Francisco, CA) according to the method of Laemmli (1970), were transferred electrophoretically to nitrocellulose overnight at 200 mA (Burnette, 1981). Molecular weight standards, obtained from Bethesda Research Laboratories (Bethesda, MD) and supplemented with purified erythrocyte spectrin (Gratzer, 1982), were stained with amido black after transfer. Strips of nitrocellulose were stored in distilled water and, before incubation with antibodies, were incubated in 3% milk solids (Carnation Products, Los Angeles, CA) in PBS supplemented with 10 mM sodium azide and 0.5% Tween-20. Samples were labeled overnight with the antibody diluted in the same solution without milk solids. Strips were then incubated for 4 h with appropriate secondary antibody conjugated to alkaline phosphatase, and bound antibodies were visualized using a detection system obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Immunofluorescence

Animals were perfused through the left ventricle with PBS. Rat sternomastoid or mouse hamstring muscle was dissected, pinned or clamped at resting length, and fixed for 10 min in 2% paraformaldehyde in PBS. In a few experiments, we perfused rats with 2% paraformaldehyde in PBS, removed the sternomastoid muscle, and fixed it for 30 min longer. Tissue was blotted dry and plunged into a slush of liquid N_2. DMD and normal human muscle samples were obtained from patients undergoing routine diagnostic muscle biopsies and frozen in liquid N_2-cooled isopentane. Sections 4-μm thick were cut on a cryostat (model 2800, Frigocut; Reichert-Jung, Cambridge Instruments, Deerfield, IL), deposited on slides coated with 0.5% gelatin, 0.05% chromium potassium sulfate, and stored at −50°C. Bundles of rabbit psoas muscle were tied to wooden sticks in situ, dissected, and stored in glycerol at 4°C. Samples were teased to single fibers in glycerol, and were washed extensively with PBS.

Sections or fibers were incubated for 15 min in PBS containing 1 mg/ml BSA and 10 mM sodium azide (PBS/BSA), to block non-specific binding of antibody, and stained for 1 h with primary antibody diluted in the same solution. Samples were washed, blocked again for 15 min in PBS/BSA, and labeled for 1 h with combinations of the following secondary antibodies: 10 μg/ml for fluoresceinated donkey anti-rabbit IgG, goat anti-rabbit IgG, or rabbit anti-mouse IgG; 1 μg/ml for biotinylated goat anti-mouse IgG or donkey anti-sheep IgG. Samples were then labeled for 1 h with 4 μg/ml rhodamine streptavidin. All incubations were performed at room temperature in a humidified chamber.

In some experiments, we labeled muscles with the lipid probe, 3,3′-di($C_{12}H_{25}$) indocarbocyanine iodide (C_{12}-diI; Wolf, 1988). Muscles fixed after dissection were labeled en bloc with 1 μg/ml C_{12}-diI for 10–30 min, rinsed in PBS, and frozen and sectioned as described above. Such samples were then labeled as above with anti-spectrin antibody. C_{12}-diI was visualized under rhodamine optics.

Samples were mounted in nine parts glycerol, one part 1 M Tris-HCl, pH 8, supplemented with 1 mg/ml *p*-phenylenediamine to reduce photobleaching (Johnson et al., 1982). Conventional immunofluorescence observations were performed on an IM-35 microscope (Carl Zeiss, Oberkochen, Germany) with a 63X/N.A. 1.4 phase objective (Leitz, Rockleigh, NJ). Photographs were taken on TMAX P3200 film (Eastman Kodak, Rochester, NY) and were processed to an ASA of 1,600. Some samples were observed with a Bio-Rad MRC 600 confocal scanning laser microscope (Bio-Rad Laboratories) at a Z axis resolution of 0.56 μm, obtained with the same objective. Images were sharpened and saved using SOM 4.61 software (Bio-Rad Microsciences Division). NIH Image 1.41 software was used to increase the contrast of the images and to generate graphs of the fluores-

cence intensity. These images were exposed to Kodak TMAX P100 film on the Montage FR1 machine (Presentation Technologies, Sunnyvale, CA).

Statistics

A correlation coefficient of the values used to plot the image intensities in Fig. 3, *e* and *f* was obtained using StatView SE + Graphics (Abacus Concepts, Inc., Berkeley, CA). A regression plot of this line was generated for Fig. 3 *g*. The slope of this line was shown to be significantly different from zero in a two-tailed *t* test using the formula: $t = r(N - 2)^{1/2}/(1 - r^2)^{1/2}$ (Bolton, 1984).

Materials

Rats were from Zivic Miller (Zelienople, PA), rabbits were from Hazelton Research Products (Denver, PA), and mice were from Jackson Laboratories (Bar Harbor, ME). C_{12}-dil was generously provided by Dr. A. Waggoner (Carnegie-Mellon University, Pittsburgh, PA). Conjugates of antibodies and streptavidin were obtained from Jackson Immuno Research Laboratories (West Grove, PA). All other chemicals, except where noted, were from Sigma Chemical Co. (St. Louis, MO).

Results

Antibodies

Sheep antibodies to mouse dystrophin (Hoffman et al., 1987) and a mouse mAb, 1808, to *Torpedo* dystrophin (Sealock et al., 1991) specifically recognized a doublet at ~400 kD in immunoblots of normal rat skeletal muscle but not of *mdx* mouse muscle (Fig. 1), as previously reported (Hoffman et al., 1987, 1988; Byers et al., 1991; Sealock et al., 1991). Antibodies to the major muscle isoform of β-spectrin recognized a doublet with an apparent molecular weight of ~270 kD, as predicted (Winkelmann et al., 1990*b*), in both normal and *mdx* muscle (Fig. 1). The spectrin doublet labeled in homogenates of *mdx* mouse muscle was more apparent in 5% polyacrylamide gels (not shown). The presence of two spectrin bands could be due to NH_2-terminal proteolysis of β-spectrin or to the existence of two isoforms of the protein, perhaps the result of alternative splicing (e.g., Winkelmann et al., 1990*b*). Both bands of the doublet are distinct from erythrocyte β-spectrin, which has a molecular weight of 246 kD (Winkelmann et al., 1990*a*).

Both monoclonal and polyclonal anti-vinculin antibodies recognized two proteins in immunoblots of rat skeletal muscle (Fig. 1). The apparent molecular masses of these bands, 116 and 133 kD, are consistent with those of vinculin and metavinculin (Coutu and Craig, 1988; Gimona et al., 1988*b*; Weller et al., 1990). An additional mAb against vinculin reacted with the same bands but failed to label sections by immunofluorescence (not shown). Although high levels of metavinculin have not been found in adult skeletal muscle before, previous reports indicate that the level of expression of metavinculin varies widely among different striated muscles in different species (Feramisco et al., 1982; Saga et al., 1985; Glukhova et al., 1986; Belkin et al., 1988*a*,*b*; Gimona et al., 1988*a*). In fact, the rabbit anti-vinculin antibody used here only recognized a single vinculin band in chicken skeletal muscle (Shear and Bloch, 1985).

Normal Muscle

Cytoskeletal Proteins in a Subsarcolemmal Lattice. We labeled teased rabbit muscle fibers and longitudinal cryosections of rat, mouse, and human skeletal muscle with these antibodies by indirect immunofluorescence. As previously reported, both anti-dystrophin antibodies stained only the sarcolemma of myofibers (Hoffman et al., 1987; Sealock et al., 1991). We occasionally saw a regularly spaced, punctate pattern at the periphery of many fibers, however. Anti-dystrophin antibodies labeled the membrane adjacent to the M lines and the I bands, but often did not label at Z lines (Fig. 2, *b* and *c*). The source of the repetitive pattern became apparent in fibers which had been sectioned tangentially such that large areas of membrane were present in a single plane of focus. These "glancing sections" showed a lattice-like pattern of label (Fig. 2 *e*), with no cytoplasmic background, suggesting a sarcolemmal location (see below). This lattice consisted of occasional longitudinal strands and periodic transverse structures. The transverse structures were of two types: one over I bands appeared as a pair of thicker lines separated by a barely discernible unstained space overlying the Z lines and the second appeared as thin lines overlying most of the M lines. The longitudinal strands and those elements above the I bands resemble a sub-membrane, two dimensional lattice of cytoskeletal proteins described previously in cardiac and skeletal muscle (see Discussion). Pardo

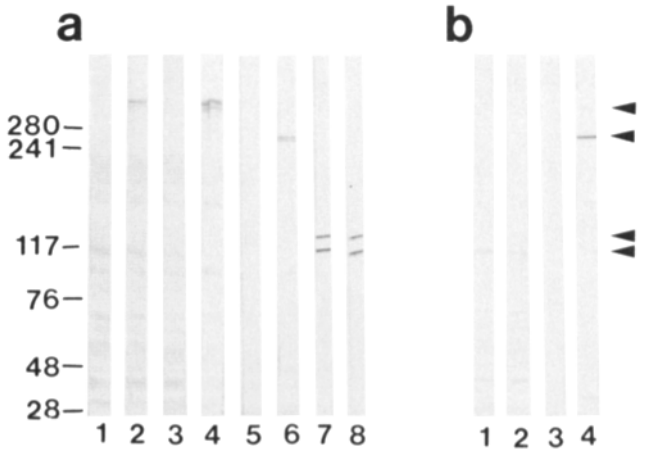

Figure 1. Specificity of antibodies to dystrophin, spectrin, and vinculin. Homogenates of normal rat (*a*) and *mdx* mouse (*b*) muscle were separated by SDS-PAGE and transferred to nitrocellulose. Strips of nitrocellulose were labeled with antibody and visualized with secondary antibodies conjugated to alkaline phosphatase. Control antibodies were mopc 21 mouse monoclonal IgG (*a*, lane 1, *b*, lane *1*), normal sheep serum (*a*, lane *3*), and normal rabbit serum (*a*, lane *5*, *b*, lane *3*). Anti-dystrophin antibodies, mab 1808 (*a*, lane *2*, *b*, lane *2*) and sheep anti-dystrophin fusion protein (*a*, lane *4*), label a doublet at ~400 kD in normal rat but not *mdx* mouse muscle. Rabbit antibody against the major muscle isoform of β-spectrin labels a doublet at ~270 kD in both normal rat, (*a*, lane *6*) and *mdx* mouse (*b*, lane *4*) muscle. Both mouse (*a*, lane *7*) and rabbit (*a*, lane *8*) anti-vinculin antibodies labeled two bands of 116 and 133 kD in normal rat muscle. Molecular weight standards ($M_r \times 10^3$) are indicated on the left. Arrows on the right indicate (in descending order) dystrophin, spectrin, metavinculin, and vinculin. Labeling of the lower molecular weight bands in *a* lanes *2* and *4*, and *b*, lane *2* can be accounted for by non-specific interactions, as shown for pre-immune or irrelevant antibodies (*a*, lane *1*, *a*, lane *3*, *b*, lane *1*). Immunofluorescence labeling is therefore likely to be specific.

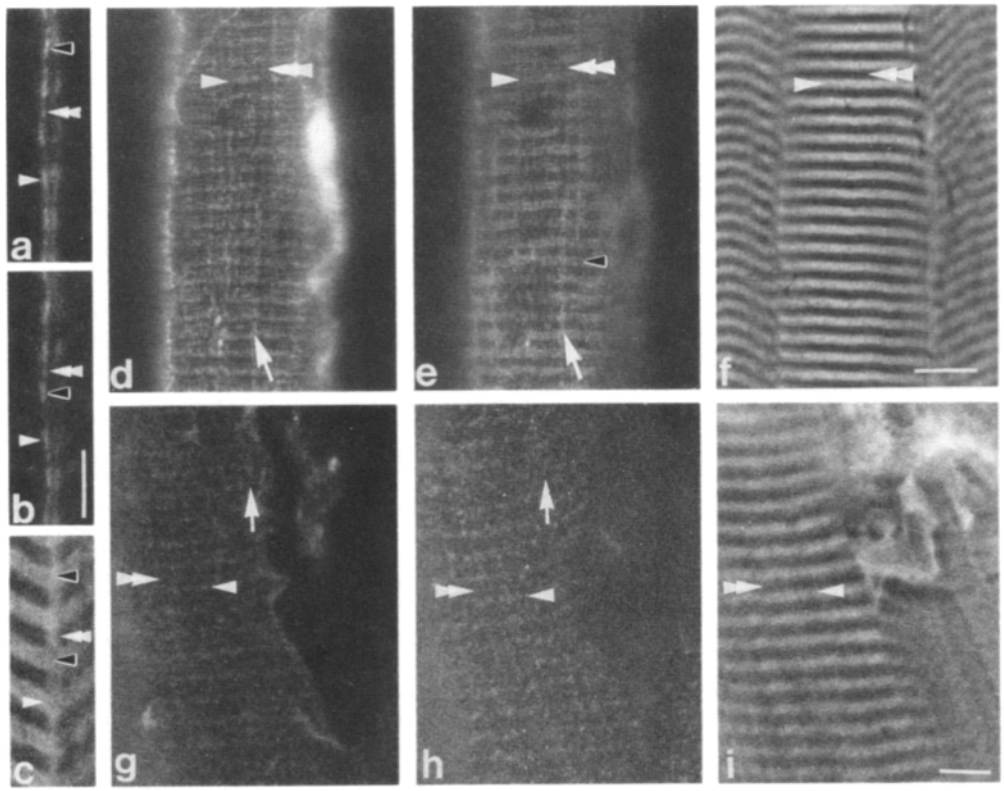

Figure 2. Dystrophin and β-spectrin are present in a sub-sarcolemmal lattice in rat and rabbit skeletal muscle. Longitudinal sections (4-μm thick) of rat sternomastoid (*a–f*) and individual fibers of rabbit psoas (*g–i*) muscle were colabeled with rabbit anti-β-spectrin (*a*, *d*, and *g*) and mAb 1808 anti-dystrophin (*b*, *e*, and *h*) followed by fluoresceinated or biotinylated anti-antibodies and rhodamine streptavidin. The corresponding phase micrographs (*c*, *f*, and *i*) are present to facilitate comparison to contractile structures. In a section perpendicular to the sarcolemma (*a–c*), spectrin (*a*), and dystrophin (*b*) are labeled on the sarcolemma in a punctate pattern corresponding to the adjacent I bands (*single white arrowheads*) and M lines (*double white arrowheads*). Note the discontinuity in the center of the I band staining which corresponds to the adjacent Z line (*black arrowheads*). In sections tangential to the sarcolemma of a myofiber, a lattice of spectrin (*d*) and dystrophin (*e*) has elements above the I bands and M lines and occasional longitudinal strands (*white arrows*). Again note the discontinuity of the I band staining above the Z line. Arrowheads label structures as in *a–c*. The lattice of spectrin (*g*) and dystrophin (*h*) seen in permeabilized rabbit psoas muscle is similar to that seen in *d–f*. Because of the curvature of the fiber, only part of the surface is in focus. Bars: (*a–c*), 5 μm; (*d–f*), 10 μm; (*g–i*), 5 μm.

and co-workers named the elements overlying the I bands "costameres" (Pardo et al., 1983*a*). The longitudinal strands have no obvious correlate in the contractile apparatus. We cannot rule out the possibility that the labeling of these strands is due to folds in the sarcolemma.

Longitudinal sections of rat skeletal muscle stained with antibodies to the major muscle isoform of β-spectrin also revealed a punctate pattern at the edges of myofibers and a lattice in glancing sections (Fig. 2, *a* and *d*). In double immunofluorescence labeling, dystrophin and the major muscle isoform of β-spectrin colocalized to these structures (Fig. 2, *a–f*). Similar colabeling of a membrane lattice was obtained in intact, permeabilized fibers from the rabbit psoas muscle (Fig. 2, *g–i*). Antibodies did not stain a lattice unless the fibers were first permeabilized with detergent (not shown), consistent with the idea that the lattice resides on the inner surface of the sarcolemma. Thus, at the level of resolution afforded by fluorescence microscopy, dystrophin and spectrin colocalize on the cytoplasmic side of the sarcolemma in a lattice with major components over the M lines and I bands.

We performed additional experiments to examine the specificity of antibody labeling. Sections stained with nonimmune IgG (mopc21), or pre-immune rabbit IgG or sheep serum, followed by secondary and tertiary reagents, showed minimal background label. Labeling by the primary, secondary, or tertiary reagents alone also gave no staining. Finally, sections labeled with a single primary antibody followed by combinations of secondary and tertiary reagents appropriate for double labeling showed that there was no species cross-reactivity of the secondary and tertiary reagents. Thus the patterns of labeling described above were not due to nonspecific labeling by primary antibodies, to species cross-reactivity of secondary antibodies, or to "bleed through" of rhodamine and fluorescein. The labeling shown in Figs. 2–5 is therefore specific.

As previous reports have localized vinculin to costameres and longitudinal strands (Craig and Pardo, 1983; Pardo et al., 1983*a*,*b*; Shear and Bloch, 1985; Terracio et al., 1990), we compared the distribution of dystrophin to that of vinculin. In longitudinal sections of rat skeletal muscle, antibodies to vinculin and dystrophin colabeled the three domains of the sub-sarcolemmal lattice (Fig. 3, *a–d*). Both proteins are enriched above I bands and M lines, with the area labeled above the I bands appearing wider than that above the M lines. In addition, a small split in the labeling above I bands is evident. Labeling for these proteins was further compared by confocal scanning laser microscopy (Fig. 3, *c–g*). A limited area of membrane with lattice labeled for both vinculin and dystrophin was digitized (Fig. 3, *c* and *d*) and scanned to obtain graphs of label intensity (Fig. 3, *e* and *f*). The graphs revealed almost identical patterns of label as described above, e.g., labeling over the M lines and I bands with a decrease at the center of the I bands above the Z lines. An analysis of correlation between the two plots (Fig. 3 *g*) yielded a correlation coefficient (*r*) of 0.885. The slope of the

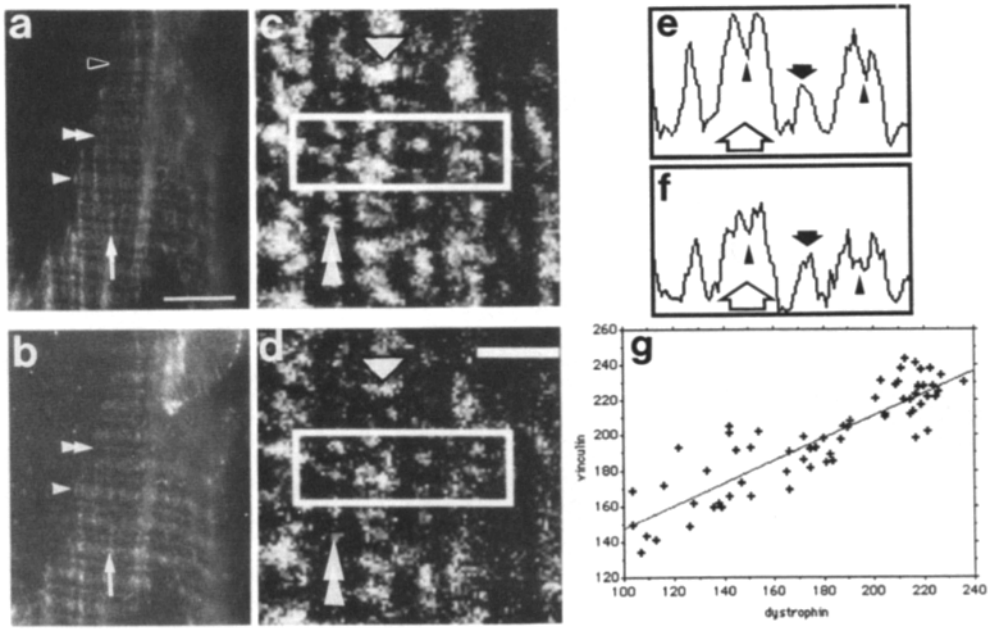

Figure 3. Vinculin codistributes with dystrophin in the subsarcolemmal lattice. Glancing sections of rat sternomastoid muscle were labeled by fluorescein and rhodamine fluorescence for dystrophin (*a* and *c*) and vinculin (*b* and *d*). For *a–d*, arrows and arrowheads denote the same structures as in Fig. 2. Dystrophin (*a* and *c*) distributes with vinculin (*b* and *d*) at the sarcolemma above the I bands, above the M lines, and in longitudinal strands. *a* and *b* were collected by conventional immunofluorescence, while *c* and *d* were collected on a Bio-Rad confocal scanning laser microscope and analyzed with NIH Image 1.41 software. The areas outlined *c* and *d* (20 × 65 pixels) were scanned for labeling intensity, and the average of each vertical column of 20 pixels was plotted. The results show that labeling for dystrophin (*e*) and vinculin (*f*) is present above the M lines (*black arrows*) and I bands (*wide outlined arrows*). The labeling above the I bands is both wider than that above the M lines and is decreased in the center above the Z lines (*small black arrowheads*). *g* shows an analysis of the lines plotted in *e* and *f* for correlation with Statview SE + Graphics which yielded a correlation coefficient (*r*) of 0.885. The slope of the line, 0.644, was significantly different from zero ($P < 0.001$). The values on the ordinate and abscissa indicate the pixel intensity on a gray scale of 0 to 255. Bars: (*a* and *b*), 10 μm; (*c* and *d*), 2.2 μm.

line, 0.644, is significantly different from zero ($P < 0.001$). The lack of a one-to-one relationship between the labeling by the two antibodies may be explained by differences in relative amounts of dystrophin and vinculin, by differences in the binding constants and stoichiometries of binding of both the primary and secondary antibodies, and by the fact that labeling for dystrophin was enhanced using biotinylated secondary antibody followed by rhodamine streptavidin. From the plots and the correlation data, we conclude that the labeling for dystrophin and vinculin is indeed coincident at the

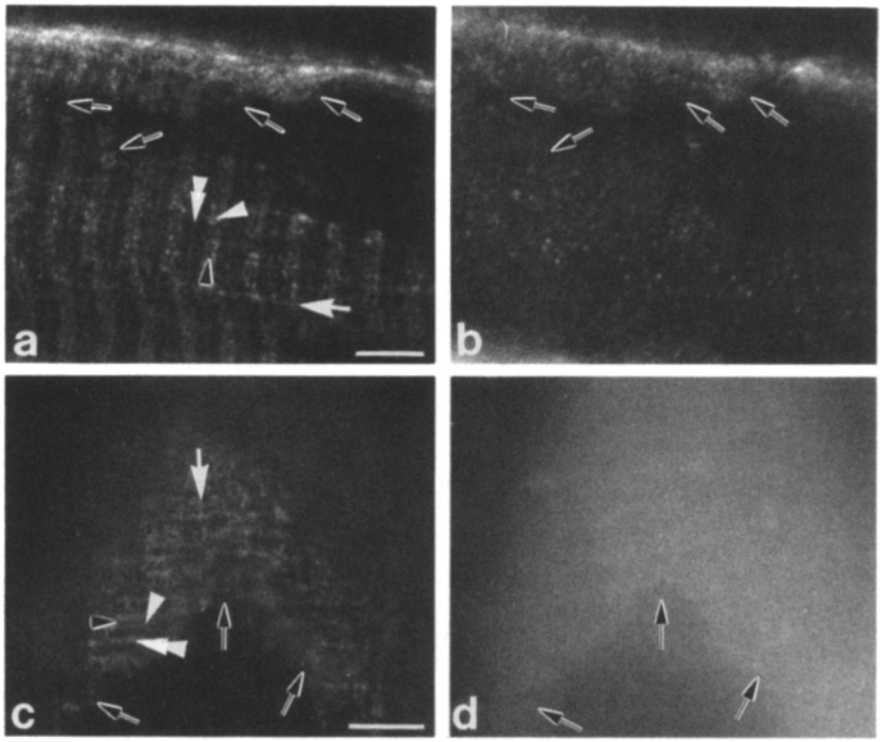

Figure 4. The dystrophin-spectrin lattice is present at intact sarcolemma. Glancing sections of rat sternomastoid muscle were colabeled with anti-dystrophin (*a*) and anti-fibronectin (*b*), and with anti-spectrin (*c*) and C_{12}-diI (*d*) as described in Materials and Methods. White arrows and all arrowheads denote the same structures as in Fig. 2. Black arrows point to the edges of the lattice (*a* and *c*) and of areas labeled with anti-fibronectin (*b*) and C_{12}-diI (*d*), where the cell surface exits the plane of section. Results suggest that the lattice of dystrophin and spectrin is present in areas of the sarcolemma that are intact, as judged by continuous labeling by C_{12}-diI and anti-fibronectin. Bars, 5 μm.

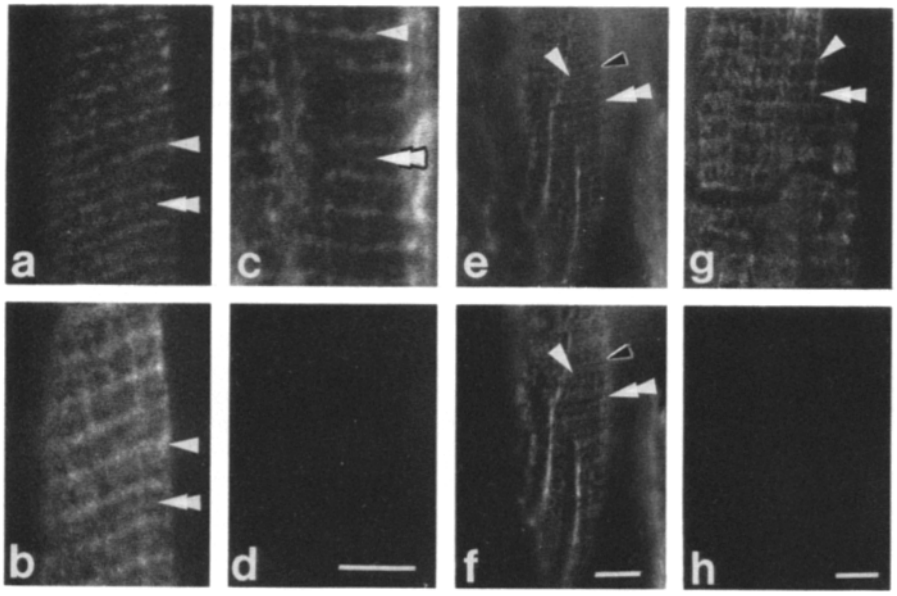

Figure 5. DMD and *mdx* muscle lack dystrophin but display a lattice of β-spectrin. Glancing sections from normal mouse hamstring (*a* and *b*) and human quadriceps (*e* and *f*) muscles display a lattice of spectrin (*a* and *e*) and dystrophin (*b* and *f*) similar to that seen in normal rat and rabbit muscle (Fig. 2). Glancing sections from *mdx* mouse (*c* and *d*) and DMD human (*g* and *h*) muscles show a lattice of spectrin (*c* and *g*) but contain no dystrophin (*d* and *h*). All samples were colabeled with anti-dystrophin and anti-spectrin. Arrows and arrowheads are as described in Fig. 2. Bars, 5 μm.

highest levels of resolution afforded by optical techniques. Therefore, like antibodies to spectrin and dystrophin, antibodies to vinculin label the sarcolemma not only at costameres and in longitudinal strands, as previously reported (Craig and Pardo, 1983; Pardo et al., 1983*a*,*b*; Shear and Bloch, 1985; Terracio et al., 1990), but also above M lines. As the anti-vinculin antibodies used in this study also label metavinculin (Fig. 1), we cannot rule out the possibility that vinculin and metavinculin distribute to different domains of the lattice.

The Subsarcolemmal Lattice Is Not Created Artifactually. The lattice was further compared to labeling for fibronectin and for the lipid bilayer of the sarcolemma. Labeling by antibodies to fibronectin appeared as an almost uniform sheath over the surface of the muscle fiber, as expected (Sanes, 1982). The lattice of dystrophin was visible at the same focal plane (Fig. 4, *a* and *b*). Neither fibronectin nor dystrophin were seen in areas where the plane of section went deeper into the sarcoplasm or into the extracellular space (denoted by black arrows in Fig. 4, *a* and *b*), suggesting that both were present close to the sarcolemma. Labeling of intact fibers with the indocarbocyanine lipid probe, C_{12}-diI (Wolf, 1988), followed by sectioning and labeling with anti-spectrin antibodies, showed the sub-sarcolemmal lattice in the same regions that stained almost uniformly with C_{12}-diI (Fig. 4, *c* and *d*). As with fibronectin, labeling with C_{12}-diI extended only as far as that for spectrin and not deeper into the sarcoplasm (Fig. 4, *c* and *d*). The slight lack of uniformity in the C_{12}-diI label was probably due to penetration of small amounts of C_{12}-diI from the sarcolemma into the T-tubules. The lattice also remained intact in sections from muscle which had been extensively fixed by perfusion followed by fixation en bloc (not shown). Since the lattice remained intact where C_{12}-diI and anti-fibronectin antibodies labeled the membrane almost uniformly and in muscle which had been extensively fixed, it is unlikely that processing of the sample was accompanied by fragmentation or reorganization of the membrane or membrane-associated proteins.

Dystrophic Muscle

Muscles from the *mdx* mouse and from patients with DMD were colabeled with antibodies to dystrophin and spectrin. Glancing sections of normal mouse and human muscles showed a lattice similar to that seen in rat muscle (Fig. 5, *a*, *b*, *e*, and *f*). Glancing sections from *mdx* and DMD muscle contained no dystrophin, as expected (Fig. 5, *d* and *h*). They did, however, contain a spectrin-rich sub-sarcolemmal lattice of transverse elements overlying the I bands and M lines and containing occasional longitudinal strands (Fig. 5, *c* and *g*). The lattices in dystrophic muscle always appeared less ordered than in controls, perhaps due to the presence of regenerating fibers commonly seen in these muscles (Carpenter and Karpati, 1979; Torres and Duchen, 1987).

Discussion

This report is the first to show that dystrophin is not homogeneously distributed under the sarcolemma of vertebrate skeletal muscle but is instead concentrated at discrete sites. The absence of periodicity reported for immunolabeling with anti-dystrophin and secondary antibody conjugated to HRP (Carpenter et al., 1990) was probably due to diffuse deposition of enzyme reaction product on nearby structures, a phenomenon noted previously (Sanes, 1982). Electron microscopic studies of muscle labeled with immunogold (Watkins et al., 1988; Cullen et al., 1990; Byers et al., 1991; Wakayama and Shibuya, 1991) afforded labeling so sparse that the pattern we describe would have been very difficult to detect. In addition, those studies did not attempt to correlate immunogold labeling on the sarcolemma to the underlying contractile apparatus. Even immunofluorescence may not detect the periodic distribution of dystrophin if longitudinal sections are too thick or if the sarcolemma is skewed such that distinct label at I bands and M lines cannot be observed. Limited regions of periodic labeling are apparent in some reports (Bonilla et al., 1988*b*; Ohlendieck et al., 1991), however.

We were concerned that the labeling of discrete domains at the sarcolemma might be a result of disruption of the membrane during processing. We tested this possibility in three experiments. Sections from extensively fixed myofibers had the same labeling patterns as those used in most of our studies, which were lightly fixed. This suggests that these patterns were not caused by inadequate fixation of the tissue. In addition, sections showed smooth labeling of the extracellular matrix by anti-fibronectin antibodies, and of the lipid bilayer by C_{12}-DiI, in the same focal plane as the periodic lattice of dystrophin and spectrin. Because there was no fragmentation of either the extracellular matrix or the lipid bilayer, it is unlikely that the cytoskeletal proteins underneath the sarcolemma were disrupted. Therefore, the labeling of dystrophin, spectrin, and vinculin that we report is probably not the result of processing artifact.

These results suggest that dystrophin and the major muscle isoform of β-spectrin are present in the structures which connect the contractile apparatus to the sarcolemma and stabilize the sarcolemma itself (see below). Dystrophin is similar in primary structure to spectrin, a major component of the erythrocyte sub-membrane network and has been shown to be present in a spectrin-rich filamentous network in acetylcholine receptor clusters of cultured myotubes (Dmytrenko et al., manuscript submitted for publication). A similar network is present under the sarcolemma of mouse skeletal muscle (Wakayama and Shibuya, 1990, 1991). We think it likely that dystrophin and β-spectrin are part of such a network in the membrane domains described in this report.

Ultrastructural experiments have shown that connections between the contractile apparatus and electron-dense areas on the sarcolemma occur at the M and Z lines in some striated muscles. Some filaments course directly from M lines to the membrane while other filaments travel from Z lines to the sarcolemma underlying the I bands in rat diaphragm and frog semitendinosis (Pierobon-Bormioli, 1981; Street, 1983). In bee flight, mammalian cardiac, and chicken tonic skeletal myofibers, however, attachments occur only at Z lines (Garamvögyi, 1965; Chiesi et al., 1981; Shear and Bloch, 1985). These connections allow for the transmission of force laterally along the myofiber to the extracellular matrix, to adjacent myofibers, and, ultimately, to the tendon (Street, 1983). In addition, these structures may stabilize the sarcolemma in two ways: (*a*) They may support the membrane in the areas where the most stress is applied—the sites of attachment between the sarcolemma and the contractile apparatus. The sub-membrane skeleton that is probably found at these sites would stabilize the membrane. It could also connect the contractile apparatus to extracellular structures through integral membrane proteins such as integrins, which may be enriched over I bands (Terracio et al., 1989, 1990), or through the glycoprotein complex associated with dystrophin (Campbell and Kahl, 1989; Ervasti et al., 1990; Yoshida et al., 1990; Ohlendieck et al., 1991), a component of which binds laminin (Ibraghimov-Beskrovnaya et al., 1992). As the disruption of the membrane skeleton of erythrocytes through mutations of the component proteins causes hereditary spherocytosis and elliptocytosis (Gallagher et al., 1990; Liu et al., 1990; Palek and Lambert, 1990), a similar disruption of a sub-sarcolemmal network could cause damage such as is seen in muscle from patients with DMD. (*b*) The connections between the sarcolemma and contractile apparatus also stabilize the sarcolemma during contraction by creating an ordered "festooning" of the membrane in which the non-attached membrane bulges into the extracellular space to maintain cell volume (Pierobon-Bormioli, 1981; Pardo et al., 1983*a*; Street, 1983; Shear and Bloch, 1985). Without some type of ordered anchorage of the membrane, the sarcolemma would fold in a disordered fashion and so might be damaged during contraction.

The absence of dystrophin from the sub-sarcolemmal lattice may account in part for the damage that occurs in Duchenne's muscular dystrophy. The primary site of damage in dystrophic muscle is the sarcolemma (Mokri and Engel, 1975; Carpenter and Karpati, 1979). The relative importance of dystrophin and β-spectrin in maintaining the integrity of the sarcolemma and the contractile apparatus has not been determined. Although a lattice of spectrin is present in dystrophic muscle, the lack of dystrophin may render it too weak to withstand the stress of normal contraction. Muscles from both *mdx* mice and patients with DMD lack dystrophin (Hoffman et al., 1987), but contain a lattice of spectrin, yet *mdx* muscle sustains less damage despite some structural modifications (Bulfield et al., 1984; Torres and Duchen, 1987; Tidball and Law, 1991). The lattice of spectrin may therefore be sufficient to protect *mdx* muscle. The amount of damage to myofibers seems to correlate to the amount of stress on the muscle (Florence et al., 1985; Webster et al., 1988), and myofibers in mice probably undergo less stress than human myofibers because of the smaller body mass of mice. The higher stress to which human muscle is exposed may render a lattice containing spectrin but not dystrophin inadequate to protect DMD muscle from irreversible damage and clinical manifestations.

In immunofluorescence studies of striated muscle, β_1 integrin, a collagen receptor, γ-actin, spectrin, ankyrin, vinculin, talin, and intermediate filament proteins have been shown to localize to a sub-membrane, two-dimensional lattice containing longitudinal strands and elements overlying the I bands (Repasky et al., 1982; Craig and Pardo, 1983; Nelson and Lazarides, 1983; Pardo et al., 1983*a*,*b*; Nelson and Lazarides, 1984; Shear and Bloch, 1985; Terracio et al., 1989, 1990). In addition, spectrin and ankyrin at the sarcolemma have been localized above M lines as well as the two-dimensional lattice of costameres and longitudinal strands (Repasky et al., 1982; Craig and Pardo, 1983; Nelson and Lazarides, 1983, 1984). The results presented above show that these three domains contain dystrophin and the major muscle isoform of β-spectrin. We have also shown that vinculin or metavinculin is present over M lines as well as over I bands and in longitudinal strands. Vinculin has only been reported under the skeletal muscle sarcolemma at the latter two structures by other investigators (Craig and Pardo, 1983; Pardo et al., 1983*a*). As we do not yet have antibodies that specifically recognize either vinculin or metavinculin, we cannot rule out the possibility that metavinculin is selectively enriched at the domains overlying the M lines. The failure to detect vinculin over M lines in earlier studies of chicken muscle (Craig and Pardo, 1983; Pardo et al., 1983*a*; Shear and Bloch, 1985) may be explained by differences in expression of vinculin and metavinculin among fiber types or species (Feramisco et al., 1982; Saga et al., 1985; Glukhova et al., 1986; Belkin et al., 1988*a*,*b*; Gimona et al., 1988*a*).

Thus the concept of costameres in mammalian muscle may have to be expanded from structures at or flanking Z lines to include all structures that mediate lateral attachment of the contractile apparatus to the sarcolemma. If that is the case, then, like vinculin, other proteins previously found in costameres and in longitudinal strands may be present above M lines. We are currently investigating the distribution of such proteins at the sarcolemma to determine if they are limited to positions above the I bands or if they are more widespread.

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