Membrane Organization of the Dystrophin–Glycoprotein Complex

James M. Ervasti and Kevin P. Campbell
Howard Hughes Medical Institute
and Department of Physiology and Biophysics
University of Iowa College of Medicine
Iowa City, Iowa 52242

Summary

The stoichiometry, cellular location, glycosylation, and hydrophobic properties of the components in the dystrophin–glycoprotein complex were examined. The 156, 59, 50, 43, and 35 kd dystrophin-associated proteins each possess unique antigenic determinants, enrich quantitatively with dystrophin, and were localized to the skeletal muscle sarcolemma. The 156, 50, 43, and 35 kd dystrophin-associated proteins contained Asn-linked oligosaccharides. The 156 kd dystrophin-associated glycoprotein contained terminally sialylated Ser/Thr-linked oligosaccharides. Dystrophin, the 156 kd, and the 59 kd dystrophin-associated proteins were found to be peripheral membrane proteins, while the 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins and the 25 kd dystrophin-associated protein were confirmed as integral membrane proteins. These results demonstrate that dystrophin and its 59 kd associated protein are cytoskeletal elements that are tightly linked to a 156 kd extracellular glycoprotein by way of a complex of transmembrane proteins.

Introduction

Dystrophin is the high molecular weight, low abundance protein product of the Duchenne muscular dystrophy (DMD) gene (Hoffman et al., 1987). The deduced amino acid sequence of dystrophin (Koenig et al., 1988) and its cellular localization (Zubrzycka-Gaarn et al., 1988; Arahata et al., 1988; Bonilla et al., 1988; Watkins et al., 1988) suggest that dystrophin is a membrane-associated cytoskeletal protein.

We have shown that dystrophin is part of a large (18S), tightly associated oligomeric complex containing five other proteins (Ervasti et al., 1990). Particularly interesting was our finding (Ervasti et al., 1990) of a marked reduction of the 156 kd dystrophin-associated glycoprotein in muscle from mdx mice and DMD patients, which suggests that the absence of dystrophin may lead to the loss of dystrophin-associated glycoprotein(s). The loss of associated proteins as a result of dystrophin's absence may initiate the degenerative cascade of muscular dystrophy.

In the present work, we have prepared specific polyclonal antibodies against the 59 kd dystrophin-associated protein and the 156 kd, 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins. We have used these antibodies to study the stoichiometry, cellular localization, glycosylation, and transmembrane/hydrophobic properties of the components in the dystrophin–glycoprotein complex. Our

results suggest that the function of the dystrophin–glycoprotein complex is to link the actin cytoskeleton with an extracellular component of skeletal muscle. A model of the dystrophin–glycoprotein complex is proposed that takes into account the available biochemical and structural data.

Results

Characterization of Polyclonal Antibodies Specific for Dystrophin-Associated Proteins

We have previously reported the preparation and characterization of monoclonal antibodies (MAbs) against dystrophin and the 156 kd and 50 kd dystrophin-associated glycoproteins (Ervasti et al., 1990; Jorgensen et al., 1990; Ohlendieck et al., 1991). However, MAb VIA4$_1$ bound very poorly to the native 156 kd dystrophin-associated glycoprotein, while MAb IVD3$_1$ stained the reduced form of the 50 kd dystrophin-associated glycoprotein very weakly on immunoblots (Ervasti et al., 1990; Ohlendieck et al., 1991). In addition, the induction of high-titered ascites from these hybridomas has yet to be successful. These limitations, coupled with the need for specific probes to the 59 kd, 43 kd, and 35 kd dystrophin-associated proteins, compelled us to prepare polyclonal antisera specific for each component of the dystrophin–glycoprotein complex. Antisera from guinea pigs immunized with purified dystrophin–glycoprotein complex (Ervasti et al., 1991) showed immunoreactivity to all components of the complex, with the exception of the 50 kd dystrophin-associated glycoprotein (not shown). Immobilon-P transfer strips containing individual components of the dystrophin–glycoprotein complex separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were used to affinity purify antibodies specific for the 156 kd, 59 kd, 43 kd, and 35 kd dystrophin-associated proteins (Figure 1). Polyclonal antibodies to the 50 kd dystrophin-associated glycoprotein were affinity purified from antisera obtained by immunizing a guinea pig with SDS–polyacrylamide gel slices containing the reduced 50 kd dystrophin-associated glycoprotein (Figure 1). Immunoblot staining of skeletal muscle microsomes, sarcolemma, and purified dystrophin–glycoprotein complex (Figure 1) demonstrated that each of the affinity-purified antibodies recognized only proteins of the same molecular weight to which they were raised and against which they were affinity purified. These data demonstrate that the 156 kd, 59 kd, 50 kd, 43 kd, and 35 kd dystrophin-associated proteins contain distinct epitopes, suggesting that they are not proteolytic fragments of larger proteins or dystrophin. The 59 kd dystrophin-associated protein appears as a single band in skeletal muscle microsomes (see Figure 5), a doublet in purified sarcolemma (Figure 1), and a triplet in the dystrophin–glycoprotein complex (Figure 1). This phenomenon is most likely due to the presence of abundant muscle proteins in the microsome and sarcolemma preparations, which compress and obscure detection of the 59 kd triplet on immunoblots.

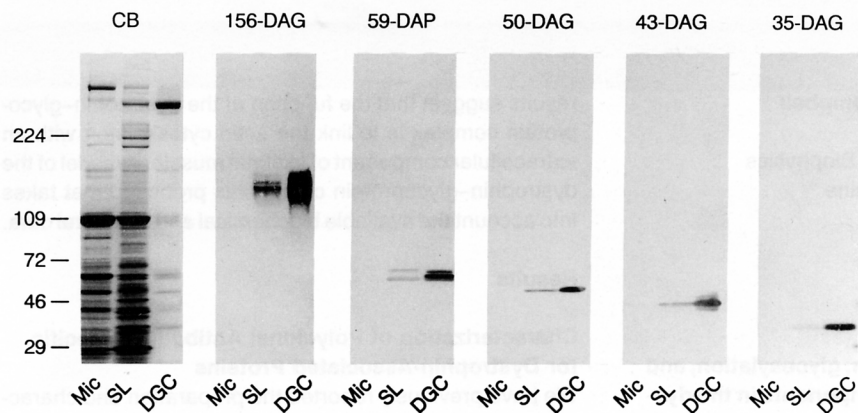In agreement with another study (Yoshida and Ozawa,

Figure 1. Coenrichment of Dystrophin-Associated Proteins with Dystrophin

Fifty micrograms of KCl-washed skeletal muscle microsomes (Mic), 50 μg of pure skeletal muscle sarcolemma (SL), and 12 μg of dystrophin–glycoprotein complex (DGC) were electrophoretically separated on 3%–12% SDS–polyacrylamide gels and either stained with Coomassie blue (CB) or transferred to nitrocellulose as described in the Experimental Procedures. Nitrocellulose transfers were stained with affinity-purified guinea pig polyclonal antibodies to the 156 kd (156-DAG), 59 kd (59-DAP), 50 kd (50-DAG), 43 kd (43-DAG), or 35 kd (35-DAG) dystrophin-associated proteins. The molecular weight standards ($\times 10^3$) are indicated on the left.

1990), densitometric analysis of Coomassie blue–stained SDS–polyacrylamide gels containing the electrophoretically separated components of six different preparations of the dystrophin–glycoprotein complex demonstrated that the 59 kd, 50 kd, 43 kd, 35 kd, and 25 kd dystrophin-associated proteins exhibited average stoichiometric ratios of 1.6 ± 0.22, 0.82 ± 0.11, 0.95 ± 0.14, 1.8 ± 0.19, and 0.36 ± 0.12, respectively, relative to dystrophin. However, the stoichiometry of the 156 kd dystrophin-associated glycoprotein relative to dystrophin has not been determined because it stains very poorly with Coomassie blue (Ervasti et al., 1990). Therefore, the antibody staining intensity was quantitated from autoradiograms of the immunoblots shown in Figure 1 after incubation with ^{125}I-labeled protein A and was compared with the Coomassie blue staining intensity of dystrophin in sarcolemma and purified dystrophin–glycoprotein complex. The 400 kd Coomassie blue–stained band in rabbit sarcolemma has been shown to be dystrophin (Ohlendieck et al., 1991). Densitometric analysis of Coomassie blue–stained gels demonstrated that dystrophin was enriched 2.5-fold in the dystrophin–glycoprotein complex versus sarcolemma. The ratios of autoradiographic densitometric intensities of dystrophin–glycoprotein complex versus sarcolemma for polyclonal antibodies against the 156 kd, 59 kd, 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins were 2.7, 2.2, 2.6, 2.3, and 3.0, respectively. These results suggest that all components of the dystrophin–glycoprotein complex quantitatively co-enrich and that the 156 kd dystrophin-associated glycoprotein is stoichiometric with dystrophin.

Immunolocalization of Dystrophin-Associated Proteins

The cellular localization of the dystrophin-associated proteins was determined by indirect immunofluorescence labeling of transverse cryostat sections of rabbit skeletal muscle (Figure 2). As previously reported (Ohlendieck et

al., 1991), the cell periphery was exclusively stained with MAb VIA4₂ against dystrophin (Figure 2). In addition, affinity-purified polyclonal antibodies specific for the 156 kd, 59 kd, 50 kd, 43 kd, and 35 kd dystrophin-associated proteins also exhibited immunofluorescent staining of the sarcolemmal membrane, demonstrating the unique association of these proteins with the muscle fiber plasma membrane or the intracellular cytoskeleton subjacent to the surface membrane.

N-Glycosidase F Treatment of the Dystrophin–Glycoprotein Complex

The diffuse antibody staining, weak Coomassie blue staining, and strong staining by peroxidase-conjugated wheat germ agglutinin (WGA) suggested that the 156 kd dystrophin-associated glycoprotein is heavily and heterogeneously glycosylated. Weak Coomassie blue staining is a noted property of heavily glycosylated mucins (Holden et al., 1970) and several erythrocyte membrane glycoproteins (Fairbanks et al., 1971). In addition, the 50 kd, 43 kd, and 35 kd dystrophin-associated proteins have been shown to stain with peroxidase-conjugated WGA and concanavalin A (Ervasti et al., 1990).

To better characterize the glycosylation of the 156 kd, 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins, purified dystrophin–glycoprotein complex was treated with N-glycosidase F. N-glycosidase F cleaves Asn-linked high mannose and hybrid and complex oligosaccharides; deglycosylation by this enzyme can be monitored by increases in electrophoretic mobility and loss of lectin staining. Dystrophin and the 59 kd dystrophin-associated protein were unaffected by N-glycosidase F treatment, while the 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins exhibited decreases of approximately 4 kd, 2 kd, and 2 kd, respectively (Figure 3). The absence of staining of the 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins by peroxidase-conjugated WGA (Figure 3) and concanavalin A (not shown) confirmed that all Asn-linked

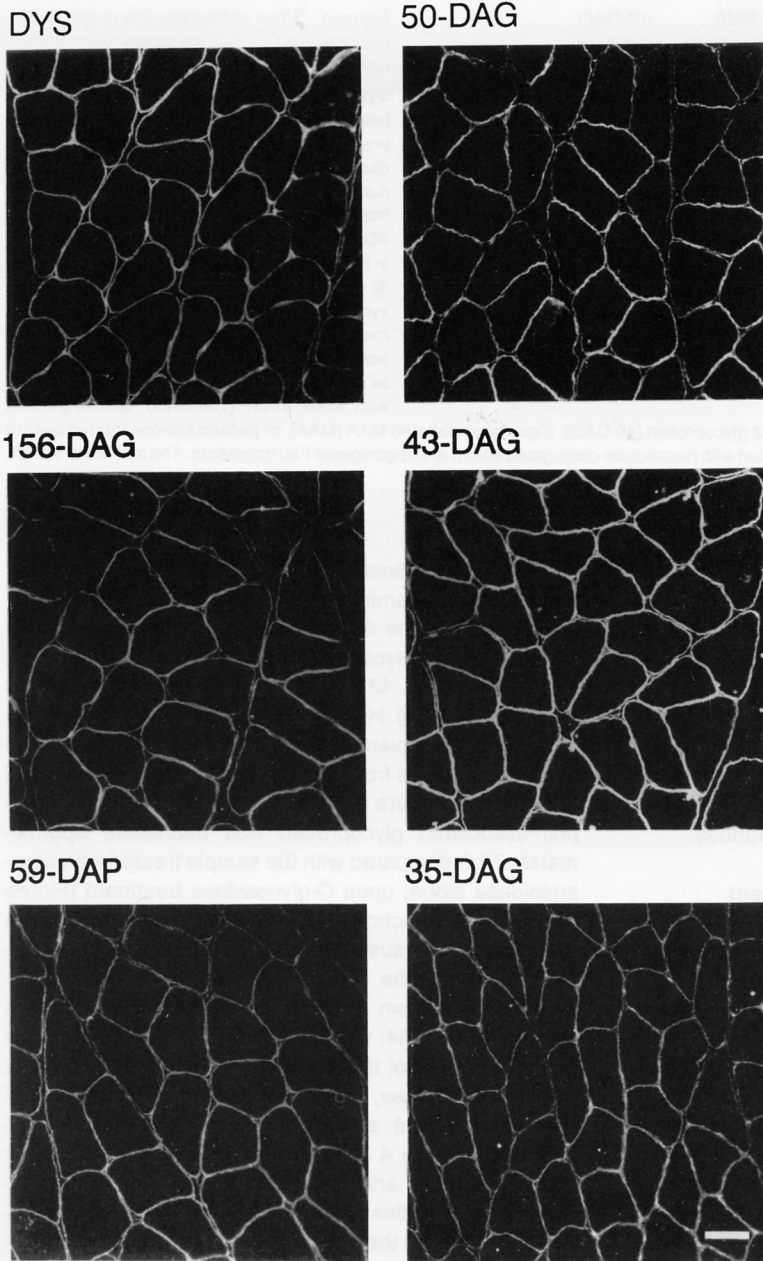DYS

50-DAG

156-DAG

43-DAG

59-DAP

35-DAG

Figure 2. Immunolocalization of Dystrophin-Associated Proteins in Skeletal Muscle

Transverse cryostat sections of rabbit skeletal muscle were labeled by indirect immunofluorescence as described in the Experimental Procedures. Sections were stained with MAb VIA4$_2$ against dystrophin (DYS) or affinity-purified guinea pig polyclonal antibodies specific for the 156 kd (156-DAG), 59 kd (59-DAP), 50 kd (50-DAG), 43 kd (43-DAG), or 35 kd (35-DAG) dystrophin-associated proteins. Bar = 40 μm.
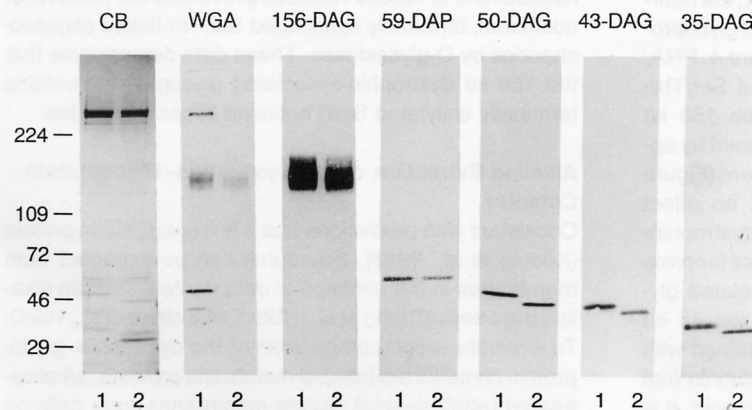

Figure 3. Effect of N-Glycosidase F on the Dystrophin–Glycoprotein Complex

Dystrophin–glycoprotein complex was SDS denatured and incubated for 1 hr at 37°C in the absence (lane 1) or presence (lane 2) of 12 U/ml N-glycosidase F as described in the Experimental Procedures. Control and enzyme-treated samples were analyzed on Coomassie blue (CB)–stained 3%–12% SDS–polyacrylamide gels (6 μg per lane) or transferred to nitrocellulose (3 μg per lane) and stained with peroxidase-conjugated WGA (WGA), MAb VIA4$_1$ (156-DAG), or with affinity-purified guinea pig polyclonal antibodies specific for the 59 kd (59-DAP), 50 kd (50-DAG), 43 kd (43-DAG), or 35 kd (35-DAG) dystrophin-associated proteins. The molecular weight standards (× 10^{-3}) are indicated on the left.
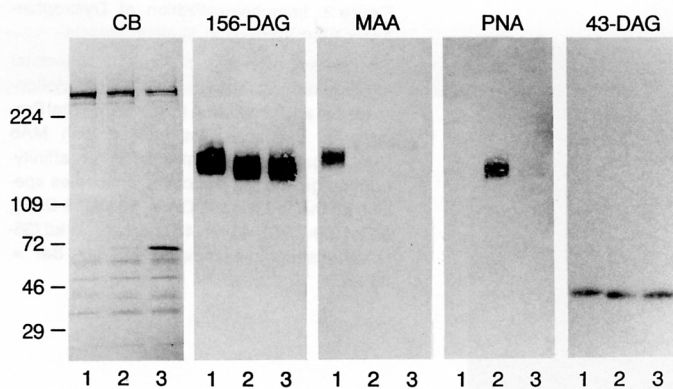
Figure 4. Effect of Neuraminidase and O-Glycosidase on the Dystrophin–Glycoprotein Complex

Dystrophin–glycoprotein complex was incubated for 1 hr at 37°C in the absence (lane 1) or presence (lanes 2 and 3) of 0.1 U/ml neuraminidase as described in the Experimental Procedures. Control and neuraminidase-treated dystrophin–glycoprotein complex was subsequently SDS denatured and incubated for 1 hr at 37°C in the absence (lanes 1 and 2) or presence (lane 3) of 17 U/ml O-glycosidase. Control and enzyme-treated samples were analyzed on Coomassie blue (CB)–stained 3%–12% SDS–polyacrylamide gels (5 μg per lane) or transferred to nitrocellulose (2.5 μg per lane) and stained with MAb VIA4$_1$ (156-DAG), affinity-purified guinea pig antibodies to the 43 kd dystrophin-associated glycoprotein (43-DAG), digoxigenin-labeled MAA (MAA), or peroxidase-conjugated peanut agglutinin (PNA). The MAA-positive proteins were detected with peroxidase-conjugated sheep anti-digoxigenin Fab fragments. The molecular weight standards ($\times$ 10^{-3}) are indicated on the left.

oligosaccharides had been removed with N-glycosidase F treatment. The 156 kd dystrophin-associated glycoprotein was decreased approximately 4 kd, while its WGA-peroxidase staining was only slightly diminished with N-glycosidase F treatment (Figure 3). These data indicate that the 156 kd, 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins contain at least one Asn-linked oligosaccharide and further suggest that the glycosylation of the 156 kd dystrophin-associated glycoprotein is more complex, perhaps containing Ser/Thr-linked oligosaccharides.

Neuraminidase and O-Glycosidase Treatment of the Dystrophin–Glycoprotein Complex

To test for the presence of Ser/Thr-linked oligosaccharides on the 156 kd dystrophin-associated glycoprotein, purified dystrophin–glycoprotein complex was treated with neuraminidase and O-glycosidase. O-glycosidase cleaves Ser/Thr-linked disaccharide galβ(1-3)GalNAc units unless the disaccharide contains terminal substituents such as sialic acid. The 156 kd dystrophin-associated glycoprotein stained positive with Maackia amurensis agglutinin (MAA) (Figure 4, MAA, lane 1), a lectin specific for sialic acid–linked α(2-3) to galactose. Upon treatment with neuraminidase, MAA staining of the 156 kd dystrophin-associated glycoprotein was lost (Figure 4, MAA, lane 2), as was WGA-peroxidase staining (not shown). However, the neuraminidase-treated 156 kd dystrophin-associated glycoprotein stained positive for peanut agglutinin (Figure 4, PNA, lane 2), which binds only to the unsubstituted Ser/Thr-linked disaccharide galβ(1-3)GalNAc unit. The 156 kd dystrophin-associated glycoprotein was decreased by approximately 10 kd upon neuraminidase treatment (Figure 4, 156-DAG). Neuraminidase treatment had no effect on dystrophin, the 59 kd, 50 kd, or 35 kd dystrophin-associated proteins, but caused a slight decrease (approximately 0.6 kd) in the 43 kd dystrophin-associated glycoprotein (Figure 4, CB or 43-DAG). Since the 43 kd dystrophin-associated glycoprotein was not stained with MAA (Figure 4, MAA) or Sambucus nigra agglutinin (not shown), a lectin specific for α(2-6)-linked sialic acid, it is probable that the 43 kd dystrophin-associated glycopro-

tein contains terminal α(2-8)-linked sialic acid, which was removed by neuraminidase treatment.

Treatment of the desialylated dystrophin–glycoprotein complex with O-glycosidase had no effect on dystrophin, the 59 kd, 50 kd, 43 kd, or 35 kd dystrophin-associated proteins (Figure 4). However, O-glycosidase treatment resulted in the complete removal of peanut agglutinin–reactive disaccharides from the 156 kd dystrophin-associated glycoprotein (Figure 4, PNA, lane 3). The 156 kd dystrophin-associated glycoprotein was decreased approximately 2 kd, compared with the sample treated with neuraminidase alone, upon O-glycosidase treatment (Figure 4, 156-DAG). Although neuraminidase and O-glycosidase treatment each caused additive increases in electrophoretic mobility of the 156 kd dystrophin-associated glycoprotein, the protein retained its diffuse staining pattern with the MAb VIA4$_1$ (Figure 4, 156-DAG, compare lanes 1–3). Treatment of the dystrophin–glycoprotein complex with neuraminidase, O-glycosidase, and N-glycosidase F had no apparent additional effect over that demonstrated in Figure 4 (not shown). The lack of effect by hexosaminidase and absence of staining by the fucose-specific lectins Ulex europaeus I and Tetragonolobus purpureas on the 156 kd dystrophin-associated glycoprotein (data not shown) suggested that neither terminal hexosamine or fucose residues prevented the removal of additional, terminally substituted Ser/Thr-linked oligosaccharides by O-glycosidase. These data demonstrate that the 156 kd dystrophin-associated glycoprotein contains terminally sialylated Ser/Thr-linked oligosaccharides.

Alkaline Extraction of the Dystrophin–Glycoprotein Complex

Consistent with predictions that it is a cytoskeletal protein (Koenig et al., 1988), dystrophin can be extracted from membranes in the absence of detergents by simple alkaline treatment (Chang et al., 1989; Ohlendieck et al., 1991). To evaluate which components of the dystrophin–glycoprotein complex are integral membrane proteins, alkaline-treated rabbit skeletal muscle membranes were pelleted (100,000 $\times$ g) and the soluble supernatant and insoluble
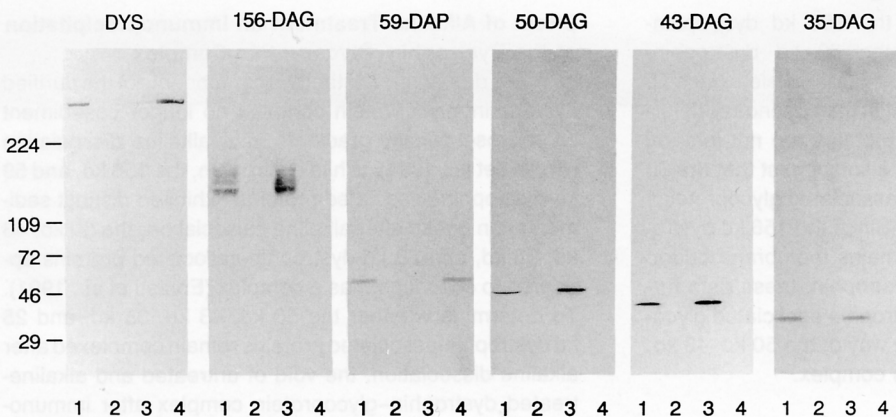
Figure 5. Alkaline Extraction of Dystrophin and 59-DAP from Skeletal Muscle Membranes

KCl-washed skeletal muscle membranes (2.5 mg) were diluted to a volume of 1 ml with 50 mM Tris–HCl (pH 7.4) and incubated for 1 hr at 22°C (pH 7.4) or titrated to pH 11 with 1 M NaOH and incubated for 1 hr at 22°C with gentle mixing as described in Experimental Procedures. The membranes were then pelleted (100,000 × g for 30 min), the supernatants decanted, and the membrane pellets resuspended to a volume of 1 ml. Equal volumes of resuspended pellets (lanes 1 and 3) and supernatants (lanes 2 and 4) from control (lanes 1 and 2) or alkaline-treated (lanes 3 and 4) samples were electrophoresed on 3% to 12% SDS–polyacrylamide gels and stained with Coomassie blue (CB) or transferred to nitrocellulose and stained with polyclonal antisera to the C-terminal decapeptide of dystrophin (DYS), MAb VIA4₁ (156-DAG), affinity-purified guinea pig antisera against the 59 kd (59-DAP), 50 kd (50-DAG), 43 kd (43-DAG), or 35 kd (35-DAG) dystrophin-associated proteins. The molecular weight standards (× 10^{-3}) are indicated on the left.

membrane pellet were analyzed by SDS–PAGE and immunoblotting (Figure 5). The supernatant of alkaline-treated membranes contained greater than 90% of all dystrophin (Figure 5, DYS), while the remaining pellet-associated dystrophin could be extracted with a second alkaline treatment (not shown). The 59 kd dystrophin-associated protein was also extracted by alkaline treatment (Figure 5, 59-DAP). On the other hand, dystrophin and the 59 kd dystrophin-associated protein remained associated with the pellet in membranes diluted in identical buffer that was not titrated to pH 11 (Figure 5, DYS and 59-DAP). The 156 kd, 50 kd, 43 kd, and 35 kd (Figure 5, 156-DAG, 50-DAG, 43-DAG, and 35-DAG, respectively) glycoproteins were retained in the membrane pellet after alkaline treatment. The supernatants obtained from skeletal muscle membranes titrated to pH 11 and pelleted at 100,000 × g were also enriched in nonperipheral or peripheral membrane proteins such as calsequestrin (Zarain-Herzberg et al., 1988), the 53 kd and 160 kd glycoproteins of the sarcoplasmic reticulum (Leberer et al., 1989, 1990), and actin, while the sarcoplasmic reticulum ryanodine receptor, an integral membrane protein (Takeshima et al., 1989), was retained in the pellet (not shown).

To determine how tightly dystrophin associates with the sarcolemma, crude rabbit surface membranes were incubated at various pH values ranging from pH 7.4 to pH 12, and the relative amount of dystrophin extracted was determined from immunoblot analysis (Figure 6). No dystrophin was extracted from surface membranes incubated at pH 7.4, 9, or 10; however, dystrophin was completely extracted when surface membranes were incubated at pH 11 or 12 (Figure 6, DYS). In addition, the 59 kd dystrophin-associated protein was only extracted upon incubation of surface membranes at pH 11 or greater (not shown). Surprisingly, the 156 kd dystrophin-associated glycoprotein,

which was not extracted from membranes incubated at pH 11 (Figures 5 and 6), was almost completely extracted from surface membranes incubated at pH 12 (Figure 6, 156-DAG). The 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins remained in the membrane pellet even after incubation of surface membranes at pH 12
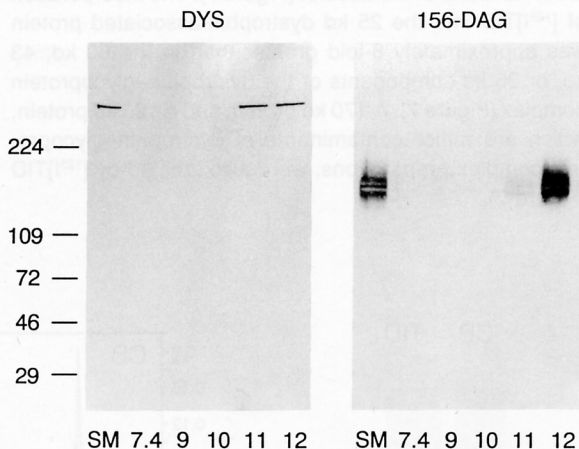

Figure 6. pH Dependence of Extraction of the 156 kd Dystrophin-Associated Glycoprotein from Skeletal Muscle Membranes

Crude surface membranes were diluted 25-fold into 200 mM Tris titrated to the indicated pH and incubated for 1 hr at 22°C with gentle mixing as described in the Experimental Procedures. The membranes were then pelleted (100,000 × g for 30 min), the supernatants decanted and electrophoresed on 3% to 12% SDS–polyacrylamide gels, transferred to nitrocellulose, and stained with polyclonal antisera to the C-terminal decapeptide of dystrophin (DYS) or MAb IIH6 (156-DAG). Shown is 50 μg of crude surface membrane (SM) or the resulting supernatant after incubation at pH 7.4 (7.4), 9 (9), 10 (10), 11 (11), or 12 (12). The molecular weight standards (× 10^{-3}) are indicated on the left.

(not shown). That dystrophin, the 156 kd dystrophin-associated glycoprotein, and the 59 kd dystrophin-associated protein can be extracted from skeletal muscle membranes by alkaline treatment in the absence of detergents demonstrates that these proteins are not integral membrane proteins. These data also suggest that the 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins are integral membrane proteins. Since the 156 kd dystrophin-associated glycoprotein remains membrane bound under conditions that extract dystrophin, these data further suggest that the 156 kd dystrophin-associated glycoprotein is linked to dystrophin by way of the 50 kd, 43 kd, and/or 35 kd components of the complex.

Incorporation of [¹²⁵I]TID into Purified Dystrophin–Glycoprotein Complex

To further assess the hydrophobic nature of the components of the dystrophin–glycoprotein complex, the hydrophobic probe 3-(trifluoromethyl)-3-(m-[¹²⁵I]iodophenyl) diazirine ([¹²⁵I]TID) was photoincorporated into purified dystrophin–glycoprotein complex (Figure 7). Hydrophobic segments (presumably transmembrane domains) of proteins can be specifically labeled with [¹²⁵I]TID (Brunner and Semenza, 1981). Dystrophin, the 156 kd dystrophin-associated glycoprotein, and the 59 kd dystrophin-associated protein were not labeled with [¹²⁵I]TID, while the 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins demonstrated roughly equal incorporation of the probe (Figure 7). The upper band of the 43 kd doublet, which is less intensely stained by Coomassie blue, exhibited an equal intensity of [¹²⁵I]TID labeling when compared with the lower band of the doublet (Figure 7). The incorporation of [¹²⁵I]TID into the 25 kd dystrophin-associated protein was approximately 8-fold greater than in the 50 kd, 43 kd, or 35 kd components of the dystrophin–glycoprotein complex (Figure 7). A 170 kd protein and a 100 kd protein, which are minor contaminants of dystrophin–glycoprotein complex preparations, were also labeled by [¹²⁵I]TID (Figure 7).

Effect of Alkaline Treatment on Immunoprecipitation of the Dystrophin–Glycoprotein Complex

We have demonstrated that the components of the purified dystrophin–glycoprotein complex no longer cosediment on sucrose density gradients after alkaline dissociation (Ervasti et al., 1991). While dystrophin, the 156 kd, and 59 kd dystrophin-associated proteins exhibited distinct sedimentation peaks after alkaline dissociation, the 50 kd, 43 kd, 35 kd, and 25 kd dystrophin-associated proteins appeared to cosediment as a complex (Ervasti et al., 1991). To determine whether the 50 kd, 43 kd, 35 kd, and 25 kd dystrophin-associated proteins remain complexed after alkaline dissociation, the void of untreated and alkaline-treated dystrophin–glycoprotein complex after immunoprecipitation by MAb XIXC2 (dystrophin)–Sepharose or MAb IVD3₁ (50-DAG)–Sepharose was analyzed by SDS–PAGE and immunoblotting (Figure 8). As previously reported (Ervasti et al., 1990), dystrophin- and 50 kd dystrophin–associated glycoprotein–antibody matrices were effective in immunoprecipitating dystrophin and the 59 kd, 50 kd, 43 kd, and 35 kd dystrophin-associated proteins from untreated dystrophin–glycoprotein complex (Figure 8, CB, lanes 2 and 4). Dystrophin- and 50 kd dystrophin–associated glycoprotein–antibody matrices immunoprecipitated 63% and 85%, respectively, of the 156 kd dystrophin-associated glycoprotein (Figure 8, 156-DAG, lanes 2 and 4). The dystrophin–antibody matrix immunoprecipitated dystrophin from the alkaline-treated dystrophin–glycoprotein complex, but the 59, 50, 43, and 35 kd dystrophin-associated proteins remained largely in the void (Figure 8, lane 3), indicating that the interaction between dystrophin and the complex was disrupted by alkaline treatment. The 50 kd dystrophin-associated glycoprotein–antibody matrix was not effective in immunoprecipitating dystrophin, the 156 kd, or 59 kd dystrophin-associated proteins from the alkaline-treated complex (Figure 8, lane 5). However, the 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins were still immunoprecipitated from the alkaline-treated complex using the 50 kd dystrophin-associated glycoprotein–antibody matrix
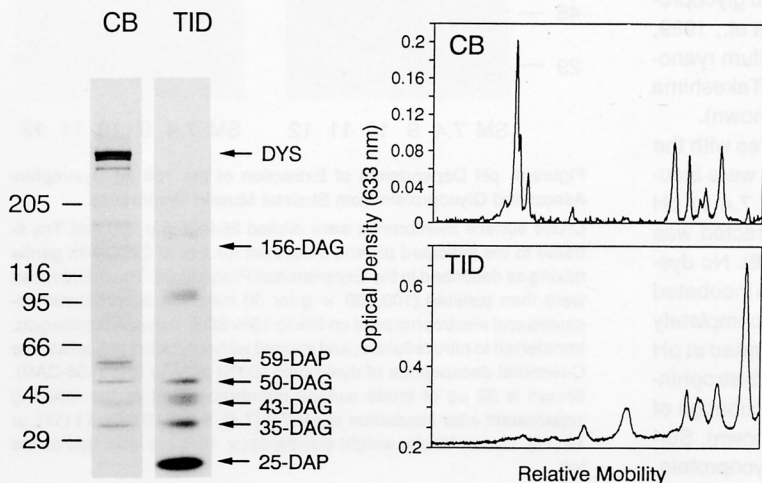

Figure 7. Hydrophobic Labeling of the Dystrophin–Glycoprotein Complex by [¹²⁵I]TID

Purified dystrophin–glycoprotein complex was photolabeled with [¹²⁵I]TID, electrophoresed on a 3% to 12% SDS–polyacrylamide gel, stained with Coomassie blue (CB), then dried and placed on Kodak XAR-5 film to obtain the corresponding autoradiogram (TID) as described in the Experimental Procedures. The densitometric scans of the Coomassie blue–stained gel (CB) and corresponding autoradiogram (TID) are shown on the right. The molecular weight standards (× 10⁻³) are indicated on the left.
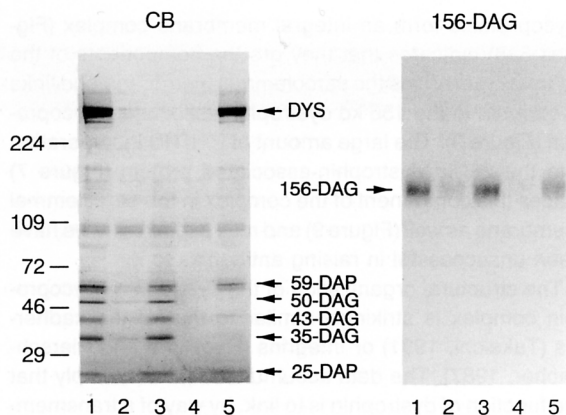
Figure 8. Effect of Alkaline Treatment on the Immunoprecipitation of Dystrophin–Glycoprotein Complex

Thirty micrograms of untreated (lanes 1, 2, and 4) or alkaline-treated (lanes 3 and 5) dystrophin–glycoprotein complex were incubated with Sepharose alone (lane 1), XIXC2–Sepharose (lanes 2 and 3), or IVD3₁– Sepharose (lanes 4 and 5) for 12 hr at 4°C with gentle mixing as described in the Experimental Procedures. After pelleting the Sepharose matrices, the supernatants were decanted and analyzed. Equal volumes (150 µl per lane) of each supernatant were electrophoretically separated on 3%–12% SDS–polyacrylamide gels and either stained with Coomassie blue (CB) or transferred to nitrocellulose and stained with MAb VIA4₁ (156-DAG). The molecular weight standards ($\times$ 10⁻³) are indicated on the left.

(Figure 8, lane 5). Both immunoaffinity matrices precipitated the 25 kd dystrophin-associated protein from native complex (Figure 8, lanes 2 and 4) but not from alkaline-dissociated complex (Figure 8, lanes 3 and 5). Thus, these data demonstrate that the 50 kd, 43 kd, and 35 kd dystrophin-associated proteins alone form an alkali-stable complex. Since the 50 kd dystrophin-associated glycoprotein–antibody matrix immunoprecipitated more of the 156 kd dystrophin-associated glycoprotein than the dystrophin–antibody matrix, these data further suggest that the 156 kd dystrophin-associated glycoprotein is directly linked to the 50 kd, 43 kd, and 35 kd glycoprotein complex rather than to dystrophin.

Discussion

In the present work, we have prepared polyclonal anti-

bodies specific to each of the proteins that exist in a complex with dystrophin in skeletal muscle. We have used these antibodies to determine several biochemical and structural properties of the dystrophin–glycoprotein complex. Our results demonstrate that dystrophin is associated with the 156 kd dystrophin-associated glycoprotein by way of the 50 kd, 43 kd, and 35 kd transmembrane glycoprotein complex and suggest that dystrophin serves as a specialized link between the actin cytoskeleton and components external to the sarcolemmal membrane.

We propose a model of the dystrophin–glycoprotein complex (Figure 9) based on the data presented here and elsewhere (Koenig et al., 1988; Koenig and Kunkel, 1990; Ervasti et al., 1990; Yoshida and Ozawa, 1990; Murayama et al., 1990; Cullen et al., 1990; Ohlendieck et al., 1991; Ervasti et al., 1991). The proposed model is presented to aid in the visualization of what is presently known about the structure of the dystrophin–glycoprotein complex, allowing the design of future experiments that test its validity. Dystrophin is modeled as a bent, antiparallel dimer with the C-terminus linked to the transmembrane components of the complex and the N-terminus binding to filamentous actin cytoskeleton. Dystrophin is depicted as such to conform to the results obtained from sequence analysis (Koenig et al., 1988), protease mapping (Koenig and Kunkel, 1990), rotary shadowed images of purified dystrophin–glycoprotein complex (Murayama et al., 1990), size estimates of purified dystrophin (Ervasti et al., 1991), and ultrastructural localization (Cullen et al., 1990).

We postulate that the 156 kd dystrophin-associated glycoprotein is located on the extracellular side of the sarcolemma (Figure 9). While it is not clear what function glycosylation serves, the presence of Ser/Thr-linked oligosaccharides provides clues to the structure of the 156 kd dystrophin-associated glycoprotein as well as to its orientation with respect to the sarcolemmal membrane. As reviewed by Jentoft (1990), Ser/Thr-linked glycosylation appears to confer protease resistance and a stiff conformation to the peptide core that protects a cell surface protein. The 156 kd dystrophin-associated glycoprotein appears to be very protease resistant, remaining intact long after identical trypsin concentrations have completely degraded dystrophin (J. M. E. and K. P. C., unpublished data). Thus, by analogy with cell surface molecules containing densely Ser/Thr-linked glycosylated regions, such
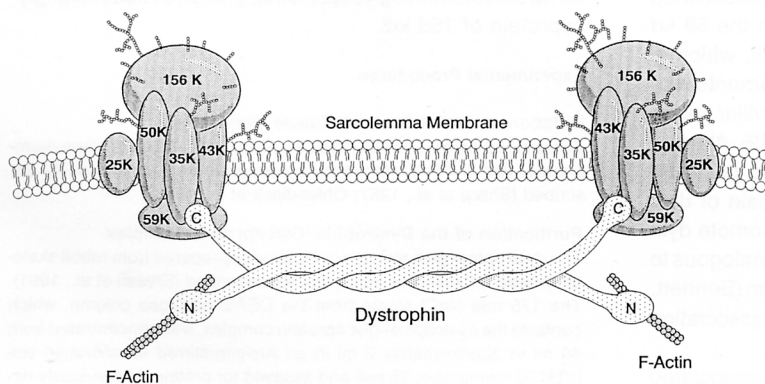

Figure 9. Model of the Dystrophin–Glycoprotein Complex

as NCAM (Walsh et al., 1989; Moore et al., 1987) and the LDL receptor (Cummings et al., 1983), we conclude that the 156 kd dystrophin-associated glycoprotein is an extracellular protein.

In contrast to the high molecular weight isoforms of NCAM (Rutishauser and Jessell, 1988) and the LDL receptor (Russell et al., 1984), the 156 kd dystrophin-associated glycoprotein does not appear to contain a transmembrane domain, as evidenced by its absence of labeling by [^{125}I]TID (Figure 7) and extraction from skeletal muscle membranes upon incubation at pH 12 (Figure 6). It has previously been shown that incubation of erythrocytes at pH 11 selectively solubilized membrane-associated cytoplasmic proteins, while all erythrocyte glycoproteins remained lipid bound (Steck and Yu, 1973). The extraction of the 156 kd dystrophin-associated glycoprotein from membranes incubated at pH 12, but not pH 11 (Figure 6), suggests that its association with the sarcolemma is unique from that of dystrophin and the 59 kd dystrophin-associated protein. It is interesting that proteoglycans were originally (Carney, 1986) extracted from connective tissues by incubation in 2% NaOH (i.e., >pH 12). This feature of the 156 kd dystrophin-associated glycoprotein coupled with its failure to focus as a sharp band after enzymatic deglycosylation (Figures 3 and 4) suggest that the 156 kd dystrophin-associated glycoprotein may also contain glycosaminoglycan chains.

The similarity in size and Ser/Thr-linked glycosylation (Figure 4) of the 156 kd dystrophin-associated glycoprotein initially suggested that it may be related to a glycosylphosphatidylinositol-linked isoform of NCAM expressed in myotubes (Walsh et al., 1989; Moore et al., 1987). However, the 156 kd dystrophin-associated glycoprotein is not released from skeletal muscle membranes by treatment with phosphatidylinositol-specific phospholipase C, does not bind heparin, and is not stained on immunoblots by a MAb (Walsh et al., 1989; Moore et al., 1987) that recognizes NCAM (J. M. E. and K. P. C., unpublished data).

The placement of the 59 kd dystrophin-associated protein in the cytoplasm in direct contact with dystrophin (Figure 9) is based on its cross-linking to dystrophin (Yoshida and Ozawa, 1990), solubilization from skeletal muscle membranes by alkaline treatment (Figure 5), and the absence of labeling by hydrophobic probe (Figure 7). Placement of the 59 kd dystrophin-associated protein in contact with the 50 kd, 43 kd, and 35 kd dystrophin-associated glycoproteins is based solely by analogy with the 58 kd protein of MAT-C1 ascite tumor cell microvilli, which is thought to stabilize the association of microfilaments with a glycoprotein complex located in the microvillar membrane (Carraway and Carothers-Carraway, 1989). Alternatively, the 59 kd dystrophin-associated protein could be located near the predicted actin-binding domain of dystrophin (Koenig et al., 1988), where it might promote dystrophin binding to actin filaments in a manner analogous to protein 4.1 promoting spectrin–actin association (Bennett, 1990) or zyxin promoting α actinin–actin association (Crawford and Beckerle, 1991).

That the 50 kd, 43 kd, and 35 kd dystrophin-associated

glycoproteins form an integral membrane complex (Figures 5–8) indicates that they are the components of the complex that spans the sarcolemmal membrane and links dystrophin to the 156 kd dystrophin-associated glycoprotein (Figure 9). The large amount of [^{125}I]TID incorporation into the 25 kd dystrophin-associated protein (Figure 7) places this component of the complex in the sarcolemmal membrane as well (Figure 9) and may explain why we have been unsuccessful in raising antibodies to it.

The structural organization of the dystrophin–glycoprotein complex is strikingly similar to that of the cadherins (Takeichi, 1991) or integrins (Ruoslahti and Pierschbacher, 1987). The data accumulated thus far imply that the function of dystrophin is to link, by way of a transmembrane glycoprotein complex, the actin cytoskeleton of a muscle cell to an extracellular component of skeletal muscle. The 156 kd dystrophin-associated glycoprotein may interact with the extracellular matrix or bind to a like molecule on an adjoining cell. The drastic reduction of the 156 kd dystrophin-associated glycoprotein (the component of the complex most distal to dystrophin) in muscle from mdx mice and DMD patients (Ervasti et al., 1990) is evidence that alteration in dystrophin expression profoundly affects components external to the muscle cell. Absence of dystrophin thus may compromise the integrity and flexibility of the sarcolemma, leading to either mechanical damage (Weller et al., 1990; Menke and Jockusch, 1991) of or alteration in specific calcium regulatory mechanisms (Franco and Lansman, 1990; Fong et al., 1990) of the sarcolemmal membrane. That dystrophin comprises 2% of sarcolemmal protein (Ohlendieck et al., 1991) and 5% of the sarcolemmal cytoskeleton (Ohlendieck and Campbell, 1991) supports the role of the dystrophin–glycoprotein complex in maintaining skeletal muscle architecture. It will be important to examine whether the absence of dystrophin affects the other components of the dystrophin–glycoprotein complex as severely as the 156 kd dystrophin-associated protein. Unfortunately, the antibodies used in this study do not appear to cross-react with mouse or human tissues. However, the present results provide new information concerning the organization of the dystrophin–glycoprotein complex with respect to the sarcolemma membrane; this information demonstrates that dystrophin and its 59 kd associated protein are cytoskeletal elements that are tightly linked to an integral membrane complex composed of a 25 kd protein, 50 kd, 43 kd, and 35 kd sarcolemmal glycoproteins, and an extracellular glycoprotein of 156 kd.

Experimental Procedures

Isolation of Rabbit Skeletal Muscle Membranes
KCl-washed rabbit skeletal muscle microsomes, crude surface membranes, and purified sarcolemma were prepared as previously described (Sharp et al., 1987; Ohlendieck et al., 1991).

Purification of the Dystrophin–Glycoprotein Complex
The dystrophin–glycoprotein complex was prepared from rabbit skeletal muscle microsomes as previously described (Ervasti et al., 1991). The 175 mM NaCl eluate from the DEAE–cellulose column, which contains the dystrophin–glycoprotein complex, was concentrated from 40 ml to approximately 2 ml in an Amicon-stirred ultrafiltration cell (YM100 membrane, 25 psi) and assayed for protein as previously de-

scribed (Ervasti et al., 1991). Alternatively, the protein concentration was estimated from a standard curve of the densitometric intensities of known amounts of dystrophin–glycoprotein complex resolved on Coomassie blue–stained SDS–polyacrylamide gels (Crawford and Beckerle, 1991).

Enzymatic Deglycosylation

Dystrophin–glycoprotein complex (0.5 mg/ml) in buffer A (Ervasti et al., 1991) treated with Flavobacterium meningosepticum N-glycosidase F (Boehringer Mannheim) was first made 1% in SDS and incubated at 100°C for 5 min, then diluted 5-fold with concentrated buffer, water, and enzyme to final concentrations of 50 mM sodium phosphate (pH 7.4), 1% Triton X-100, 0.1% SDS, and 12 U/ml N-glycosidase F. After incubation at 37°C for 2 hr, samples were analyzed by SDS–PAGE.

Samples (0.313 mg/ml) treated with Diplococcus pneumoniae O-glycosidase (Boehringer Mannheim) were first incubated for 1 hr at 37°C with 0.1 U/ml Vibrio cholerae neuraminidase (Boehringer Mannheim). Samples were then prepared as described above for N-glycosidase F treatment, except O-glycosidase was present at a final concentration of 17 mU/ml.

Alkaline Treatment of Skeletal Muscle Membranes

KCl-washed skeletal muscle microsomes (2.5 mg) were diluted 20-fold to a volume of 1 ml with 4% (w/v) sucrose, 50 mM Tris–HCl (pH 7.4), 0.1 mM PMSF, 0.75 mM benzamidine, 2.5 μg/ml aprotinin, 93 μg/ml iodoacetamide, 2.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A and either titrated to pH 11 with 1 M NaOH or diluted with a volume of H_2O equal to NaOH added (control). After a 1 hr incubation at 22°C with mixing, the samples were centrifuged for 30 min at 100,000 × g and the supernatants were decanted from the membrane pellets. The membrane pellets were resuspended to 1 ml. Equal volumes of control and alkaline-treated supernatants and resuspended pellets were compared by SDS–PAGE analysis.

The effect of pH on the extraction of components of the dystrophin–glycoprotein complex from rabbit skeletal muscle membranes was examined by diluting 150 μg of crude surface membranes 25-fold into buffer containing 0.1 mM PMSF, 0.75 mM benzamidine, and 200 mM Tris buffer titrated to the indicated pH. After a 1 hr incubation at room temperature with mixing and centrifugation (100,000 × g, 30 min), the resulting supernatants were compared by SDS–PAGE and immunoblot analysis.

Incorporation of [125I]TID into Purified Dystrophin–Glycoprotein Complex

Purified dystrophin–glycoprotein complex was centrifuged through 5% to 20% sucrose gradients (Ervasti et al., 1991) containing 0.1% CHAPS as detergent instead of digitonin. The dystrophin–glycoprotein complex–containing gradient fractions were pooled, concentrated with a Centricon 100, and photolabeled with 50 μCi/ml [125I]TID (Amersham) as previously described (Jay et al., 1991). Incorporation of [125I]TID into the components of the dystrophin–glycoprotein complex was detected by autoradiography following SDS–PAGE.

Immunoprecipitation of the Dystrophin–Glycoprotein Complex

Anti-dystrophin and 50 kd dystrophin-associated glycoprotein immunoaffinity matrices were prepared as previously described (Ervasti et al., 1990). In brief, 1 vol of packed goat anti-mouse IgG Sepharose (Organon Teknika, Durham, NC) was incubated with 10 vol of tissue culture supernatant alone or with media containing either MAb XIXC2 or IVD3₁, washed extensively with phosphate-buffered saline (PBS), then equilibrated in buffer A containing 0.5 M NaCl. Untreated and alkaline-dissociated (Ervasti et al., 1991) dystrophin–glycoprotein complex (0.3 ml, 30 μg) was incubated with 0.2 ml of goat anti-mouse IgG Sepharose, MAb XIXC2–Sepharose, or IVD3₁–Sepharose at 4°C overnight with gentle mixing. After pelleting the Sepharose (500 × g for 10 s), the supernatants were removed and compared by SDS–PAGE analysis.

Polyclonal Antibodies

Polyclonal antisera against chemically synthesized peptide representing the last 10 C-terminal amino acids (PGKPMREDTM) of the predicted human dystrophin sequence (Koenig et al., 1988) were raised in New Zealand White rabbits (Ervasti et al., 1990; Ohlendieck et al.,

1991; Campbell et al., 1991). Polyclonal antisera specific for various components of the dystrophin–glycoprotein complex were prepared by two methods. In the first method (Sharp and Campbell, 1989), individual components of the dystrophin–glycoprotein complex (~500 μg) were separated by SDS–PAGE in the presence of 1% 2-mercaptoethanol. The gels were stained for 10 min with Coomassie blue in 10% acetic acid, 25% isopropanol and destained in distilled water. Individual bands were cut from the gel and frozen in 1 ml of PBS until being used for immunization of guinea pigs. Alternatively, 50 μg of dystrophin–glycoprotein complex in buffer A was used as immunogen. Animals were boosted on day 14 with 5 μg of the appropriate antigen and monthly thereafter. Antisera were collected weekly after sufficient titers had been achieved. Antisera specific for each component of the dystrophin–glycoprotein complex were affinity purified using Immobilon-P transfers of individual dystrophin-associated proteins separated by SDS–PAGE (Sharp and Campbell, 1989).

MAbs

The preparation and characterization of MAbs IVD3₁ and VIA4₁, specific for the 50 kd and 156 kd dystrophin–associated glycoproteins, respectively, and the dystrophin-specific MAbs VIA4₂ and XIXC2 were previously described (Ervasti et al., 1990; Jorgensen et al., 1990; Ohlendieck et al., 1991). MAb IIH6, specific for the 156 kd dystrophin-associated glycoprotein, was obtained from female BALB/c mice immunized with purified rabbit skeletal muscle sarcolemma membranes and boosted with dystrophin–glycoprotein complex by previously described methods (Leung et al., 1987).

SDS–PAGE, Lectin, and Immunoblotting

SDS–PAGE (Laemmli, 1970) was carried out on 3% to 12% gradient gels in the presence of 1% 2-mercaptoethanol and stained with Coomassie blue or transferred to nitrocellulose (Towbin et al., 1979). Molecular weight standards shown in the figures were purchased from BRL or Sigma (Figure 7 only). Nitrocellulose transfers were stained with 1 μg/ml peroxidase-conjugated lectins (Sigma Chemical Co., St. Louis, MO) by the same method previously described using 125I-labeled WGA (Campbell and Kahl, 1989) or stained with digoxigenin-conjugated MAA as described in the instructions for the Boehringer Mannheim Glycan Differentiation Kit and detected with affinity-purified peroxidase-conjugated sheep anti-digoxigenin antibodies. Immunoblots were stained with affinity-purified polyclonal antisera or MAbs as previously described (Campbell et al., 1987). Coomassie blue–stained gels and autoradiograms were analyzed densitometrically using a Molecular Dynamics Model 300A scanning densitometer.

Immunofluorescence Microscopy

Immunofluorescence staining of transverse cryosections (8 μm) from rabbit skeletal muscle (soleus and gastrocnemius) was carried out as previously described (Ervasti et al., 1990; Ohlendieck et al., 1991). Cryosections were blocked for 20 min with 5% rabbit serum in PBS (50 mM sodium phosphate [pH 7.4], 0.9% NaCl), followed by a 1 hr incubation at 37°C with the affinity-purified guinea pig polyclonal antibody. After washing in PBS, the sections were further incubated for 30 min at 37°C in PBS with a 1:50 dilution of FITC-labeled rabbit anti-guinea pig (Boehringer-Mannheim) and subsequently examined in a Zeiss Axioplan fluorescence microscope.

References

Arahata, K., Ishiura, S., Ishiguro, T., Tsukahara, T., Suhara, Y., Eguchi, C., Ishihara, T., Nonaka, I., Ozawa, E., and Sugita, H. (1988). Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy peptide. Nature 333, 861–866.

Bennett, V. (1990). Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. Physiol. Rev. 70, 1029–1065.

Bonilla, E., Samitt, C. E., Miranda, A. F., Hays, A. P., Salviati, G., DiMauro, S., Kunkel, L. M., Hoffman, E. P., and Rowland, L. P. (1988). Duchenne muscular dystrophy: deficiency of dystrophin at the muscle cell surface. Cell 54, 447–452.

Brunner, J., and Semenza, G. (1981). Selective labeling of the hydrophobic core of membranes with 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl) diazirine, a carbene-generating reagent. Biochemistry 20, 7174–7181.

Campbell, K. P., and Kahl, S. D. (1989). Association of dystrophin and an integral membrane glycoprotein. Nature 338, 259–262.

Campbell, K. P., Knudson, C. M., Imagawa, T., Leung, A. T., Sutko, J. L., Kahl, S. D., Raab, C. R., and Madson, L. (1987). Identification and characterization of the high affinity [^{3}H]ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. J. Biol. Chem. 262, 6460–6463.

Campbell, K. P., Ervasti, J. M., Ohlendieck, K., and Kahl, S. D. (1991). The dystrophin–glycoprotein complex: identification and biochemical characterization. In Frontiers in Muscle Research, E. Ozawa, T. Masaki, and Y. Nabeshima, eds. (Amsterdam: Elsevier), pp. 321–340.

Carney, S. L. (1986). Proteoglycans. In Carbohydrate Analysis: A Practical Approach, M. F. Chaplin and J. F. Kennedy, eds. (Oxford: IRL Press), pp. 97–141.

Carraway, K. L., and Carothers-Carraway, C. A. (1989). Membrane-cytoskeleton interactions in animal cells. Biochim. Biophys. Acta 988, 147–171.

Chang, H. W., Bock, E., and Bonilla, E. (1989). Dystrophin in electric organ of Torpedo californica homologous to that in human muscle. J. Biol. Chem. 264, 20831–20834.

Crawford, A. W., and Beckerle, M. C. (1991). Purification and characterization of zyxin, an 82,000-dalton component of adherens junctions. J. Biol. Chem. 266, 5847–5853.

Cullen, M. J., Walsh, J., Nicholson, L. V. B., and Harris, J. B. (1990). Ultrastructural localization of dystrophin in human muscle by using gold immunolabelling. Proc. R. Soc. Lond. (B) 240, 197–210.

Cummings, R. D., Kornfeld, S., Schneider, W. J., Hobgood, K. K., Tolleshaug, H., Brown, M. S., and Goldstein, J. L. (1983). Biosynthesis of the N- and O-linked oligosaccharides of the low density lipoprotein receptor. J. Biol. Chem. 258, 15261–15273.

Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature 345, 315–319.

Ervasti, J. M., Kahl, S. D., and Campbell, K. P. (1991). Purification of dystrophin from skeletal muscle. J. Biol. Chem. 266, 9161–9165.

Fairbanks, G., Steck, T. L., and Walloch, D. F. H. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10, 2606–2617.

Fong, P., Turner, P. R., Denetclaw, W. F., and Steinhardt, R. A. (1990). Increased activity of calcium leak channels in myotubes of Duchenne human and mdx mouse origin. Science 250, 673–676.

Franco, A., and Lansman, J. B. (1990). Calcium entry through stretch-inactivated ion channels in mdx myotubes. Nature 344, 670–673.

Hoffman, E. P., Brown, R. H., Jr., and Kunkel, L. M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 51, 919–928.

Holden, K. G., Yim, N. C. F., Griggs, L. J., and Weisbach, J. A. (1970). Gel electrophoresis of mucous glycoproteins. I. Effect of gel porosity. Biochemistry 10, 3105–3109.

Jay, S. D., Sharp, A. H., Kahl, S. D., Vedvick, T. S., Harpold, M. M., and Campbell, K. P. (1991). Structural characterization of the dihydro-

pyridine-sensitive calcium channel α_2 subunit and the associated peptides. J. Biol. Chem. 266, 3287–3293.

Jentoft, N. (1990). Why are proteins O-glycosylated? Trends Biochem. Sci. 15, 291–294.

Jorgensen, A. O., Arnold, W., Sheen, A. C.-Y., Yuan, S., Gaver, M., and Campbell, K. P. (1990). Identification of novel proteins unique to either transverse tubules (TS28) or the sarcolemma (SL50) in rabbit skeletal muscle. J. Cell Biol. 110, 1173–1185.

Koenig, M., and Kunkel, L. M. (1990). Detailed analysis of the repeat domain of dystrophin reveals four potential hinge segments that may confer flexibility. J. Biol. Chem. 265, 4560–4566.

Koenig, M., Monaco, A. P., and Kunkel, L. M. (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell 53, 219–228.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leberer, E., Charuk, J. H. M., Clarke, D. M., Green, N. M., Zubrzycka-Gaarn, E., and MacLennan, D. H. (1989). Molecular cloning and expression of cDNA encoding the 53,000-dalton glycoprotein of rabbit skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 264, 3484–3493.

Leberer, E., Timms, B. G., Campbell, K. P., and MacLennan, D. H. (1990). Purification, calcium binding properties, and ultrastructural localization of the 53,000- and 160,000 (sarcalumenin)-dalton glycoproteins of the sarcoplasmic reticulum. J. Biol. Chem. 265, 10118–10124.

Leung, A. T., Imagawa, T., and Campbell, K. P. (1987). Structural characterization of the dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle: evidence for two distinct high molecular weight subunits. J. Biol. Chem. 262, 7943–7946.

Menke, A., and Jockusch, H. (1991). Decreased osmotic stability of dystrophin-less muscle cells from the mdx mouse. Nature 349, 69–71.

Moore, S. E., Thompson, J., Kirkness, V., Dickson, J. G., and Walsh, F. S. (1987). Skeletal muscle neural cell adhesion molecule (N-CAM): changes in protein and mRNA species during myogenesis of muscle cell lines. J. Cell Biol. 105, 1377–1386.

Murayama, T., Osamu, S., Kimura, S., Shimizu, T., Sawada, H., and Maruyama, K. (1990). Molecular shape of dystrophin purified from rabbit skeletal muscle myofibrils. Proc. Japan. Acad. 66, 96–99.

Ohlendieck, K., and Campbell, K. P. (1991). Dystrophin constitutes five percent of membrane cytoskeleton in skeletal muscle. FEBS Lett. 283, 230–234.

Ohlendieck, K., Ervasti, J. M., Snook, J. B., and Campbell, K. P. (1991). Dystrophin–glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. J. Cell Biol. 112, 135–148.

Ruoslahti, E., and Pierschbacher, M. D. (1987). New perspectives in cell adhesion: RGD and integrins. Science 238, 491–497.

Russell, D. W., Schneider, W. J., Yamamoto, T., Luskey, K. L., Brown, M. S., and Goldstein, J. L. (1984). Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor. Cell 37, 577–585.

Rutishauser, U., and Jessell, T. M. (1988). Cell adhesion molecules in vertebrate neural development. Physiol. Rev. 68, 829–857.

Sharp, A. H., and Campbell, K. P. (1989). Characterization of the 1,4-dihydropyridine receptor using subunit-specific polyclonal antibodies: evidence for a 32,000 Da subunit. J. Biol. Chem. 264, 2816–2825.

Sharp, A. H., Imagawa, T., Leung, A. T., and Campbell, K. P. (1987). Identification and characterization of the dihydropyridine binding subunit in the skeletal muscle dihydropyridine receptor. J. Biol. Chem. 262, 12309–12315.

Steck, T. L., and Yu, J. (1973). Selective solubilization of proteins from red blood cell membranes by protein perturbants. J. Supramol. Struct. 1, 220–232.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science 251, 1451–1455.

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and

Numa, S. (1989). Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature *339*, 439–445.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Walsh, F. S., Parekh, R. B., Moore, S. E., Dickson, G., Barton, C. H., Gower, H. J., Dwek, R. A., and Rademacher, T. W. (1989). Tissue specific O-linked glycosylation of the neural cell adhesion molecule (NCAM). Development *105*, 803–811.

Watkins, S. C., Hoffman, E. P., Slayter, H. S., and Kunkel, L. M. (1988). Immunoelectron microscopic localization of dystrophin in myofibres. Nature *333*, 863–866.

Weller, B., Karpati, G., and Carpenter, S. (1990). Dystrophin-deficient mdx muscle fibers are preferentially vulnerable to necrosis induced by experimental lengthening contractions. J. Neurol. Sci. *100*, 9–13.

Yoshida, M., and Ozawa, E. (1990). Glycoprotein complex anchoring dystrophin to sarcolemma. J. Biochem. *108*, 748–752.

Zarain-Herzberg, A., Fliegel, L., and MacLennan, D. H. (1988). Structure of the rabbit fast-twitch skeletal muscle calsequestrin gene. J. Biol. Chem. *263*, 4807–4812.

Zubrzycka-Gaarn, E. E., Bulman, D. E., Karpati, G., Burghes, A. H. M., Belfall, B., Klamut, H. J., Talbot, J., Hodges, R. S., Ray, P. N., and Worton, R. G. (1988). The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. Nature *333*, 466–469.