Volume 283, number 2, 230–234 FEBS 09765 June 1991
© 1991 Federation of European Biochemical Societies 00145793/91/$3.50
ADONIS 0014579391004743

Dystrophin constitutes 5% of membrane cytoskeleton in skeletal muscle

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Received 20 March 1991

Dystrophin, which is absent in skeletal muscle of Duchenne muscular dystrophy patients, has not been considered to play a major structural role in the cell membrane of skeletal muscle because of its low abundance (~0.002% of total muscle protein). Here, we have determined the relative abundance of dystrophin in a membrane cytoskeleton preparation and found that dystrophin constitutes approximately 5% of the total membrane cytoskeleton fraction of skeletal muscle sarcolemma. In addition, dystrophin can be removed from sarcolemma by alkaline treatment. Thus, our results have demonstrated that dystrophin is a major component of the subsarcolemmal cytoskeleton in skeletal muscle and suggest that dystrophin could play a major structural role in the cell membrane of skeletal muscle.

Dystrophin; Sarcolemma; Membrane cytoskeleton; Skeletal muscle

1. INTRODUCTION

Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene, is absent in skeletal muscle of DMD patients and mdx mice [1–3]. The predicted structure [4] and cellular localization [1,2,5] of dystrophin indicate that it is a membrane-associated cytoskeletal protein and further suggest that DMD is a disease of the membrane cytoskeleton in skeletal muscle. Studies of other genetic diseases involving the cytoskeleton have shown that a deficiency in a major cytoskeletal component can severely affect the integrity of the cell membrane. For example, spectrin deficiency results in fragile erythrocyte membranes causing severe hemolytic anemia [6,7]. In comparison, dystrophin has not been considered to play a major structural role in the membrane cytoskeleton of skeletal muscle because of its low abundance of approximately 0.002% of total muscle protein [8]. The precise functional or structural role and the relative amount of dystrophin in muscle cytoskeleton are still unknown.

Recently, we have applied a wheat germ agglutination technique to isolate skeletal muscle sarcolemma and have demonstrated by immunoblot analysis using monoclonal antibodies that this membrane preparation is highly enriched in sarcolemma markers and the dystrophin/glycoprotein complex [9]. SDS-PAGE analysis of this preparation indicated that a protein band of approximately 400 kDa (which co-migrates with dystrophin on immunoblots) was an identifiable component of isolated sarcolemma [9]. To learn more about the cytoskeletal properties and relative abundance of dystrophin in muscle, we characterized the membrane cytoskeleton fraction of highly purified sarcolemma from rabbit skeletal muscle.

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2. MATERIALS AND METHODS

Sarcolemma vesicles from rabbit and age-matched normal and mdx mouse skeletal muscle were isolated by a wheat germ agglutination procedure as described previously [9]. For further purification, agglutinated sarcolemma vesicles were mildly washed with detergent by incubation for 10 min on ice with 0.1% Triton X-100, 0.3 M sucrose, 20 mM Tris-Cl, pH 7.4, and pelleted for 90 s at $14000 \times g$. Subsequently the pellets were resuspended in the above buffer without the Triton X-100 and deagglutinated as described [9].

The cytoskeletal fraction of sarcolemma was isolated by an established procedure [10]. Purified rabbit sarcolemma vesicles (1 mg/ml) were treated for 10 min with 0.5% Triton X-100, 4 mM EGTA, 2 mM $MgCl_2$, 0.1 M KCl, 60 mM PIPES, pH 6.9, 0.75 mM benzamidine and 0.1 mM PMSF and then centrifuged for 20 min at $150000 \times g$. The cytoskeleton fraction remains as an insoluble pellet. Treatment of sarcolemma membranes with alkaline solutions was performed as described previously [11]. Rabbit sarcolemma vesicles (1 mg/ml) were incubated at room temperature in 20 mM Tris-buffer, 0.303 M sucrose, 0.75 mM benzamidine, 0.1 mM PMSF at pH 11 for 1 h and then centrifuged for 20 min at $150000 \times g$.

Protein samples were fractionated on 3–12% gradient SDS polyacrylamide gels [12] and stained with Coomassie blue or transferred to nitrocellulose paper [13] and stained with antibodies as described [14]. The specificity of the rabbit antisera against the C-terminal decapeptide of dystrophin was previously demonstrated by the fact that it does not label muscle biopsy samples from DMD patients, but strongly stains the cell periphery of normal human muscle [9]. Monoclonal antibody McB2 was a generous gift from Dr Kathleen Sweadner (Harvard Medical School, Boston). Densitometric scanning of Coomassie blue-stained SDS-PAGE gels was carried out on a Molecular Dynamics 300S computing densitometer. Immunofluorescence microscopy was performed as outlined in [14].

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3. RESULTS AND DISCUSSION

To establish that the 400 kDa protein band in isolated sarcolemma is exclusively dystrophin we applied a lectin agglutination procedure to isolate sarcolemma from control mouse muscle and from mdx mouse muscle, which is known from immunological studies to be missing dystrophin [1]. The overall SDS-PAGE profile of control and mdx sarcolemma appears to be very similar (Fig. 1). The major difference between the control and mdx sarcolemma is the absence of the 400 kDa protein band (Fig. 1a,e), which is stained in immunoblotting (Fig. 1b,f) by antisera against the C-terminal decapeptide of dystrophin in normal mouse muscle sarcolemma. The absence of the 400 kDa protein band in mdx sarcolemma was observed in sarcolemma preparations from 5–6-, 8–10-, 10–12- and 20–30-week-old mice. Restricted immunofluorescence labeling of the cell periphery in normal mouse muscle cryosections in comparison to no staining of mdx muscle cells established the specificity of the polyclonal rabbit antisera against the C-terminal decapeptide of dystrophin (Fig. 1c,d). A very faint Coomassie blue-stained protein band in mdx mouse sarcolemma with a slightly higher M_r than dystrophin was observed in mdx mouse sarcolemma (Fig. 1a,e). Immunoblot staining with affinity-purified rabbit antibodies against the C-terminus of a dystrophin-related protein, which is encoded by a different gene [15,16], indicated that the protein of slightly higher M_r than dystrophin observed in mdx sarcolemma could be the dystrophin-related protein (results not shown). Thus, the analysis of con-

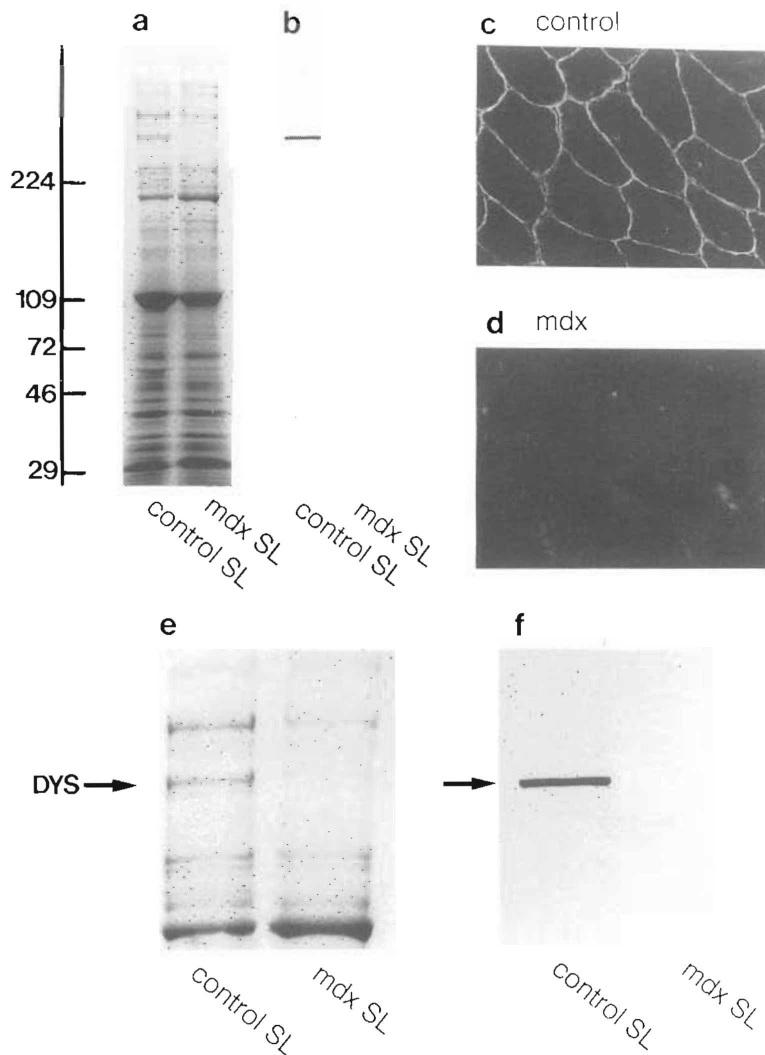

Fig. 1. Comparison of control and mdx mouse muscle sarcolemma membranes. Shown are a Coomassie blue-stained gel (a, e) of isolated sarcolemma (SL) and an immunoblot (b, f) of an identical gel stained with polyclonal antisera against the C-terminal decapeptide of dystrophin. Parts (e) and (f) are enlargements of the upper portion of the gel and corresponding immunoblot (DYS, dystrophin; arrow). Molecular weight standards are from top to bottom ($M_r \times 10^{-3}$) 224, 109, 72, 46, and 29. Transverse cryostat sections of normal (c) and mdx (d) mouse skeletal muscle were labelled by indirect immunofluorescence with polyclonal antisera against the C-terminal decapeptide of dystrophin.

trol and mdx sarcolemma has demonstrated that the 400 kDa Coomassie blue-stained protein band in isolated sarcolemma is exclusively dystrophin.

The fact that dystrophin could be identified as a distinct band on Coomassie blue-stained gels of isolated sarcolemma suggested that it was not a minor component of the sarcolemma membrane from skeletal muscle. In order to determine the density of dystrophin in the sarcolemma membrane, the Coomassie blue-stained gels of the isolated sarcolemma were analyzed by densitometric scanning (Fig. 2). Sarcolemma isolated from rabbit skeletal muscle was used for this analysis and subsequent experiments because of the difficulty in obtaining large amounts of mouse muscle sarcolemma and because rabbit sarcolemma was less contaminated with sarcoplasmic reticulum. In addition, since thin section electron microscopy indicated that sarcoplasmic reticulum contamination was likely from the entrapment of small sarcoplasmic reticulum vesicles within larger sarcolemma vesicles (results not shown), we treated lectin-agglutinated sarcolemma vesicles with low concentrations of the non-ionic detergent Triton X-100 (0.1%) with the aim of removing these contaminating, trapped vesicles. The Coomassie blue-stained protein pattern of crude surface membrane (lane 1), isolated sarcolemma (lane 2),

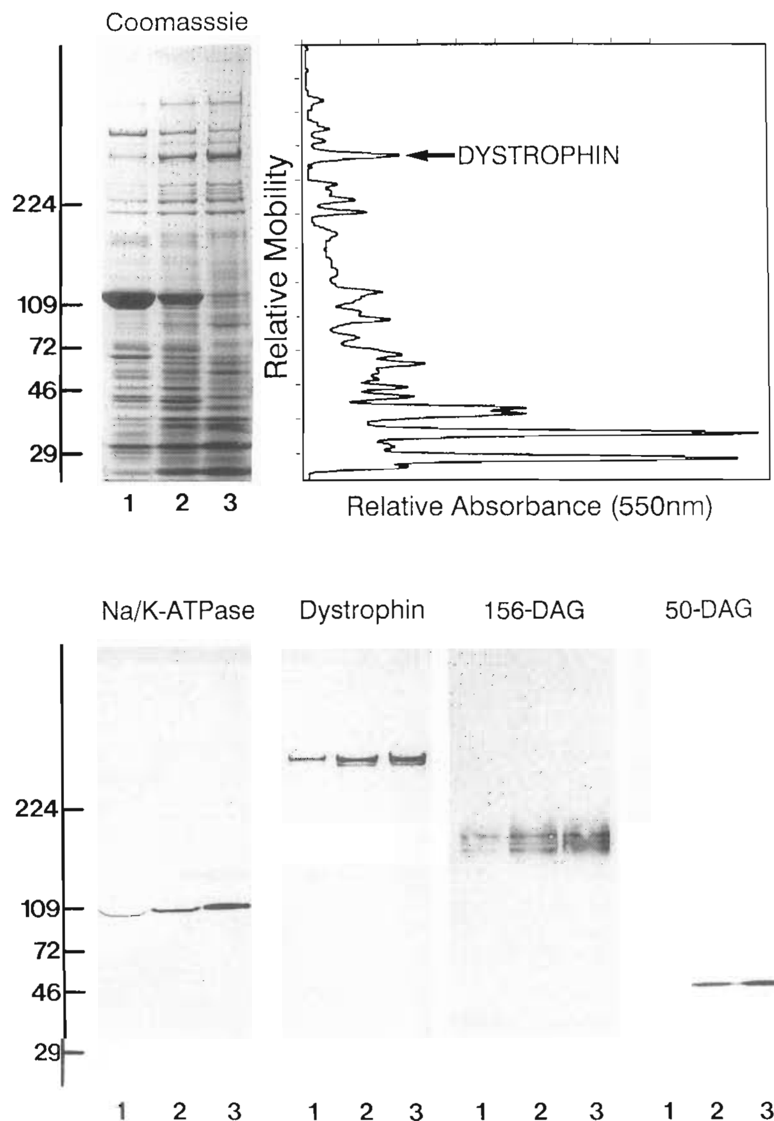

Fig. 2. Immunoblot analysis of purified sarcolemma from rabbit skeletal muscle. Shown are a Coomassie blue-stained gel and identical immunoblots of crude surface membrane (lane 1), isolated sarcolemma (lane 2) and detergent-washed sarcolemma (lane 3). The densitometric scan of lane (3) demonstrates the relative abundance of dystrophin (arrow) in the purified sarcolemma fraction. Immunoblots were stained with monoclonal antibody McB2 against Na/K-ATPase, monoclonal antibody XIXC2 against dystrophin (DYS), monoclonal antibody $VIA4_1$ against dystrophin-associated glycoprotein of 156 kDa (156-DAG) and monoclonal antibody $IVD3_1$ against dystrophin-associated glycoprotein of 50 kDa (50-DAG). Molecular weight standards are as described in Fig. 1.

and detergent-washed sarcolemma (lane 3) demonstrated that the low concentrations of Triton X-100 were very effective in removing the 110000 Da Ca^{2+}-ATPase (a sarcoplasmic reticulum marker) while enriching in dystrophin and the sarcolemma marker Na^{+},K^{+}-ATPase (Fig. 2). Removal of the Ca^{2+}-ATPase was not due to solubilization of the membranes since electron microscopy of the detergent-washed preparation demonstrated sealed vesicles (results not shown). Peak integration of the densitometric scan of the detergent-washed sarcolemma (lane 3) revealed that dystrophin accounts for 4.8 ± 0.8% (n = 6) of the total protein. Thus, the density of dystrophin in highly purified sarcolemma membranes is approximately 2400-fold higher than its density in whole muscle and is comparable to the density of spectrin in brain membranes [17]. Dystrophin-associated glycoproteins of 50 kDa and 156 kDa [14,18] are also enriched in the highly purified sarcolemma (Fig. 2, immunoblots) although the 50 kDa and 156 kDa glycoproteins cannot be distinguished as distinct bands in the Coomassie blue-stained gel.

In order to identify the cytoskeletal components of skeletal muscle sarcolemma, established methods [10,11,19] for the isolation of cytoskeletal fractions from cell membranes were employed. Lectin-agglutinated sarcolemma and not detergent-washed sarcolemma was used for these experiments in order to be consistent with previously published procedures. Extraction of plasma membranes with high concentrations of the non-ionic detergent Triton X-100 (0.5%) leaves the cytoskeleton as an insoluble residue while solubilizing the membrane proteins not associated with the cytoskeleton [10,19]. SDS-PAGE and immunoblot analysis of the Triton-extracted sarcolemma are shown in Fig. 3a. Dystrophin is exclusively found in the insoluble pellet comprising 5.1 ± 1.0% (n = 6) of the total cytoskeleton protein. Dystrophin-associated

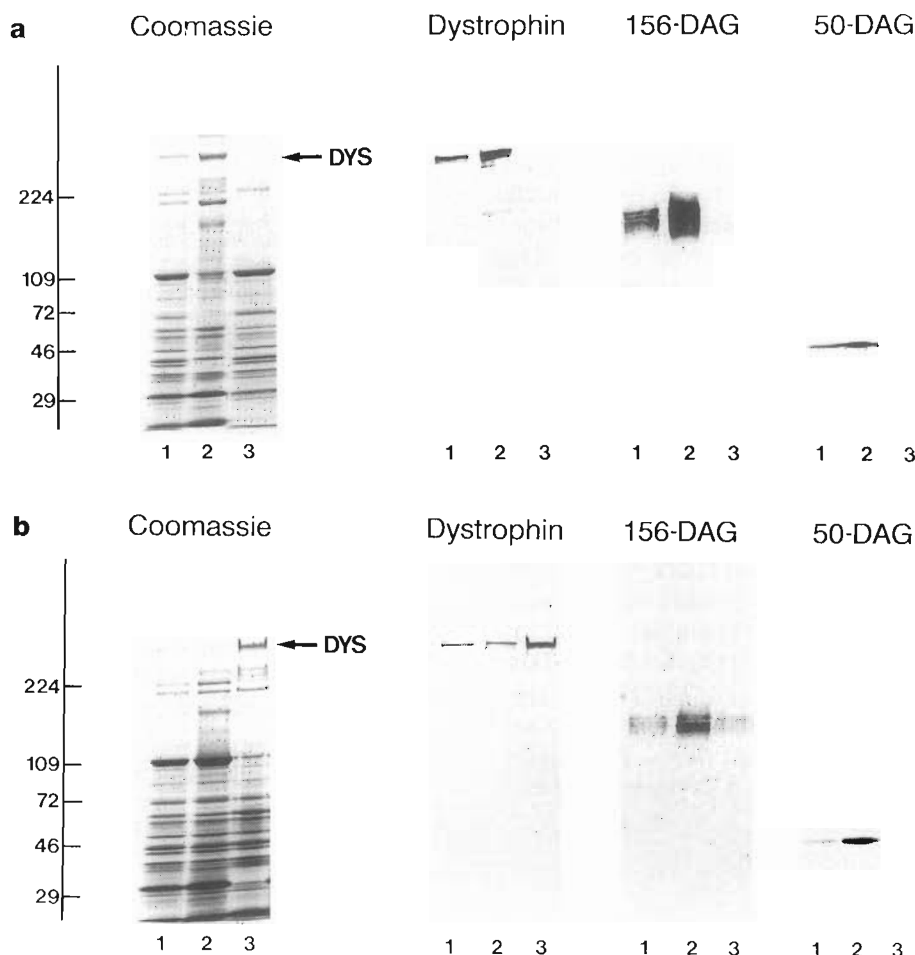

Fig. 3. Characterization of dystrophin in rabbit skeletal muscle sarcolemma. Shown are a Coomassie blue-stained gel and immunoblots of identical gels stained with antibodies against components of the dystrophin complex: monoclonal antibody XIXC2 against dystrophin (DYS), monoclonal antibody VIA4$_1$ against the 156 kDa glycoprotein (156-DAG) and monoclonal antibody IVD3$_1$ against the 50 kDa glycoprotein (50-DAG). (a) Lane 1, sarcolemma; lane 2, cytoskeleton fraction; lane 3, detergent solubilized fraction. (b) Lane 1, sarcolemma; lane 2, pH 11 pellet; lane 3, pH 11 extracted supernatant. The dystrophin protein band is indicated by an arrow. Molecular weight standards are as described in Fig. 1.

glycoproteins of 50 kDa and 156 kDa also remain with the cytoskeletal fraction (Fig. 3a) while Na^+,K^+-ATPase is found in the supernatant (results not shown). In contrast to the treatment with Triton X-100, treatment of membranes with strong alkaline solutions is known to remove tightly associated cytoskeletal components (i.e. spectrin, ankyrin, band 4.2) [20] from membranes while leaving the integral membrane proteins with the bilayer [11,19]. Fig. 3b shows that most of dystrophin is extracted by alkaline treatment, while the 50 kDa and 156 kDa dystrophin-associated glycoproteins are not removed. Actin and myosin are also found in the supernatant, while Na^+,K^+-ATPase remains with the bilayer (results not shown). Thus, established procedures for the characterization of the cytoskeleton demonstrate that dystrophin and its associated glycoproteins of 50 and 156 kDa are integral components of the cytoskeleton of the sarcolemma in skeletal muscle. In addition, the fact that dystrophin and not the dystrophin-associated glycoproteins of 50 and 156 kDa can be removed from sarcolemma membranes by alkaline treatment suggests that dystrophin is tightly associated to the sarcolemma membrane through its interactions with dystrophin-associated glycoproteins.

Our findings demonstrate that although dystrophin is a minor protein when compared to the total muscle protein [8], it is a major component of the subsarcolemmal cytoskeletal network in skeletal muscle. The high density of dystrophin in the cytoskeleton of skeletal muscle sarcolemma strongly suggests that dystrophin plays an important structural role in skeletal muscle sarcolemma like spectrin does for the erythrocyte membrane [21]. Since the absence of spectrin from the cytoskeleton of red blood cells leads to a fragile erythrocyte membrane [6,7] it is likely that the absence of dystrophin could also lead to an unstable sarcolemma membrane. Whether this alone is enough to initiate the pathogenesis of DMD is not known. The high density of dystrophin in the cytoskeleton also supports the hypothesis that dystrophin is an important factor for the flexibility and integrity of the surface membrane during muscle contraction [22]. Finally, our results could give a molecular explanation for the recent finding that dystrophin-less fibres from mdx mouse are more fragile and have a decreased osmotic stability [23].

Acknowledgements: We would like to acknowledge the expertise of Steven D. Kahl and Cynthia J. Leveille in maintaining the mdx and normal mouse colony and we also wish to thank Dr James M. Ervasti for his helpful comments on this paper. K.P.C. is an Investigator of the Howard Hughes Medical Institute.

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