Subcellular fractionation of dystrophin to the triads of skeletal muscle

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Duchenne muscular dystrophy (DMD) is a human X-linked biochemical defect resulting in the progressive wasting of skeletal muscle of affected individuals[1]. It is the most common and is considered to be the most devastating of the muscular dystrophies, affecting about 1 in 3,500 live-born males[2]. The gene that, when defective, results in this disorder was recently isolated[3-6]. Using the cloned complementary DNA sequences corresponding to the DMD gene, antibodies have been produced that react with a protein species of relative molecular mass (M_r) ~400,000 (400K) which was absent in two DMD-affected individuals and in *mdx* mice[7]. This protein species is called dystrophin because of its identification by molecular-genetic analysis of affected individuals. Here we show that dystrophin is associated with the triadic junctions in skeletal muscle, and is therefore probably involved with Ca^{2+} homoeostasis. We also show that the ~450K ryanodine receptor/sarcoplasmic reticulum Ca^{2+} channel[8], which has the large size and subcellular distribution characteristics of dystrophin, is an immunologically distinct protein species.

Dystrophin messenger RNA[3,5,6,9] and protein[7] are present in all types of mature, terminally differentiated muscle tissue.

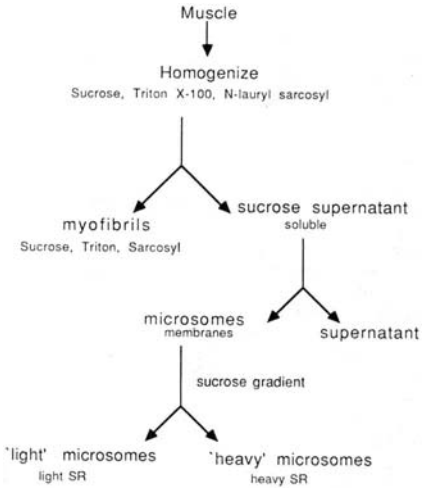

Fig. 1 Schematic diagram of subcellular fractionations, showing the procedure for fractionation of mouse skeletal muscle.
Methods. For detergent-insoluble myofibrils, freshly dissected muscle (2 g) was homogenized in a Waring blender at full speed in 15ml homogenization buffer (10 mM HEPES *p*H 7.1, 10 mM EGTA, 1 mM iodoacetamide, 0.8 mM benzamidine, 0.5 μg ml^{-1} aprotinin, 0.5 μg ml^{-1} leupeptin, 0.25 μg ml^{-1} pepstatin A, supplemented with solid phenylmethylsulphonyl fluoride) containing either 0.25% Triton X-100 or 0.1% *N*-lauryl sarcosyl. Insoluble proteins were pelleted at 10,000 r.p.m. in a Beckman JA-13 rotor, pellets resuspended in homogenization buffer, and the protein concentration of the suspension determined using the Bio-Rad protein assay. Samples were diluted with sample buffer and boiled before fractionation (see Fig. 2, lanes 2,3,11). Sucrose homogenate fractions (see Fig. 2, lanes 4-9,12-13) were prepared as described[11]. Mouse rear-leg skeletal muscle and heart was dissected and immediately homogenized as above, supplemented with 0.25% sucrose. Myofibrillar fractions were isolated by centrifugation as above for 20 min. The skeletal muscle myofibrillar pellet contained two layers (Fig. 2; lanes 4, 5), whereas the heart myofibrillar pellet was homogeneous (Fig. 2; lane 12). The pellets were resuspended in homogenization buffer without sucrose. An aliquot of the low-speed supernatant was reserved (Fig. 2; lane 6) and the remainder was centrifuged at 40,000 r.p.m. in a Beckman SW-40 rotor for 20 min. An aliquot of the pellet was resuspended in homogenization buffer and reserved (microsomes. Fig. 2; lanes 7, 13). The rest of the microsomal pellets were resuspended and fractionated on a discontinuous sucrose gradient for the isolation of light and heavy microsomes (Fig. 2; lanes 8, 9) as described for mouse skeletal muscle[12], except that protease inhibitors were added.

Dystrophin is thought to have a structural role in myofibres because of its high degree of conservation between mice and humans, the 'structural' characteristics of the amino-terminal one-third of the primary amino-acid sequence[4,5], and the significant similarity of the amino terminus to the actin-binding site of the cytoskeletal protein α-actinin[10]. Consistent with this hypothesis, the protein is generally insoluble in the detergent Triton, but the very low abundance of dystrophin in myofibres (~0.002% of total muscle protein[7]) indicates that it is probably not a prominent component of the myofibrillar matrix. We sought to determine the subcellular compartment in which dystrophin is localized. We decided to perform fractionation of subcellular components followed by Western analysis rather than immunofluorescent localization because of the artefacts inherent to immunocytological techniques when detecting very low abundance proteins with polyclonal antisera.

We dissected skeletal muscle from normal, adult mice and fractionated it into detergent-insoluble (Triton X-100, *N*-lauryl sarcosyl) and sucrose-homogenized myofibrillar, soluble and microsomal fractions[11]. We further separated the microsomal fraction into 'light' and 'heavy' microsomes using a sucrose-step gradient[12] (see Fig. 1). We also performed similar fractionations for mouse heart. We incubated identical Western blots with antibodies directed against dystrophin[7] (Fig. 2*a*); the (Ca^{2+}+ Mg^{2+})ATPase[13] (Fig. 2*b*); the ryanodine receptor[8,14] (Fig. 2*c*); or tropomyosin (Fig. 2*d*). As expected, tropomyosin and cross-reacting myosin are enriched in the myofibrillar fractions[15] (Fig. 2*d*; lanes 2-5, 11-12), whereas the (Ca^{2+} + Mg^{2+})ATPase and ryanodine receptor are both enriched in the microsomal fractions (Fig. 2*b*,*c*; lanes 7-9, 13)[12,14,16]. The ATPase monoclonal antibodies used do not cross-react with the cardiac form of this protein (Fig. 2*b*; lanes 10-13)[13].

Dystrophin parallels the localization of the ryanodine receptor and (Ca^{2+} + Mg^{2+})ATPase, being enriched in the microsomal fractions (Fig. 2*a*; lanes 7-9, 13), with a particularly strong enrichment in the heavy fraction (Fig. 2*a*; lane 9). The heavy microsomal fraction was originally developed to enrich sarcoplasmic reticulum vesicles derived from the terminal cisternae of the triad junction. Proteins known to be contained in this fraction are the (Ca^{2+}+Mg^{2+})ATPase[16], calsequestrin[16] and the ryanodine receptor[14]. The enrichment of dystrophin in the same fraction, and the lack of enrichment in other fractions, suggests a similar association with the triad region.

To define further the subcellular compartmentalization of dystrophin within the triad junctional region of skeletal muscle, and to determine whether dystrophin and the ryanodine receptor are the same protein, we performed a second subcellular fractionation series using rabbit skeletal muscle. Triadic fractions can be efficiently isolated from rabbit skeletal muscle using the pyrophosphate variant protocol[11]. Such fractions have been found to be enriched in high-affinity [^{3}H]PN200-110 (radiolabelled dihydropyridine) binding[17,18] (a junctional T-system marker) and high-affinity [^{3}H]ryanodine binding[14] (a junctional sarcoplasmic reticulum marker). We collected aliquots from all steps of the purification to demonstrate the co-purification of ryanodine receptor, the dihydropyridine receptor and dystrophin in all fractions. Figure 3*a* shows the Coomassie blue staining of the fractionated muscle, with the myofibrillar proteins myosin, nebulin and titin indicated, as well as the sarcoplasmic reticulum ryanodine receptor, (Ca^{2+}+Mg^{2+})ATPase and calsequestrin. Western blot analysis of gels identical to Fig. 3*a* using affinity-purified anti-dystrophin antibodies, monoclonal antibody against the 170K subunit of the dihydropyridine receptor or polyclonal antibodies against the ryanodine receptor indicate that ail three detected proteins have a very similar subcellular fractionation pattern (Fig. 3*b*-*d*). All three of these proteins were highly concentrated within fractions enriched for triadic structures, [^{3}H]ryanodine binding and [^{3}H]PN200-110 binding (Fig. 3*b*-*d*; lanes 4-6). Our additional experiments (not shown) show that extraction of triads with 0.6 M KCl does not remove dystrophin; and immunoblot analysis of heavy sarcoplasmic reticulum vesicles[16] compared with isolated triads[11] shows the enrichment of dystrophin and the ryanodine receptor in isolated triads. The co-purification of dystrophin with other, well-characterized triad-associated proteins indicates that dystrophin is tightly associated with the triad structures of skeletal muscle.

To test the relationship between the ryanodine receptor and dystrophin directly, we performed further immunoblot experiments using an affinity-purified ryanodine receptor preparation containing Ca^{2+}-channel activity[8]. We stained isolated triads and the purified ryanodine receptor with polyclonal anti-ryanodine receptor and anti-dystrophin antibodies (Fig. 4*b*,*c*). We found that anti-dystrophin antibodies do not react with the affinity-purified 450K. ryanodine receptor and we did not detect dystrophin in the purified ryanodine receptor preparation. Also, the polyclonal antibodies to the ryanodine receptor do not react with dystrophin (Fig. 4*b*; lane 1).

The comparison of the immunostaining (Fig. 4*b*,*c*) and amido black staining (Fig. 4*a*) of the triadic fractions also reveals that the ryanodine receptor is larger and much more abundant than

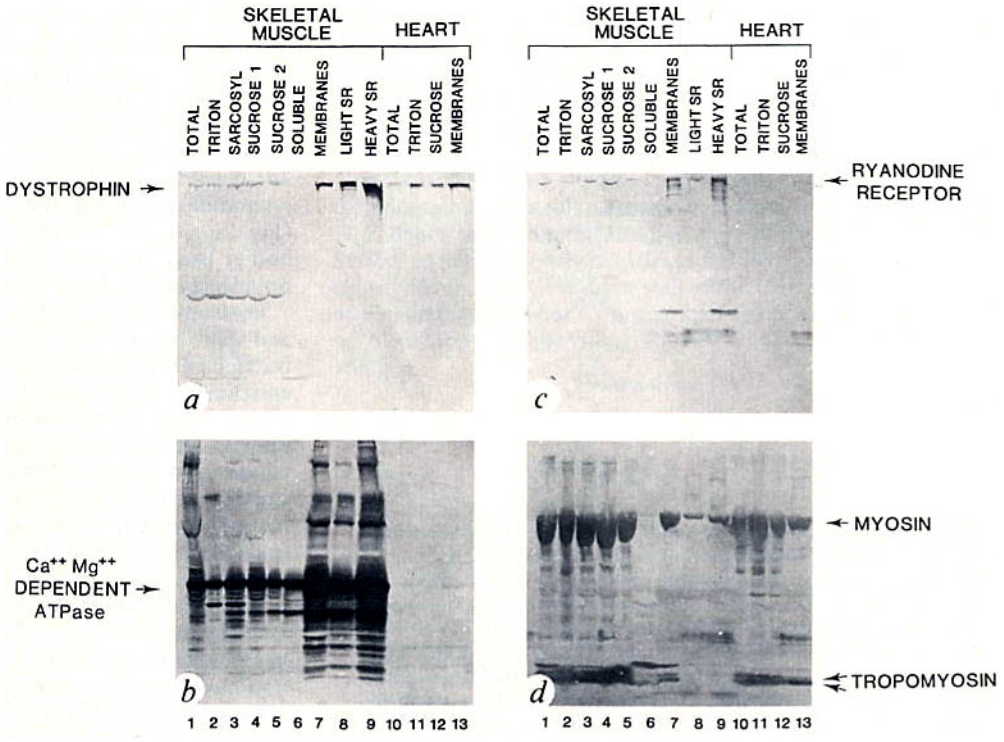

Fig. 2 Localization of dystrophin to the microsomal fractions of mouse skeletal and cardiac muscle by Western blot analysis of the subcellular protein fractions described in Fig. 1. Mouse skeletal muscle. lane 1, total SDS-solubilized (50 µg) (total); lane 2, Triton X-100-insoluble myofibrils (50 µg) (triton); lane 3, *N*-lauryl sarcosyl-insoluble myofibrils (50 µg) (sarcosyl); lane 4, sucrose-insoluble myofibrils (dense fraction) (50 µg) (sucrose 1); lane 5, sucrose-insoluble myofibrils (50 µg) (sucrose 2); lane 6, soluble/microsomes (50 µg) (soluble); lane 7, microsomes (50 µg) (membranes); lane 8, light microsomes (25 µg) (light SR); lane 9, heavy microsomes (50 µg) (heavy SR). Mouse cardiac muscle: lane 10, total SDS-solubilized (50 µg) (total); lane 11, Triton X-100-insoluble myofibrils (50 µg)(triton); lane 12, sucrose-insoluble myofibrils (50 µg) (sucrose); lane 13, microsomes (25 µg) (membranes). Antibodies used: *a*, anti-mouse dystrophin (affinity-purified sheep anti-60K and rabbit anti-30K polyclonals[7]; *b*, mouse anti-chicken fast (Ca^{2+} + Mg^{2+}) ATPase monoclonal (sarcoplasmic reticulum Ca^{2+} pump)[13]; *c*, guinea pig anti-rabbit ryanodine receptor polyclonal (sarcoplasmic reticulum Ca^{2+} channel); *d*, rabbit anti-chicken smooth-muscle tropomyosin antisera (myofibrillar marker with cross-reacting myosin shown). Dystrophin (400K) (*a*) parallels the localization of the sarcoplasmic reticulum markers, the 105K (Ca^{2+}+Mg^{2+})ATPase[13] (*b*) and the 450K. ryanodine receptor[8,14] (*c*). Disassociation of the triads from myofibrils is very inefficient[27], explaining the presence of the sarcoplasmic reticulum markers in myofibrillar preparations. Also in *a* is the 100K, skeletal muscle-specific, myofibrillar protein species that cross-reacts with the rabbit anti-30K dystrophin antibodies[7]. The ATPase monoclonal is specific for the fast muscle form of this protein and does not react with the cardiac muscle form (panel *b*, lanes 10-13).

Methods. Total SDS-solubilized muscle (lanes 1,10). Freshly dissected muscle was minced, boiled in 10 vol sample-loading buffer (10% SDS, 50 mM dithiothreitol, 0.1 M Tris *p*H 8.0, 10 mM EDTA). SDS-insoluble proteins were precipitated and discarded. All other samples were prepared as described in Fig. 1. Samples were boiled and SDS-insoluble proteins precipitated before gel loading. Protein samples were fractionated on 3.5-12.5% gradient SDS-polyacrylamide gels[30], using a 3% stacking gel. Western blotting was as described[7]. Polyclonal antibodies against the purified ryanodine receptor were prepared according to Tung[29] by immunization of guinea pigs with gel slices of the purified 450K ryanodine receptor protein.

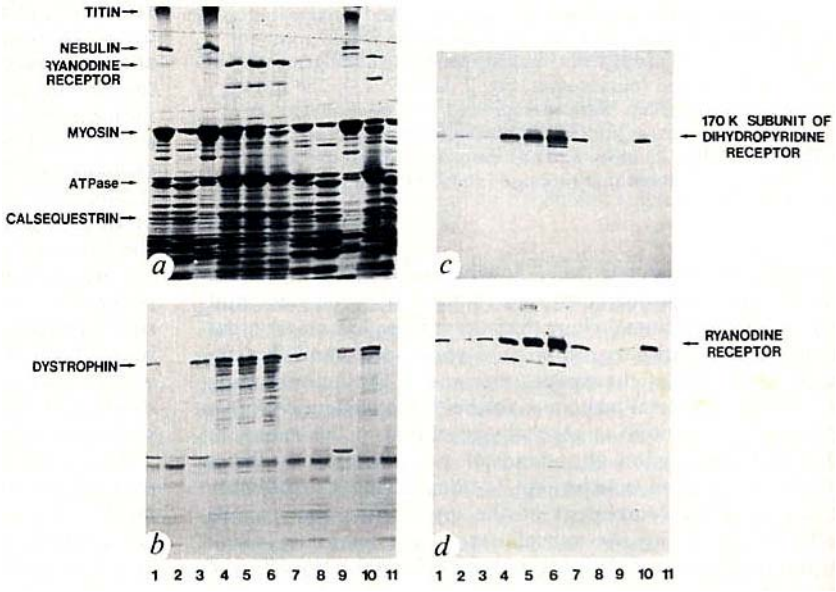

Fig. 3 Association of dystrophin with triadic junctions of rabbit skeletal muscle. Shown are the following subcellular protein fractions from rabbit skeletal muscle. Numbers in parentheses represent [^{3}H]PN200-110 and [^{3}H]ryanodine binding, respectively. Binding (in pmol mg^{-1}) was performed as described[12,15]. Lane 1, initial muscle homogenate (0.39, 0.09); lane 2, soluble/microsomal fraction of initial homogenate (0-32, 0.12); lane 3, rehomogenized myofibrillar fraction (second homogenate) (0.51,0.15); lane 4, heavy microsomes from initial homogenate (7.61, 2.53); lane 5, heavy microsomes from second homogenate (10.60,3.27); lane 6, isolated triads (12.03, 4.25); lane 7, soluble/microsomal fraction from second homogenate (0.46, 1.62); lane 8, soluble/light microsomal fraction from lane 7 (0, 0.05); lane 9, myofibrillar fraction of second homogenate (0.41, 0.11); lane 10, light microsomes from lane 8 (2.50, 0.78); lane 11, soluble fraction (cytosol) (0,0). *a*, Coomassie blue-stained gel (100 µg protein per lane); *b*, Western blot using affinity-purified sheep anti-mouse dystrophin antibodies with alkaline phosphatase conjugated donkey anti-sheep IgG secondary. (50 µg per lane); *c*, Western blot using monoclonal antibody IIF7 directed against the 170K subunit of the dihydropyridine receptor with peroxidase-conjugated goat anti-mouse IgG secondary (50 µg per lane). *d*, Western blot using polyclonal guinea pig anti-rabbit ryanodine receptor antibodies with peroxidase-conjugated rabbit anti-guinea pig IgG secondary. (50 µg per lane). In *a*, myofibrillar markers are titin (apparent M_r ~1,500K[31]), nebulin (apparent M_r ~700K[32]), myosin (205K) and the sarcoplasmic reticulum marker (Ca^{2+}+Mg^{2+}ATPase (105K). Dystrophin (*b*) parallels the localization of the ryanodine receptor (sarcoplasmic reticulum Ca^{2+} channel, *c*), being most highly concentrated in the purified triad fraction (lane 6). Because of differences in the second antibodies used and differences in the sensitivities of the various antisera, the intensity of the bands does not reflect the relative quantity of dystrophin present.

Methods. Total rabbit skeletal muscle was dissected and fractionated into myofibrils, heavy microsomes and triads using a modification[18] of the pyrophosphate-variant procedure[11]. Light microsomes were isolated as described[16]. Protein content of samples was determined[33], and aliquots were solubilized in SDS and fractionated using 3-12% gradient SDS-polyacrylamide gels[30] using a 3% stacking gel. Western blot analysis was performed as described[14].

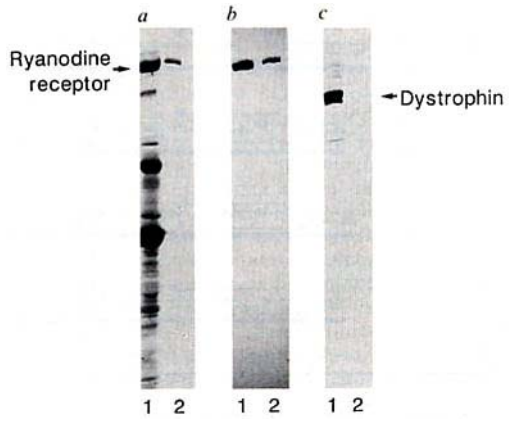

Fig. 4 Dystrophin is unrelated to the ryanodine receptor. Shown are three similar Western blots containing isolated triad structures and purified ryanodine receptor[8]. *a*, Amido black staining. Lane 1, triads (50 μg); lane 2, purified ryanodine receptor (1.5 μg), *b*, Ryanodine receptor seen using guinea pig anti-rabbit ryanodine receptor antibodies with peroxidase-conjugated rabbit anti-guinea pig IgG secondary antibodies. Lane 1, triads (10 μg); lane 2, purified ryanodine receptor (0.7 μg). *c*, Dystrophin seen using sheep anti-mouse dystrophin antibodies with alkaline phosphatase-conjugated donkey anti-sheep IgG. Lane 1, triads (50 μg); lane 2, purified ryanodine receptor (1.5 μg).

dystrophin. The amido black-stained protein species migrating immediately below the abundant ryanpdine receptor (Fig. 4*a*; see also Fig. 3*a*) could potentially represent dystrophin. But there are differences between the fractionation of this amido black-stained protein species and the fractionation of dystrophin; the stained protein is not evident in lane 9 of Fig. 3*a*, whereas dystrophin is present in this lane (Fig. 3*b*). Furthermore, in contrast to dystrophin (Fig. 3*b*), the stained protein shows a substantial enrichment in the light microsomal fraction (Fig. 3*a*; lane 10) relative to the heavy microsomal fractions (Fig. 3*a*; lanes 4-6). Dystrophin either co-migrates with this amido black-stained protein species, or is not abundant enough to be stained at all. In either case, dystrophin presumably is a very minor component of the triads. This observation is consistent with previous estimates of the very low relative abundance of dystrophin in striated muscle[5,7]. The differences in size and abundance between the ryanodine receptor and dystrophin indicate that they are indeed distinct, immunologically unrelated proteins.

The triadic structures of muscle consist of transverse sarcolemmal invaginations (t-tubules) interdigitated between two terminal cisternae of the longitudinally oriented sarcoplasmic reticulum (junctional sarcoplasmic reticulum). These structures occur at the A-I junction of every half sarcomere in higher vertebrates and are responsible for the signal transduction of membrane depolarization into sarcoplasmic reticulum Ca^{2+} release, resulting in myofibrillar contraction (excitation-contraction coupling). Membrane depolarizations triggered by motomeurons are transmitted to the internal portions of myofibres via the t-tubule system, where voltage sensors in the triadic structures (possibly the dihydropyridine receptor of the t-tubules[19-21]) are thought to initiate Ca^{2+} release from the sarcoplasmic reticulum through the sarcoplasmic reticulum Ca^{2+} channel (the ryanodine receptor)[8,22], resulting in myofibril contraction. The junctional sarcoplasmic reticulum is also responsible, together with the non-junctional sarcoplasmic reticulum, for the active uptake and storage of intracellular Ca^{2+}, which is accomplished via the very abundant intrinsic (Ca^{2+}+ Mg^{2+})ATPase and calsequestrin, the extrinsic Ca^{2+}-binding protein.

Here we have shown that the protein product of the DMD locus, dystrophin, is a protein associated with triadic structures in myofibres. Because these structures are essential for the maintenance of calcium homoeostasis within the muscle fibre, one can speculate that the absence of one of its potential components, dystrophin, would disrupt this process. It has been reported[23] that increased iritracellular Ca^{2+} levels could initiate myofibre death by the activation of phospholipase A, resulting in sarcolemmal dissolution. It is possible that altered Ca^{2+} homoeostasis resulting from dystrophin deficiency leads to such a Ca^{2+}-mediated phospholipase activation. This process could explain the very high. levels of soluble sarcoplasmic enzymes in the serum of the DMD-affected individual[23].

The ammo-terminal 200 amino acids of dystrophin exhibit striking similarity to the actin filament-binding domain of chicken cytoskeletal α-actinin[10]. This actin filament-binding domain is highly conserved between the widely divergent *Dictyostelium* and chicken α-actinin, presumably due to the highly conserved nature of the actin filaments to which both proteins bind[24]. The similarity of the amino terminus of dystrophin to this same conserved region suggests -that this similarity represents a functional homology. It is therefore probable that dystrophin interacts with actin filaments in myofibres. We have shown that dystrophin is associated with the triadic Structures of muscle. It is tempting to suggest that dystrophin might serve as an anchor for the triads to the myofibrillar cytpskeleton through the binding of actin filaments at its amino terminus. Supporting this hypothesis, DMD-affected muscle (which lacks dystrophin) has been seen to contain poorly aligned triads[25] and disorganized t-tubule system networks[26].

Clearly there are many other hypothetical roles for dystrophin in the triads. The localization of dystrophin to the triadic junctions, however, implies that Ca^{2+} homoeostasis is the most likely process disrupted in DMD. The precise function of dystrophin should soon be elucidated, as its subcellular localization is refined by immunoelectron microscopy and production of monoclonal antibodies, and as its biochemistry is defined by more extensive purifications and binding studies.

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