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Size and localization of dystrophin molecule: immunoelectron microscopic and freeze etching studies of muscle plasma membranes of murine skeletal myofibers

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Abstract. The ultrastructure and mode of existence of the dystrophin molecule and its relations to actin filaments were examined in murine skeletal myofibers. Electron microscopy of freeze-etched replicas of gold-labelled dystrophin molecules in quick-freeze, deep-etch, rotary-shadow preparations revealed rod-like structures 108.2 ± 16.3 nm long and 3.1 ± 1.5 nm thick. Some dystrophin molecules appeared to link their ends to form anastomosing networks; others were separate from each other. The dystrophin molecules were parallel or nearly parallel to the inner surface of the muscle plasma membrane. Double immuno-labelling transmission electron microscopy using N- and C-terminal dystrophin antibodies showed that the group mean distances of the N- and C-terminal signals from the muscle plasma membrane were 52.7 ± 8.1 nm and 45.9 ± 11.3 nm, respectively, which were not significantly different. Histograms of the distribution of the N- and C-terminal distances from the muscle plasma membrane had similar patterns with peaks 10 ~ 20 nm from the membrane. This was consistent with the findings of the mode of existence of dystrophin molecules seen in freeze-etched replicas. Finally, the dystrophin molecules were linked with the most peripheral sarcoplasmic actin like filaments, end to side as well as end to end.

Key words: Size and localization of dystrophin – Relation to actin filament – Skeletal myofibers – Freeze-etched replica – Double immuno-labelling electron microscopy

Dystrophin is a large molecular weight, low abundance membrane cytoskeletal protein encoded by the Duchenne muscular dystrophy (DMD) gene [12, 25]. Previously, we and others have presented electron microscopic data showing the presence of dystrophin at the inner surface of muscle plasma membrane [6, 26, 30, 36–38, 41]. Electron microscopy of replicas of deeply etched, antibody-labelled muscle samples demonstrated the presence of rod-shaped dystrophin molecules on the inner surface of muscle plasmalemma [37, 38]. However, the size and mode of existence of dystrophin molecules at the inner surface of the muscle plasmalemma has not yet been fully elucidated. Thus, in the present study we investigated the size and situation of dystrophin molecules on the inside surface of sarcolemmal plasmalemma. We first examined the various rod-shaped dystrophin molecules and their mutual interactions, and measured their lengths and diameters. Most of these molecules lay parallel to the inner surface of the muscle plasma membranes. However, the possibility cannot be ruled out that some dystrophin molecules that lie at an angle to the inner surface of muscle plasma membranes might have been scraped away and eliminated by the process of freeze fracturing. To examine this possibility, the second part of this study was to undertake electron microscopy of double-immunolabelled muscle samples using the N- and C-terminal dystrophin antibodies, and compare the distances of two different sizes of gold particles from the muscle plasma membranes. The dystrophin molecules were thought to link with other membrane and sarcoplasmic cytoskeletons for their functional roles. We previously suggested the probable connection of actin filaments with dystrophin molecules that were decorated by biotinilated secondary antibody [37]. Here we also show connection of dystrophin molecules to actin-like filaments of muscle fibers in immunogold-labelled muscle samples.

Materials and methods

C-terminal peptide synthesis and antibody production

General procedures for peptide synthesis and antibody generation were similar to those described previously [40]. Briefly the

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synthetic peptide of the C-terminal end of the human dystrophin molecule [16] was predicted by amino acid residues 3,676–3,685 and the extra cysteine was added to the 3′ terminus in this peptide (PGKPMREDTM-C). The synthetic peptide has no amino acid sequence homology with chick α-actinin [2] or slime mold α-actinin [16]. Solid-phase enzyme-linked immunosorbent assay (ELISA) was used to determine the rabbit polyclonal antibody titer, which was × 128,000.

Immunohistochemistry and Western blot analysis of antibody against C-terminal synthetic peptide

Western blot analysis of the quadriceps femoris muscles from normal control mice(C57BL/10ScSn) and mdx mice (C57BL/10ScSn-mdx) was carried out by a previously described method [40] with minor modifications. The protein was transferred from gel to a clear blot P membrane (ATTO) sheet by horizontal electrophoresis at 108 mA for 90 min at room temperature.

For immunohistochemistry the quadriceps femoris muscles from control mice and mdx mice were excised and immediately frozen in isopentane cooled by liquid nitrogen. Frozen 6-μm cross sections of the muscles were placed on coverslips and primary antipeptide antiserum, which was diluted 1:400, was used for immunohistochemistry. Indirect immunofluorescent staining was performed according to methods previously described [40].

Transmission and freeze-etching electron microscopy of antibody-decorated dystrophin molecules

The quadriceps femoris muscles of control mice were put in a U-shaped muscle clamp and immersed for fixation in chilled 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 1 h. The fixed muscles were washed three times in phosphate-buffered saline (PBS) and frozen in liquid nitrogen-cooled isopentane. The muscles were then cut into thin (1 ~ 3 μm) slices in a cryostat and washed three times in PBS. To eliminate nonspecific reaction, the slices were incubated for 30 min at room temperature in PBS with 5% normal donkey or goat serum according to the primary anti-dystrophin antibody. For immunolabelling we used two polyclonal antibodies, a 60-kDa antibody and an anti-6–10 antibody (kindly provided by Drs. Hoffman, Byers and Kunkel) as primary antibodies. The 60-kDa antibody was raised in sheep against a 60-kDa fusion protein containing mouse DMD homologues [12], while the anti-6–10 antibody was raised in rabbit against fusion proteins containing human DMD cDNA (residue number 6,181 to 9,544) [4, 19]. Thinly sliced quadriceps femoris muscle samples from control mice were incubated for 24 h in primary anti-dystrophin antibody solution diluted 1:1000 in PBS and washed four times in PBS. The sections were then incubated for 24 h in the secondary antibody, either 10-nm-gold-labelled donkey anti-sheep antibody (Bio cell, UK; code no. EMDAS10) or 10-nm-gold-labelled goat anti-rabbit antibody (Amersham, UK; code no. RPN421) diluted 1:20 in PBS for the respective primary antibody and washed four times. The antibody-labelled muscle samples were further fixed in chilled 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 30 min. Control sections were incubated with nonimmune sheep or rabbit serum. These muscle samples were divided into two portions: one for immuno-electron microscopy, and the other for quick-freeze, deep-etch, rotary-shadow replica study. The muscle samples for the immuno-electron microscopy were post-fixed in chilled 2% OsO_4 solution for 1 h, dehydrated in an ascending series of ethanol and propylene oxide, and embedded in Epon. The unstained ultrathin sections were observed under the electron microscope.

Samples for the quick-freeze, deep-etch, rotary-shadow replica study were processed as follows; since the secondary antibody contained glycerol as a cryoprotectant, the antibody-labelled muscle samples were washed four times and left overnight in PBS to eliminate glycerol. The antibody-labelled muscle samples, including immunocontrols, were washed four times in distilled water to eliminate the salt components of PBS and were then transferred to 40% methanol before freezing. The dystrophin antibody-labelled muscle samples and immunocontrol muscle samples were quickly frozen in liquid helium in an Eiko RF23 metal contact-type rapid-freeze apparatus. The frozen muscles were fractured in a vacuum of $1 \times 10^{-7} \sim 2 \times 10^{-7}$ Torr at a temperature of −150°~ −160°C in an Eiko FD 5A freeze-fracture apparatus (Eiko Engineering, Inc., Mito, Japan), and deep-etched at −90°C for 30 min. The specimens were then cooled to −150°~ −160°C and rotary-shadowed with electron beam-gunned platinum and carbon at a shadow angle of 30°. The 1.5-nm platinum thickness of the replicas was ensured by a quartz crystal thin-film monitor. The tissue was digested with a commercial bleaching solution. The detached replicas were washed twice in distilled water and finally picked up on uncoated 200-mesh grids. The replicas were examined and micrographed in an electron microscope. The measurement of length and diameter of dystrophin molecules was carried out in the electron micrographs enlarged to × 160,000 by a digitizing machine. The platinum thickness of 3 nm was subtracted from the individual dystrophin molecules to determine their length and diameter. The mean length and diameter of 50 dystrophin molecules were calculated.

Double-immunolabelling electron microscopy using N- and C-terminal dystrophin antibodies

Quadriceps femoris muscles from six control mice were put in a U-shaped muscle clamp and immersed for fixation in chilled 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for

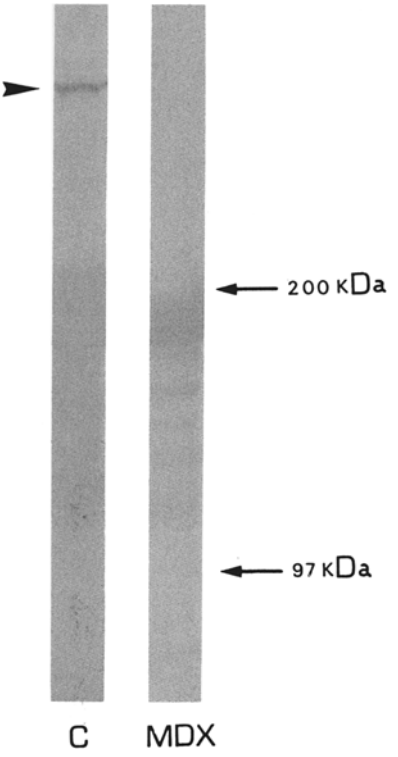


Fig. 1. Western blots of dystrophin in muscle tissues from control (*C*) and mdx mouse (*MDX*). Electrophoresis and blotting were performed by the methods described in Materials and methods. Immunostaining was with anti-dystrophin C-terminal peptide antiserum (1:600). Dystrophin band is indicated by *arrowhead* at the *left*. The numbers to the *right* indicate the molecular masses of standards; myosin (200 kDa) and phosphorylase (97 kDa)

1 h. The fixed muscles were washed three times in PBS and frozen in liquid nitrogen-cooled isopentane. The muscles were then cut into 1- to 3-μm sections in a cryostat and washed three times in PBS. To eliminate nonspecific reaction, the slices were incubated for 30 min at room temperature in PBS containing 5 % normal donkey and 5 % normal goat sera. For double-immunolabelling experiments, we used two polyclonal primary antibodies, an affinity-purified sheep antibody (1–2) against N-terminal fusion protein of human fetal skeletal muscle dystrophin [13] (a generous gift of Drs. Hoffman and Kunkel) and a rabbit antibody against the synthetic peptide of the dystrophin C terminus. The sheep and rabbit primary antibodies, which were diluted 1:200, were mixed and applied together to the section for 24 h at 4 °C. After thorough rinsing, the two secondary antibodies, which were 10-nm-gold-labelled donkey anti-sheep antibody (Bio cell) and 5-nm-gold-labelled goat anti-rabbit antibody (Amersham), were diluted 1:20 in PBS, applied together to the sections for 24 h at 4 °C, and washed four times. Control sections were incubated with nonimmune sheep and rabbit sera and processed similarly. The antibody-labelled muscle samples and control samples were further processed into Epon as mentioned above.

For the measurement of distance from the center of two different sized gold particles to the inner surface of muscle plasma membranes the electron micrographs were taken at random in 10 muscle fibers from each of six mice and were printed at a final magnification of × 160,000. The distances from the 5-nm-gold particles to the muscle plasma membranes and from the 10-nm particles to the membranes were measured in a total of 60 muscle fibers, and statistically analyzed by the two tailed *t*-test.

Results

Western blot analysis and immunofluorescence

Western blot analysis showed that antiserum against the C-terminal portion of dystrophin reacted with a protein of molecular mass of about 400 kDa in muscle extracts of control mice (Fig. 1). This antiserum did not react with any proteins in muscle extract from mdx mice (Fig. 1).

Immunohistochemically, rabbit antibody against the C-terminal portion of dystrophin showed strong and clear staining of the cross-sectioned myofiber surface membrane in control mice (Fig. 2a), but not in the mdx dystrophin deficient mice (Fig. 2b).

Transmission and freeze-etching electron microscopy of antibody-decorated dystrophin molecule

Immunoelectron microscopy of Epon-embedded muscle samples of control mice showed the presence of 10-nm gold particles just inside the plasma membrane (Fig. 3a,b) and gold particles were also observed inside the myofiber a short distance from the plasma mem-

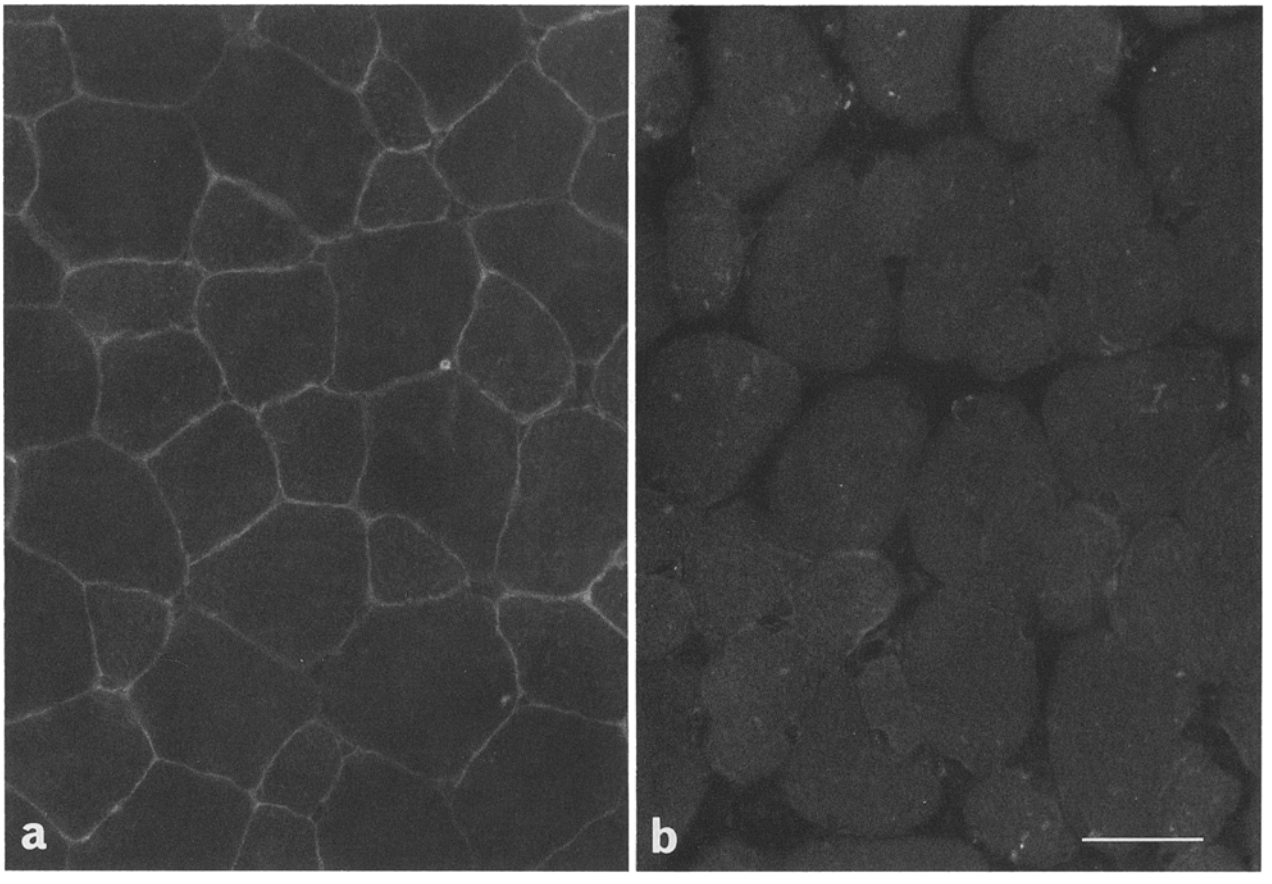


Fig. 2a,b. Binding of anti-dystrophin C-terminal peptide antiserum in skeletal muscle of (**a**) control mouse and (**b**) mdx mouse. Dystrophin immunoreaction is observed at the surface membranes of myofibers in control mouse and is absent in mdx mouse. *Bar* for **a** and **b** = 50 μm

brane. No immunoreaction was evident in cellular organelles such as transverse tubules or triadic junctions of control myofibers. The immunoreaction pattern of the sheep anti-60-kDa antibody was identical to that of the rabbit anti-6–10 antibody, but the signal density was higher with the rabbit anti-6–10 antibody. No gold particles were observed in the myofibers incubated in the nonimmune sheep or rabbit serum.

In the quick-freeze, deep-etch, rotary-shadow preparations, fracture lines proceeded at random through the muscle samples. Cytoskeletons associated with muscle plasma membrane were easily visualized on the exposed cytoplasmic surface of the muscle plasma membrane (Fig. 4a). At higher magnification, most of the plasma membrane-associated cytoskeletons formed an anastomosing network on this cytoplasmic surface, whereas a few membrane cytoskeletons were present but separate from other membrane – associated cytoskeletons (Fig. 4b). Dystrophin molecules were easily recognized by their associated 10-nm gold particles. These are indicated by the arrow (Fig. 4c). Although some dystrophin molecules were thickened due to decoration by antibodies, and it was difficult to observe whole molecules, the overall shapes of dystrophin molecules were rod like. These included straight or curved, or coiled rods, with or mostly without enlarged end(s) (Figs. 4b,c;

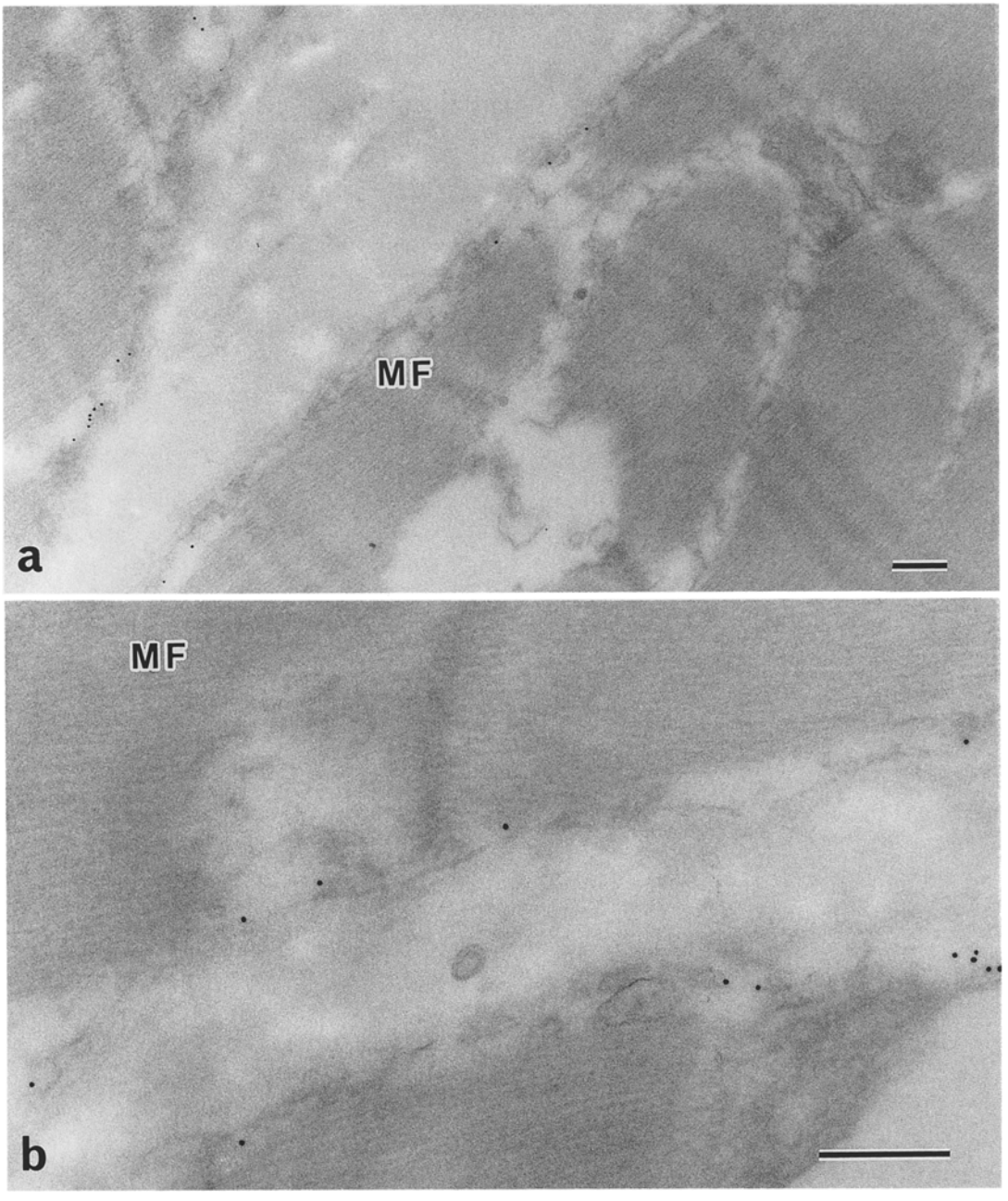


Fig. 3a,b. Electron micrographs of rabbit anti-dystrophin (6–10) antibody binding in myofibers from control mouse. The gold immunoreaction particles are seen at the inside surface of the muscle plasma membrane in both figures. Reaction is absent deep inside the myofiber (**a**). *MF*, myofilaments. *Bars* **a,b** = 0.25 μm

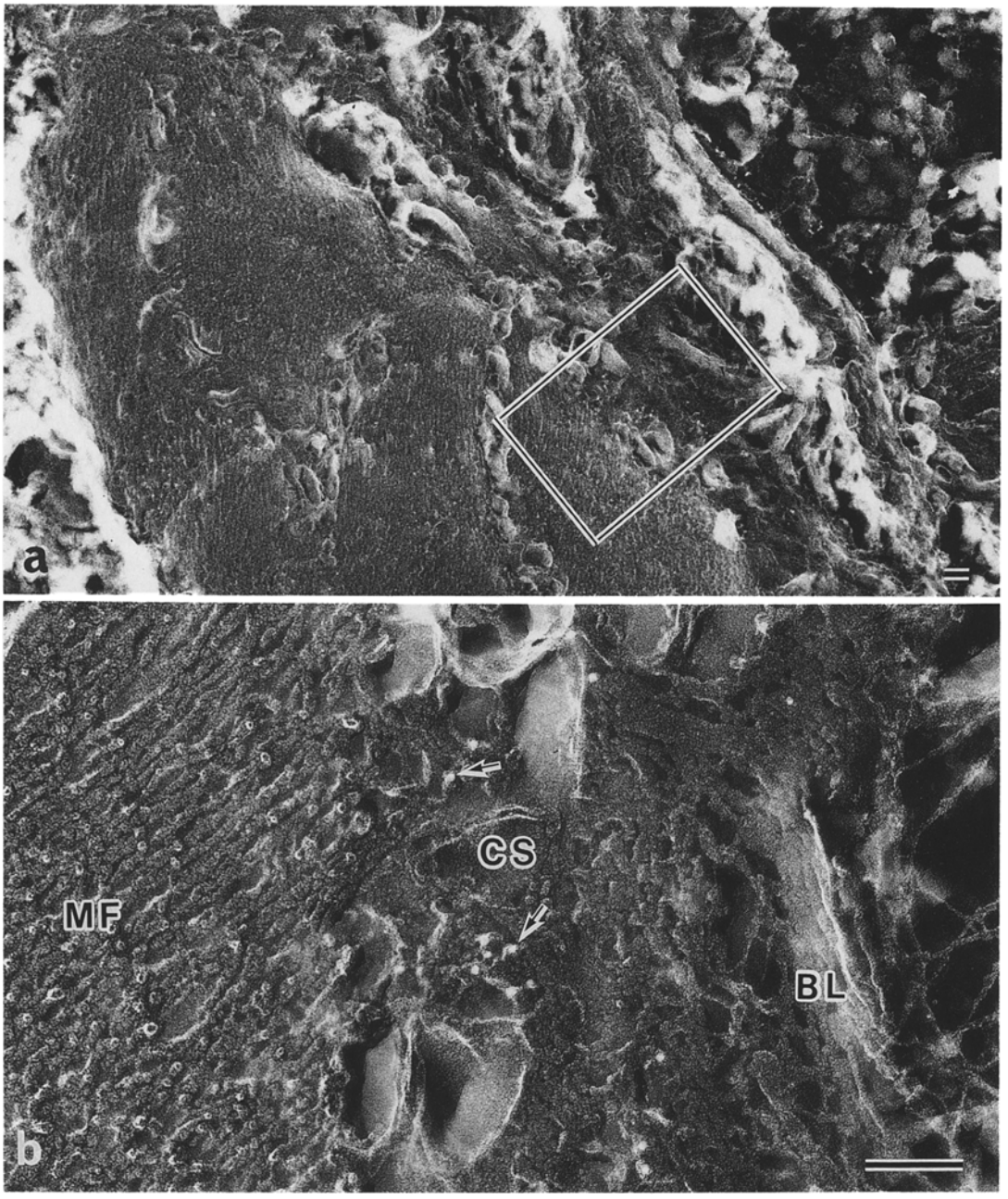


Fig. 4a–d. Electron micrographs of replica of the rabbit anti-dystrophin (6–10) immunogold-labelled control muscle. Low-magnification micrograph (**a**) shows the transitional zone (*rectangle*) between the outside and inside of myofiber. **b** Higher magnification of *rectangle* of **a** demonstrates the presence of gold (*arrows*)-labelled structures at the cytoplasmic surface (*CS*) of a myofiber. *MF*, Myofilaments, *BL*, Basal lamine. **c** Higher magnification of cytoplasmic surface area (around *CS*) of **b** shows structures (*small arrows*) associated with gold particles (*large arrows*). These structures (*small arrows*) are considered to be dystrophin molecules. The enlarged end (*arrowhead*) of a dystrophin molecule is evident. **d** Drawing of the dystrophin network present in the lower right quadrant of **c** The putative N and C termini are indicated by the locations of gold particles on the dystrophin molecule. *Bars* **a–c** = 0.1 μm

5a–e; 6a–e). The ends of some dystrophin molecules appeared to link and form anastomosing networks, and others remained separate from each other (Fig. 4b,c). Most dystrophin molecules were present singly, and not as double-helical heterodimers. In the drawing (Fig. 4d), the putative N and C termini of each dystrophin molecule are indicated by the location of gold particles of secondary antibody against anti-6–10 primary antibody. From this finding, the N and C termini of neighboring dystrophin molecules appear to interact with each other to form an anastomosing network (Fig. 4c). The dystrophin molecules were calculated to be 108.2 ± 16.3 nm ($n = 50$) long and 3.1 ± 1.5 nm ($n = 50$) in diameter. All of the dystrophin molecules appeared to be parallel or nearly parallel to the cytoplasmic surface of the muscle plasma membrane. This is more easily recognizable in the conventional print (Fig. 5a) than in the white-black reversed print in which

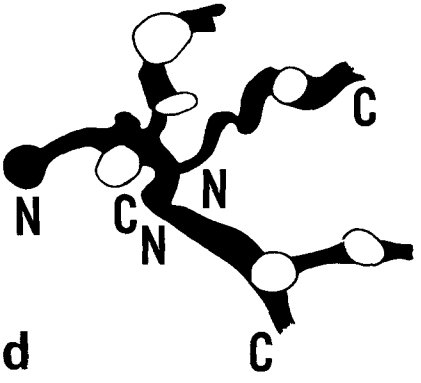


Fig. 4c,d.

the cytoskeletal elements are more obvious while background texture often vanishes (asterisks, Fig. 5a,b). Some dystrophin molecules showed connections with the farthest-out myofibrillar actin-like filament which displayed a characteristic surface contour that repeated approximately every 5 nm (arrows, Figs. 5f, 6f). The end of the dystrophin molecule was associated with the end of an actin-like filament (Fig. 5f), and the dystrophin molecule in Fig. 6f was connected end to side with an actin-like filament.

Double-immunolabelling electron microscopy using N- and C-terminal dystrophin antibodies

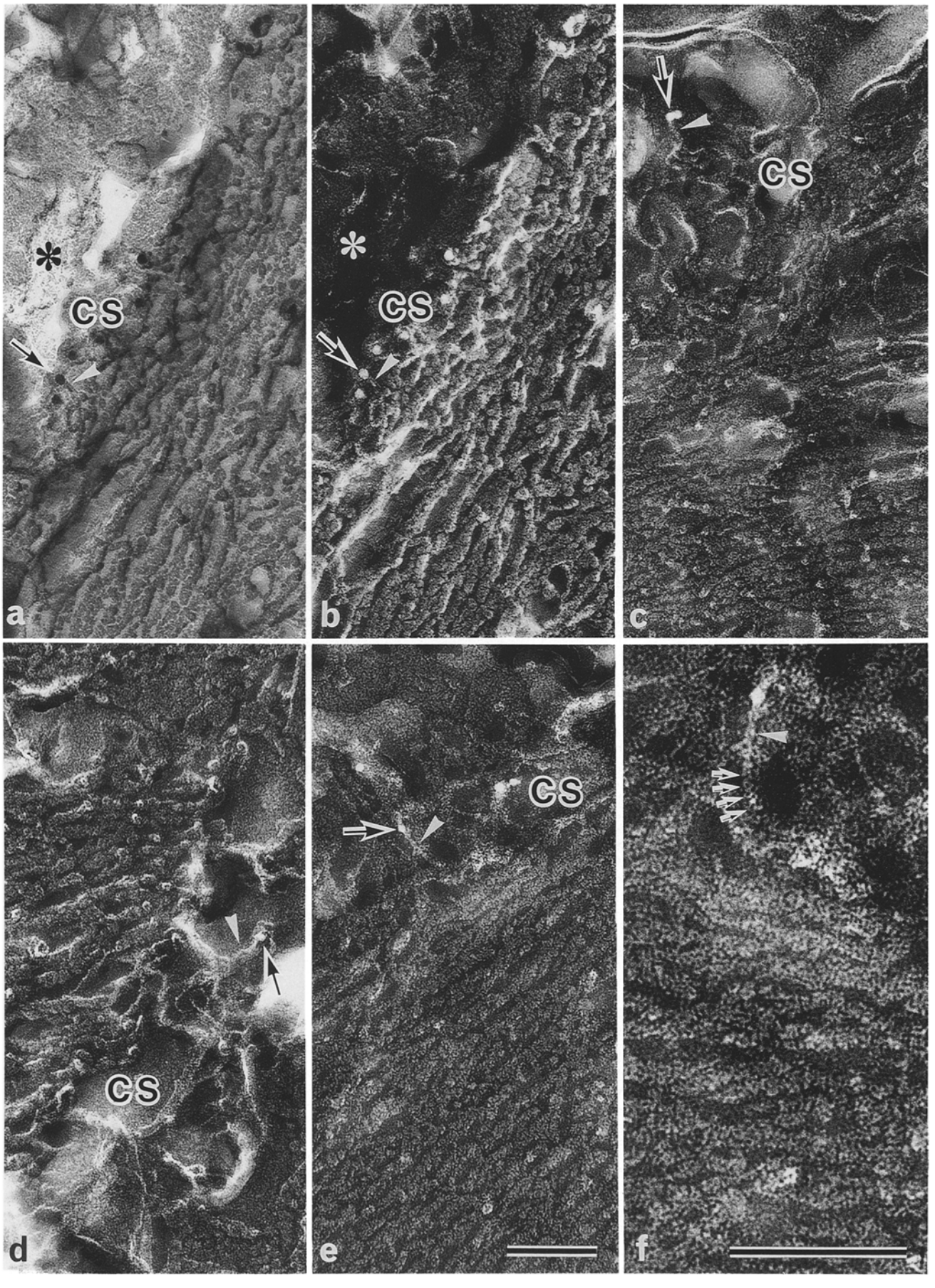
Immunoreaction was seen at muscle cell peripheries, and higher magnification of these areas revealed the presence of 5- and 10-nm gold particles, mostly confined to the inner surface of the muscle plasma membranes (Fig. 7). The 5-nm gold particles were more numerous than the 10-nm particles. Qualitative analysis did not place the 10-nm particles farther from the muscle plasma membranes than the 5-nm particles. No gold particles were noted in the myofibers incubated in the nonimmune sheep and rabbit sera. Quantitatively the mean distance of the 5-nm gold particles from the muscle plasma membrane was 45.9 ± 11.3 nm and the distance of the 10-nm particles was 52.7 ± 8.1 nm; a difference that was not statistically significant ($P > 0.1$, two tailed t-test). Distribution of the two kinds of gold particles are shown in Fig. 8.

Discussion

Cytoskeletons associated with muscle plasma membrane were revealed by freeze-etching electron microscopy to have various shapes [15, 36–38], and they contained various kinds of substrates such as dystrophin [1, 3, 12, 20–22, 32, 33, 35, 39, 44], spectrin [28], fodrin [23], actin [37] and vinculin [29]. Among these, dystrophin is reported to constitute 5% of the total cytoskeletons that are associated with muscle plasma membrane [25]. Since dystrophin molecules are considered to be incorporated in the subsarcolemmal network, it is interesting and important to investigate the nature of their presence, the relations between dystrophin and other cytoskeletal elements, and their ultrastructures.

Several shapes of dystrophin molecule have been reported. Pons et al. [27] reported rods about 175 nm long isolated from chicken gizzard. Murayama, et al. [24] and Sato et al. [31] described dumbbell-shaped dystrophin, about 110 nm long, isolated from rabbit skeletal muscle. We previously reported various kinds of rods of gold-labelled dystrophin molecules in mouse skeletal muscles [38]. In the present study, we further show extensive gold-labelled dystrophin molecules about 110 nm long and 3 nm thick. They were rod-like with minor variations, such as straight, curved or coiled. This information was more easily obtainable in the immunogold-labelled muscle samples than the biotinilated secondary antibody-labelled muscle samples in the present study because of the easy identification of gold particles. In the biotinilated secondary antibody-labelled samples [26, 37] it was often difficult to differentiate real antibody decoration from unevaporated ice crystals. However, the advantage of using biotinilated

Fig. 5a–f. Electron micrographs of replica of rabbit anti-dystrophin (6–10) immunogold-labelled control muscles. At the cytoplasmic surface (*CS*), structures (*arrowheads*) associated with gold particles (*arrows*) of homogeneous density are shown. These structures (*arrowheads*) are considered to be dystrophin molecules and appear rodlike (**a–e**). **f** Higher magnification of **e**. The gold-labelled rod-like structure (*arrowhead*) is connected end-to-end with a filament which shows a characteristic surface contour with periodicity of about 5 nm (series of *four arrows*). *Asterisks* of **a** and **b** point out the loss of back ground texture by white-black reversal. *Bars* **e** (for **a–e**), **f** = 0.1 μm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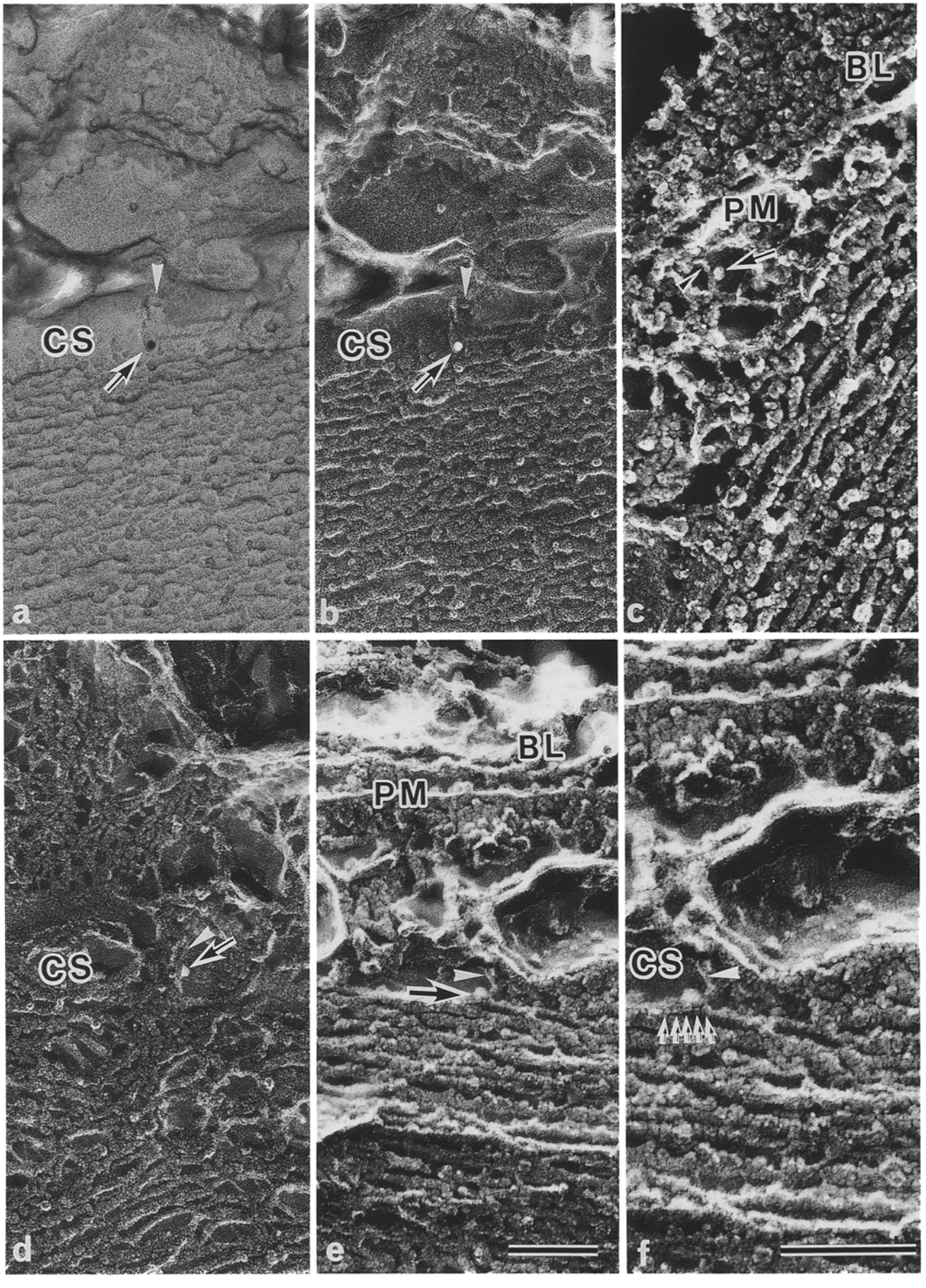
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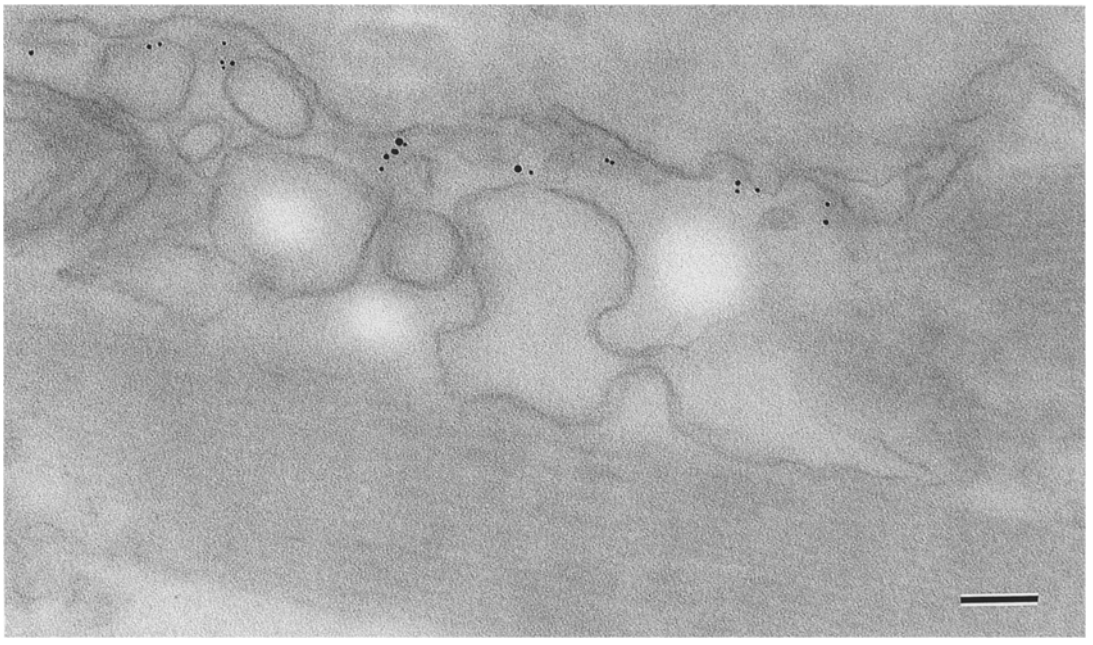

Fig. 7. Electron micrograph showing results of double-immunolabelling cytochemistry using anti-dystrophin N-terminal (10-nm gold) and C-terminal (5-nm gold) antibodies. Two different sized gold particles are evident along the inside surface of the muscle plasma membrane. *Bar* = 0.1 μm

secondary antibody was the easy penetration of antibody into the deep muscle samples.

The freeze-etched electron micrographs showed the dystrophin molecules to be parallel to the cytoplasmic surface of the muscle plasma membrane. Although the white and black reversed pictures have the advantage of easy identification of the cytoskeletal elements, this reversal introduces an artifact in which the membrane cytoskeletons appear to be protruding, and the spacial relation between the membrane cytoskeletons and the cytoplasmic surface is often distorted because of the frequent disappearance of the cytoplasmic surface texture as background. Our present results in which the mean distances between N- and C-dystrophin terminal signals and the cytoplasmic surface of the muscle plasma membranes were not significantly different were additional evidence that the dystrophin molecules are parallel, or nearly parallel, to the cytoplasmic surface of the muscle plasma membranes. Dystrophin molecules are reported to link with the anchoring proteins of intramembranous particles in muscle plasma membrane [5, 43]. The main linking point has been thought to be in the C-terminal end [7, 11], or the cysteine-rich domain and the first half of the C-terminal domain [34]. Interestingly, our present study revealed connections of N-terminal ends of dystrophin molecules with the C-terminal ends of neighboring dystrophin molecules, forming a subsarcolemmal network. According to Ohlendieck et al. [25], dystrophin constitutes about 5 % of the total membrane cytoskeletons, and it may, thus, be impossible to form a subsarcolemmal network with dystrophin molecules alone. It would be interesting to see with what kinds of membrane cytoskeletons the dystrophin molecules are linking, but thus far there is little such information. However, linking of dystrophin with an actin protein has been suggested biochemically and biogenetically by several groups of investigators [8, 9, 17, 18, 42]. A few of the earlier papers [26, 37] presented ultrastructural evidence that dystrophin links with myofibrillar actin-like filaments, and we present additional ultrastructural evidence of dystrophin connection with myofibrillar actin-like filaments which display a characteristic surface contour that is repeated approximately every 5.5 nm [10]. The muscle plasma membrane contains various kinds of membrane cytoskeletons, including dystrophin, and further investigation of the three-dimensional relations of dystrophin to other membrane and sarcoplasmic cytoskeletons, and dystroglycan [14] may clarify the precise functional roles of dystrophin molecules.

Fig. 6a–f. Electron micrographs of replica of sheep anti-dystrophin (60 kDa) immunogold-labelled muscles of control mice. At the cytoplasmic surface (*CS*) or near the plasma membrane (*PM*), structures (*arrowheads*) associated with gold particles (*arrows*) of homogeneous density are shown. These structures (*arrowheads*) are considered to be dystrophin molecules and appear rodlike (**a–e**). **f** Higher magnification of **e**. The gold-labelled structure (*arrowhead*) is connected end-to-side with a farthest-out filament which shows a surface contour with about 5-nm periodicity (series of *five arrows*). *BL,* Basal lamina. Occasionally a structure with gold particle is more easily identified in conventional print than in white-black reversed print (**a,b**). *Bars* **e** (for **a–e**), **f** = 0.1 μm

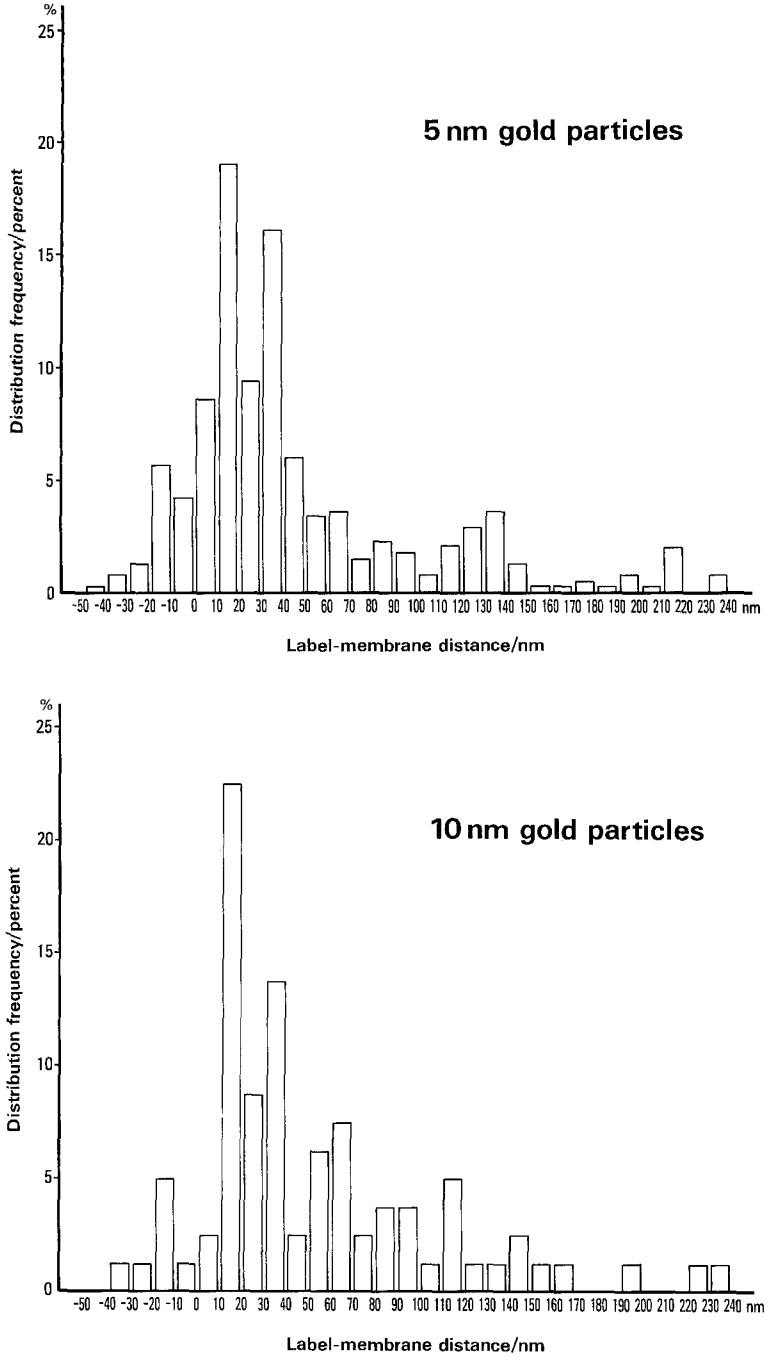

Fig. 8. Histograms depicting the distance from the plasma membrane of two different size gold particles. The patterns of distribution of the 5- and 10-nm gold particles are quite similar, and the peaks of distribition are in the range 10~20 nm from the muscle plasma membrane for both the 5- and 10-nm particles

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