THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 263, No. 17, Issue of June 15, pp. 8480-8484, 1988
Printed in U.S.A.

Evidence for the Association of Dystrophin with the Transverse Tubular System in Skeletal Muscle*

(Received for publication, January 4, 1988)

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Polyclonal antibodies to dystrophin (the protein product of the human Duchenne muscular dystrophy gene) were used to identify and characterize dystrophin in isolated triads from rabbit skeletal muscle. Anti-dystrophin antibodies recognize an ~400,000-Da protein in isolated triads or heavy microsomes from skeletal muscle. Treatment of heavy microsomes with buffers containing high salt or EDTA to remove peripheral or extrinsic membrane proteins does not remove dystrophin; however, treatment of intact triads with trypsin shows that dystrophin is extremely sensitive to mild proteolytic digestion. Isolation of junctional complexes from skeletal muscle triads indicates that dystrophin is tightly associated with the triadic junction. Fractionation of the triadic junction into junctional transverse tubular membranes and junctional sarcoplasmic reticulum membranes has shown that dystrophin is enriched in junctional transverse tubular membranes. Thus, our results suggest that dystrophin is a component of the triad junction which is exposed to the cytoplasm and embedded in or attached to the transverse tubular membrane.

Duchenne muscular dystrophy is the most common and devastating of the muscular dystrophies and is caused by a defective gene found on the human X chromosome (1). This gene has been isolated and shown to encode for a large mRNA of approximately 14 kilobases (2–5). These cloned cDNA sequences have been used to produce antibodies which react with the ~400,000-Da protein product of the Duchenne muscular dystrophy gene, which is absent in Duchenne patients and *mdx* mice (6). This protein has been called "dystrophin" due to its identification via the molecular genetic analysis of individuals with Duchenne muscular dystrophy. Although dystrophin's precise function remains unknown, dystrophin has recently been shown to fractionate with protein components of the triadic junction in skeletal muscle (7).

The triadic junction in skeletal muscle is the site where electrical excitation of the transverse tubular membrane results in Ca^{2+} release from the terminal cisternae of the sarcoplasmic reticulum. The molecular mechanism of excitation-contraction coupling is not known, but two of the major components of the triad junction which function in excitation-contraction coupling have recently been identified and purified (8–12). One is the dihydropyridine receptor which consists of four protein components of 175,000, 170,000, 52,000, and 32,000 Da (8–11) and has been implicated as the voltage sensor in the transverse tubular membrane (13). The other is the ryanodine receptor which consists of a single polypeptide of ~450,000 Da and has been shown to be the Ca^{2+} release channel in junctional sarcoplasmic reticulum membrane (12).

In this report, polyclonal antibodies to dystrophin have been used to identify and characterize dystrophin in isolated triads and transverse tubular membrane vesicles from skeletal muscle. Our results demonstrate that dystrophin is tightly associated with the triad junction and embedded in or attached to the junctional transverse tubular membrane.

EXPERIMENTAL PROCEDURES

Isolation of Skeletal Muscle Triads and Transverse Tubular Membranes—Skeletal muscle membrane fractions were isolated from adult rabbit or rat skeletal muscle in the presence of the following protease inhibitors: aprotinin (76.8 nM), benzamidine (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 μM), pepstatin A (0.7 μM), and PMSF[1] (0.23 mM). Heavy microsomes and triads were purified by a modification of the method of Mitchell *et al.* (14, 15) as described previously (10). Transverse tubular membranes were isolated from rat or rabbit skeletal muscle using the method of Meissner (16) with minor modifications. Transverse tubular vesicles were also isolated from rabbit skeletal muscle according to Rosemblatt *et al.* (17). All membrane preparations were characterized for high affinity [^{3}H]PN200-110 binding (8) and high affinity [^{3}H]ryanodine binding (9) and stored frozen at −135 °C in 0.25 M sucrose, 20 mM Tris-maleate (pH 7.4), 0.83 mM benzamidine, 1 mM iodoacetamide, and 58 μM PMSF. Heavy microsomes were treated for 30 min on ice with either 0.6 M KCl to remove myofilament proteins (18) or 10 mM EDTA, pH 8.0, to remove extrinsic membrane proteins such as calsequestrin (19). Heavy microsomes were also treated for 30 min at 37 °C with 0.3 mM sodium phosphate, 0.1 mM EDTA, 0.2 mM PMSF, pH 7.4, which is a procedure that removes spectrin from red blood cell ghosts (29). In all treatments membranes were recovered by centrifugation at 100,000 × *g* in a Beckman type 45 Ti rotor. Protein was determined by the method of Lowry *et al.* (20) as modified by Peterson (21). Protein samples were analyzed by SDS-PAGE (3–12% gradient gels) using the buffer system of Laemmli (22) and either stained with Coomassie Blue or transferred to nitrocellulose according to Towbin (23).

Monoclonal and Polyclonal Antibodies—Polyclonal antibodies to

* This work was supported by grants from the National Institutes of Health (to K. P. C. and L. M. K.) and the Muscular Dystrophy Association (to K. P. C. and L. M. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; [^{3}H]PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([^{3}H]methoxycarbonyl)pyridine 3-carboxylate.

distinct 60- and 30-kDa antigens derived from the NH_2-terminal third of dystrophin have been previously prepared (6). Polyclonal antibodies to the purified ryanodine receptor have been previously prepared (7) using guinea pigs immunized with gel slices of the purified ~450,000-Da ryanodine receptor polypeptide according to the method of Tung (24). Monoclonal antibodies against the dihydropyridine receptor from rabbit skeletal muscle, monoclonal antibodies against the junctional sarcoplasmic reticulum proteins (including calsequestrin), and monoclonal antibodies against the skeletal (type II) (Ca^{2+} + Mg^{2+})-ATPase have been previously prepared (8, 9, 11, 25). Indirect immunoperoxidase staining of nitrocellulose blots was performed using nonfat dry milk as a blocking agent as previously described (8).

Tryptic Digestion of Isolated Triads—Isolated triads were diluted with buffer A (0.3 M sucrose, 20 mM Tris-HCl, pH 7.4) and centrifuged for 30 min at 230,000 × *g* to remove protease inhibitors. The pellet was resuspended in buffer A, frozen in liquid nitrogen, and stored at −135 °C. Triads (5.0 mg/ml) were digested for 5 min at 30 °C with various amounts of trypsin in the presence of 1.0 M sucrose and 100 mM KCl according to the methods developed by Stewart *et al.* (26). Digestion was inhibited by the addition of an equal volume of buffer containing 1.0 mM PMSF and 0.2 mM benzamidine.

Isolation of Junctional Complexes from Triads—Junctional complexes containing both the ryanodine receptor and the dihydropyridine receptor were isolated from triads using 0.5% Triton X-100 in the presence of calcium (27, 28). Isolated triads (2.5 mg/ml) were incubated with 1 mM $CaCl_2$ for 10 min at 0 °C. Solubilization of the (Ca^{2+} + Mg^{2+})-ATPase on these Ca^{2+}-treated triads (1 mg/ml) was performed in the presence of 0.5% Triton X-100, 0.3 M sucrose, 20 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine, 0.1 mM PMSF, 0.6 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, and 185 μg/ml iodoacetamide. The sample was incubated for 30 min at 0 °C and centrifuged for 30 min at 100,000 × *g* in a Beckman type 45 Ti rotor. Pellets were resuspended in 0.3 M sucrose, 20 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine, 0.1 mM PMSF, and incubated at 1 mg/ml with 2 mM EDTA for 20 min at 0 °C. The mixture was centrifuged for 30 min at 100,000 × *g* and resuspended in the above resuspension buffer. Samples were frozen in liquid nitrogen and stored at −135 °C.

Materials—[^{3}H]PN200-110 was from Amersham Corp., and [^{3}H] ryanodine was from Du Pont-New England Nuclear. Electrophoretic reagents were obtained from Bio-Rad and molecular weight standards from Bethesda Research Laboratories. Protease inhibitors were obtained from Sigma. Peroxidase-conjugated secondary antibodies were obtained from Boehringer Mannheim and Bio-Rad. All other reagents were of reagent grade quality.

RESULTS AND DISCUSSION

Triads isolated from rabbit skeletal muscle have been shown to be enriched in high affinity dihydropyridine binding (junctional transverse tubular membrane marker), high affinity ryanodine binding (junctional sarcoplasmic reticulum membrane marker), and dystrophin, the protein absent in patients with Duchenne muscular dystrophy (7). The triad preparation used has been well characterized and is known to be practically devoid of contractile elements, mitochondria, and free sarcolemma (14). This evidence alone suggests that dystrophin is likely to be an intrinsic protein or at least tightly associated with the membranes of the triad junction. To further test this hypothesis we used treatments which remove peripheral or contaminating myofibrillar proteins from heavy microsomes identical to those used in the preparation of triads. Rabbit microsomes were treated with 0.1 mM EDTA at 37 °C or 10 mM EDTA at 0–4 °C, or 0.6 M KCl at 0–4 °C, centrifuged at 100,000 × *g*, and the pellets were resuspended. Fig. 1*A* shows a Coomassie Blue-stained gel of untreated (*lane 1*) and treated microsomes (*lanes 2–4*). It is clear that the two EDTA treatments remove calsequestrin while treatment with KCl removes a majority of the myosin. All three treatments remove phosphorylase which is a soluble enzyme loosely associated with skeletal muscle microsomes. Both the ryanodine receptor and the (Ca^{2+} + Mg^{2+})-ATPase are known intrinsic membrane proteins and are not removed by any of the treatments (Fig. 1*A*). Since dystrophin cannot

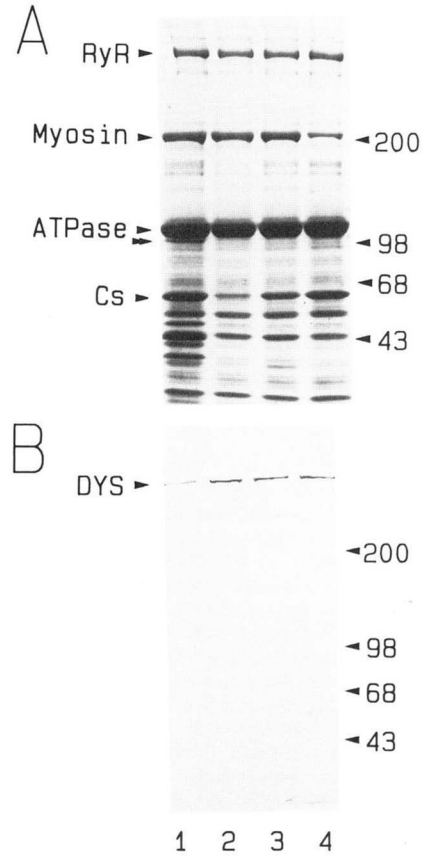

FIG. 1. **SDS-PAGE and immunoblot analysis of treated rabbit skeletal muscle microsomes.** Isolated microsomes were treated as described under "Experimental Procedures." Samples were subjected to SDS-PAGE on a 3–12% gradient gel and stained with Coomassie Blue (*Panel A*, 75 μg/lane) or transferred electrophoretically to nitrocellulose (*Panel B*, 250 μg/lane) for indirect immunoperoxidase staining using polyclonal sheep anti-dystrophin antibodies (7, 8). Control microsomes (*lane 1*) and microsomes treated with 0.1 mM EDTA at 37 °C (*lane 2*), 10 mM EDTA at 0–4 °C (*lane 3*), and 0.6 M KCl at 0–4 °C (*lane 4*) are shown. Dystrophin, ~400,000 Da, (*DYS*), myosin, ~200,000 Da; (Ca^{2+} + Mg^{2+})-ATPase, 105,000 Da (*ATPase*), ryanodine receptor, ~450,000 Da (*RyR*); and calsequestrin, 63,000 Da (*Cs*) are indicated by *arrows*. The *double arrow* indicates phosphorylase. Molecular weight standards are indicated on the right.

be definitively identified on Coomassie Blue-stained SDS gels, immunoblots were used to assay for dystrophin in the treated microsomes. Fig. 1*B* shows an immunoblot of the identical lanes in *panel A* stained with a polyclonal antibody directed against dystrophin. No clear reduction of dystrophin (~400,000 Da) due to any of the treatments is discernible on this blot. Furthermore, solubilization of triads with digitonin shows that dystrophin is solubilized at 0.5–1.0% digitonin (not shown). These concentrations are nearly identical to those required to solubilize two intrinsic membrane proteins, the ryanodine receptor and the dihydropyridine receptor. These results suggest that dystrophin is not an extrinsic membrane protein or a contaminating myofilament protein in isolated triads and is likely an intrinsic membrane protein or tightly associated membrane protein in the transverse tubular or sarcoplasmic reticulum membranes of the triad junction.

The predicted primary amino acid sequence of the amino-terminal third of dystrophin has been shown to share characteristics common to many structural proteins (3, 4). It was expected, therefore, that only a portion of dystrophin could be attached to or embedded in the membrane and that a

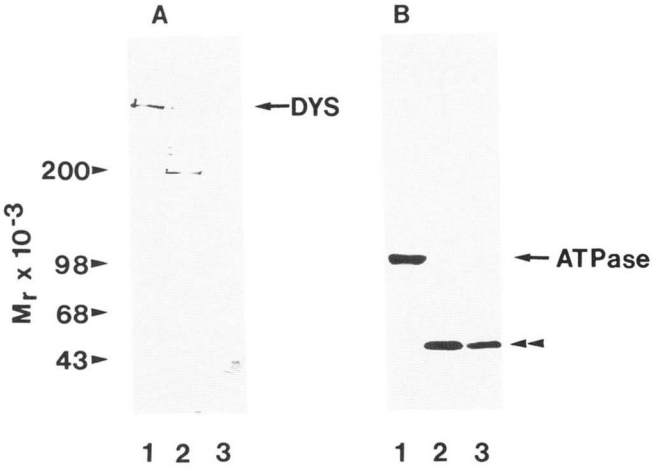

FIG. 2. **Immunoblot staining of rabbit skeletal muscle triads digested with trypsin.** Triads (5 mg/ml), free of protease inhibitors, were treated with various amounts of trypsin for 5 min at 30 °C as described under "Experimental Procedures." Treatment with trypsin is as follows: *lane 1*, no trypsin; *lane 2*, 1:800 ratio of trypsin to protein; *lane 3*, 1:160 ratio of trypsin to protein. Various quantities of protein samples were subjected to SDS-PAGE on a 3–12% gradient gel and transferred electrophoretically to nitrocellulose. *Panel A* (150 μg/lane of triads) was stained with polyclonal sheep anti-dystrophin antibodies. No tryptic fragments of dystrophin (*DYS*) were visualized in *lane 3*. *Panel B* (5 μg/lane of triads) was stained with monoclonal antibody IIH11 against the $(Ca^{2+} + Mg^{2+})$-ATPase. The *double arrowhead* denotes the two major tryptic fragments of the ATPase (A and B fragments) which have nearly identical mobility on Laemmli gels. Molecular weight standards are indicated on the left.

majority of dystrophin is exposed to the extracellular space, intraluminal sarcoplasmic reticulum, or the cytoplasm. To distinguish among these possibilities we have used mild trypsin digestion of isolated triads under the conditions developed by Stewart *et al.* (26) for the partial digestion of the $(Ca^{2+} + Mg^{2+})$-ATPase. Mild digestion of isolated sarcoplasmic reticulum vesicles has previously been shown to result in the formation of two to four fragments of the ATPase without affecting calsequestrin, an intraluminal protein (26). Therefore, proteins found in the lumen of the T-system or within the terminal cisternae of the sarcoplasmic reticulum should be resistant to mild tryptic digestion of the isolated triad. Dystrophin was found to be extremely sensitive to digestion by trypsin with the complete disappearance of the ~400,000-Da polypeptide using a trypsin to protein ratio of 1:160 (Fig. 2*A*, *lane 3*). Under these conditions the $(Ca^{2+} + Mg^{2+})$-ATPase was only cleaved once (Fig. 2*B*), while calsequestrin, an intraluminal sarcoplasmic reticulum protein, was not digested (not shown). Since the anti-dystrophin antibodies used are directed against the NH_2-terminal third of the protein these results suggest that at least the NH_2-terminal third of dystrophin is exposed to the cytoplasm. Noncytoplasmic domains of the protein may exist and be resistant to digestion but could not be identified with the antibodies used. Interestingly, two other junctional specific proteins, the ryanodine receptor and the dihydropyridine receptor, were also extremely sensitive to trypsin digestion.[2]

Electron microscopic characterization of both triad structures in intact muscle cells and isolated triads suggests that the junctional sarcoplasmic reticulum membrane and the transverse tubular membranes are tightly associated via "junctional feet" (14, 15, 31–33). The region of membrane contact has been called a junctional complex (33) and is devoid of the $(Ca^{2+} + Mg^{2+})$-ATPase (32, 34). We have used a procedure to solubilize the $(Ca^{2+} + Mg^{2+})$-ATPase and free sarcoplasmic reticulum from the junctional membranes using Triton X-100 (27, 28). The isolated junctional complex contains the ryanodine receptor, the dihydropyridine receptor, and dystrophin (Fig. 3, *B–D*), and is deplete of $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 3*E*). These results demonstrate that dystrophin is located at or near the junction between the sarcoplasmic reticulum and the transverse tubular membrane. Since the ryanodine receptor and the dihydropyridine receptor are both thought to be involved in Ca^{2+} release from the sarcoplasmic reticulum these results suggest that dystrophin may play an active role or a passive structural role in excitation-contraction coupling.

[2] A. T. Leung and K. P. Campbell, manuscript in preparation.

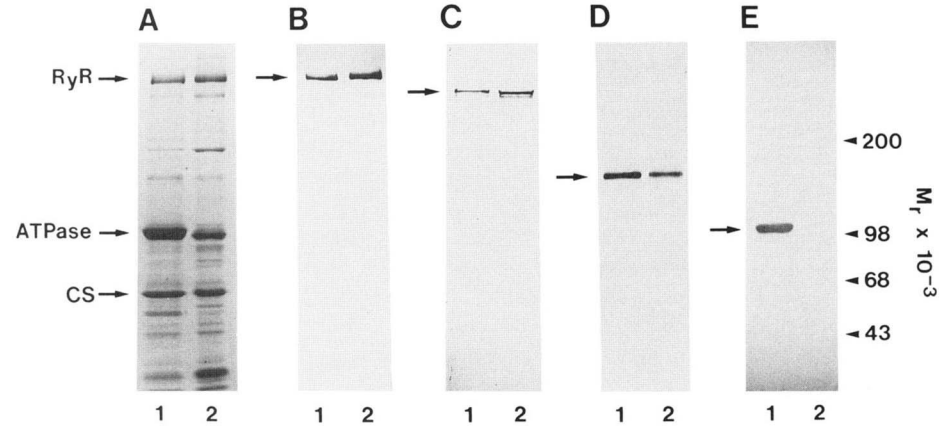

FIG. 3. **Immunoblot staining of rabbit skeletal muscle triads and isolated junctional complexes.** Rabbit skeletal muscle triads (*lane 1*) or isolated junctional complexes (*lane 2*) were prepared as described under "Experimental Procedures" and subjected to SDS-PAGE on a 3–12% gradient gel and transferred electrophoretically to nitrocellulose. Immunoperoxidase staining of nitrocellulose transfers was performed as previously described (7, 8). *Panel A* shows a Coomassie Blue-stained gel indicating the position of the ~450,000-Da ryanodine receptor (*RyR*), $(Ca^{2+} + Mg^{2+})$-ATPase (*ATPase*), and calsequestrin (*CS*). Nitrocellulose transfers were stained with polyclonal anti-ryanodine receptor antibodies (*B*), polyclonal anti-dystrophin antibodies (*C*), anti-170,000-Da dihydropyridine receptor monoclonal antibody IIF7 (*D*), and anti-$(Ca^{2+} + Mg^{2+})$-ATPase monoclonal antibody IIH11 (*E*). Proteins amounts are *A*, 50 μg; *B*, 10 μg; *C*, 100 μg, *D*, 50 μg; *E*, 10 μg. *Arrows* indicate the position of the ~450,000-Da ryanodine receptor (*B*), dystrophin (*C*), 170,000-Da dihydropyridine receptor (*D*), and the $(Ca^{2+} + Mg^{2+})$-ATPase (*E*). Molecular weight standards are indicated on the right.

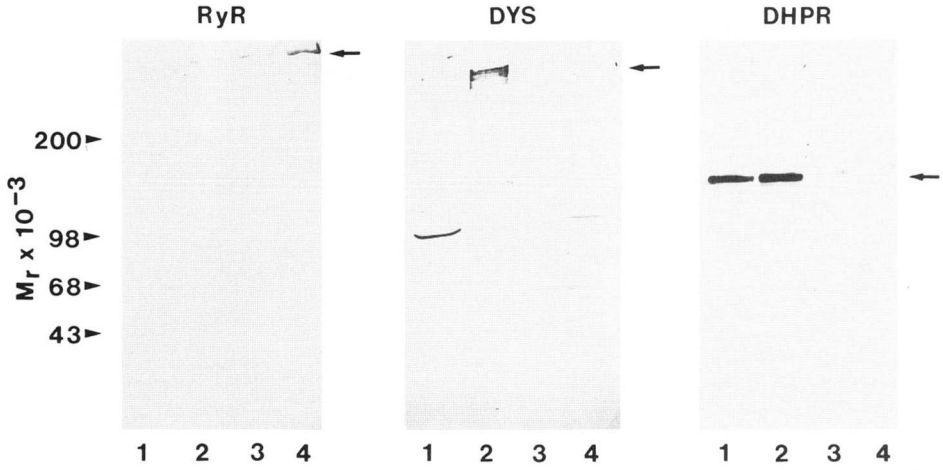

FIG. 4. **Dystrophin fractionates with the junctional transverse tubular membranes.** Junctional transverse tubular membranes and junctional sarcoplasmic reticulum membranes were prepared according to Meissner (16) by fractionating heavy rat skeletal muscle microsomes on discontinuous sucrose gradients (10, 20, 25, 30, 35, and 40%). Four distinct membrane fractions were collected from the gradients and analyzed for [^{3}H]ryanodine and [^{3}H]PN200-110 binding. Immunoblots of the various membrane fractions named TS_1 (*lane 1*), TS_2 (*lane 2*), JSR_1 (*lane 3*), and JSR_2 (*lane 4*) are shown. For each *panel* equal quantities of protein samples were subjected to SDS-PAGE on a 3–12% gradient gel and transferred electrophoretically to nitrocellulose. The *left panel* contains 50 μg/lane protein and was stained with polyclonal guinea pig anti-ryanodine receptor (*RyR*) antibody. [^{3}H]Ryanodine binding data for these fractions were as follows: TS_1 = 0.57 pmol/mg, TS_2 = 0.75 pmol/mg, JSR_1 = 0.97 pmol/mg, JSR_2 = 3.30 pmol/mg. The *center panel* contains 100 μg/lane and is stained with polyclonal sheep anti-dystrophin (*DYS*) antibodies. Dystrophin is enriched in the TS_2 fraction (*lane 2*) and could not be detected in *lanes 3* or *4*. The 95-kDa protein stained in the *center panel* is an abundant myofibrillar protein which is only recognized by this particular antibody preparation and is not related to dystrophin (6). The *right panel* contains 50 μg/ml and is stained with three monoclonal antibodies directed against the 170-kDa subunit of the dihydropyridine receptor (*DHPR*). [^{3}H]PN200-110 binding data for these fractions were as follows: TS_1 = 15.6 pmol/mg, TS_2 =32.2 pmol/mg, JSR_1 = 5.28 pmol/mg, JSR_2 = 2.62 pmol/mg. Molecular weight standards are indicated on the left.

Finally, in order to examine whether the membrane portion or attachment of dystrophin is found in the junctional transverse tubular membrane and/or the junctional sarcoplasmic reticulum membrane we have isolated junctional transverse tubular membrane vesicles and junctional sarcoplasmic reticulum vesicles using a preparation developed by Meissner (16). Rat skeletal muscle was used for this procedure since it yields junctional transverse tubular membrane vesicles with the highest [^{3}H]PN200-110 binding and highest ratio of [^{3}H]PN200-110 to [^{3}H]ryanodine binding. To compare the quantity of dystrophin, ryanodine receptor, and dihydropyridine receptor present in each fraction, the samples were subjected to SDS-PAGE, transferred to nitrocellulose, and stained with the appropriate antibody. Fig. 4, *left panel*, shows that the ryanodine receptor is most abundant in *lane 4* which is the fraction collected at the bottom of the sucrose gradient and is termed JSR_2. Fig. 4, *right panel*, shows that dihydropyridine receptor is most abundant in *lanes 1* and *2* collected from the top of the sucrose gradient. These fractions have been termed TS_1 and TS_2, respectively. These results are consistent with the [^{3}H]ryanodine and [^{3}H]PN200-100 binding data determined for these fractions (data in figure legend). Fig. 4, *center panel*, shows that dystrophin is most abundant in the TS_2 fraction and was not detected in the JSR_2 fraction. Similar results were obtained with rabbit skeletal muscle using the procedures of Meissner (16) or Rosemblatt *et al.* (17) for the transverse tubular membrane preparation. These results suggest that dystrophin is more tightly associated with the junctional transverse tubular membrane than the junctional sarcoplasmic reticulum membrane.

Two possible roles of dystrophin consistent with our results are a functional role in excitation-contraction coupling and/or a structural role in stabilization of the triadic structure (7). A direct role in excitation-contraction coupling seems less likely since muscle cells from Duchenne patients and from mdx mice (which both lack dystrophin) seem capable of excitation-contraction coupling (4, 6). The predicted amino acid sequence of dystrophin is consistent with a structural role for the protein. First, the amino-terminal third of dystrophin is probably rich in α-helix secondary structure typical of many structural proteins (4). Second, the high conservation of dystrophin between widely divergent species is more characteristic of a structural protein than a globular or enzymatic protein (4). Finally, the amino-terminal 200 amino acids of dystrophin are highly homologous to the amino terminus of α-actinin which is a cytoskeletal protein that binds actin (30).

Our data suggest that dystrophin is a component of the transverse tubular/sarcoplasmic reticulum junction which is exposed to the cytoplasm and embedded in or attached to the junctional transverse tubular membrane. Our results strongly support the hypothesis that dystrophin has a structural role in the stabilization of the triadic junction, since it appears to be anchored in junctional transverse tubular membrane and its NH_2 terminal (actin binding region) is exposed to the cytoplasm. The evidence presented here is consistent with dystrophin "anchoring" the junctional transverse tubular system to its location at the A-I junction by binding to the myofibrillar or cytoskeletal matrix.

Acknowledgments—We would like to thank Dr. Donald Wood of the Muscular Dystrophy Association for his role in initiating the collaboration between our two groups. We thank Dr. Toshiaki Imagawa and Albert Leung for the anti-ryanodine receptor antibody and anti-dihydropyridine receptor antibodies, respectively, Shawn Rogers for the excellent technical assistance, and Joanne Schmitz for the excellent secretarial assistance.

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