

## **The dystrophin-glycoprotein complex: identification and biochemical characterization**

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### **Summary**

Dystrophin, the high molecular weight protein product of the Duchenne muscular dystrophy (DMD) gene, is localized to the sarcolemma of normal skeletal muscle but is absent from the skeletal muscle of patients with DMD and mdx mice. The predicted amino acid sequence of dystrophin suggests that dystrophin is involved in the anchoring of sarcolemmal proteins to the underlying cytoskeleton. However, the sarcolemmal proteins which are associated with or bound to dystrophin are not known and the status of these proteins in muscle where dystrophin is absent is also unknown.

Here, we review the purification of a dystrophin-glycoprotein complex and the identification of five dystrophin-associated proteins, including four dystrophin-associated glycoproteins. The dystrophin-glycoprotein complex was isolated following digitonin-solubilization of rabbit skeletal muscle membranes using WGA-Sepharose and DEAE-cellulose and further purified by sucrose density gradient centrifugation. In addition to dystrophin, the complex contains a 59 kDa protein triplet and four glycoproteins of 156 kDa, 50 kDa, 43 kDa and 35 kDa. Indirect immunofluorescence with monoclonal antibodies specific for dystrophin, the 156 kDa glycoprotein or the 50 kDa glycoprotein demonstrated a restricted localization of the dystrophin-glycoprotein complex to the sarcolemma of skeletal muscle. Immunoaffinity beads specific for dystrophin or the 50 kDa glycoprotein selectively absorb the dystrophin-

glycoprotein complex, indicating that the components of the complex are tightly associated.

A dramatic (~90%) deficiency of the 156 kDa dystrophin-associated glycoprotein was also observed in muscle from mdx mice and DMD patients. Thus, the marked reduction of the 156 kDa glycoprotein in dystrophic muscle, and possibly other dystrophin-associated proteins, may be the initial step(s) involved in the molecular pathogenesis of muscular dystrophy. The elucidation of the function of dystrophin-associated glycoproteins should help to define the function of dystrophin and explain how its absence results in DMD.

### **Introduction**

Duchenne muscular dystrophy (DMD) is caused by a defective gene found on the X-chromosome. Dystrophin is the large molecular weight protein product of the DMD gene [1] which is localized to the sarcolemmal membrane of normal skeletal muscle [2-5] but is absent from the skeletal muscle of DMD humans [1,2,6], xmd dogs [7], and mdx mice [1,5]. The predicted amino acid sequence of dystrophin suggests that dystrophin is a membrane cytoskeletal protein [8,9] involved in the anchoring of sarcolemmal proteins to the underlying cytoskeleton. However, neither the exact function of dystrophin, nor its precise role in the resulting fiber necrosis of dystrophic muscle has been determined. In studies of other genetic diseases involving cytoskeleton proteins [10,11], the absence of one component of the cytoskeleton is sometimes accompanied by the loss of another component of the membrane cytoskeleton. Thus, in order to understand the molecular pathogenesis of DMD, it is imperative to identify the proteins which are associated and/or linked to dystrophin and to characterize the status of these proteins in muscle where dystrophin is absent.

We have previously shown that dystrophin could be isolated from detergent-solubilized skeletal muscle membranes using wheat germ agglutinin (WGA) chromatography because of its tight association with WGA-binding glycoproteins [12]. Our results suggested that the localization of dystrophin to the cytoplasmic face of the sarcolemma [2-5] results from a tight association of dystrophin with an integral membrane glycoprotein.

In the present work, we discuss our report [13] of the purification of a large (~18S) oligomeric complex containing dystrophin and identify four glycoproteins of apparent molecular mass 156 kDa, 50 kDa, 43 kDa and 35 kDa as integral components of the dystrophin complex. The 156 kDa

glycoprotein and the 50 kDa glycoprotein are shown to be sarcolemmal glycoproteins by indirect immunofluorescence. Immunoaffinity beads against dystrophin and the 50 kDa glycoprotein selectively adsorb the dystrophin-glycoprotein complex. Of particular interest is our finding of a marked reduction of the 156 kDa glycoprotein in muscle from mdx mice and DMD patients. In dystrophic muscle, the absence of dystrophin may lead to the net loss of dystrophin-associated protein(s) thus indicating the degenerative cascade typifying muscular dystrophy.

### **Experimental procedures**

#### *Polyclonal antisera to the dystrophin C-terminal decapeptide*

A ten amino acid residue peptide representing the carboxy-terminal of dystrophin (Pro-Gly-Lys-Pro-Met-Arg-Glu-Asp-Thr-Met) [8] was synthesized on an Applied Biosystems Model 430A peptide synthesizer by the Alberta Peptide Institute, University of Alberta, Edmonton, Alberta, Canada, using methods previously described [14]. The purified peptide was photochemically coupled to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) at peptide to protein ratios of 17:1 for the BSA-peptide conjugate and 8:1 for the KLH-peptide conjugate [15].

New Zealand white rabbits (8-week-old females) were bled from the outer marginal ear vein prior to immunization with 125 µg of the KLH-peptide conjugate in Freund's complete adjuvant. Rabbits were boosted as above using Freund's incomplete adjuvant 14 days after the initial immunization. Serum was collected on day 28 and every 2 weeks thereafter.

#### *Monoclonal antibodies against dystrophin and dystrophin-associated glycoproteins*

Production of monoclonal antibodies (mAbs) against components of the dystrophin-glycoprotein complex was carried out by immunizing female BALB/c mice with rabbit skeletal muscle membrane preparations and boosting with WGA eluate obtained from digitonin-solubilized rabbit skeletal muscle membranes as described previously [16-18]. An immunodot assay was used for the initial screening of the hybridoma supernatants, followed by immunoblot and immunofluorescence analysis to further characterize the specificity of the new mAbs.

#### *Isolation of the dystrophin-glycoprotein complex*

Heavy microsomes were prepared from rabbit skeletal muscle [19] and washed twice with 0.6 M KCl in 50 mM Tris-HCl, pH 7.4, 0.165 M

sucrose, 0.1 mM PMSF and 0.75 mM benzamidine to remove contractile proteins. One gram of KCl-washed membranes were solubilized in 1.0% digitonin, 0.5 M NaCl, and protease inhibitors as previously described [12]. After removal of the ryanodine receptor by immunoaffinity chromatography [20], the digitonin-solubilized membranes were circulated overnight on a 40 ml WGA-Sepharose column, washed extensively, then eluted with 3 column volumes of 0.3 M *N*-acetylglucosamine. Eluted fractions containing dystrophin were applied to a 3 ml DEAE-cellulose column and sequentially eluted with the following NaCl concentrations in Buffer A (0.1% digitonin, 50 mM Tris-HCl, pH 7.4, 0.75 mM benzamidine, 0.1 mM PMSF): 0 mM, 25 mM, 50 mM, 75 mM, 100 mM, 110 mM and 175 mM.

#### *Sucrose gradient sedimentation of the dystrophin-glycoprotein complex*

Sucrose gradients (12.5 ml linear 5% to 20% sucrose) containing 0.5 M NaCl and 0.01% NaN<sub>3</sub> in Buffer A were prepared using a Beckman density gradient former. Partially purified dystrophin complex, which eluted in fraction 2 (3 ml) from the DEAE-column 175 mM NaCl wash, was concentrated to 0.5 ml in a Centricon-100 (Amicon), layered on a sucrose gradient, and overlaid with 0.5 ml of Buffer A containing 175 mM NaCl and 0.01% NaN<sub>3</sub>. Gradients were centrifuged at 4°C in a Beckman VTi 65.1 vertical rotor for 90 min at 200 000 × *g*. Fractions (0.6 ml) were collected from the top of the gradients using an ISCO Model 640 density gradient fractionator. Sucrose concentrations of individual fractions were determined using a refractometer. Gradient fractions were analysed by SDS-PAGE [21] and immunoblotting [22]. Gel lanes were scanned with a Molecular Dynamics 300A computing densitometer and analysed using Image Quant Version 2.0 data analysis software. Immunoblots for mAb IVD3 were prepared from gels run in the absence of reducing agent plus 10 mM *N*-ethylmaleimide.

#### *Indirect immunofluorescence of skeletal muscle*

The indirect immunofluorescence labelling of fixed 8 µm transverse cryostat sections from rabbit gastrocnemius was carried out as described previously [17]. Sections were preincubated for 20 min with 5% normal goat antiserum in PBS, followed by a 2 h incubation at 37° C with the primary antibody (hybridoma supernatants or 1:1000 diluted antiserum). After washing in PBS the sections were further incubated for 30 min at 37°C in PBS with a 1:50 dilution of FITC-labelled goat F(ab')<sub>2</sub> anti-mouse IgG or anti-rabbit IgG and subsequently examined in a Leitz fluorescence microscope.

*Immunoaffinity adsorption of dystrophin-glycoprotein complex*

Immunoaffinity beads [23] were equilibrated with Buffer A containing 0.5 M NaCl and then incubated overnight (12 h) with 0.75 ml of fraction 2 from the 175 mM NaCl wash of the DEAE-cellulose column described above. After pelleting, the supernatants were decanted (voids) and the affinity beads were washed with five  $\times$  0.7 ml aliquots of Buffer A containing 0.5 M NaCl. The void from each affinity column and the five washes were pooled and concentrated to 375  $\mu$ l in a Centricon 100 (Amicon). In addition, 0.75 ml of Fraction 2, which was diluted to 4.2 ml concentrated to 375  $\mu$ l, served as a control. Column voids were analysed by SDS-PAGE [21] and immunoblotting [22].

*Immunoblot analysis of mdx muscle membranes*

Membranes from control and mdx mice were prepared in 10% sucrose, 76.8 nM aprotinin, 0.83 mM benzamidine, 1 mM iodoacetamide, 1.1  $\mu$ M leupeptin, 0.7  $\mu$ M pepstatin A, 0.23 mM PMSF, 20 mM Tris-maleate, pH 7.0 by centrifuging muscle homogenates for 15 min at 14 000  $\times$  g and subsequently pelleting the supernatant for 30 min at 125 000  $\times$  g followed by KCl washing. Control and mdx mouse muscle membranes were analyzed by SDS-PAGE [21] and immunoblotting [22]. The amount of 156 kDa glycoprotein in each preparation was estimated densitometrically from autoradiographs of identical blots incubated with  $^{125}$ I-labelled sheep anti-mouse secondary antibody [24].

*Immunoblot analysis of DMD muscle*

Frozen muscle biopsy samples (50 mg) were crushed in liquid nitrogen using a mortar and pestle and then prepared for electrophoresis as described by Hoffman et al. [6]. The pulverized muscle samples were transferred to 10 volumes of SDS-PAGE sample buffer (10% SDS, 2 M sucrose, 4% 2-mercaptoethanol, 0.002% bromophenol blue, 260 mM Tris-HCl, pH 6.8), vortexed, and precipitated material allowed to settle. Aliquots (50  $\mu$ l) of the SDS-extracted muscle samples were analysed by SDS-PAGE [21] and immunoblotting [22].

**Results***Production of antibodies to DMD gene product, dystrophin*

Rabbits were immunized with the KLH-conjugated sequence corresponding to the last ten amino acids in the predicted sequence of human dystrophin. Pre- and post-immune sera screened against BSA and BSA conjugates indicated that preimmune serum did not react with BSA or

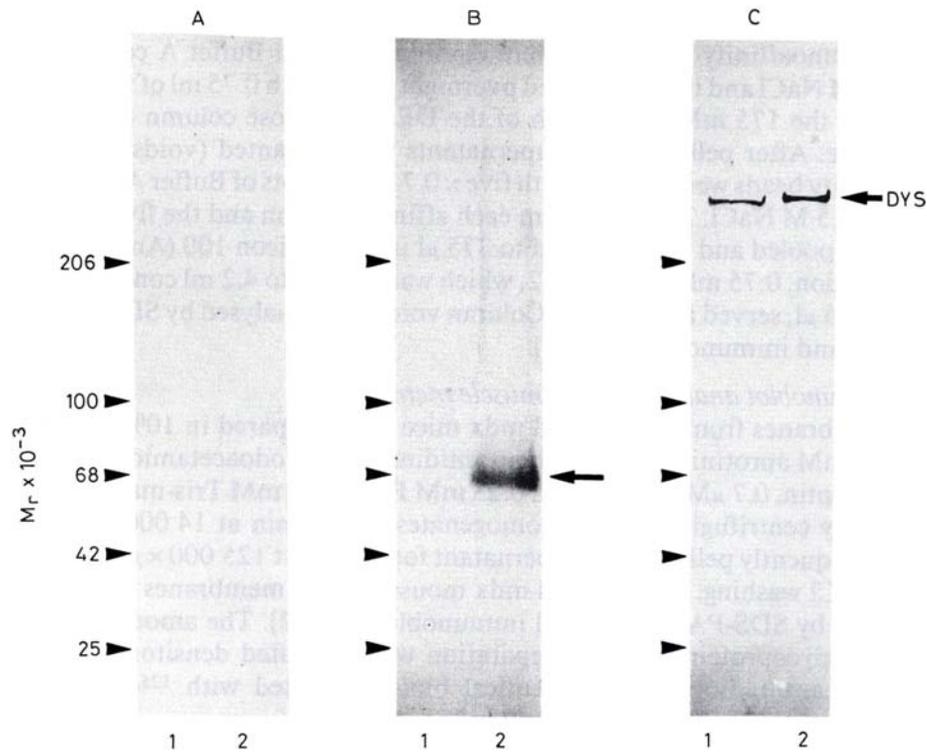


Fig. 1. Antibodies to the C-terminal of dystrophin. One microgram of BSA (A and B, lane 1), 0.5  $\mu$ g BSA-peptide conjugate (A and B, Lane 2), 150  $\mu$ g rabbit skeletal muscle membranes (C, lane 1) or 2  $\mu$ g WGA eluate (C, lane 2) were separated by SDS-PAGE on a 3-12% gradient gel, transferred electrophoretically to nitrocellulose and analysed using indirect immunoperoxidase staining as described under 'Experimental Procedures'. (A) Stained with a 1:1000 dilution of rabbit serum obtained on day 0 prior to immunization. (B and C) Stained with a 1:1000 dilution of postimmune rabbit serum obtained on day 56. The arrow in B indicates the position of the BSA-peptide conjugate and the arrow in C indicates the position of dystrophin (DYS). Molecular weight standards ( $M_r \times 10^{-3}$ ) are indicated by arrow heads on the left.

BSA conjugate, whereas postimmune serum reacted strongly with BSA conjugate that had the C-terminal decapeptide of dystrophin linked to it (Fig. 1). Peptide conjugates from non-related sequences were not detected by the post-immune serum (not shown). The specific binding of C-terminal antibodies to dystrophin was demonstrated using immunoblots of rabbit skeletal muscle membranes and WGA-Sepharose-isolated dystrophin (Fig. 1C).

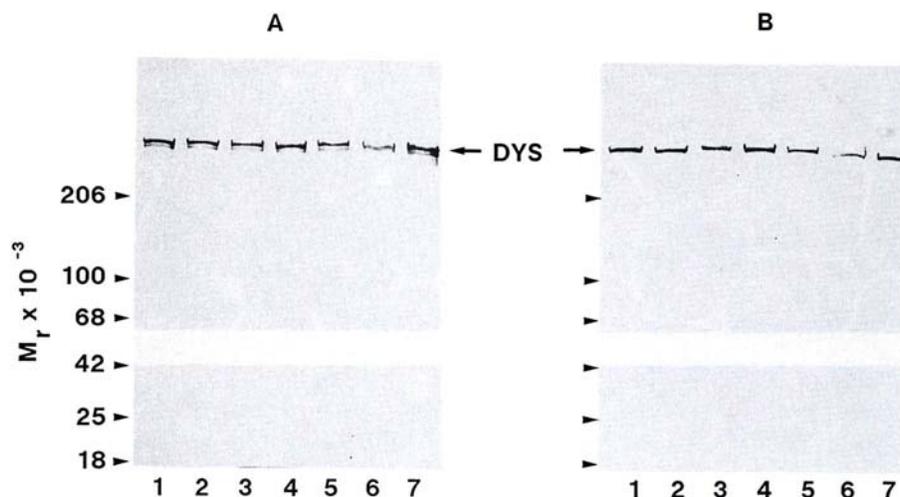


Fig. 2. Immunoblot analysis of N- and C-terminal domains of dystrophin in various muscle membranes. Membranes from various muscle tissue sources were prepared as described in 'Experimental Procedures', separated on 3-12% SDS-PAGE and transferred electrophoretically to nitrocellulose. (A) Stained with sheep polyclonal anti-dystrophin antibodies [1] and (B) was stained with rabbit polyclonal anti-dystrophin antibodies against the C-terminal decapeptide of dystrophin. Lane 1, rabbit skeletal; lane 2, guinea pig skeletal; lane 3, canine skeletal; lane 4, human skeletal; lane 5, canine cardiac; lane 6, mouse cardiac; lane 7, rat cardiac. All lanes contain 200  $\mu\text{g}$  of membrane protein. The arrow represents the position of dystrophin (DYS). Molecular weight standards ( $M_r \times 10^{-3}$ ) are indicated on the left.

The immunoblot staining of isolated dystrophin from muscle membranes of various species, including human skeletal muscle, was compared using sheep polyclonal antisera to dystrophin developed by Hoffman and Kunkel [1] and the rabbit polyclonal antisera to the C-terminal decapeptide of dystrophin (Fig. 1). The sheep polyclonal antisera recognized both the higher and lower molecular weight forms of dystrophin at almost equal intensity (Fig. 2A). This is most evident in the cardiac sample. The C-terminal decapeptide antibodies appear to preferentially react with the higher molecular weight of dystrophin and in some cases do not react with the lower molecular weight form of dystrophin (Fig. 2B).

The antisera against the C-terminal decapeptide of dystrophin showed immunofluorescence staining only on the cell periphery of normal mouse (Fig. 3B) and human (Fig. 3A) skeletal muscle which indicates a restricted localization of dystrophin to the sarcolemma. However, no staining of mdx mouse (Fig. 3D) or DMD muscle (Fig. 3C) was observed with the C-terminal-specific antisera. It was important to test for absence of staining in DMD muscle, since recent work in the field of DMD research has

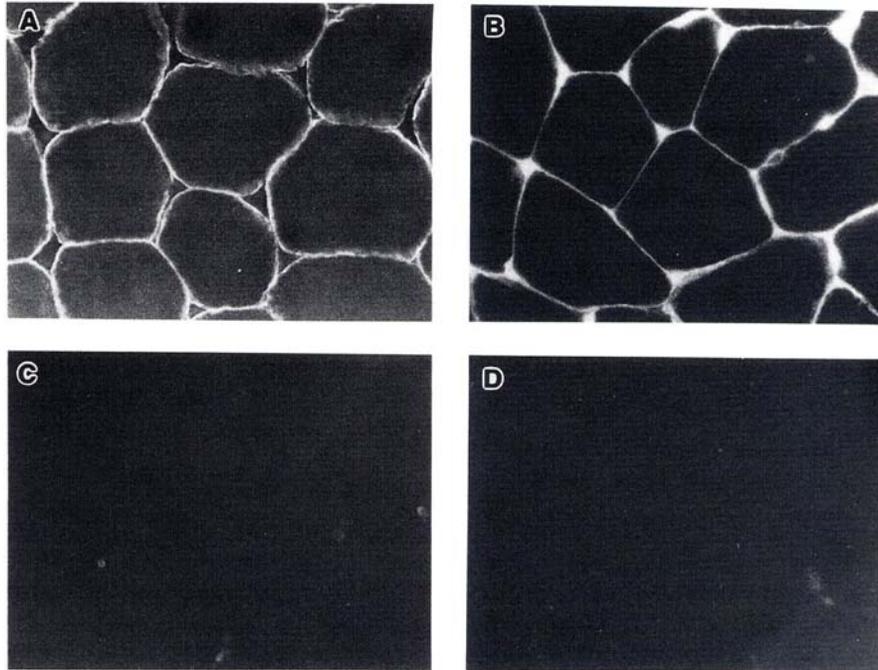


Fig. 3. Immunofluorescence labelling of human and mouse muscle with antibodies against C-terminal decapeptide of dystrophin. Shown are transverse cryosections of normal human (A), normal mouse (B), dystrophic human (C) and mdx mouse (D) skeletal muscle labelled with rabbit antisera against the C-terminal decapeptide of dystrophin. The antibody localizes dystrophin to the cell periphery of normal human and mouse muscle cells and exhibits no labelling of human dystrophic or mdx mouse muscle. The dystrophic muscle sample from a patient with Duchenne muscular dystrophy was obtained from the Department of Neuropathology, University of Iowa (magnification,  $\times 250$ ).

indicated the existence of dystrophin-related proteins which have sequence homology with dystrophin, are encoded by a different gene and may be present in DMD [25-27].

#### *The dystrophin-glycoprotein complex*

The dystrophin-glycoprotein complex was isolated following digitonin-solubilization of rabbit skeletal muscle membranes using WGA-Sepharose and DEAE-cellulose [12] and further purified by sucrose density gradient centrifugation in the presence of 0.1% digitonin (Fig. 4). It is evident from the Coomassie-blue-stained gel of sequential gradient fractions (Fig. 4B) that the dystrophin-glycoprotein complex was clearly separated from the voltage-sensitive sodium channel and the dihydropyridine

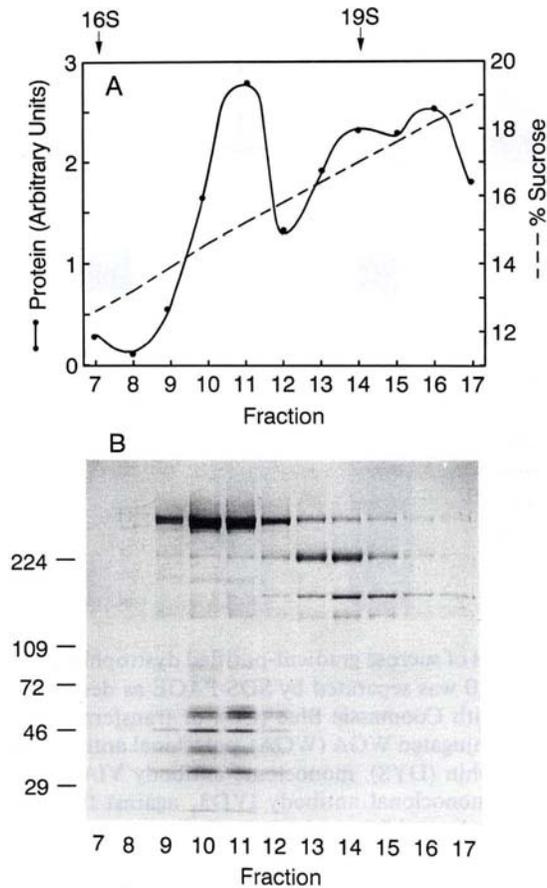


Fig. 4. Sedimentation of the dystrophin-glycoprotein complex through sucrose gradients. DEAE cellulose-purified dystrophin-glycoprotein complex was sedimented through a 5 to 20% linear sucrose gradient containing 0.1% digitonin as described in 'Experimental Procedures'. (A) Total protein (—) and sucrose concentrations (---) of gradient fractions 7 through 17. The sedimentation of  $\beta$ -galactosidase (16S) and thyroglobulin (19S) are indicated by arrows. (B) Coomassie-blue stained gel of sucrose gradient fractions 7 through 17. The molecular weight standards ( $\times 10^3$ ) are indicated on the left.

receptor. The size of the dystrophin complex was estimated to be  $\sim 18S$  by comparing its migration to that of the standards  $\beta$ -galactosidase (16S), thyroglobulin (19S) and the dihydropyridine receptor (20S). Densitometric scanning of the peak dystrophin containing gradient fractions (fractions 10 and 11 in Fig. 4B) revealed several proteins which co-sedimented with dystrophin: a broad, diffusely staining component with

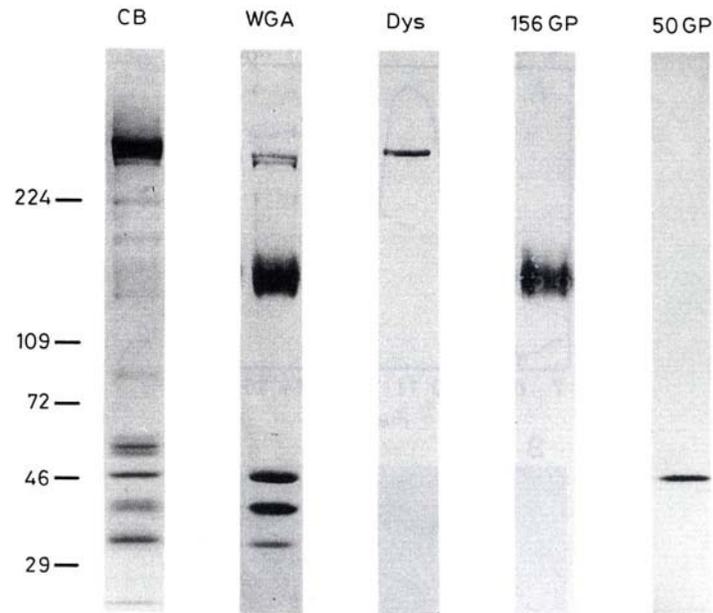


Fig. 5. Immunoblot analysis of sucrose gradient-purified dystrophin-glycoprotein complex. Sucrose gradient fraction 10 was separated by SDS-PAGE as described in 'Experimental Procedures' and stained with Coomassie Blue (CB), or transferred to nitrocellulose and stained with peroxidase-conjugated WGA (WGA), polyclonal antisera against the C-terminal decapeptide of dystrophin (DYS), monoclonal antibody VIA4<sub>1</sub> against the 156 kDa glycoprotein (156GP) or monoclonal antibody IVD3<sub>1</sub> against the 50 kDa glycoprotein (50GP). The molecular weight standards ( $\times 10^{-3}$ ) are indicated on the left.

an apparent molecular mass of 156 kDa, an 88 kDa protein, a triplet of proteins centered at 59 kDa, a 50 kDa protein, a protein doublet at 43 kDa, a 35 kDa protein and a 25 kDa protein.

In order to identify the glycoprotein constituents of the dystrophin-glycoprotein complex, electrophoretically separated nitrocellulose blots of sucrose gradient fraction 10 was stained with peroxidase-conjugated WGA (Fig. 5). Four WGA-binding proteins with apparent molecular mass of 156 kDa, 50 kDa, 43 kDa and 35 kDa were found to strictly co-sediment with dystrophin. All four of the WGA-binding proteins were also stained with peroxidase-conjugated Concanavalin A (not shown). In addition, the lower molecular mass component of the 43 kDa protein doublet, apparent with Coomassie-blue staining, was also stained with Concanavalin A (not shown).

The dystrophin-glycoprotein complex was further characterized with antibodies raised against various components of the complex. A library of

monoclonal antibodies against muscle proteins eluted from WGA-Sepharose were also screened for reactivity against components of the dystrophin-glycoprotein complex and by indirect immunofluorescence staining of rabbit skeletal muscle. Of six hybridomas which showed immunofluorescence staining only on the sarcolemma, monoclonal antibodies XIXC2 (Fig. 6) and VIA4<sub>2</sub> (not shown) were found to stain dystrophin on immunoblots. Both dystrophin monoclonal antibodies are IgM subtypes, and recognized both native and denatured dystrophin.

Two of the other sarcolemma-specific monoclonal antibodies were specific for components of the dystrophin-glycoprotein complex. The 50 kDa glycoprotein stained with monoclonal antibody IVD3<sub>1</sub> (Fig. 5) and has been localized exclusively to the sarcolemmal membrane of rabbit skeletal muscle (Fig. 6). Monoclonal antibody IVD3<sub>1</sub> recognized only the non-reduced form of the 50 kDa glycoprotein and it is not highly cross-reactive. Monoclonal antibody VIA4<sub>1</sub> stained the 156 kDa glycoprotein which co-purified with dystrophin (Fig. 5). Monoclonal antibody VIA4<sub>1</sub> recognized the denatured form of the 156 kDa glycoprotein (Fig. 5) and is highly cross-reactive. Monoclonal antibody VIA4<sub>1</sub> exhibited weak, but specific immunofluorescent staining of the sarcolemmal membrane (Fig. 6), consistent with its low affinity for the native 156 kDa glycoprotein. In agreement with the immunofluorescence results, a rabbit membrane preparation greatly enriched in sarcolemmal proteins also exhibits a substantial enrichment in dystrophin, the 156 kDa and 50 kDa glycoproteins (not shown). Immunofluorescence staining for dystrophin, 50 kDa glycoprotein or the 156 kDa glycoprotein was equally distributed in fast and slow muscle fibers (not shown).

The association of the dystrophin-glycoprotein complex was also assessed by immunoaffinity absorption. Immuno-affinity beads were prepared with the monoclonal antibodies XIXC2 (anti-dystrophin) and IVD3<sub>1</sub> (anti-50 kDa GP) and incubated with the partially purified dystrophin-glycoprotein complex. After pelleting the immunoaffinity beads, the supernatants were removed and the beads were washed extensively. The supernatants and washes were pooled (voids), concentrated and analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting. The voids from the XIXC2 (anti-dystrophin) and the IVD3<sub>1</sub> (anti-50 kDa GP) immunoaffinity beads contained no dystrophin, 59 kDa triplet, 50 kDa 43 kDa doublet or 35 kDa proteins as detected by Coomassie-blue staining (Fig. 7). It is also apparent from Fig. 7 that both the XIXC2 (anti-dystrophin) and IVD3<sub>1</sub> (anti-50 kDa GP) immunoaffinity beads quantitatively removed dystrophin from the starting material. Analysis of the voids for the 156 kDa (Fig. 7) and 50 kDa (Fig. 7) glycoproteins revealed

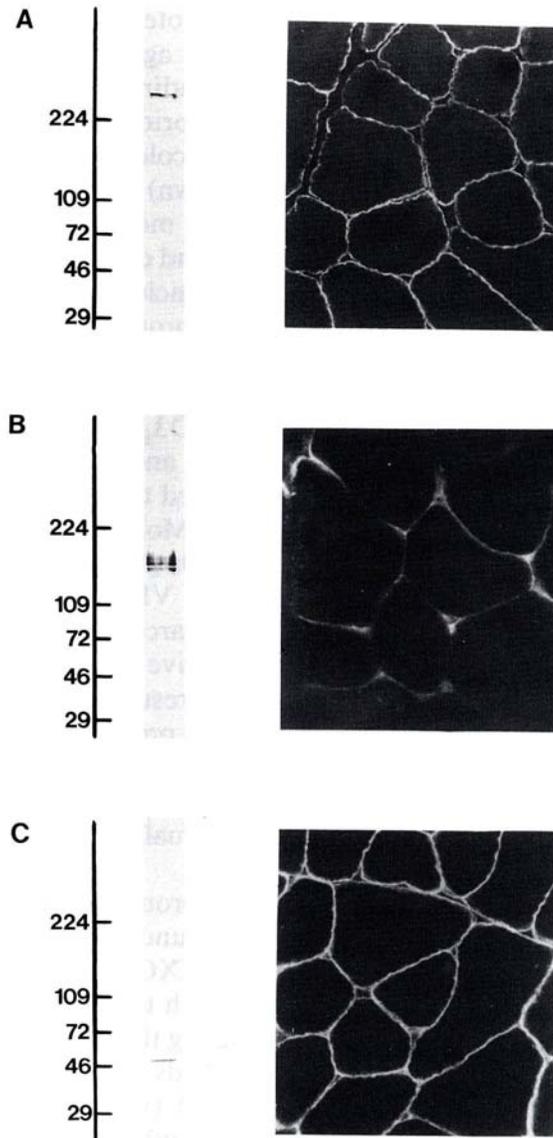


Fig. 6. Immunoblot analysis and immunolocalization of components of the dystrophin-glycoprotein complex. Immunoblots of crude surface membrane, separated by 3-12% SDS-PAGE, and transverse cryosections of rabbit skeletal muscle were labelled with mAb XIXC2 against dystrophin (A), mAb VIA4<sub>1</sub> against the 156 kDa glycoprotein (B) and mAb IVD3<sub>1</sub> against the 50 kDa glycoprotein. All three mAbs strongly label crude surface membrane and stain specifically the cell periphery of skeletal muscle cells. Molecular weight standards ( $\times 10^{-3}$ ) are indicated on the left and the magnification of the micrographs is  $\times 250$ .

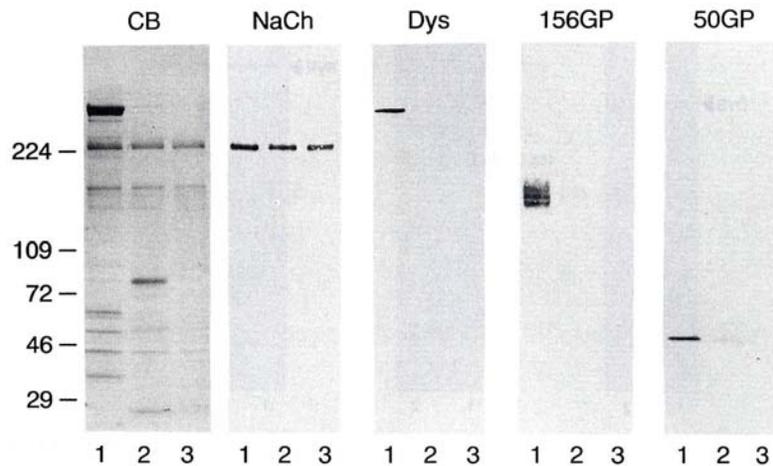


Fig. 7. Immunoadsorption of the dystrophin-glycoprotein complex. Dystrophin-glycoprotein complex was partially purified by WGA-Sepharose and DEAE-cellulose chromatography as described in Experimental Procedures. Portions of DEAE cellulose-purified dystrophin-glycoprotein complex were left untreated (Lane 1), or incubated with XIXC2 (anti-dystrophin) immuno-affinity beads (Lane 2), or IVD3<sub>1</sub> (anti-50GP) immuno-affinity beads (Lane 3) and the supernatants analysed by SDS-PAGE after pelleting of immunobeads. Polyacrylamide gels were either stained with Coomassie Blue (CB), or transferred to nitrocellulose and stained with monoclonal antibody G/C6 against the sodium channel (NaCh), polyclonal antisera to the C-terminal decapeptide of dystrophin (DYS), monoclonal antibody VIA4<sub>1</sub> (156GP), or monoclonal antibody IVD3<sub>1</sub> (50GP). Molecular weight standards ( $\times 10^{-3}$ ) are indicated on the left. Monoclonal antibody G/C6 against the skeletal muscle sodium channel [28] was the generous gift of Dr. Robert Barchi (University of Pennsylvania).

that both the XIXC2 and IVD3<sub>1</sub> immunoaffinity beads selectively adsorbed all but a trace of each of these glycoproteins from the voids. The skeletal muscle voltage-sensitive sodium channel [28] (Fig. 7) and the  $\alpha_1$  and  $\alpha_2$  subunits of the dihydropyridine receptor (not shown) remained in the voids. As detected by peroxidase-conjugated WGA (not shown), the 43 kDa and 35 kDa glycoproteins were also adsorbed from the voids. Immunoblots of immunoaffinity beads separated on gels indicated that dystrophin, the 156 kDa and 50 kDa glycoproteins were retained by the beads and not selectively proteolysed (not shown). Dystrophin, the 156 kDa glycoprotein and the 50 kDa glycoprotein could also be immunoadsorbed from digitonin-solubilized skeletal muscle membranes (not shown). Initial experiments with monoclonal antibody VIA4<sub>1</sub> (anti-156 kDa GP) have indicated that it has too low an affinity for the native 156 kDa glycoprotein to be successful in this type of experiment.

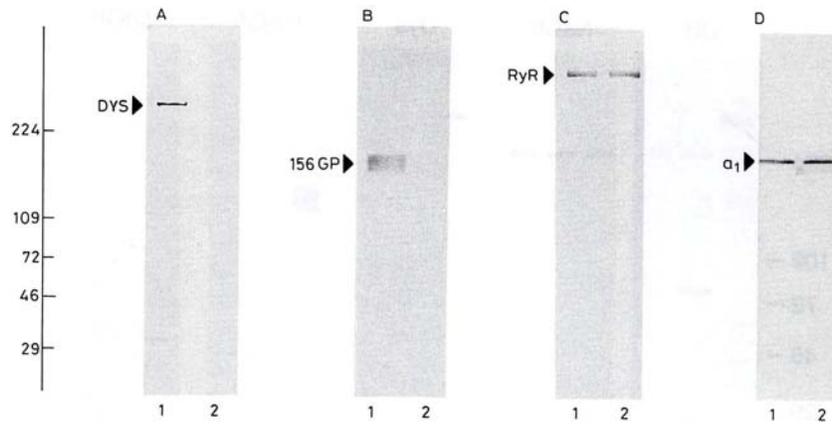


Fig. 8. Immunoblot analysis of control and mdx mouse muscle membranes. Shown are immunoblots labelled with polyclonal rabbit antisera against the C-terminal decapeptide of dystrophin (DYS) (A), mAb VIA4<sub>1</sub> against the 156 kDa glycoprotein (156 GP) (B), polyclonal sheep anti-ryanodine receptor antibody (RyR) (C) and sheep polyclonal antibody to the dihydropyridine receptor ( $\alpha_1$ ) (D). Lanes (1) and (2) for each panel consist of equal amounts of muscle membrane protein from control and mdx mice, respectively (300  $\mu$ g membrane protein per lane in (A) and (B), 150 Mg protein per lane in (C) and (D)). Molecular weight standards ( $\times 10^{-3}$ ) are indicated on the left.

#### *Analysis of dystrophic muscle*

To investigate whether either of the dystrophin-linked 156 kDa or 50 kDa glycoproteins is affected by the absence of dystrophin, immunoblots of skeletal muscle membranes were prepared from control and mdx mice and stained with the various antibodies (Fig. 8). Staining with polyclonal antisera against the C-terminal decapeptide of dystrophin revealed that dystrophin was completely absent from mdx mouse membranes (Fig. 8). In addition, comparison of normal and mdx mouse with immunostaining by monoclonal antibody VIA4<sub>1</sub> against the 156 kDa glycoprotein revealed that the 156 kDa glycoprotein was absent or greatly reduced in mdx mouse membranes (Fig. 8). Staining of identical transfers with sheep polyclonal antisera against either the ryanodine receptor (Fig. 8) or the dihydropyridine receptor (Fig. 8) did not differ between control and mdx mouse muscle membranes. Monoclonal antibody IVD3i against the 50 kDa glycoprotein did not cross-react with normal mouse membranes and thus could not be evaluated. The absence of the 156 kDa glycoprotein was also confirmed using SDS muscle extracts (not shown) instead of isolated membranes from control and mdx mice. Estimation of the amount of 156 kDa glycoprotein remaining in the mdx muscle membranes using <sup>125</sup>I-labelled secondary antibodies and total membrane preparations from

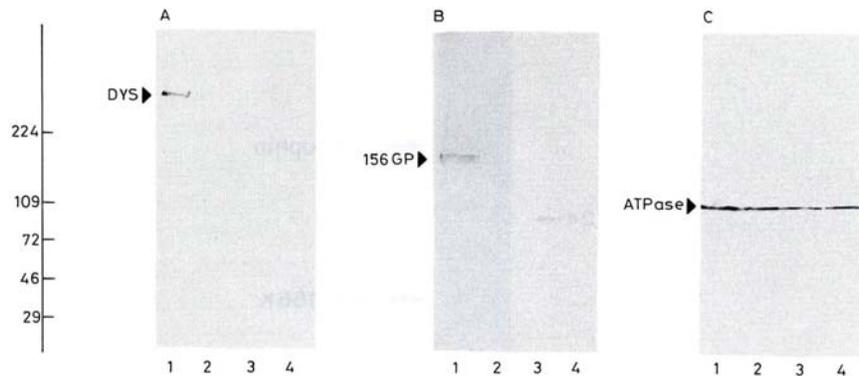


Fig. 9. Immunoblot analysis of normal and dystrophic human muscle biopsies. Shown are immunoblots labelled with mAb XIXC2 against dystrophin (DYS) (A), mAb VIA4<sub>1</sub> against the 156 kDa glycoprotein (156 GP) (B) and mAb IID8 against the Ca<sup>2+</sup>-ATPase (ATPase) (C). Lane 1 consists of normal human muscle extract and lanes 2-4 are dystrophic muscle extracts from three patients with Duchenne's muscular dystrophy, obtained from the Department of Neuropathology, University of Iowa. Molecular weight standards ( $\times 10^{-3}$ ) are indicated on the left.

four different control and four different mdx mice revealed an average reduction of 85% in mdx muscle.

Total muscle extracts were also prepared from biopsy samples of normal controls and patients with Duchennes muscular dystrophy (obtained from the Department of Neuropathology, University of Iowa). The dystrophic samples, exhibited no staining with antibodies against dystrophin by indirect immunofluorescence microscopy (not shown) and immunoblotting (Fig. 9). In contrast to the normal muscle extract the three DMD samples showed greatly reduced staining for the 156 kDa glycoprotein (Fig. 9). On the other hand, identical immunoblots stained with monoclonal antibodies against the Ca<sup>2+</sup>-dependent ATPase (Fig. 9) revealed no difference in the staining intensity between normal and dystrophic muscle samples. Again, the amount of 156 kDa glycoprotein was estimated to be reduced by approximately 90% in DMD samples.

## Discussion

We have presented evidence for the existence of a large oligomeric complex (~ 18S) containing dystrophin, a 59 kDa triplet and four sarcolemmal glycoproteins with apparent molecular masses of 156 kDa, 50 kDa, 43 kDa and 35 kDa (Fig. 10). At least one of the proteins in the complex is an

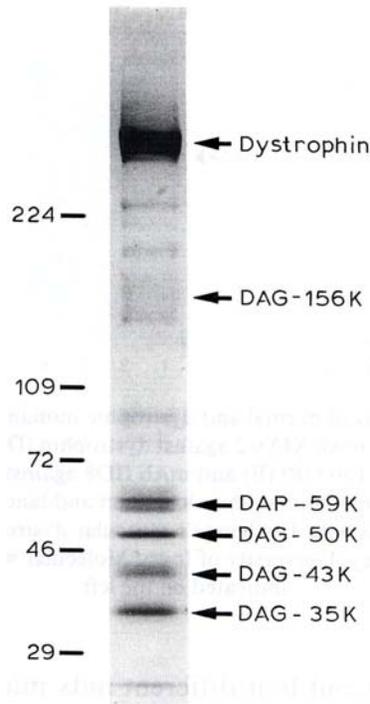


Fig. 10. Components of the dystrophin-glycoprotein complex. Coomassie Blue-stained SDS polyacrylamide gel of purified dystrophin-glycoprotein complex. Dystrophin, the 59 kDa dystrophin-associated protein (DAP-59K) and the 156 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins (DAG-156K, 50K, 43K, 35K) are indicated on the right. Molecular mass standards ( $\times 10^{-3}$ ) are indicated on the left.

integral membrane protein since 1.0% digitonin was necessary to solubilize the dystrophin-glycoprotein complex. The immunoaffinity experiments demonstrate that the complex is tightly associated. To date, a large number of antibodies specific for extracellular matrix proteins, cytoskeletal proteins, plasma membrane pump, channel and receptor proteins have been screened for reactivity against the dystrophin-glycoprotein complex. None of these antibodies to known proteins has demonstrated cross-reactivity to any component of the dystrophin-glycoprotein complex. The elucidation of primary sequences by recombinant DNA techniques should provide clues to the function of the dystrophin-associated glycoproteins. Until the function of the proteins is understood, we have chosen to designate them dystrophin-associated proteins (DAP) or dystrophin-associated glycoproteins (DAG) followed by the molecular mass of each component (Fig. 10).

The surface of DMD myofibers have been reported to exhibit altered [29] or decreased [30] lectin binding, and a 370 kDa glycoprotein is apparently missing from DMD muscle [31]. However, our work is the first to demonstrate the marked deficiency of a glycoprotein which is closely linked to dystrophin. Our report of the substantial reduction of the 156 kDa glycoprotein from muscle of mdx mice and DMD patients is analogous to findings in erythrocytes of individuals afflicted with Hereditary Elliptocytosis [32]. In Hereditary Elliptocytosis, the absence of the cytoskeletal protein 4.1 [11,33,34] is accompanied by greatly diminished steady-state levels of glycophorin C [10,11]. In both diseases, the disruption of the cytoskeleton appears to destabilize the plasma membrane and associated proteins. As antibody probes to other components of the dystrophin complex become available, it will be interesting to determine whether any other proteins are also affected in mdx and DMD muscle.

How the absence of dystrophin leads to the clinical manifestation of muscular dystrophy is an unanswered question. Clearly there could be many steps in the dystrophic process. Here, we may have identified the first step which is the loss of a dystrophin-associated glycoprotein due to the absence of dystrophin. For example, muscle from mdx mice exhibits elevated intracellular ionized  $\text{Ca}^{2+}$  levels and corresponding higher net degradation of muscle proteins [35]. Dystrophin could be modulating stretch-regulated calcium channels [36], either directly or indirectly, by way of its associated glycoproteins. Loss of a dystrophin-anchored protein with a role in the regulation of intracellular calcium or in the maintenance of membrane tension could thus result in elevated intracellular calcium levels and therefore lead to the reported activation of calcium-dependent protease activities [37]. Such a mechanism could explain the abnormal muscle protein degradation and fiber necrosis of dystrophic muscle.

The absence of dystrophin-associated proteins in dystrophic muscle may complicate the therapeutic efficacy of myoblast transfer [38] as the reintroduction of dystrophin synthesis might not lead to recovery of associated protein levels and thus could necessitate treatment only at one particular developmental stage.

Perhaps a deficiency in a dystrophin-associated glycoprotein could explain the DMD-like symptoms observed in suspected autosomal recessive patients [39,40] that express apparently normal dystrophin.

In conclusion, dystrophin has been shown to exist in a large oligomeric complex, and four glycoproteins of apparent molecular mass 156 kDa, 50 kDa, 43 kDa and 35 kDa have been shown to be integral components of the dystrophin complex. The elucidation of the function of these glycoproteins which are linked to dystrophin should help to define the

exact function of dystrophin and explain how its absence results in the pathologies observed in Duchenne muscular dystrophy.

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