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REVIEW ARTICLE

DEFICIENCY OF DYSTROPHIN-ASSOCIATED PROTEINS: A COMMON MECHANISM LEADING TO MUSCLE CELL NECROSIS IN SEVERE CHILDHOOD MUSCULAR DYSTROPHIES

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Abstract—Dystrophin is a large cytoskeletal protein encoded by the Duchenne muscular dystrophy (DMD) gene. Dystrophin is associated with a large oligomeric complex of sarcolemmal glycoproteins, including the novel laminin-binding glycoprotein called dystroglycan, which provides a linkage to the extracellular matrix. In DMD, the absence of dystrophin leads to a drastic reduction in all of the dystrophin-associated proteins. In severe childhood autosomal recessive muscular dystrophy with DMD-like phenotype (SCARMD), a specific deficiency of the 50 kDa dystrophin-associated glycoprotein is found. Thus, the disruption/dysfunction of the dystrophin–glycoprotein complex due to the deficiency of one or more of the dystrophin-associated proteins is presumed to cause the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix. This may render muscle cells susceptible to necrosis in two forms of severe childhood muscular dystrophy, DMD and SCARMD.

Key words: Dystrophin–glycoprotein complex, dystrophin, dystrophin-associated proteins, dystroglycan, DMD, SCARMD.

INTRODUCTION

The identification of the Duchenne muscular dystrophy (DMD) gene and its protein product, dystrophin [1, 2], has led to the discovery of a large oligomeric complex of novel sarcolemmal glycoproteins associated with dystrophin [3–9]. The 156 kDa component of these dystrophin-associated proteins binds the extracellular matrix protein, laminin [9]. These results indicate that the dystrophin–glycoprotein complex (a protein complex consisting of dystrophin and the sarcolemmal glycoproteins associated with dystrophin) spans the sarcolemma to provide a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix [8, 9]. In this review, we will discuss the potential role of the dystrophin–glycoprotein complex in both normal and dystrophic skeletal muscle.

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DYSTROPHIN–GLYCOPROTEIN COMPLEX: A TRANS-SARCOLEMMAL LINKER BETWEEN THE SUBSARCOLEMMAL CYTOSKELETON AND THE EXTRACELLULAR MATRIX

Dystrophin is predicted to be a rod-shaped cytoskeletal protein of 427 kDa, composed of four structural domains: (1) the amino-terminal domain with high homology to the actin-binding region of such actin-binding proteins as α-actinin, β-spectrin and dictyostelium actin-binding protein 120; (2) a series of 24 repeats of a 109 amino acid motif in the form of a triple helix; (3) a cysteine-rich domain homologous to the calcium-binding region of α-actinin; and (4) the carboxyl-terminal domain with no homology to previously described protein sequences [2]. Dystrophin does not have transmembrane domains [2].

Immunohistochemical and immunoelectron microscopic analyses using specific antibodies demonstrate that dystrophin is localized to the cytoplasmic face of normal skeletal and cardiac sarcolemma, and immunoblot analysis detects a

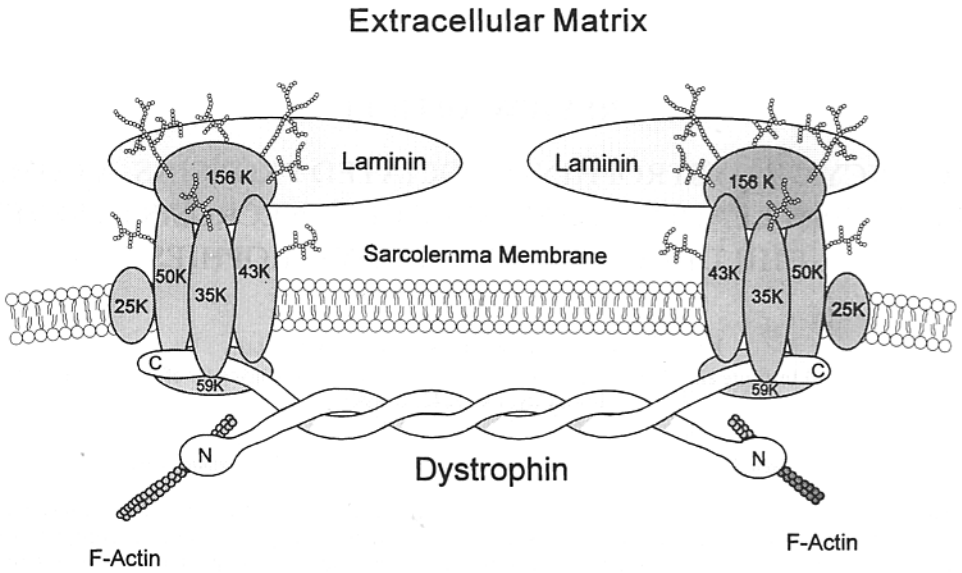

Fig. 1. Schematic model of the dystrophin–glycoprotein complex as a trans-sarcolemmal link between the subsarcolemmal cytoskeleton and the extracellular matrix (modified from [8]). Abbreviations as follows: 156K, 156 kDa dystroglycan; 59K, 59 kDa dystrophin-associated protein; 50K, 50kDa dystrophin-associated glycoprotein; 43K, 43 kDa dystroglycan; 35K, 35 kDa dystrophin-associated glycoprotein; 25K, 25 kDa dystrophin-associated protein.

protein of approximately 400 kDa in normal skeletal and cardiac muscles [10–15]. Dystrophin is absent in the skeletal and cardiac muscles of DMD patients [10, 11, 13–15]. Although dystrophin is a minor component of the total skeletal muscle proteins, it constitutes 2% of the total sarcolemmal proteins and 5% of sarcolemmal cytoskeletal proteins, indicating that dystrophin is a major structural component of the subsarcolemmal cytoskeleton [6, 16]. Recently the amino-terminal domain of dystrophin was shown to interact with F-actin, as predicted from the primary structure [17, 18]. Since dystrophin is localized to the cytoplasmic face of the sarcolemma, it probably interacts with cytoskeletal actin, such as γ-actin, rather than α-actin of thin filaments.

Dystrophin is associated with a large oligomeric complex of novel sarcolemmal glycoproteins. The dystrophin–glycoprotein complex has been isolated and its components have been characterized. The dystrophin–glycoprotein complex consists of two cytoskeletal proteins, dystrophin and a 59 kDa protein (59 kDa dystrophin-associated protein), three transmembrane glycoproteins of 50 kDa (50 kDa dystrophin-associated glycoprotein), 43 kDa (43 kDa dystrophin-associated glycoprotein) and 35 kDa (35 kDa dystrophin-associated glycoprotein), a transmembrane protein of 25 kDa (25 kDa dystrophin-associated protein) and an extracellular glycoprotein of 156 kDa (156 kDa dystrophin-associated glycoprotein) (Fig. 1) [3–9]. Dystrophin and these dystrophin-associated proteins are co-localized throughout the sarcolemma [8], including the sarcolemma of the intrafusal muscle fibers and the neuromuscular and myotendon junctions, two specialized regions of the sarcolemma where dense dystrophin staining is observed (Matsumura and Campbell, unpublished results) [19–21].

Recently, a single cDNA encoding two of the dystrophin-associated proteins, the 156 kDa dystrophin-associated glycoprotein and the 43 kDa dystrophin-associated glycoprotein, was identified. Post-translational processing of a 97 kDa precursor protein results in production of the two dystrophin-associated proteins [9]. The carboxyl-terminal portion of the precursor protein, which is processed into the 43 kDa dystrophin-associated glycoprotein, has three potential *N*-glycosylation sites, a single potential transmembrane domain and a 120-amino-acid-long cytoplasmic tail [9]. The amino-terminal portion of the precursor protein, corresponding to the approximately 56 kDa core protein of the 156 kDa dystrophin-associated glycoprotein, has no transmembrane domains, but has one potential *N*-glycosylation site and many potential *O*-glycosylation sites [9]. Carbohydrate residues constitute two-thirds of the molecular mass, suggesting that the 156 kDa dystrophin-associated glycoprotein may be a proteoglycan [9]. The heavy glycosylation could explain the

high resistance of the 156 kDa dystrophin-associated glycoprotein to proteolysis [8]. Based on these properties and its association with dystrophin, the 43 kDa/156 kDa dystrophin-associated glycoproteins were named dystroglycan [9].

The dystrophin–glycoprotein complex was initially hypothesized to provide a linkage of the subsarcolemmal cytoskeleton to the extracellular matrix [8]. The primary structure of the 156 kDa dystroglycan indicated that it was localized on the extracellular face of the sarcolemma and, thus, was a strong candidate for interaction with components of the extracellular matrix [9]. However, the demonstration of this interaction and the identification of the component of the extracellular matrix which interacts with the dystrophin–glycoprotein complex required biochemical analysis, since the sequence of the 156 kDa dystroglycan indicated that it was a novel protein [9]. ^{125}I laminin-binding demonstrated that laminin, a major protein component of the extracellular matrix, bound to the 156 kDa dystroglycan but not to the other components of the dystrophin–glycoprotein complex [9]. Further experiments have demonstrated that the 156 kDa dystroglycan is a highly specific laminin-receptor and does not bind other well-characterized extracellular matrix components, such as several forms of collagen, fibronectin or heparan sulphate proteoglycan [22]. In cardiac muscle, dystrophin, all of the dystrophin-associated proteins and laminin, co-localize to the sarcolemma and transverse tubules [23].

These recent findings indicate that the dystrophin–glycoprotein complex spans the sarcolemma to link the subsarcolemmal cytoskeleton to the extracellular matrix (Fig. 1) [8, 9]. The dystrophin–glycoprotein complex is expected to provide a structural support to the sarcolemma during muscle contraction and stretch. In addition to a structural role, the dystrophin–glycoprotein complex may have far more diverse biological functions, such as signal transduction and regulation of the intracellular calcium concentration. At present, the primary structure and function of the dystrophin-associated proteins, other than dystroglycan, are unknown. However, analysis of partial amino acid sequences indicates that the dystrophin-associated proteins are likely to be novel proteins (Ervasti and Campbell, unpublished results). Characterization of each of the dystrophin-associated proteins at both the molecular biological and biochemical levels is required for the complete understanding of the structural organization and function of the dystrophin–glycoprotein complex.

MOLECULAR PATHOGENESIS OF DMD AND RELATED DISEASES

DMD

How does the absence of dystrophin lead to muscle cell necrosis in DMD? The answer to this question is crucial for the development of effective therapies for this devastating disease. To begin to address this issue, our laboratory has investigated the status of the dystrophin-associated proteins in dystrophin-deficient muscles. Specific antibodies against each component of the dystrophin–glycoprotein complex were produced and used for analysis of the components of the complex in skeletal muscle from DMD patients [24, 25]. Immunohistochemistry revealed that all of the dystrophin-associated proteins are drastically reduced in the DMD sarcolemma (Fig. 2a) [4, 9, 24, 25]. The reduction of the dystrophin-associated proteins was confirmed by immunoblot analysis which showed approximately an 80–90% reduction in all of these proteins in DMD muscle membranes compared with normal membranes (Fig. 2b) [4, 24, 25].

The loss of the dystrophin-associated proteins in DMD is considered to be a direct consequence of the absence of dystrophin, and not to be due to the non-specific secondary effects of muscle fiber degeneration based on the following observations: (1) all of the dystrophin-associated proteins are preserved in a variety of other neuromuscular diseases in which muscle fiber necrosis occurs; (2) the loss of the dystrophin-associated proteins is common in all DMD patients, irrespective of age; (3) the loss of the dystrophin-associated proteins is found in all muscle fibers of the biopsied skeletal muscles, independent of the severity of degeneration of individual fibers; (4) the abundance of many other glycoproteins of the sarcolemma is not affected in DMD muscle; (5) other proteins, including the membrane cytoskeletal protein spectrin, remain well preserved in DMD; and (6) the dystrophin-associated proteins are well preserved in the neuromuscular junctions of DMD skeletal muscle due to their association with dystrophin-related protein (DRP or utrophin), an autosomal homologue of dystrophin [21, 25–27].

Based on the aforementioned structural organization of the dystrophin–glycoprotein complex, we proposed that the disruption of the complex

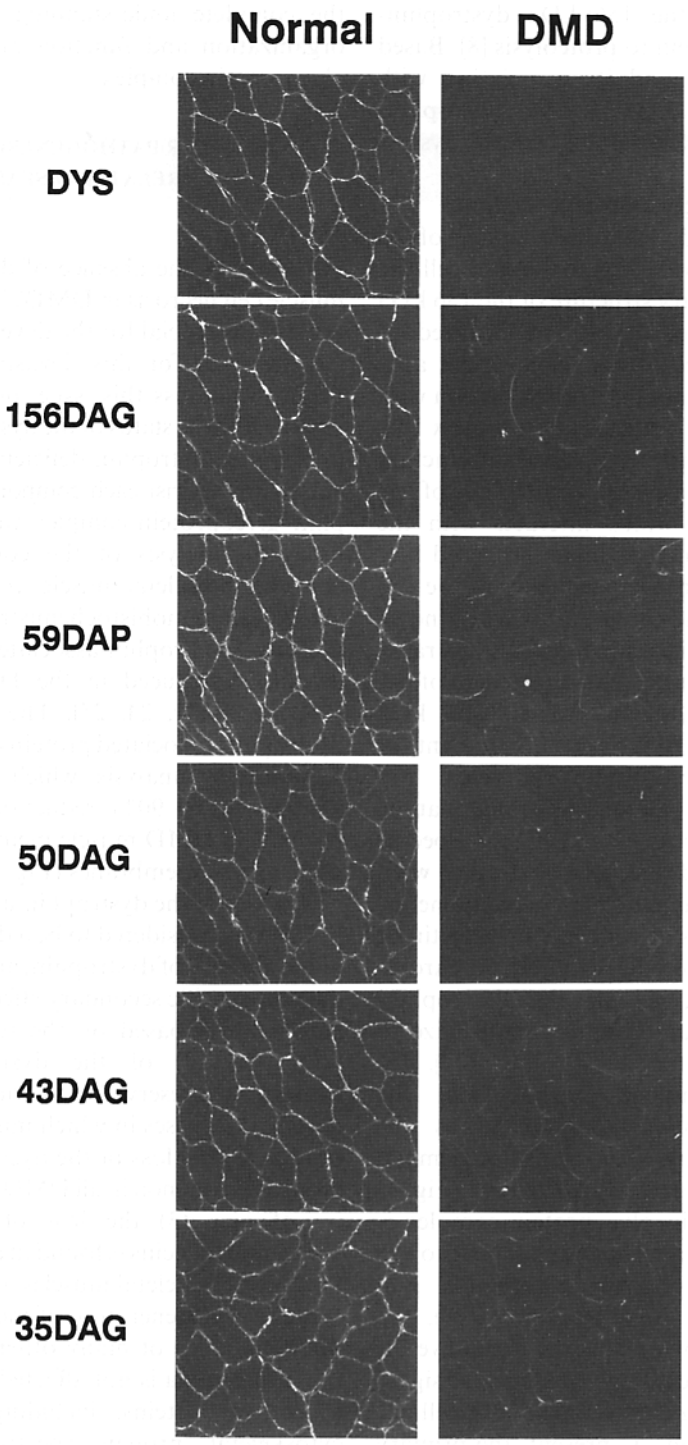

Fig. 2a.

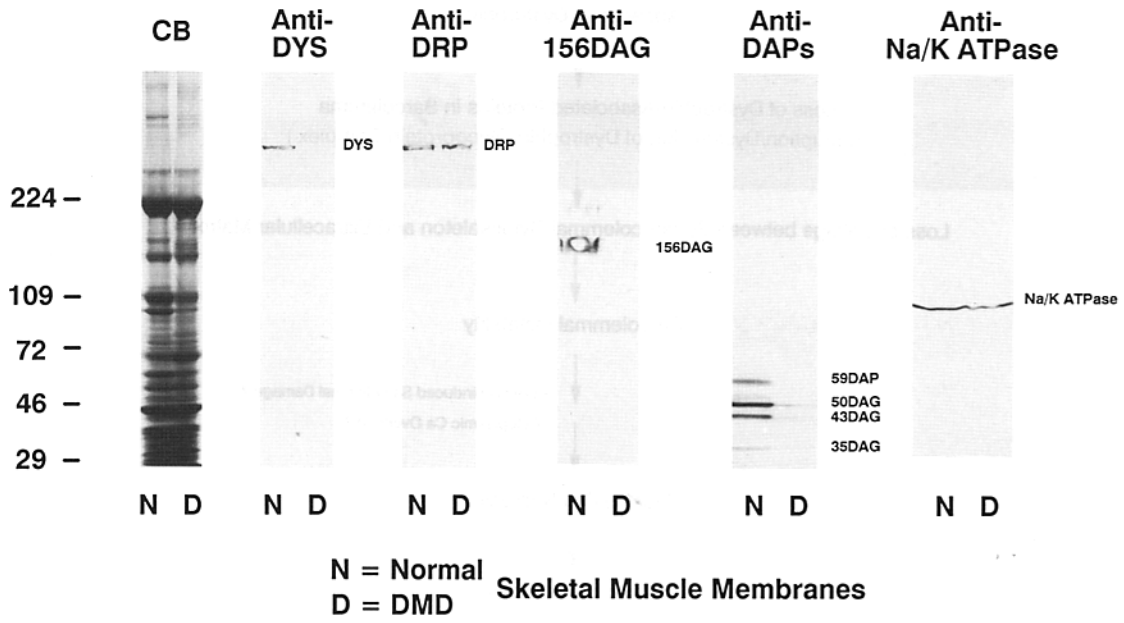

Fig. 2b.

Fig. 2. (a) Immunohistochemical analysis of the components of the dystrophin–glycoprotein complex in normal and DMD skeletal muscle. Abbreviations as follows: 156DAG, 156 kDa dystroglycan; 59DAP, 59 kDa dystrophin-associated protein; 50DAG, 50 kDa dystrophin-associated glycoprotein; 43DAG, 43 kDa dystroglycan; 35DAG, 35 kDa dystrophin-associated glycoprotein. Dystrophin and all the dystrophin-associated proteins are co-localized to the sarcolemma in normal muscle. In DMD, dystrophin is absent and all the dystrophin-associated proteins are greatly reduced in the sarcolemma. (b) Immunoblot analysis of the components of the dystrophin–glycoprotein complex in normal and DMD skeletal muscle membranes. CB shows a gel stained with Coomassie blue. Identical blots were immunostained with different antibodies (Anti-DYS, antibody against dystrophin; Anti-DRP, antibody against dystrophin-related protein (DRP); Anti-156DAG, antibody against the 156 kDa dystroglycan; Anti-DAPs, a cocktail of antibodies against the dystrophin-associated proteins; Anti-Na/K ATPase, antibody against Na/K ATPase). Dystrophin is absent and all the dystrophin-associated proteins are greatly reduced in the DMD membranes. In contrast, DRP, an autosomal homologue of dystrophin, and Na/K ATPase, a sarcolemmal protein, are well preserved in the DMD membranes.

plays a key role in the cascade of events leading to muscle cell necrosis in DMD (Fig. 3) [25]. The absence of dystrophin leads to a drastic reduction in all of the dystrophin-associated proteins in the sarcolemma, causing the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix, which, in turn, may lead to sarcolemmal instability and eventually to muscle cell necrosis [25]. This may be the case, especially during muscle contraction, which may cause physical breaks or tears of the sarcolemma.

DMD patients with dystrophin lacking the cysteine-rich and carboxyl-terminal domains

The domain of the dystrophin molecule which interacts with the dystrophin-associated proteins remains unclear. The C-terminal domains (cysteine-rich and carboxyl-terminal domains) of dystrophin were hypothesized to be involved in the interaction with the dystrophin-associated proteins, based on the following observations: (1) the lack of significant homology between the carboxyl-terminal domain and proteins of known function, except dystrophin-related protein (DRP) [2, 26, 27]; (2) the conservation of the C-terminal domains among different species [28]; and (3) the observation that the phenotype of the patients with mutations in the C-terminal domains is severe [29]. This hypothesis is consistent with both the results of immunogold labeling studies [30, 31] and the results of limited calpain digestion of the dystrophin–glycoprotein complex [32]. The recent demonstration that DRP, which has the C-terminal domains highly homologous to those of dystrophin, is associated with the dystrophin-associated proteins in *mdx* muscle also supports this hypothesis [21]. However, dystrophin lacking the C-terminal domains was reported to be properly localized to the sarcolemma in four unusual patients with DMD, leading to speculation that the C-terminal domains may not be involved in the interaction with the sarcolemma [33–37]. The reason why the phenotype of these patients is severe, in spite of the presence of truncated dystrophin, was unknown [33–37].

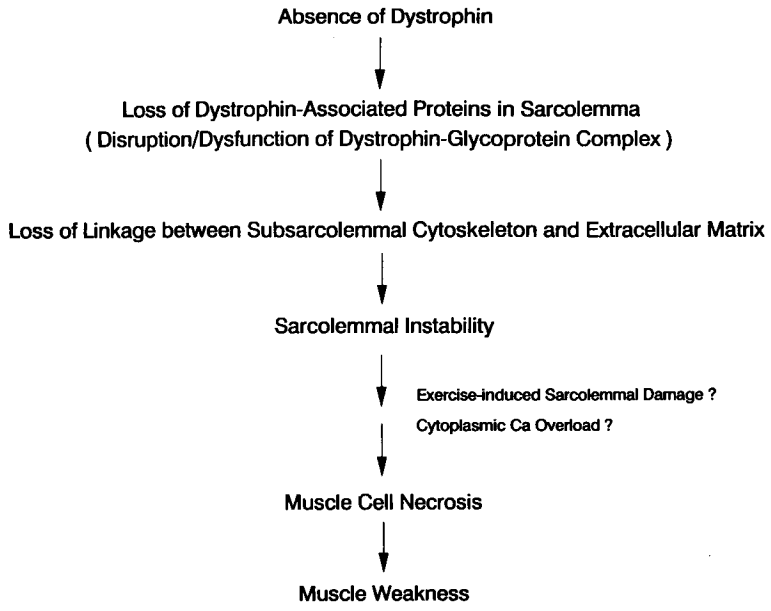

Fig. 3. Hypothetical scheme of the mechanism causing muscle cell necrosis in DMD (modified from [25]).

To address these issues, we investigated the status of the dystrophin-associated proteins in the skeletal muscle from such patients [38]. Immunohistochemistry revealed that all the dystrophin-associated proteins were lost in the sarcolemma, even though dystrophin lacking the C-terminal domains was present and localized to the sarcolemmal region [38]. The results suggest that the domain of dystrophin which interacts with the dystrophin-associated proteins is missing in these patients and, thus, the C-terminal domains of dystrophin are essential for proper stabilization of the dystrophin-associated proteins in the sarcolemma. The disruption of the linkage between the subsarcolemmal cytoskeleton and extracellular matrix, due to the loss of the dystrophin-associated proteins in the sarcolemma, is presumed to be the cause of severe muscular dystrophy in spite of the expression and proper localization of truncated dystrophin [38]. Thus, the status of the dystrophin-associated proteins in the sarcolemma could be more directly correlated with the phenotype of the patients than the status of expression of dystrophin itself [38].

These results also support our hypothesis that dystrophin is associated with both the subsarcolemmal cytoskeleton and the dystrophin-associated proteins in the sarcolemma [8]. Dystrophin lacking the C-terminal domains is presumed to be localized to the sarcolemmal region by the association of its intact actin-binding site in the amino-terminal domain with the sub-sarcolemmal cytoskeletal component(s), such as γ-actin, even when it is not associated with the dystrophin-associated proteins in the sarcolemma.

Immunochemical analysis of the dystrophin-associated proteins in Becker muscular dystrophy (BMD) patients with various deletions or duplications in the dystrophin gene is currently underway in our laboratory. This study could give us insight into not only the molecular pathogenesis of BMD, but also the intramolecular domain of dystrophin involved in the interaction with the dystrophin-associated proteins and the possible role of the actin-binding domain in BMD.

DMD carriers

A mosaic of dystrophin-positive and -negative muscle fibers is observed in DMD carriers [39, 40]. However, the precise mechanism by which the partial deficiency of dystrophin leads to muscle fiber degeneration is unclear. We recently analyzed the status of the dystrophin-associated proteins in a young girl presumed to be a symptomatic DMD carrier [41]. We found that all the dystrophin-associated proteins were lost in the dystrophin-deficient muscle fibers [41]. Thus, the same sarcolemmal instability as in DMD is possibly responsible for the muscle fiber degeneration and high serum creatine kinase level in this patient [41]. Further investigation on

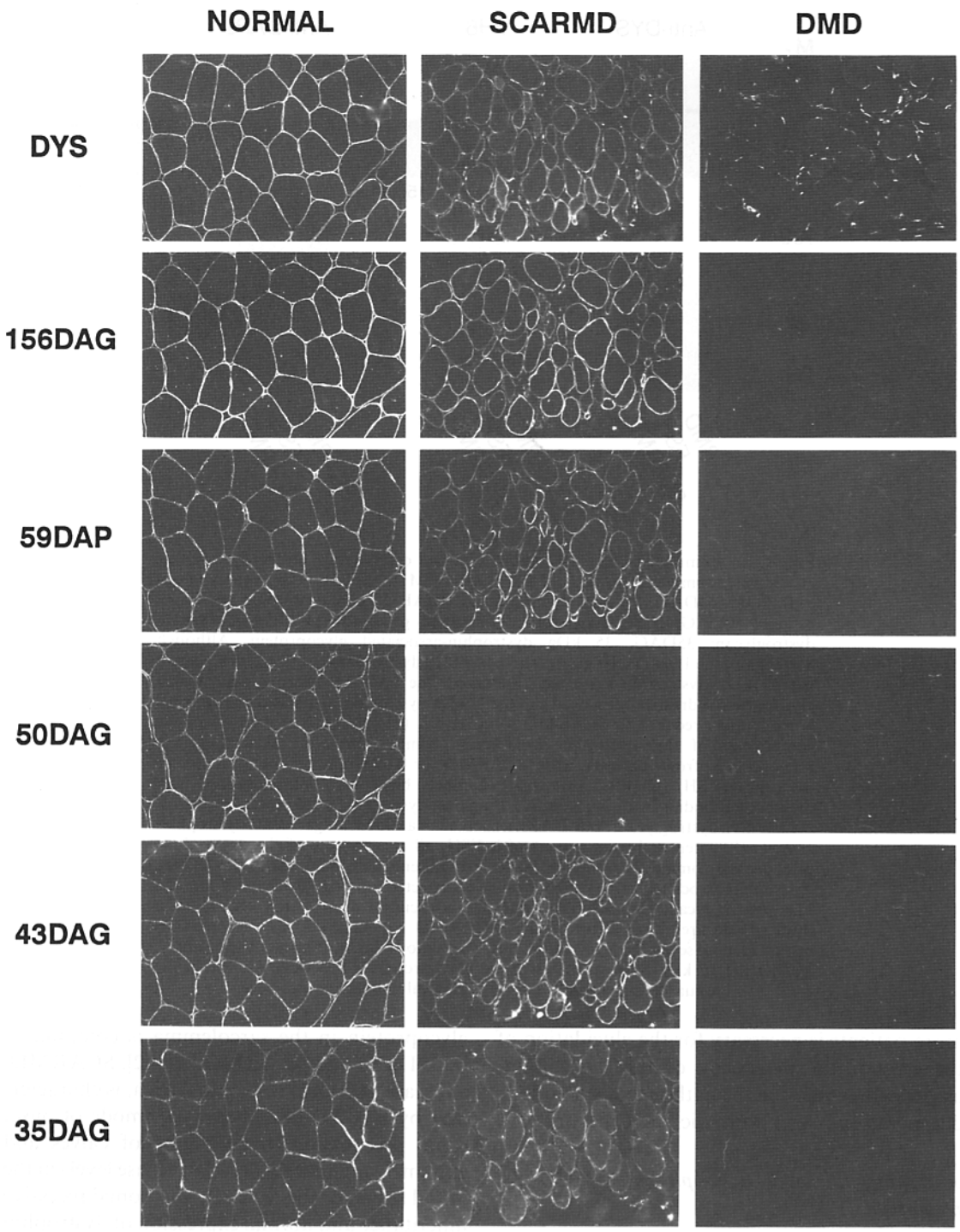

Fig. 4a.

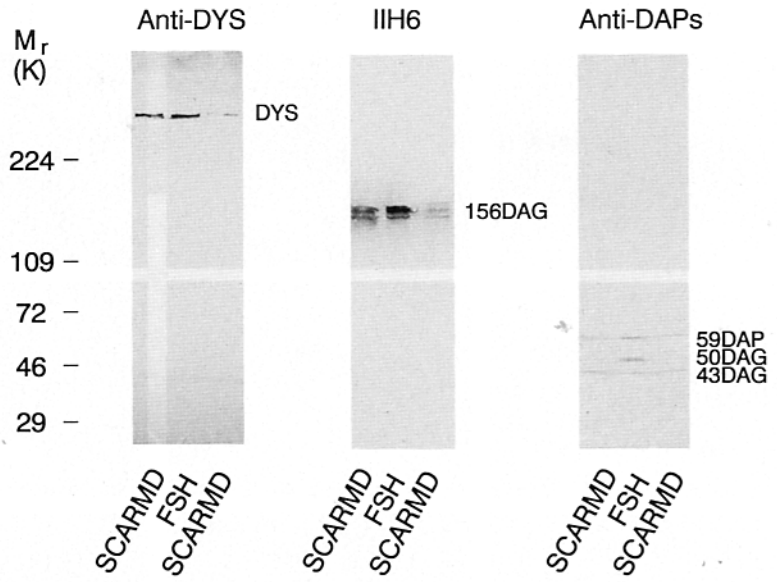

Fig. 4b.

Fig. 4. (a) Immunohistochemical analysis of the components of the dystrophin–glycoprotein complex in normal, SCARMD and DMD skeletal muscle. Abbreviations as follows: 156DAG, 156 kDa dystroglycan; 59DAP, 59 kDa dystrophin-associated protein; 50DAG, 50 kDa dystrophin-associated glycoprotein; 43DAG, 43 kDa dystroglycan; 35DAG, 35 kDa dystrophin-associated glycoprotein. Although dystrophin, the 156 kDa dystroglycan, the 59 kDa dystrophin-associated protein and the 43 kDa dystroglycan are well preserved, the 50 kDa dystrophin-associated glycoprotein is drastically reduced and the 35 kDa dystrophin-associated glycoprotein is moderately reduced in SCARMD sarcolemma. (b) Immunoblot analysis of the components of the dystrophin–glycoprotein complex in crude skeletal muscle homogenates from patients with SCARMD or facioscapulohumeral muscular dystrophy (FSH) (modified from [42]). Identical blots were immunostained with different antibodies (Anti-DYS, antibody against dystrophin; IIH6, antibody against the 156 kDa dystroglycan; Anti-DAPs, a cocktail of antibodies against the dystrophin-associated proteins). In the patient with FSH, all components of the dystrophin–glycoprotein complex are present. In the two patients with SCARMD, the 50 kDa dystrophin-associated glycoprotein cannot be detected although dystrophin, the 156 kDa dystroglycan, the 59 kDa dystrophin-associated protein and the 43 kDa dystroglycan are clearly detected. The SCARMD patient shown in the third lane exhibited more severe symptoms than the patient shown in the first lane. The antibody against the 35 kDa dystrophin-associated glycoprotein is too weak for use in the immunoblot analysis of the crude skeletal muscle homogenates.

more patients is necessary for the elucidation of the role of the deficiency of the dystrophin-associated proteins in the pathogenesis of muscle degeneration in symptomatic DMD carriers.

Autosomal muscular dystrophy with DMD-like phenotype

The structural organization of the dystrophin–glycoprotein complex raised the possibility that the deficiency of a dystrophin-associated protein could be the cause of an autosomal muscular dystrophy with DMD-like phenotype. Recently, we demonstrated the specific deficiency of the 50 kDa dystrophin-associated glycoprotein in severe childhood autosomal recessive muscular dystrophy (SCARMD) (Fig. 4) [42]. Thus, the deficiency of the 50 kDa dystrophin-associated glycoprotein in the sarcolemma is common to both DMD and SCARMD [25, 42]. SCARMD, a disease prevalent in North Africa, is characterized by a DMD-like phenotype (mode of onset rapid progression, hypertrophy of calves and extremely high serum creatine kinase levels in the initial stages of the disease), autosomal recessive inheritance and normal expression of dystrophin [43, 44]. Dysfunction of the dystrophin–glycoprotein complex caused by deficiency of the 50 kDa dystrophin-associated glycoprotein could play a key role in the cascade of events leading to muscle cell necrosis in SCARMD [42].

Does the deficiency of the 50 kDa dystrophin-associated glycoprotein affect the other components of the dystrophin–glycoprotein complex? Immunohistochemistry has demonstrated that the 35 kDa dystrophin-associated

glycoprotein is moderately reduced in the SCARMD sarcolemma, suggesting that the 50 kDa dystrophin-associated glycoprotein and the 35 kDa dystrophin-associated glycoprotein may be closely associated with each other in the dystrophin–glycoprotein complex and that the deficiency of the 50 kDa dystrophin-associated glycoprotein may lead to a secondary reduction of the 35 kDa dystrophin-associated glycoprotein in SCARMD (Fig. 4a). In the advanced stages of SCARMD, all components of the dystrophin–glycoprotein complex, including dystrophin, seem to be reduced (Fig. 4b) [47].

This could raise a serious problem for the differential diagnosis between BMD and SCARMD by the immunoblot analysis of dystrophin. A significant percentage of patients with myopathy are diagnosed as BMD on the basis of immunoblots showing reduced amounts of dystrophin rather than abnormalities in size [15]. Using this diagnostic criterion, the SCARMD patient shown in the third lane of Fig. 4b could have been diagnosed as having BMD. This is especially the case since the phenotype of SCARMD is slightly milder than that of DMD and, thus, is very close to that of severe BMD or so-called "outliers" [15]. Indeed, the phenotype of the BMD patients having quantitative abnormalities of dystrophin was reported to be more severe than those having qualitative abnormalities [15]. Therefore, the immunochemical analysis of the dystrophin-associated proteins, in addition to dystrophin, is needed for the accurate diagnosis of certain muscular dystrophies.

The primary defect causing the deficiency of the 50 kDa dystrophin-associated glycoprotein in SCARMD is unknown. It could be caused by a primary defect in the structure or expression of the gene for the 50 kDa dystrophin-associated glycoprotein or could be due to a secondary effect of unknown primary defect. Recently, the defective gene causing Tunisian autosomal recessive Duchenne-like muscular dystrophy (DLMD) has been localized to the pericentromeric region of chromosome 13q [45]. At present, it is unclear if SCARMD and DLMD are an identical disease. If this is the case, however, it would be of utmost importance to clarify if the DLMD gene encodes the 50 kDa dystrophin-associated glycoprotein itself or a protein which influences the expression of, or functionally interacts with, the 50 kDa dystrophin-associated glycoprotein.

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