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# Dystrophin-associated glycoproteins: their possible roles in the pathogenesis of Duchenne muscular dystrophy

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## 6.1 INTRODUCTION

The application of 'positional cloning' to the analysis of inherited disorders has led to the identification of an impressive and rapidly growing list of gene defects which are responsible for some of the most debilitating and scientifically perplexing human diseases (Collins, 1992). For example, Duchenne muscular dystrophy (DMD) is caused by a defective gene found on the X-chromosome which was identified by two variations of positional cloning (Monaco *et al.*, 1985; Ray *et al.*, 1985). The DMD gene encodes for a large protein named dystrophin which has a predicted primary structure that is similar to a number of cytoskeletal proteins (Koenig *et al.*, 1988). Knowledge of dystrophin's predicted primary sequence has enabled the production of specific antibodies, which in turn have been very useful in confirming its size (Hoffman *et al.*, 1987), determining its tissue distribution (Hoffman *et al.*, 1988b) and cellular localization (Zubrzycka-Gaarn *et al.*, 1988; Arahata *et al.*, 1988; Bonilla *et al.*, 1988; Watkins *et al.*, 1988). Dystrophin has been localized to the cytoplasmic face of the sarcolemma in skeletal muscle (Zubrzycka-Gaarn *et al.*, 1988; Arahata *et al.*, 1988; Bonilla *et al.*, 1988; Watkins *et al.*, 1988), including the neuromuscular junction (Ohlendieck *et al.*, 1991a; Yeadon *et al.*, 1991; Byers *et al.*, 1991). Dystrophin is completely absent in skeletal muscle of DMD patients, *mdx* mice and *GRMD* dogs (Hoffman *et al.*, 1987; Zubrzycka-Gaarn *et al.*, 1988; Watkins *et al.*, 1988; Hoffman *et al.*, 1988a; Cooper *et al.*, 1988).

Thus, positional cloning techniques have enabled scientists to identify

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abnormal genes, pinpoint the mutations that occur and make powerful predictions concerning the structure of the gene products. However, identification of the defect of a genetic disorder may not explain the function of the normal gene product or the complete molecular mechanism by which the disorder progresses. In the case of DMD, neither the exact function of dystrophin, nor the precise mechanism of fibre necrosis which occurs in its absence in dystrophic muscle, has been determined.

The function of a protein may be better understood through its interactions with other proteins. For example, previous studies of genetic diseases involving cytoskeletal proteins (Alloisio *et al.*, 1985; Mueller and Morrison, 1981) have demonstrated that the absence of one component of the cytoskeleton is sometimes accompanied by the loss of an associated component of the membrane cytoskeleton. Thus, in order to understand the function of dystrophin and the molecular pathogenesis of DMD, it is imperative to identify the proteins which are associated with dystrophin and to characterize the status of these proteins in muscle where dystrophin is absent.

In this chapter, we review recent advancement in the understanding of the structure of dystrophin, its relative subcellular abundance, the identification and characterization of six proteins that form a complex with dystrophin, and the fate of these dystrophin-associated proteins in dystrophic tissues. Of particular interest is the marked reduction of dystrophin-associated proteins in muscle from *mdx* mice and DMD patients. These results indicate that the first step in the molecular pathogenesis of Duchenne muscular dystrophy is the loss of the dystrophin-associated glycoproteins, which leads to the loss of linkage between the sarcolemmal cytoskeleton and the extracellular matrix, ultimately rendering muscle fibres more susceptible to necrosis.

## 6.2 MEMBRANE PROPERTIES OF DYSTROPHIN

Based on its deduced primary structure, dystrophin was originally predicted to consist of four distinct regions, dominated by a large rod-shaped domain with a length of 125 nm (Koenig *et al.*, 1988). Immunogold labelling studies of skeletal muscle using site-specific antibodies have reported sarcolemmal periodicities ranging from 100 to 140 nm (Watkins *et al.*, 1988; Cullen *et al.*, 1990, 1991) while the length of rotary-shadowed images of dystrophin have varied from 100 to 180 nm (Murayama *et al.*, 1990; Pons *et al.*, 1990). The rod-shaped domain is flanked on its N-terminus by 240 amino acids with high homology to the actin binding domain of $\alpha$-actinin, spectrin and *Dictyostelium* actin binding protein 120 (Koenig *et al.*, 1988; Karinch *et al.*, 1990; Bresnick *et al.*, 1990). From this sequence homology, it has been hypothesized that the N-terminal 240 amino acids comprise a filamentous actin binding site. In support of this hypothesis, Hemmings *et al.* (1992)

recently demonstrated that a fusion protein corresponding to the first 233 amino acids was able to bind filamentous actin *in vitro*. Immediately C-terminal to the rod-shaped domain of dystrophin is a cysteine-rich region with significant homology to a domain of *Dictyostelium* $\alpha$-actinin that contains two potential $Ca^{2+}$-binding sites. However, there is currently no evidence that this putative $Ca^{2+}$-binding domain is functional in skeletal muscle dystrophin. The last C-terminal 420 amino acids comprise the fourth distinct domain of dystrophin and exhibit no homology with any known sequence. The lack of significant homology with proteins of known function has led to speculation that these last 420 amino acids may be involved in dystrophin's interaction with the sarcolemmal membrane.

Immunogold labelling studies (Cullen *et al.*, 1991) with an antibody against the extreme C-terminus of dystrophin suggest that the C-terminal domain is closely apposed or inserted into the plasma membrane of skeletal muscle, thus providing the strongest evidence to date that the C-terminal domain is the membrane-binding region of dystrophin. However, three patients clinically diagnosed as DMD have recently been found to express a truncated form of dystrophin lacking the cysteine-rich and C-terminal domains (Hoffman *et al.*, 1991; Recan *et al.*, 1992; Helliwell *et al.*, 1992). Indirect immunofluorescence analysis of biopsies from these patients have demonstrated sarcolemmal localization of the truncated dystrophin, indicating that its interaction with the membrane cytoskeleton is important in determining dystrophin's location in the muscle cell.

Dystrophin's predicted primary structure suggests that it shares many features with abundant structural proteins of the membrane cytoskeleton such as $\alpha$-actinin and spectrin (Koenig *et al.*, 1988). However, dystrophin is an extremely minor component of skeletal muscle, representing only about 0.002% of the total muscle protein (Hoffman *et al.*, 1987). For this reason, dystrophin has not been considered to play a major structural role in the membrane cytoskeleton of skeletal muscle. On the other hand, sarcolemmal proteins constitute only a minute fraction of total muscle protein. Since it has long been apparent that the initial degenerative processes leading to DMD are associated with the surface membrane of skeletal muscle (Mokri and Engel, 1975), it was important to be able to study the structure of isolated sarcolemma in order to identify the normal protein composition of the sarcolemma membrane from skeletal muscle and to determine the relative abundance of dystrophin to other sarcolemma proteins.

For the biochemical characterization of surface membrane components, sarcolemma vesicles have to be prepared in a sufficient yield and with a high degree of purity. A variety of procedures have been employed to isolate skeletal muscle fractions enriched in sarcolemma, most involving density gradient centrifugation (Barchi *et al.*, 1979; Moczydlowski and Latorre, 1983; Seiler and Fleischer, 1982, 1988). However, previous attempts to

obtain pure sarcolemma have been hindered by the lack of specific and well defined markers for the sarcolemma and the low abundance of sarcolemma in comparison to other subcellular membranes in skeletal muscle. Wheat germ agglutinin is a homodimeric lectin which crosslinks terminally linked *N*-acetyl-D-glucosamine and/or sialic acid (Bhavanandan and Katlic, 1979). Thus, lectin agglutination is expected to aggregate specifically sealed right-side-out sarcolemma vesicles because the carbohydrate chains of membrane glycoproteins are extracellular (Charuk *et al.*, 1989). In fact, Charuk *et al.* (1989) employed a wheat germ agglutination procedure following density gradient centrifugation for the subfractionation of cardiac sarcolemma.

Our approach was to first identify specific markers for the sarcolemma using immunofluorescence localization of a library of monoclonal antibodies isolated from mice immunized with a crude preparation of rabbit skeletal muscle membranes. We then set out to isolate highly purified sarcolemmal vesicles from rabbit skeletal muscle, using sucrose density step gradient centrifugation and wheat germ agglutination, and to characterize the isolated sarcolemmal vesicles by immunoblot analysis using the subcellular membrane-specific monoclonal antibodies as probes (Ohlendieck *et al.*, 1991b). SDS-polyacrylamide gel analysis of the purified sarcolemma preparation revealed a protein band of approximately 400 kDa which was detectable with Coomassie Blue. This 400 kDa protein comigrated with dystrophin detected on immunoblots.

To establish that the 400 kDa protein band in isolated sarcolemma was exclusively dystrophin, the lectin agglutination procedure was used to isolate sarcolemma from control and *mdx* mouse muscle, which is known from immunological studies to be missing dystrophin (Bonilla *et al.*, 1988). The overall SDS-PAGE profile (Figure 6.1) of control and *mdx* sarcolemma was very similar (Ohlendieck and Campbell, 1991b). The major difference between the control and *mdx* sarcolemma is the absence in the latter of the 400 kDa protein band which is stained in immunoblotting by antiserum against the C-terminal decapeptide of dystrophin in normal mouse muscle sarcolemma (Figure 6.1). Restricted immunofluorescence labelling 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 antiserum against the C-terminal decapeptide of dystrophin (Figure 6.1). A faint Coomassie Blue stained protein band in *mdx* mouse sarcolemma with a slightly higher molecular weight than dystrophin was identified as dystrophin-related protein that is encoded by a different gene (Love *et al.*, 1989; Khurana *et al.*, 1990; Ohlendieck *et al.*, 1991a). Thus, the analysis of control and *mdx* sarcolemma demonstrated that the 400 kDa Coomassie Blue stained protein band in isolated sarcolemma was exclusively dystrophin. Furthermore, immunoadsorption experiments with

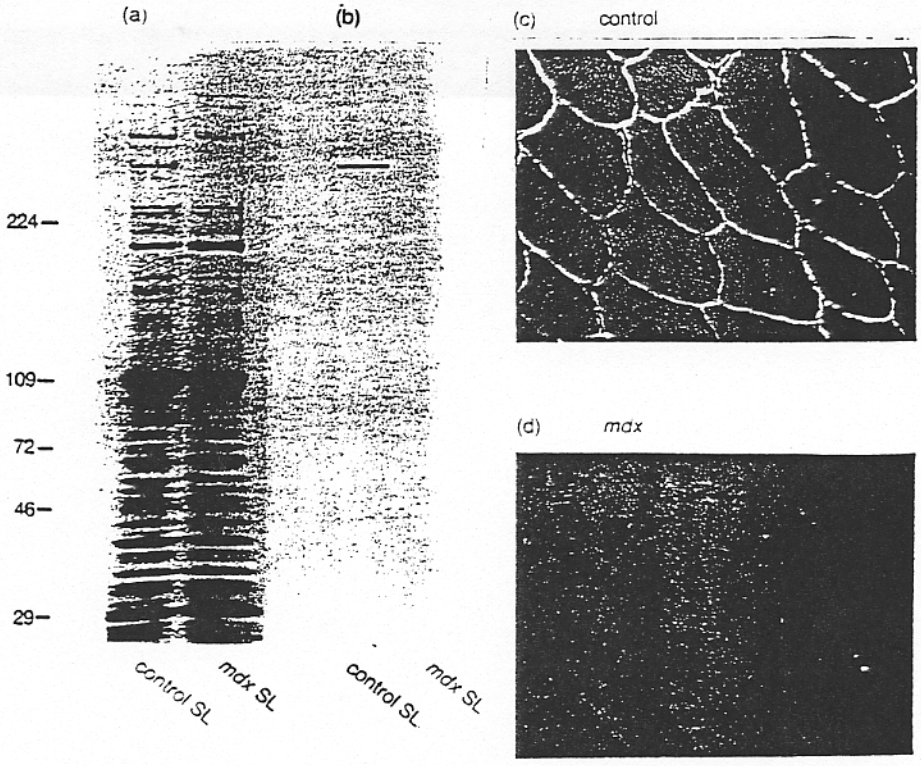


Figure 6.1 Dystrophin in control and *mdx* sarcolemma membranes. Shown are a Coomassie Blue stained gel (a) of isolated sarcolemma (SL) and an immunoblot (b) of an identical gel stained with polyclonal antiserum against the C-terminal decapeptide of dystrophin. Molecular weight standards ($\times 10^{-3}$) are indicated on the left. Transverse cryosections of normal (c) and *mdx* (d) mouse skeletal muscle were labelled by indirect immunofluorescence with polyclonal antiserum against the C-terminal decapeptide of dystrophin. After Ohlendieck and Campbell (1991a).

anti-dystrophin immunoaffinity beads quantitatively removed the 400 kDa band from digitonin-solubilized sarcolemma, thus confirming the 400 kDa band as dystrophin (Ohlendieck *et al.*, 1991b).

To estimate the amount of dystrophin in the skeletal muscle plasma membrane, purified sarcolemma were analysed by densitometric scanning of Coomassie Blue stained gels. To account for possible variations in Coomassie Blue staining intensity relative to the amount of total protein separated by SDS-PAGE, gels with different amounts of sarcolemmal protein were analysed by densitometric scanning and the values averaged. Since thin section electron microscopy indicated entrapment of small

sarcoplasmic reticulum vesicles within larger sarcolemma vesicles, lectin-agglutinated sarcolemma vesicles were additionally treated with low concentrations of the non-ionic detergent Triton X-100 (0.1%) with the aim of removing trapped sarcoplasmic reticulum vesicles. The Coomassie Blue stained protein pattern of crude surface membrane, isolated sarcolemma, and detergent-washed sarcolemma demonstrated that the low concentrations of Triton X-100 were very effective in removing the sarcoplasmic reticulum $Ca^{2+}$-ATPase while enriching the dystrophin and sarcolemma marker $Na^+/K^+$ ATPase content (Ohlendieck and Campbell, 1991b). 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. Peak integration of densitometric scans of isolated rabbit skeletal muscle sarcolemma revealed that the protein band of apparent 400 kDa accounted for 2.1 ± 0.7% (n = 8) of the total protein of this membrane preparation (Ohlendieck *et al.*, 1991b). Peak integration of the densitometric scan of the detergent-washed sarcolemma revealed that dystrophin accounted for 4.8 ± 0.8% (n = 6) of the total protein (Ohlendieck and Campbell, 1991b). 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 (Bennett *et al.*, 1982).

One criterion for determining whether a protein is a component of the cytoskeleton is its relative insolubility in Triton X-100 (Salas *et al.*, 1988; Carraway and Carothers-Carraway, 1989). Extraction of plasma membranes with high concentrations (0.5%) of the non-ionic detergent Triton X-100 leaves the cytoskeleton as an insoluble residue while solubilizing the membrane proteins not associated with the cytoskeleton (Salas *et al.*, 1988; Carraway and Carothers-Carraway, 1989). Dystrophin was exclusively found in the Triton-insoluble pellet, comprising 5.1 ± 1.0% (n = 6) of the total cytoskeleton protein while $Na^+/K^+$-ATPase was found in the supernatant (Ohlendieck and Campbell, 1991b). In contrast to the treatment with Triton X-100, treatment of membranes with strong alkaline solutions is known to remove tightly associated cytoskeletal components (Korsgren and Cohen, 1986) from membranes while leaving the integral membrane proteins with the bilayer (Steck and Yu, 1973; Carraway and Carothers-Carraway, 1989). Dystrophin is completely extracted by alkaline treatment (Chang *et al.*, 1989; Ohlendieck and Campbell, 1991b; Ervasti and Campbell, 1991). Thus, established biochemical methods for the identification of cytoskeletal proteins demonstrate that dystrophin is an integral component of the cytoskeleton of the sarcolemma in skeletal muscle.

Although dystrophin is a minor protein when compared to the total muscle protein (Hoffman *et al.*, 1987), it appears to be a major component of the subsarcolemmal cytoskeletal network in skeletal muscle. These find-

ings throw new light on the possible function and relative abundance of dystrophin in the membrane skeleton of skeletal muscle cells, comparable to that of the major membrane skeleton component spectrin in other cell types (Bennett *et al.*, 1982).

## 6.3 DYSTROPHIN–GLYCOPROTEIN COMPLEX

The ability of alkaline treatment to extract dystrophin from membrane preparations (Chang *et al.*, 1989; Ohlendieck and Campbell, 1991b) indicated that dystrophin was tightly associated with the plasma membrane through strong protein–bilayer or protein–protein interactions. In order to understand the function of dystrophin and its role in the molecular pathogenesis of DMD, it was imperative to identify the proteins which are associated with dystrophin. Our approach to identifying dystrophin-associated proteins was to solubilize and purify dystrophin from rabbit skeletal membranes using medium stringency conditions which retain specific, high affinity protein–protein interactions while minimizing non-specific aggregation. The detergent digitonin, in combination with 0.5 M NaCl, had previously been shown to optimally solubilize intact skeletal muscle dihydropyridine receptor, a hetereotetrameric, integral membrane glycoprotein complex (Leung *et al.*, 1987). In contrast to dystrophin's insolubility in Triton X-100 concentrations as high as 1% (Chang *et al.*, 1989; Ohlendieck and Campbell, 1991b), dystrophin was quantitatively solubilized from rabbit skeletal muscle membranes using 1% digitonin and 0.5 M NaCl (Campbell and Kahl, 1989). Interestingly, it was discovered that dystrophin could be purified 17 000-fold from digitonin-solubilized skeletal muscle membranes using immobilized wheat germ agglutinin. The interaction of dystrophin with wheat germ agglutinin was disrupted by agents that dissociate cytoskeletal proteins from membranes, indicating that dystrophin itself was not a glycoprotein but, rather, was tightly linked to one or several integral membrane glycoproteins (Campbell and Kahl, 1989).

The dystrophin–glycoprotein complex was initially purified from digitonin-solubilized rabbit skeletal muscle membranes using wheat germ agglutinin–Sepharose, ion exchange chromatography and sucrose density gradient centrifugation (Ervasti *et al.*, 1990). It was later found (Ervasti *et al.*, 1991) that substituting succinylated wheat germ agglutinin–agarose for wheat germ agglutinin–Sepharose resulted in dystrophin–glycoprotein complex preparations of the same purity and yield as previously reported (Ervasti *et al.*, 1990) while obviating the sucrose gradient step. The size of the dystrophin complex was estimated to be ~18S by comparing its sedimentation through a sucrose density gradient to that of the standards beta-galactosidase (16S), thyroglobulin (19S) and the 20S dihydropyridine receptor (Ervasti *et al.*, 1990). Densitometric scanning of the peak dystro-

phin containing gradient fractions revealed several proteins which cosedimented with dystrophin: a broad, diffusely staining component with an apparent molecular weight of 156 kDa, a triplet of proteins centered at 59 kDa, a 50 kDa protein, a protein doublet at 43 kDa, 35 kDa protein and a 25 kDa protein (Figure 6.2a). Immunoaffinity beads against dystrophin or the 50 kDa protein each selectively immunoprecipitated dystrophin, the 156 kDa, 59 kDa, 50 kDa, 43 kDa, 35 kDa and 25 kDa proteins (Ervasti *et al.*, 1990; Ervasti and Campbell, 1991). Since the 50 kDa dystrophin-associated glycoprotein-antibody matrix immunoprecipitated more of the 156 kDa dystrophin-associated glycoprotein than the dystrophin-antibody matrix, these data further suggested that the 156 kDa dystrophin-associated glycoprotein was directly linked to the 50 kDa glycoprotein rather than to dystrophin (Ervasti and Campbell, 1991). As

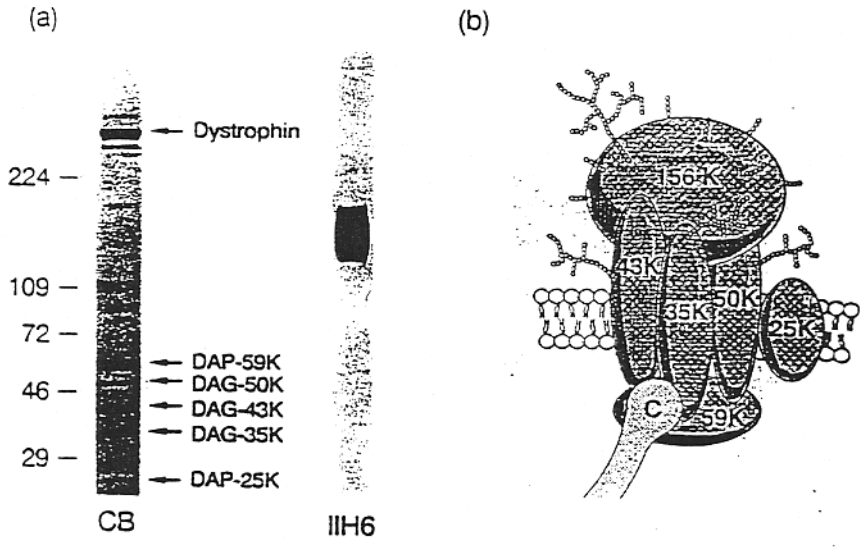


Figure 6.2 Dystrophin-glycoprotein complex: SDS PAGE analysis and proposed structural model.(a) Shown are Coomassie Blue stained SDS polyacrylamide gel (CB) and corresponding nitrocellulose transfer stained with monoclonal antibody IIH6 against the 156 kDa dystrophin-associated glycoprotein of purified 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 weight standards ($\times 10^{-3}$) are indicated on the left. (b) Proposed structural model of the dystrophin–glycoprotein complex. The C denotes the cysteine-rich and C-terminal domains of dystrophin. After Ervasti and Campbell (1991).

expected from the alkaline extraction results (Chang *et al.*, 1989; Ohlendieck and Campbell, 1991b), the components of the alkaline-treated complex no longer cosedimented on sucrose gradients but each sedimented as much smaller entities (Ervasti *et al.*, 1991). These data indicate that the dystrophin–glycoprotein complex can be dissociated by alkaline treatment. The alkaline-treated dystrophin co-sedimented with the 11S standard catalase (Ervasti *et al.*, 1991) which is in good agreement with the sedimentation of tetrameric spectrin (Bennett *et al.*, 1982). This result indicates that alkaline-treated dystrophin sediments as a dimer. The 59 kDa dystrophin-associated protein sedimented near the top of the gradient and was completely separated from dystrophin (Ervasti *et al.*, 1991). The 156 kDa dystrophin-associated glycoprotein and the upper band of the 43 kDa doublet cosedimented with a peak in fraction 5. However, the 50 kDa, the lower band of the 43 kDa doublet, 35 kDa and 25 kDa dystrophin-associated proteins cosedimented even after alkaline treatment, possibly as a complex intermediate in size between the 43 kDa/156 kDa dystrophin-associated glycoprotein peak and dimeric dystrophin (Ervasti *et al.*, 1991).

To determine whether the 50 kDa, 43 kDa, 35 kDa and 25 kDa dystrophin-associated proteins remain complexed after alkaline dissociation, alkaline-treated dystrophin–glycoprotein complex was immunoprecipitated by anti-dystrophin or anti-50 kDa dystrophin-associated glycoprotein immunoaffinity beads and the void analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting (Ervasti and Campbell, 1991). The dystrophin-antibody matrix immunoprecipitated dystrophin from the alkaline-treated dystrophin–glycoprotein complex but the 59 kDa, 50 kDa, 43 kDa and 3k5 kDa dystrophin-associated proteins remained largely in the void indicating that the interaction between dystrophin and the complex was disrupted by alkaline treatment. The 50 kDa dystrophin-associated glycoprotein-antibody matrix was not effective in immunoprecipitating dystrophin, the 156 kDa or 59 kDa dystrophin-associated proteins from the alkaline-treated complex. However, the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins were still immunoprecipitated from the alkaline-treated complex by the anti-50 kDa dystrophin-associated glycoprotein-antibody matrix. Neither of the immunoaffinity matrices precipitated the 25 kDa dystrophin-associated protein from alkaline-dissociated complex. Thus, these data demonstrate that the 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins alone form an alkali-stable complex.

Densitometric analysis of Coomassie Blue stained SDS-polyacrylamide gels containing the electrophoretically separated components of six different preparations of dystrophin–glycoprotein complex demonstrated that the 59 kDa, 50 kDa, 43 kDa, 35 kDa and 25 kDa dystrophin-associated pro-

teins 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 relative to dystrophin (Ervasti and Campbell, 1991). However, the stoichiometry of the 156 kDa dystrophin-associated glycoprotein relative to dystrophin could not be determined in this manner because it stains very poorly with Coomassie Blue (Ervasti *et al.*, 1990). Therefore, the dystrophin-associated glycoprotein-specific antibody staining intensity was quantitated from autoradiograms of immunoblots containing pure rabbit sarcolemma and dystrophin–glycoprotein complex after incubation with [$^{125}$I]-Protein A and was compared to the Coomassie Blue staining intensity of dystrophin. The 156 kDa, 59 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins each possess unique antigenic determinants, as none of the antibodies specific for a particular component of the complex cross-reacts with any other component of the complex (Ervasti *et al.*, 1990; Ohlendieck *et al.*, 1991b; Campbell *et al.*, 1991; Ervasti and Campbell, 1991). Densitometric analysis of Coomassie Blue stained gels demonstrated that dystrophin was enriched 2.5-fold in dystrophin–glycoprotein complex versus sarcolemma. The ratios of autoradiographic densitometric intensities of dystrophin–glycoprotein complex versus sarcolemma for polyclonal antibodies against each of the dystrophin-associated glycoproteins varied between 2.2 and 3.0 (Ervasti and Campbell, 1991). These results suggest that all components of the dystrophin–glycoprotein complex quantitatively co-enrich and that the 156 kDa dystrophin-associated glycoprotein is stoichiometric with dystrophin.

The cellular localization of the dystrophin-associated proteins was determined by indirect immunofluorescence labelling of transverse cryostat sections of rabbit skeletal muscle. The 50 kDa glycoprotein was previously identified as a very convenient sarcolemma marker (Jorgensen *et al.*, 1990). In addition, antibodies specific for the 156 kDa, 59 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins also exhibited immunofluorescent staining of the sarcolemmal membrane, demonstrating the unique association of these proteins with the muscle fibre plasma membrane or the intracellular cytoskeleton subjacent to the surface membrane (Ervasti *et al.*, 1990; Ohlendieck *et al.*, 1991b; Ervasti and Campbell, 1991). Immunoblot analysis of subcellular fractions from rabbit skeletal muscle confirmed that components of the dystrophin–glycoprotein complex are highly enriched in sarcolemma vesicles (Ohlendieck *et al.*, 1991b).

Fast-twitch skeletal muscle fibres are earlier affected in muscle from DMD patients than slow-twitch fibres (Webster *et al.*, 1988). Immunofluorescence localization studies of Schafer and Stockdale (Schafer and Stockdale, 1987) identified sarcolemma-associated antigens with different distribution in fast and slow skeletal muscle fibres. Variability of staining intensity among fibres were also found for a sarcolemmal $Na^+/K^+$-ATPase

in chicken muscle (Fambrough and Bayne, 1983). Therefore, the fibre type distribution of the dystrophin–glycoprotein complex was examined (Ohlendieck *et al.*, 1991b). Dystrophin and the 50 kDa component of the dystrophin–glycoprotein complex were equally distributed between fast- and slow-twitch fibres. It remains to be determined why the fibre type plays a role in the early steps of abnormal muscle protein degradation and fibre necrosis in dystrophic muscle.

The 156 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated proteins were found to contain Asn-linked oligosaccharides, as determined by specific lectin staining (Ervasti *et al.*, 1990; Ervasti and Campbell, 1991) and enzymatic deglycosylation (Ervasti and Campbell, 1991). In addition, the 156 kDa dystrophin-associated glycoprotein contained alpha(2,3)-linked sialic acid residues and Ser/Thr-linked oligosaccharides (Ervasti and Campbell, 1991). Dystrophin, the 59 kDa and the 25 kDa dystrophin-associated proteins do not appear to be glycosylated (Ervasti *et al.*, 1990; Ervasti and Campbell, 1991).

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 and Campbell, 1991b). The 59 kDa dystrophin-associated protein was also extracted by alkaline treatment while the 156 kDa, 50 kDa, 43 kDa and 35 kDa glycoproteins were retained in the membrane pellet after alkaline treatment (Ervasti and Campbell, 1991). Surprisingly, the 156 kDa dystrophin-associated glycoprotein, which was not extracted from membranes incubated at pH 11, was almost completely extracted from surface membranes incubated at pH 12 while the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins remained in the membrane pellet even after incubation of surface membranes at pH 12 (Ervasti and Campbell, 1991). That dystrophin, the 156 kDa dystrophin-associated glycoprotein and the 59 kDa 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 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins are integral membrane proteins. Since the 156 kDa dystrophin-associated glycoprotein remains membrane-bound under conditions which extract dystrophin, these data further suggest that the 156 kDa dystrophin-associated glycoprotein is linked to dystrophin by way of the 50 kDa, 43 kDa and/or 35 kDa components of the complex. The 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins and the 25 kDa dystrophin-associated protein were further confirmed as integral membrane proteins by covalent labelling with a hydrophobic probe (Ervasti and Campbell, 1991).

We recently proposed a model of the dystrophin–glycoprotein complex

(Ervasti and Campbell, 1991) to aid in visualizing what is presently known about its structure (Figure 6.2b). Dystrophin was modelled as a bent, antiparallel dimer with the cysteine-rich and C-terminal domains linked to the transmembrane components of the complex and the amino terminus binding to the filamentous actin cytoskeleton. Dystrophin was 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), ultrastructural localization (Cullen *et al.*, 1991) and recent filamentous actin cosedimentation results (Hemmings *et al.*, 1992).

We postulated that the 156 kDa dystrophin-associated glycoprotein is located on the extracellular side of the sarcolemma on the basis of the presence of Ser/Thr-linked oligosaccharides and its resistance to proteolysis (Ervasti and Campbell, 1991). By analogy with cell surface molecules containing densely Ser/Thr-linked glycosylated regions, such as NCAM (Walsh *et al.*, 1989; Moore *et al.*, 1987) and the LDL receptor (Cummings *et al.*, 1983), we conclude that the 156 kDa dystrophin-associated glycoprotein is an extracellular protein.

The extraction of the 156 kDa dystrophin-associated glycoprotein from membranes incubated at pH 12, but not pH 11 (Ervasti and Campbell, 1991) suggests that its association with the sarcolemma is distinct from that of dystrophin and the 59 kDa 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 kDa dystrophin-associated glycoprotein coupled with its failure to focus as a sharp band after enzymatic deglycosylation suggest that the 156 kDa dystrophin-associated glycoprotein may also contain glycosaminoglycan chains.

The placement of the 59 kDa dystrophin-associated protein in the cytoplasm in direct contact with dystrophin was based on its cross-linking to dystrophin (Yoshida and Ozawa, 1990), solubilization from skeletal muscle membranes by alkaline treatment and the absence of labelling by hydrophobic probe (Ervasti and Campbell, 1991). Placement of the 59 kDa dystrophin-associated protein in contact with the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins is solely by analogy with the 58 kDa of MAT-Cl ascite tumour 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 kDa dystrophin-associated protein could be located near the predicted actin-binding domain of dystrophin (Koenig *et al.*, 1988) where it might stabilize dystrophin binding to actin filaments in a manner analogous to protein 4.1 promoting spectrin–actin association

(Bennett, 1990) or zyxin promoting alpha-actinin/actin association (Crawford and Beckerle, 1991).

That the 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins form an integral membrane complex (Ervasti and Campbell, 1991) indicates that they are the components of the complex which span the sarcolemmal membrane and link dystrophin to the 156 kDa dystrophin-associated glycoprotein. The large amount of hydrophobic probe incorporation into the 25 kDa dystrophin-associated protein places this component of the complex in the sarcolemmal membrane as well.

The structural organization of the dystrophin–glycoprotein complex (Figure 6.2b) 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 the extracellular matrix of skeletal muscle. That dystrophin comprises 2% of sarcolemmal protein (Ohlendieck *et al.*, 1991b) and 5% of the sarcolemmal cytoskeleton (Ohlendieck and Campbell, 1991b) supports the role for the dystrophin–glycoprotein complex in maintaining skeletal muscle architecture.

## 6.4 STRUCTURE AND FUNCTION OF DYSTROGLYCAN (43/156 DYSTROPHIN-ASSOCIATED GLYCOPROTEIN)

The complete amino acid sequence of the 43 kDa and 156 kDa dystrophin-associated glycoproteins have been deduced from isolated cDNAs (Ibraghimov-Beskrovnaya *et al.*, 1992). A 0.6 kb cDNA clone, the protein product of which was recognized by two polyclonal antibodies against the 43 kDa dystrophin-associated glycoprotein, was isolated from a rabbit skeletal muscle cDNA expression library. This cDNA clone hybridized with a 5.8 kb transcript in mRNA preparations from a variety of rabbit tissues. Overlapping clones covering the entire coding region of the mRNA were isolated by rescreening cDNA libraries and the full length sequence determined. Sequence analysis of cDNAs revealed an open reading frame of 2685 bases encoding a precursor polypeptide of 895 amino acids, with a predicted molecular weight of 97 kDa, which exhibited no significant sequence similarity with any known proteins. The predicted amino acid sequence of the 43 kDa and 156 kDa dystrophin-associated glycoproteins revealed structural characteristics (Figure 6.3) that were in good agreement with the native proteins' biochemical properties (Ervasti and Campbell, 1991). The N-terminal portion of the precursor polypeptide encodes 56 kDa core protein for the 156 kDa dystrophin-associated glycoprotein because antibodies specific for a fusion protein corresponding to this region of the message also recognize the native 156 kDa dystrophin-associated

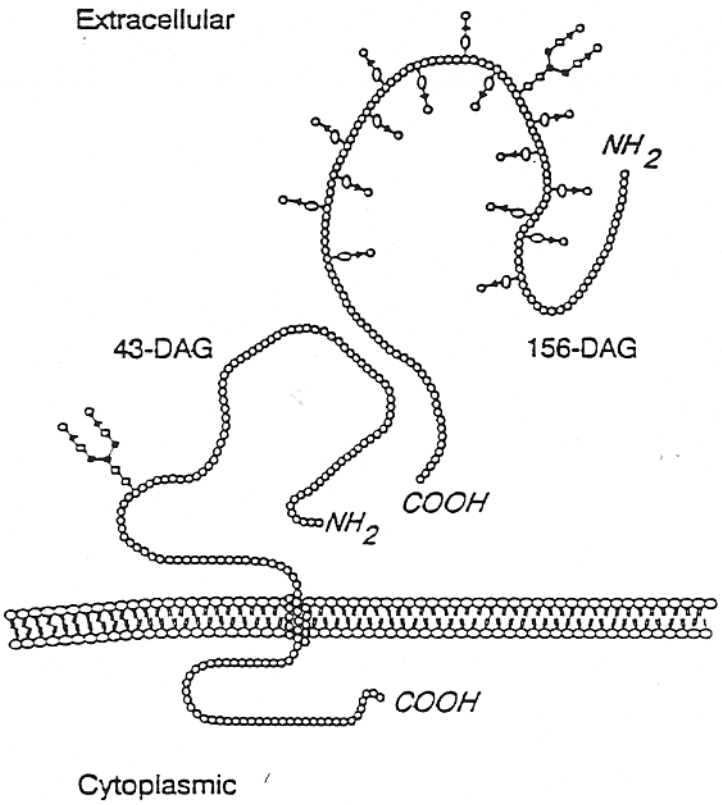


Figure 6.3 Model of dystroglycan.

glycoprotein. The 56 kDa core protein contains a single consensus sequence for Asn-linked glycosylation with many potential attachment sites for O-linked carbohydrates. Carbohydrate moieties appear to constitute up to two-thirds of the molecular mass of the 156 kDa dystrophin-associated glycoprotein which suggests that it may be a proteoglycan. The exact modifications involved in the processing of the N-terminal portion of the precursor polypeptide to the 156 kDa dystrophin-associated glycoprotein are not known but the 56 kDa core protein contains several Ser-Gly repeats for the possible addition of glycosaminoglycan chains (Bourdon *et al.*, 1987). The C-terminal portion of the precursor polypeptide is processed into the mature 43 kDa dystrophin-associated glycoprotein with four potential N-glycosylation sites, a single transmembrane domain and 120 amino acid cytoplasmic tail. The C-terminal half of the message was determined to encode for the 43 kDa dystrophin-associated glycoprotein

because an 11 amino acid sequence determined directly from protein exactly matched the predicted sequence from cDNA.

Northern and Western blot analyses have demonstrated that the 43 kDa and 156 dystrophin-associated glycoproteins are expressed in both muscle and non-muscle tissues. A prominent 5.8 kb transcript was detected in mRNA from rabbit skeletal muscle, cardiac muscle and lung (Ibraghimov-Beskrovnaya *et al.*, 1992). Thus, the 5.8 kb transcript for the 43/156 dystrophin-associated glycoproteins is present in various muscle and non-muscle tissues, most likely originating from the one gene. Identification of the 43/156 kDa dystrophin-associated glycoproteins in muscle and non-muscle tissues was performed using immunoblots of membranes from different tissues and affinity-purified antibodies. The 43 kDa dystrophin-associated glycoprotein was detected in isolated membranes from skeletal muscle, brain, cardiac muscle and lung. The 156 kDa dystrophin-associated glycoprotein was detected in skeletal and cardiac muscle membranes, but was slightly lower in molecular weight in cardiac membranes. In brain and lung membranes, the molecular weight of the '156 kDa' dystrophin-associated glycoprotein reactive protein was ~120 kDa. The variability in molecular weight for the '156 kDa' reactive protein may be due to differential glycosylation of the core protein. The broad tissue distribution of the 43/156 kDa dystrophin-associated glycoprotein precursor argues for a significant role of both glycoproteins in membrane organization within different tissues and might indicate the existence of the entire glycoprotein complex in non-muscle tissues. Absence of significant amounts of dystrophin in the examined non-muscle tissues suggests that in non-muscle tissues 43/156 kDa dystrophin-associated glycoprotein is involved in formation of a different type of complex where dystrophin may be replaced by another cytoskeleton component.

The organization (Ervasti and Campbell, 1991), available primary sequence (Ibraghimov-Beskrovnaya *et al.*, 1992) and abundance in purified sarcolemma (Ohlendieck *et al.*, 1991b; Ohlendieck and Campbell, 1991b) of the dystrophin–glycoprotein complex suggest that the complex plays a structural role and functions to link the cytoskeleton with the extracellular matrix. To evaluate this hypothesis, we designed experiments which tested for an interaction between the 156 kDa dystrophin-associated glycoprotein and several well characterized proteins of the extracellular matrix. Rabbit skeletal muscle surface membranes and pure dystrophin–glycoprotein complex were electrophoretically separated, transferred to nitrocellulose membranes and overlaid with $^{125}$I-labelled extracellular matrix proteins (Ibraghimov-Beskrovnaya *et al.*, 1992). A single laminin-binding band, corresponding to the 156 kDa dystrophin-associated glycoprotein, was detected in surface membranes and pure dystrophin–glycoprotein complex. Binding of $^{125}$I-labelled laminin to the 156 kDa dystrophin-associated

glycoprotein was completely inhibited by inclusion of an excess of unlabelled laminin to the incubation medium. $^{125}$I-labelled fibronectin did not label any component of the dystrophin–glycoprotein complex, nor did an excess of unlabelled fibronectin have any effect on the binding of $^{125}$I-labelled laminin to the 156 kDa dystrophin-associated glycoprotein. The interaction of the 156 kDa dystrophin-associated glycoprotein with laminin was also shown by co-immunoprecipitation using anti-laminin antibodies. These results suggest that the 156 kDa dystrophin-associated glycoprotein specifically binds laminin and may mediate interaction of the dystrophin–glycoprotein complex with the extracellular matrix.

A number of laminin binding proteins have previously been identified in skeletal muscle (Lesot *et al.*, 1983; Clegg *et al.*, 1988; Mecham, 1991) but the 156 kDa dystrophin-associated glycoprotein does not appear to be related to any of these. In addition, the sequence of the 43/156 kDa dystrophin-associated glycoprotein indicates that it is not related to integrins or cadherin. It is also interesting that the conditions used to identify the 156 kDa dystrophin-associated glycoprotein as a laminin binding protein are similar to that which have been used for the identification of cranin as a laminin binding protein (Smalheiser and Schwartz, 1987). The apparent molecular weight of cranin is also very similar to the protein we have identified in brain membranes with the '156 kDa'-specific antibody.

We have proposed the name 'dystroglycan' (Ibraghimov-Beskrovnaya *et al.*, 1992) because of the 43/156 kDa dystrophin-associated glycoproteins' identification via dystrophin and its extensive glycosylation.

## 6.5 MOLECULAR PATHOGENESIS OF DUCHENNE MUSCULAR DYSTROPHY

Early histopathological events in DMD are characterized by persistent skeletal muscle necrosis. A central question of current muscular dystrophy research is how the absence of dystrophin causes muscle cell necrosis. In comparison, the *mdx* mouse is completely missing dystrophin (Bonilla *et al.*, 1988; Hoffman *et al.*, 1987) and also exhibits necrosis of skeletal muscle fibres. The absence of dystrophin accompanied by skeletal muscle necrosis make the *mdx* mouse a good model system in which to study how muscle fibre necrosis is caused by the absence of dystrophin. To learn more about the early events in the molecular pathogenesis of muscular dystrophy, we investigated the relative abundance of all of the components of the dystrophin–glycoprotein complex in skeletal muscle membranes from *mdx* mice. Initially, we only investigated whether the 156 kDa dystrophin-associated glycoprotein was affected by the absence of dystrophin, because the monoclonal antibody to it was the only non-dystrophin probe to the complex which cross-reacted with mouse and human (Ervasti *et al.*, 1990).

Immunoblots of skeletal muscle membranes were prepared from control and *mdx* mice and stained with the various antibodies. Staining with polyclonal antiserum against the C-terminal decapeptide of dystrophin revealed that this protein was completely absent from *mdx* mouse membranes. In addition, comparison of normal and *mdx* mouse by immunostaining with the monoclonal antibody against the 156 kDa glycoprotein revealed that this too was absent or greatly reduced in *mdx* mouse membranes. The absence of the 156 kDa glycoprotein was also confirmed using SDS muscle extracts, instead of isolated membranes, from control and *mdx* mice. Estimation of the amount of 156 kDa glycoprotein remaining in the *mdx* muscle membranes using $^{125}$I-labelled secondary antibodies and total membrane preparations from four different control and four different *mdx* mice revealed an average reduction of 85% in *mdx* muscle (Ervasti *et al.*, 1990).

Total muscle extracts were also prepared from biopsy samples of normal controls and patients with Duchenne muscular dystrophy. The dystrophic samples exhibited no staining with antibodies against dystrophin by indirect immunofluorescence microscopy and immunoblotting. In contrast to the normal muscle extract the three DMD samples showed greatly reduced staining for the 156 kDa glycoprotein (Ervasti *et al.*, 1990). Identical immunoblots stained with monoclonal antibodies against the sarcoplasmic reticulum $Ca^{2+}$-dependent ATPase revealed no difference in the staining intensity between normal and dystrophic muscle samples. As in the case of *mdx* mouse muscle, the amount of 156 kDa glycoprotein was estimated to be reduced by approximately 90% in DMD samples. The drastic reduction of the 156 kDa dystrophin-associated glycoprotein (the component of the complex most distal to dystrophin) in muscle from *mdx* mice and DMD patients is evidence that alteration in dystrophin expression profoundly affects components external to the muscle cell.

In evaluating studies of other diseases, it is apparent that loss of a protein due to genetic defect often results in the loss of associated proteins. For example, spectrin deficiency in hereditary elliptocytosis is also associated with a reduced abundance in protein-4.1 and minor sialoglycoproteins (Alloisio *et al.*, 1985). Skeletal muscle phosphorylase kinase deficiency, which is caused by a single gene defect on the X-chromosome, is characterized by the combined loss of all four subunits of this enzyme (Cohen *et al.*, 1976). However, a generalized loss of components in a protein complex is not observed in the genetic disease muscular dysgenesis. This disorder results in a complete absence of skeletal muscle contraction due to the failure of depolarization of the transverse tubular membrane to trigger calcium release from sarcoplasmic reticulum. Interestingly, only the alpha$_1$-subunit of the dihydropyridine receptor is absent in dysgenic mice while the alpha$_2$-subunit of the receptor is present (Knudson *et al.*, 1989). In view of

these findings, it was important to investigate the status of all the dystrophin-associated proteins in *mdx* skeletal muscle. The relative abundance of each of the components of the dystrophin–glycoprotein complex in skeletal muscle was determined from normal and *mdx* mice using antibodies specific for each of the dystrophin-associated glycoproteins (Ohlendieck and Campbell, 1991a). Immunoblot analysis using total muscle membranes from control and *mdx* mice found that all of the dystrophin-associated proteins were greatly reduced in *mdx* mouse skeletal muscle. The specificity of the loss of the dystrophin-associated glycoproteins was demonstrated by the finding that the major glycoprotein composition of skeletal muscle membranes from normal and *mdx* mice was identical. Densitometric scanning of $^{125}$I-Protein A-labelled immunoblots revealed an average 84% reduction for the 156 kDa, 59 kDa, 50 kDa, 43 kDa and 35 kDa dystrophin-associated glycoproteins in *mdx* muscle membranes when compared to control membranes (Ohlendieck and Campbell, 1991a). The comparative densitometric scanning was performed with individually isolated membranes from five 10-week-old control mice and five 10-week-old *mdx* mice. A similarly reduced expression of dystrophin-associated proteins was also observed in membranes isolated from 1, 2, 5, 20 and 30-week-old *mdx* mice as compared to age-matched control mice. Immunofluorescence microscopy confirmed that the density of dystrophin-associated proteins is greatly reduced in skeletal muscle cryosections from *mdx* mice. These findings strongly suggest that the deficiency of dystrophin-associated proteins in *mdx* mouse muscle is a primary event following the absence of dystrophin and that a reduction in dystrophin-associated proteins may initiate muscle cell necrosis.

The murine mutant *dystrophia muscularis dy/dy* which has an autosomal-recessive mode of inheritance is another animal model for muscular dystrophy which exhibits progressive and severe degeneration of skeletal muscle fibres (Bray and Banker, 1970). Coomassie Blue staining revealed no apparent differences between membranes isolated from control and *dy/dy* mouse skeletal muscle and the density of dystrophin and dystrophin-related protein is also comparable between both membrane preparations (Ohlendieck and Campbell, 1991a). Most importantly, antibodies to the different dystrophin-associated proteins showed approximately equal amounts of these proteins in skeletal muscle membranes from control and *dy/dy* mice (Ohlendieck and Campbell, 1991a). These findings demonstrate that dystrophin-associated proteins are not affected by secondary events in necrotic muscle and suggest that the reduced density of dystrophin-associated proteins in skeletal muscle membranes from *mdx* mice is most likely due to the absence of dystrophin from the membrane cytoskeleton of *mdx* muscle.

One could envision three different mechanisms to account for the loss of

dystrophin-associated proteins from the cell surface of dystrophin-deficient muscle. First, point mutations, deletions or duplications in the DMD gene which result in the absence or abnormal structure of dystrophin could affect the transcription, processing or stability of dystrophin-associated protein mRNAs. Second, an absence or abnormality in dystrophin could cause a decrease in translation and/or assembly of the components of the dystrophin–glycoprotein complex. Third, loss of dystrophin-associated proteins could be due to an increase in degradative pathways.

To begin to address which of these three possible mechanisms may account for the loss of dystrophin-associated proteins in dystrophin-less tissues, Northern blots of skeletal muscle mRNA from control and *mdx* mice of different ages were probed using radiolabelled cDNA corresponding to the 43/156 kDa dystrophin-associated glycoprotein (Ibraghimov-Beskrovnaya *et al.*, 1992). Northern blot analysis revealed no reduction of 43/156 kDa dystrophin-associated glycoprotein mRNA in *mdx* mice vs. control mice. Thus, the absence of dystrophin causes no change in the mRNA for the 43/156 kDa dystrophin-associated glycoprotein but leads to dramatic reductions in the amount of the 43 kDa and 156 kDa dystrophin-associated glycoproteins in skeletal muscle. Analysis of mRNA from control and DMD skeletal muscle also showed no difference in 43/156 kDa dystrophin-associated glycoprotein mRNA expression. Thus, the 43/156 kDa dystrophin-associated glycoprotein encoding gene is transcribed and specific mRNA is still present at the normal level in dystrophic muscle, but the amount of 43 kDa and 156 kDa dystrophin-associated glycoproteins are greatly reduced in dystrophic muscle.

Since the 43/156 kDa dystrophin-associated glycoproteins are expressed in non-muscle tissues, we also examined expression of the 43 kDa dystrophin-associated glycoprotein in non-muscle tissues of control and *mdx* mice. Immunoblot analysis of brain and kidney membranes from control and *mdx* mice, stained with polyclonal anti-43 kDa dystrophin-associated glycoprotein antibodies, revealed no reduction in the amount of 43 kDa dystrophin-associated glycoprotein in these *mdx* tissues. Thus, the dramatic reduction of the 43 kDa dystrophin-associated glycoprotein that is found in *mdx* mice appears to be restricted to skeletal muscle and is not found in non-muscle tissues.

The loss of dystrophin-associated proteins from the muscle cell surface could principally occur in two different ways: (1) Translation of dystrophin-associated proteins may be downregulated or dystrophin-associated proteins could be synthesized in normal amounts but may not be properly assembled into an oligomeric complex due to the lack of dystrophin; (2) dystrophin-associated proteins may be synthesized and assembled correctly but due to the deficiency in dystrophin the membrane complex will lack the proper interaction with the actin cytoskeleton, resulting in greater mobility

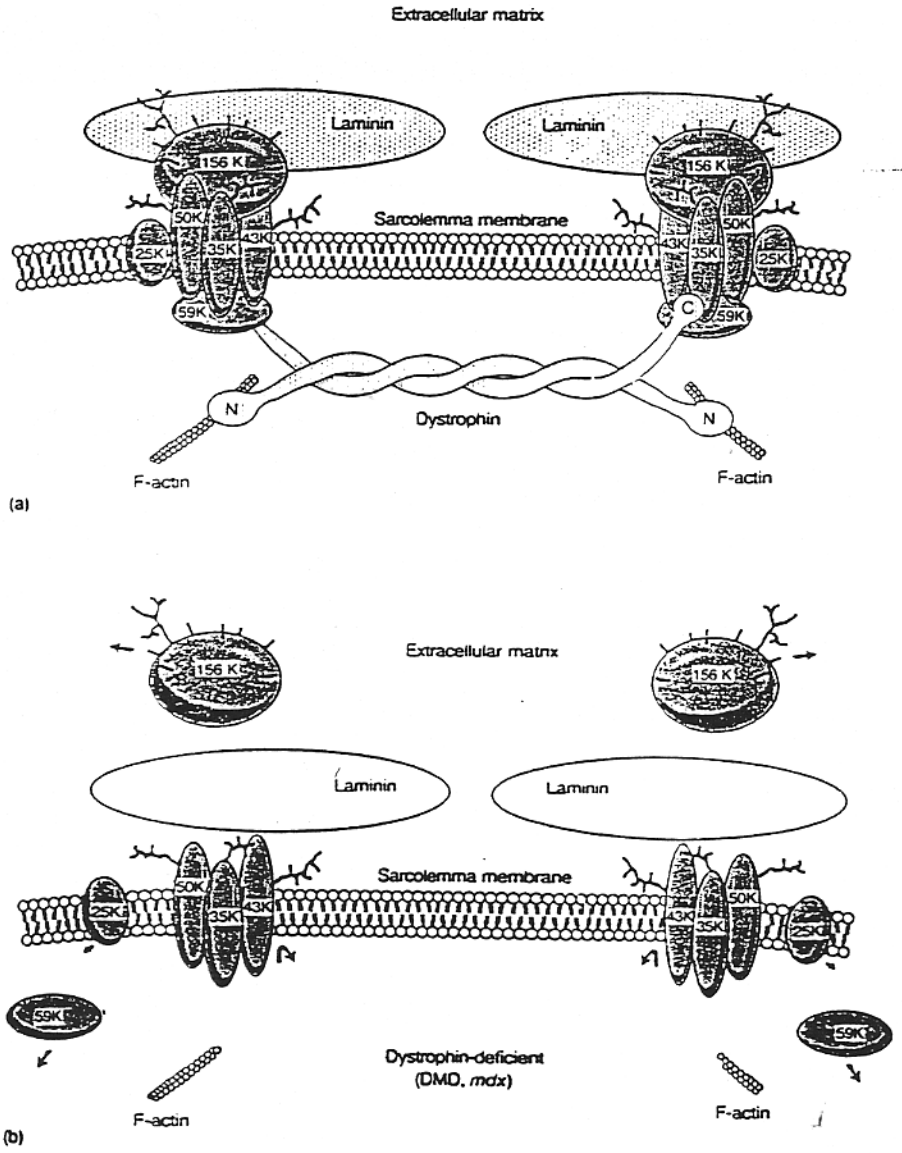


**Figure 6.4** Model of dystrophin-associated glycoproteins in the absence of dystrophin. Shown is the proposed model of the dystrophin–glycoprotein complex in normal skeletal muscle (a) and in dystrophin-deficient skeletal muscle (b). In the absence of dystrophin, dystrophin-associated proteins are present in greatly reduced amounts as a result of either down-regulation of synthesis or increased protein degradation. After Ervasti and Campbell (1991).

of the membrane complex which may render the protein components of the complex more vulnerable to degradation (Figure 6.4).

Our findings suggest that the function of dystrophin is to link the subsarcolemma membrane cytoskeleton through a transmembrane complex to an extracellular glycoprotein which binds laminin. Since the absence of dystrophin leads to the loss of all the dystrophin-associated proteins (Ervasti *et al.*, 1990; Ohlendieck and Campbell, 1991a) our results suggest that dystrophin-deficient muscle fibres may lack the normal interaction between the sarcolemma and the extracellular matrix. Disruption of the various components involved in the structural link between subsarcolemmal cytoskeleton and extracellular matrix may severely weaken the flexibility of the sarcolemma membrane during skeletal muscle contraction (Figure 6.5).

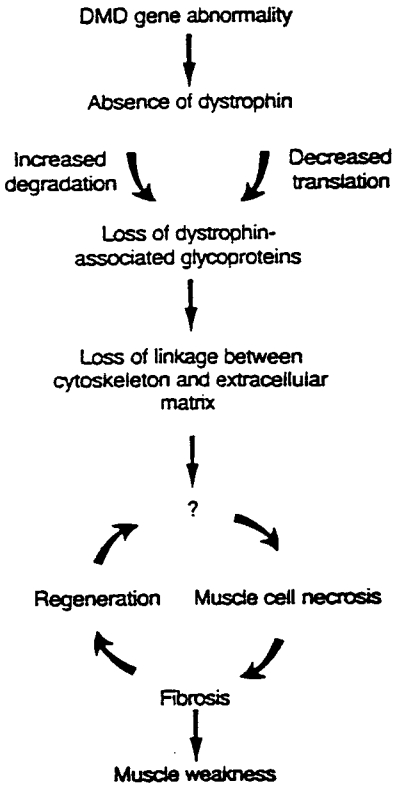


**Figure 6.5** Pathway for molecular pathogenesis of Duchenne muscular dystrophy. Proposed sequence of events leading to the molecular pathogenesis of Duchenne muscular dystrophy. The sequence of events leading from loss of linkage between the cytoskeleton and the extracellular matrix to muscle cell necrosis (?) are presently unresolved. Please refer to the text for discussion of the current hypotheses regarding these unresolved steps.

This hypothesis is supported by the histopathological finding that DMD muscle fibres exhibit an early separation between muscle cell surface and basal lamina (Bonilla and Moggio, 1986).

Comprehensive analysis of DMD skeletal muscle shows that muscle cell necrosis is preceded by a breakdown of the plasma membrane (Carpenter and Karpati, 1979; Engel and Banker, 1986; Mokri and Engel, 1975). In addition, skeletal muscle fibres from *mdx* mice exhibited in contraction experiments an enhanced vulnerability which may render the sarcolemma more susceptible to suffer focal breaks (Weller *et al.*, 1990). Recent findings demonstrated that skeletal muscle fibres from *mdx* mouse are more fragile and have a decreased osmotic stability (Menke and Jockusch, 1991). It is not known if the osmotic fragility is based only on the absence of dystrophin in the sarcolemma membrane cytoskeleton or if it is also linked to the reduced density of dystrophin-associated proteins or other secondarily affected proteins in *mdx* skeletal muscle. Alternatively, the disruption of this linkage between the sarcolemma and the extracellular matrix may be responsible for the alteration of specific $Ca^{2+}$ regulatory mechanisms (Franco and Lansman, 1990) which may lead to excessive influx of $Ca^{2+}$ ions in dystrophic muscle (Turner *et al.*, 1991). Since the extracellular matrix of the adult tissue is a scaffold that is required to allow repair following injury, the absence of the interaction between the sarcolemma and the extracellular matrix may also render dystrophic muscle fibres more prone to injury and less able to repair such injury.

The normal production of the mRNA for the 43/156 kDa dystrophin-associated glycoprotein in dystrophic muscle is important for potential DMD therapies. Prior to these findings, it was unclear how the absence of dystrophin led to the loss of the dystrophin-associated glycoproteins. Our work now indicates that dystrophin-associated glycoproteins are produced in dystrophic muscle but that the dystrophin-associated glycoproteins may not be properly assembled and/or integrated into the sarcolemma or may be degraded. These results suggest that restoring dystrophin by myoblast transfer (Partridge *et al.*, 1989; Gussoni *et al.*, 1992) or gene therapy (Lee *et al.*, 1991; Ascadi *et al.*, 1991) may stabilize and restore normal dystrophin-associated glycoprotein levels in DMD muscle.

Finally, it would be interesting to examine the status of the dystrophin–glycoprotein complex in neuromuscular diseases which have yet to be characterized at the molecular level. For example, perhaps a deficiency or abnormality in a dystrophin-associated glycoprotein could explain the DMD-like symptoms observed in suspected autosomal recessive patients (Arahata *et al.*, 1989; Francke *et al.*, 1989; Vainzof *et al.*, 1990; Ben Jelloun-Dellagi *et al.*, 1990) that express apparently normal dystrophin.

SUMMARY

Dystrophin constitutes approximately 5% of the cytoskeletal protein of skeletal muscle sarcolemma, suggesting that dystrophin could play a major structural role in skeletal muscle. We have presented evidence for the existence of a large oligomeric complex containing dystrophin, a 59 kDa triplet, a 25 kDa protein and four sarcolemmal glycoproteins with apparent $M_r$ of 156 kDa, 50 kDa, 43 kDa and 35 kDa. All components of the dystrophin–glycoprotein complex were localized to the skeletal muscle sarcolemma. Dystrophin, the 156 kDa and 59 kDa dystrophin-associated protein were found to be peripheral membrane proteins while the 50 kDa, 43 kDa, 35 kDa and 25 kDa dystrophin-associated proteins were confirmed as integral membrane proteins. The primary sequences of the 43 kDa and 156 kDa dystrophin-associated glycoproteins have been established by recombinant DNA techniques. Both the 43 and 156 kDa dystrophin-associated glycoproteins are encoded by a single 5.8 kb mRNA which is expressed in a variety of tissues in addition to skeletal muscle. The 156 kDa dystrophin-associated glycoprotein binds laminin, a well characterized component of the extracellular matrix. Finally, the dystrophin–glycoprotein complex is specifically and greatly reduced in Duchenne-afflicted and *mdx* mouse skeletal muscle, suggesting that the loss of dystrophin-associated proteins is due to the absence of dystrophin and not due to secondary effects of muscle fibre degradation. Taken together, these data support the hypothesis that the absence of dystrophin leads to a loss of the linkage between the subsarcolemmal cytoskeleton and extracellular matrix and that this may initiate muscle fibre necrosis.

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