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Cell fractionation studies indicate that dystrophin is a protein of surface membranes of skeletal muscle

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We studied the subcellular localization of dystrophin in rabbit skeletal muscle. In Western-blot analysis of membrane preparations, dystrophin was associated with the sarcolemmal fraction, as indicated by cholesterol content and co-purification with ouabain-binding activity and β-adrenergic receptor. Dystrophin was also found with junctional T-tubules, but not with 'free' T-tubules, longitudinal portions or terminal cisternae of the sarcoplasmic reticulum. Dystrophin was not solubilized by high salt solutions, but it was solubilized by low concentrations of detergents (Triton X-100 and deoxycholate), suggesting that it is a peripheral membrane protein.

INTRODUCTION

Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene (Hoffman *et al.*, 1987*a*), is found in skeletal and cardiac muscle, and, at a lower concentration, in smooth muscle (Hoffman *et al.*, 1987*a*,*c*; Chelly *et al.*, 1988). In striated muscle, dystrophin accounts for 0.001 % of total muscle protein. Dystrophin mRNA has also been found in the brain of rat, mouse and rabbit (Nudel *et al.*, 1988) and in several human tissues (Chelly *et al.*, 1988). In muscle culture, dystrophin mRNA or dystrophin were not found in unfused myocells but were present, after fusion, in myotubes (Lev *et al.*, 1987; Miranda *et al.*, 1988). Dystrophin has a molecular mass of about 400 kDa. The gene has been sequenced (Koenig *et al.*, 1988); the predicted protein sequence indicates a strong propensity for an α-helical secondary structure. The protein is similar to other sarcomere proteins, such as myosin rod and tropomyosin, with a pattern of alternating charged and hydrophobic residues in heptad repeats, suggesting a coil–coil tertiary structure (Hoffman *et al.*, 1987*c*; Koenig *et al.*, 1988). Furthermore, the amino acid sequence of a portion of dystrophin is similar to that of both α-actinin, an actin-binding protein (Hammonds, 1987), and spectrin (Koenig *et al.*, 1988). Thus, the primary sequence suggests that dystrophin may interact with other muscle proteins.

Hoffman *et al.* (1987*c*) and Koenig *et al.* (1988) have pointed out that dystrophin might be associated peripherally or integrally with muscle membranes. However, stretches of hydrophobic amino acids similar to those of proteins typically spanning the lipid bilayer have not yet been identified (Koenig *et al.*, 1988).

In subcellular fractionation experiments, Hoffman *et al.* (1987*b*) and Knudson *et al.* (1988) found that dystrophin was associated with triads, membrane structures that are formed by the convergence of the T-tubules (invaginations of sarcolemma) and pairs of terminal cisternae of the sarcoplasmic reticulum. In cell fractionation studies, dystrophin seemed to be bound to the T-tubule component of the triads (Knudson *et al.*, 1988). However, in immunocytochemical studies, dystrophin was localized to the sarcolemma, the surface-membrane system of the muscle cell (Arahata *et al.*, 1988; Bonilla *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988). Very little immunofluorescence was found intracellularly, at the site of the triads. These studies were carried out using different antibodies against dystrophin, including the antibody employed by Hoffman *et al.* (1987*a*) in their fractionation studies.

Together, these results suggest that dystrophin is bound to the surface membrane, including the plasma membrane and at least part of the T-tubules. To better define the localization of dystrophin in muscle membranes, we prepared membranes separated from the longitudinal and the terminal cisternae of the sarcoplasmic reticulum (SR), the triads, the T-tubules and the plasma membrane. Dystrophin co-purified with the plasma membrane and the junctional T-tubules, but was not found in the SR membranes. Furthermore, dystrophin did not seem to be tightly bound to the membrane because it was extracted by low concentrations of detergent.

MATERIALS AND METHODS

Materials

High-purity sucrose was from Beckmann, Palo Alto, CA, U.S.A. Protease inhibitors (pepstatin, leupeptin, phenylmethanesulphonyl fluoride and trypsin soybean inhibitor), alkaline-phosphatase-conjugated antibodies [anti-(sheep IgG)] were from Sigma, St. Louis, MO, U.S.A. Radioactive ligands (ouabain, dihydroprenolol) were from Amersham International, Amersham, Bucks., U.K. All reagents were analytical grade.

Abbreviation used: SR, sarcoplasmic reticulum.

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Preparation of subcellular membrane fractions

Membranes were isolated from predominantly fast-twitch skeletal muscle of the hind limbs of New Zealand rabbit.

Preparation of SR membrane fractions. Crude SR membrane preparations were frationated by density gradient centrifugation into longitudinal tubules (LSR or R2) and terminal cisternae (TC or R4) as described by Saito *et al.* (1984).

Preparation of T-tubule membrane fractions. The T-tubular system consists of free T-tubules and junctional T-tubules (Franzini-Armstrong, 1986). Junctional T-tubules are the innermost portions of the T-tubular system, abutting the terminal cisternae of the SR. The junctional T-tubules can be separated from free T-tubules because they are bound to SR cisternae, and therefore migrate into the high-density sucrose region of the gradient, whereas the free T-tubules are recovered in the very light region of the sucrose gradient. Free and junctional T-tubules are also structurally different; the free T-tubules lack the large particles on the cytoplasmic leaflet of the junctional membrane and which are probably related to the junctional feet (Franzini-Armstrong, 1986), the 1,4-dihydropyridine receptor (Leung *et al.*, 1988), or both. The activity of Mg-dependent basal ATPase activity is higher in free T-tubules (Martonosi, 1984; Horgan & Kuypers, 1987).

Membrane fractions enriched in free T-tubules (R1 in Saito's nomenclature) were obtained by the centrifugation procedure of Saito *et al.* (1984). Junctional T-tubules were prepared by the method of Horgan and Kuypers (1987).

Triads were purified according to either Mitchell *et al.* (1983) or Horgan & Kuypers (1987). Sarcolemma was prepared by the method of Seiler & Fleischer (1982), except that we omitted the last purification step on a dextrane gradient. All solutions used for muscle fractionation contained the following protease inhibitors: 2 mg of phenylmethanesulphonyl fluoride/l, 0.1 μM-pepstatin, 0.5 mg of leupeptin/l and 50 μg of soybean trypsin inhibitor/l.

Assays

Mg-ATPase, Ca-ATPase, and Na,K-ATPase activities were determined spectrophotometrically with an enzyme-coupled ADP-release assay (Warren *et al.*, 1974) by measuring the oxidation of NADH at 340 nm and 37 °C as previously described (Salviati *et al.*, 1982). The binding of [^{3}H]ouabain was measured according to Jones *et al.* (1980). The concentration of β-adrenergic receptors was measured as described by Caswell *et al.* (1978) using 1-[propyl-2,3-^{3}H]dihydroalprenolol. Cholesterol was measured as described by Gamble *et al.* (1978). Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

SDS/polyacrylamide-gel electrophoresis was carried out according to Laemmli (1970) on 3.5–10% polyacrylamide linear gradient gel. After electrophoresis, the protein bands were transferred to a nitrocellulose sheet by the method of Towbin *et al.* (1979). The nitrocellulose sheets were stained with Ponceau Red, photographed and destained with several changes of distilled water. Thereafter, the nitrocellulose sheets were immunoreacted (Biral *et al.*, 1984) with sheep anti-(60 kDa mouse dystrophin peptide) (Hoffman *et al.*, 1987*a*) diluted 1:1000. The second antibody was donkey anti-(sheep IgG) conjugated with alkaline phosphatase (1:1000).

RESULTS

Triads prepared by the pyrophosphate method (Mitchell *et al.*, 1983) contained a protein band that reacted selectively with the anti-dystrophin antibody (Fig. 1*a*). In agreement with the results of Hoffman *et al.* (1987*a*) this protein had an estimated molecular mass of about 400 kDa, and was a minor component (less than 0.1%) of the triad (see Ponceau Red-stained filter in Fig. 1*a*). The protein migrated just below the 450 kDa protein,

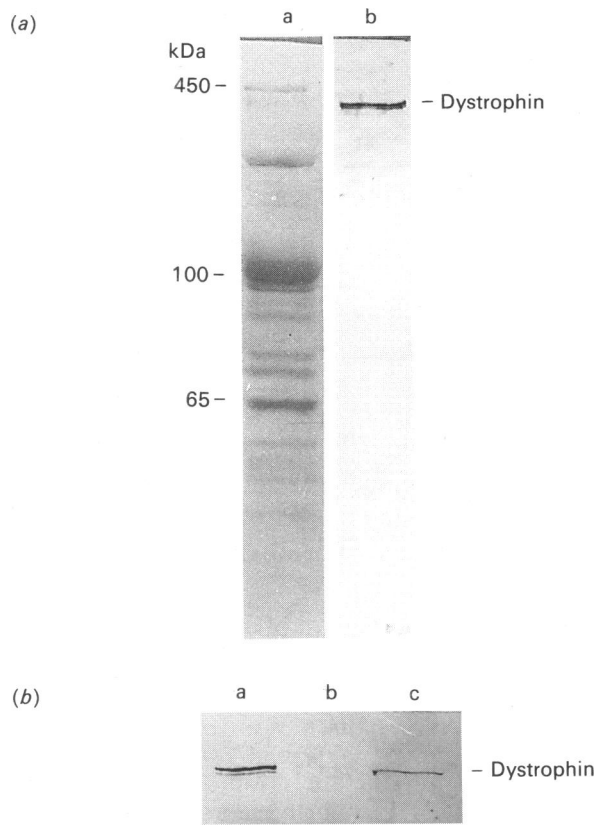

Fig. 1. Association of dystrophin with rabbit triads and with junctional T-tubules

(*a*) Rabbit triads were prepared according to Mitchell *et al.* (1983). SDS/polyacrylamide-gel electrophoresis and Western blotting were carried out as reported in the Materials and methods section. Lane *a*, 100 μg of protein stained with Ponceau Red; lane *b*, Western blotting of the same filter with anti-dystrophin antibody (anti-60 kDa). (*b*) Triads were dissociated by centrifugation on ion-free linear sucrose gradients (Horgan & Kuypers, 1987). A low-density fraction (at about 28% sucrose: junctional T-tubules) and a heavy fraction (at 40–45% sucrose: SR terminal cisternae) were collected. SDS/polyacrylamide-gel electrophoresis and Western blotting were carried out on 100 μg of membrane protein as described in (*a*). Lane a, intact triads; lane b, SR terminal cisternae; lane c, junctional T-tubules. Only the high-molecular-mass region is shown.

i.e. the ryanodine-binding protein which is known to be a component of the feet-structures (Inui *et al.*, 1987).

Triads are composed of two membrane systems, the terminal cisternae of the SR and the T-tubules, junctionally connected by the feet (Franzini-Armstrong, 1970; Somlyo, 1979). Therefore dystrophin could be bound to either the SR or the T-tubules, or to both. We therefore prepared two membrane fractions, one derived selectively from the junctional T-tubules (Horgan & Kuypers, 1987), and the other from the terminal cisternae of the SR (Saito *et al.*, 1984). T-tubules dissociated from the triads on ion-free sucrose gradient (junctional T-tubules) contained almost all the protein reacting with the anti-dystrophin antibody that was present in intact triads (Fig. 1*b*, lane c). On the other hand, the highly purified preparations of terminal cisternae did not contain a protein that reacted with the anti-dystrophin antibody (Fig. 2). In addition, the longitudinal SR membranes were also devoid of dystrophin (Fig. 2). Therefore, dystrophin seemed to be bound to the T-tubule membrane and not the SR.

The low-density membrane fraction (R1) is enriched in membranes derived from free T-tubules (Saito *et al.*, 1984). In Western blots, this membrane preparation was devoid of dystrophin (Fig. 2), implying that only junctional T-tubules contain dystrophin.

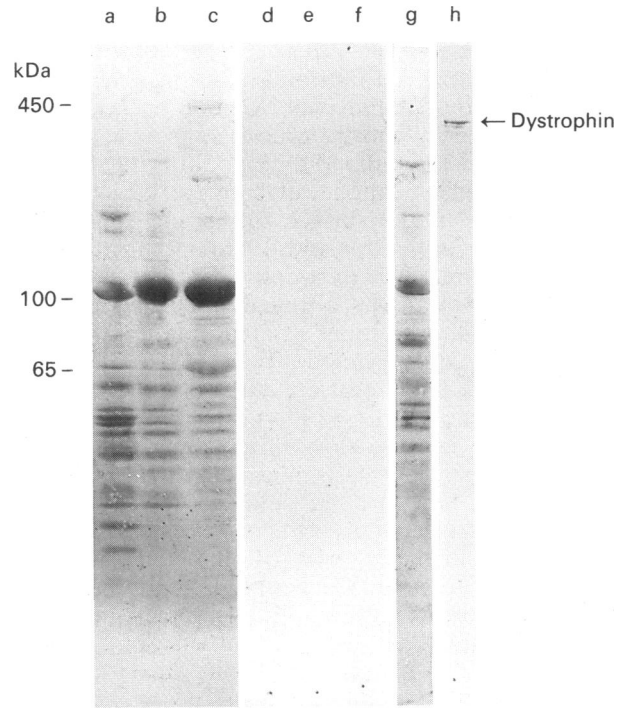

Fig. 2. Dystrophin is associated with the plasma membrane but not with the SR and free T-tubules of rabbit skeletal muscle

Rabbit-muscle subcellular fractions were obtained as described in the Materials and methods section. Lanes a, b, c and g, nitrocellulose sheet stained with Ponceau Red; lanes d, e, f, h, Western blotting with anti-dystrophin antibody (anti-60 kDa). Lanes a and d, T-tubule-enriched membrane fraction (R1); lanes b and e, SR longitudinal tubules (R2); lanes c and *f*, SR terminal cisternae (R4) [R1, R2, R4 are defined by Saito *et al.* (1984)]; lanes g and h, plasma membrane fraction.

Table 1. Plasma membrane marker concentration in subcellular membrane preparations from rabbit muscle

R1, R2 and R4 are, according to the Saito's *et al.* (1984) nomenclature, the T-tubule-enriched low-density fraction, longitudinal SR tubules and the SR terminal cisternae respectively. SL, sarcolemma. Membrane fraction isolation and the activity measurements were carried out as described in the Materials and methods section.

	Membrane fraction				
	R1	R2	R4	Triads	SL
Na,K-ATPase (μmol/h per mg of protein)	–	–	–	–	12.0
Ouabain binding (pmol/mg of protein)	13.8	3.2	0.7	18.3	42.0
Receptor (pmol/mg of protein)	–	0.03	0.03	–	0.52
Cholesterol (μmol/mg of protein)	0.21	0.005	0.005	–	0.46

Finally, we prepared a purified membrane fraction derived from plasma membranes (Seiler & Fleischer, 1982). The sarcolemmal preparation was highly enriched in markers of the muscle plasma membrane (cholesterol, β-adrenergic receptor, ouabain-binding, Na,K-ATPase activity) (Table 1), and it was reasonably free of contaminating SR membranes, as indicated by the low Ca^{2+}-ATPase activity (0.07 μmol/min per mg of protein). Western blots showed that the plasma membrane contained a protein that reacted with the anti-dystrophin antibody (Fig. 2, lanes *g*, *h*). The molecular mass of that protein (400 kDa) was identical to that of the protein found in the triads (Fig. 1).

During the isolation procedure, our sarcolemma preparation has been washed several times with 0.6 M-KCl to extract myosin and actin, but leaving dystrophin (Knudson *et al.*, 1988). However, dystrophin was consistently solubilized by low concentrations of Triton X-100 (at detergent-to-protein ratios of 0.5 and 1) (Fig. 3). Deoxycholate, at the same ratio of detergent-to-protein, was less effective in solubilizing dystrophin.

DISCUSSION

We found that dystrophin was localized in the surface membranes, including both plasma membrane and T-tubules, of rabbit skeletal muscle.

The failure of immunocytochemical methods to demonstrate dystrophin in T-tubules is not in disagreement with our data because the muscle content of dystrophin is only 0.001 % of total protein. In addition, dystrophin accounts for only 0.05–0.1 % of the total protein of the triads. Therefore, immunocytochemical methods are probably not sensitive enough to demonstrate the dystrophin which is bound to intracellular structures. Comparison with other SR proteins, such as the Ca^{2+}-pump protein, is not appropriate because triads contain about one hundred times more Ca^{2+}-pump protein than dystrophin. Hoffman *et al.* (1987*b*) did not find dystrophin in any membrane fraction other than triads. However, the anti-dystrophin antibody stained a

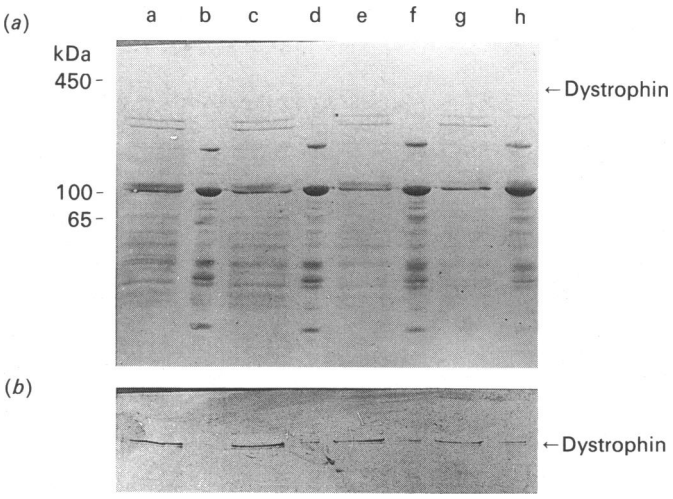

Fig. 3. Solubilization of dystrophin from the plasma membrane by deoxycholate and Triton X-100

About 200 μg of protein of sarcolemma membrane preparations were incubated with detergent at 0 °C for 30 min. Samples were centrifuged at 100000 *g* for 15 min in a Beckman Airfuge. The supernatant and the pellet were separately electrophoresed as described in the Materials and methods section. (*a*) Filter stained with Ponceau Red; (*b*) the same filter after Western blotting with anti-dystrophin antibody (anti-60 kDa). Only the high-molecular-mass region is shown. Lanes a, c, e and g are pellets; lanes b, d, f and h are supernatants. Lanes a and b, DOC:protein 0.5; lanes c and d, DOC:protein 1.0; lanes e and f, Triton X-100:protein 0.5; lanes g and h, Triton X-100:protein 1.0.

protein band of high molecular mass in the light microsomal fraction (Fig. 3; lane 10 of Hoffman *et al.*, 1987*b*) which usually contains contaminating plasma and T-tubule membranes.

Our data suggest that dystrophin is localized in junctional T-tubules and not in free T-tubules. Because we prepared both membrane fractions in the presence of protease inhibitors, there was probably no selective proteolysis of dystrophin during the preparation of free T-tubules. Furthermore, no low-molecular-mass peptide in the electrophoretic gel reacted with the anti-dystrophin antibody, suggesting that there was no proteolytic product of dystrophin. In contrast, in aged preparations of sarcolemma, we found low-molecular-mass peptides produced by the action of endogenous proteases (results not shown; see also Fig. 3 of Hoffman *et al.* 1987*b*).

Finally, we found that dystrophin was so tightly bound to the surface membrane that it was not removed by solutions of high ionic strength (Knudson *et al.*, 1988). On the other hand, dystrophin was easily extracted from the membrane by mild detergents, suggesting that it is not an intrinsic membrane protein. Similarly, sequence analysis has not shown the stretches of hydrophobic amino acids that are characteristic of transmembrane proteins (Koenig *et al.*, 1988).

If dystrophin is not a transmembrane protein, it is probably not one of the integrins (Zubrzycka-Gaarn *et al.*, 1988), the family of membrane proteins implicated in the linkage of the cell membrane to proteins of the extracellular matrix (Hynes, 1987; Niggli & Burger, 1987; Ruoslahti & Pierschbacher, 1987). The immunoelectron microscopy data of Watkins *et al.* (1988), and immunocytochemical data in living cultured human myotubes (Miranda *et al.*, 1988) indicate that dystrophin is located on the cytoplasmic site of the plasma membrane.

The role of dystrophin in muscle may be similar to that of ankyrin and band 4.1 in the red blood cell (Koenig *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988). These proteins form the 'membranoskeleton' (Marchesi, 1985) linking intrinsic membrane proteins, such as band 3 and glycophorin, to spectrin and actin. Spectrin is also found in muscle sarcolemma (Nelson & Lazarides, 1983), and dystrophin could link the surface membrane to internal structures such as intermediate filaments of the exosarcomeric cytoskeleton or myofibrils (Koenig *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988). Cables of intermediate filaments link the Z- and M-discs to the plasma membrane (Lazarides, 1980; Pierobon-Bormioli, 1981). Stereological observations in Golgi-stained SR of rabbit skeletal muscle (Yamasura & Scales, 1982) indicate that, during contraction, the shape of the SR of the I band and that of the fenestrated region of the M band do not change. Both SR regions maintained the alignment with the Z- and M-lines. In contrast, the longitudinal tubules of the SR of the A band were no longer arranged longitudinally in contracted fibres (Yamasura & Scales, 1982). These results suggest that there are connections between the contractile apparatus and the structures responsible for excitation–contraction coupling. Morphological evidence also suggests that the intermediate filaments connect the Z-disc to the triads (SR or T-tubules) (Nunzi & Franzini-Armstrong, 1980).

Therefore, dystrophin may function to preserve the structural integrity and the crucial alignment of plasma membrane and T-tubules to the myofibrils during muscle contraction and relaxation. Furthermore, the localization of dystrophin in the surface membrane supports the 'membrane theory' (Rowland, 1980) of the pathogenesis of Duchenne muscular dystrophy.

It is still not clear why, although in Duchenne patients dystrophin is lacking in all striated muscles, the disease largely spares cardiac muscle (Engel, 1986) and, among skeletal muscle fibres, fast are affected earlier than slow fibres (Webster *et al.*, 1988). Physical activity cannot play a crucial role because slow skeletal muscle fibres and cardiac muscle are normally more active than fast skeletal muscle fibres. One striking difference between cardiomyocytes and skeletal muscle fibres is cell-size, especially the transverse diameter. A large fibre requires more rigid structures to maintain the perfect lateral alignment of myofibrils and membranes (SR, T-tubules and plasma membrane) in skeletal muscle. The absence of dystrophin, a constituent of the cytoskeleton, the intracellular structure responsible for this task (Koenig *et al.*, 1988), might impede the generation of tension (Wood *et al.*, 1978), and might render the membranes more susceptible to rupture (delta lesions). Ingress of Ca^{2+} could activate proteases to initiate necrosis (Engel, 1986).

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