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Sarcoglycan Complex Is Selectively Lost in Dystrophic Hamster Muscle

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We recently reported that the dystrophin-associated glycoprotein (DAG) complex is biochemically divided into two subcomplexes: one is the dystroglycan complex comprised of 156DAG and 43DAG and the other is the sarcoglycan complex comprised of 50DAG, A3b, and 35DAG. A3b is a novel dystrophin-associated glycoprotein with an approximate molecular mass of 43 kd but is distinct from 43DAG. In the present study, we examined the striated muscles of the dystrophic hamster with anti-A3b antibody in addition to anti-50DAG, anti-43DAG, anti-35DAG, anti-dystrophin, and anti-laminin antibodies by both immunohistochemistry and immunoblot analysis and found that 50DAG, A3b, and 35DAG are selectively lost. This selective defect of the sarcoglycan complex in dystrophic hamster muscles may give rise to dystrophic changes in striated muscles. Thus, the differentiation of the dystrophin-associated glycoprotein complex into the dystroglycan and sarcoglycan complexes is important not only from a biochemical standpoint but also in understanding the cause of muscular dystrophy in the hamster. Our findings further show that the dystrophic hamster may serve as an animal model for a human disease, severe childhood autosomal recessive muscular dystrophy, which has recently been shown to result from a selective defect in the sarcoglycan complex. (Am J Pathol 1995, 146:530–536)

Dystrophin, a loss of which causes Duchenne muscular dystrophy (DMD),[1,2] is present in normal muscle at the inner surface of the sarcolemma[3] and is associated with actin filament[4] and dystrophin-associated proteins (DAPs)[5] at its amino- and carboxyl-terminal regions, respectively. DAPs are classified into membrane-integrated and nonintegrated proteins.[6,7] The former are the membranous dystrophin-associated glycoproteins (DAGs) that are known to exist in the large oligomeric glycoprotein complex[6,8] and the latter are the peripheral proteins,[6,7] that is, α-[9] and β1-syntrophin[10] (collectively 59DAP) and A0.

Ervasti et al[11] and Ohlendieck et al[12] reported that all DAPs are greatly reduced by up to 90% in amount in DMD muscles. In contrast to these results, we found that in DMD muscle, although 43DAG is decreased in amount it is still fairly well preserved,[13,14] whereas 50DAG and 35DAG are decreased to a greater degree than 43DAG,[14,15] showing that the extent of reduction differs depending on the glycoprotein. Matsumura et al[16] observed that a deficiency of 50DAG and a reduction of 35DAG give rise to a DMD-like disease called severe childhood autosomal recessive muscular dystrophy (SCARMD). These observations suggest that a loss of certain DAPs can cause dystrophic changes in muscle fibers. Meanwhile, Roberds et al[17] reported that 43DAG is present in apparently equal amounts in control and dystrophic hamster skeletal muscles whereas 50DAG is undetectable and 35DAG appears to be slightly decreased in dystrophic muscle. Their findings differ slightly from ours.[18] We found that, in dystrophic hamster skeletal muscle, not only 50DAG but also 35DAG is greatly reduced whereas 43DAG is present at an almost normal level.[18] There are qualitative differences in the composition of dystrophin-associated glycoprotein complex. We found that 43DAG is ubiquitously present in a variety of tissues[19] whereas 50DAG and 35DAG are present only in striated muscles.[15,19]

Very recently, by gel filtration after treatment of the purified dystrophin-DAP complex with octyl β-D-

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glucoside[20] we found that the glycoprotein complex is divided into two subcomplexes. One complex consists of 156DAG and 43DAG, which we named the dystroglycan complex after Ibraghimov-Beskrovnaya et al[21] who reported that both glycoproteins are encoded by a single mRNA and then derived post-translationally from a single precursor protein, dystroglycan.[21] Our report shows that these glycoproteins bind to form a complex after post-translational processing. The other, composed of 50DAG, A3b, and 35DAG, was named the sarcoglycan complex by us[20] because of its selective presence in striated muscles, although we did not determine the tissue distribution of A3b. A3b was originally described as being the smaller component of the 43DAG doublets[8,13] but was recently found to be a novel DAG distinct from 43DAG.[20] However, its relation to dystrophic changes of the muscle fiber has not yet been elucidated.

Purified A3b was obtained by the gel filtration method mentioned above, which allowed us to raise a polyclonal antibody against it.[20] In this study, we examined whether structural defects in the glycoprotein complex are involved in the dystrophic changes in hamster skeletal and cardiac muscles. We found that the defect in the dystrophic hamster is mainly confined to the sarcoglycan complex and that dystrophin, 43DAG, and laminin are well preserved. Therefore, it is tempting to speculate that the gene defect in the dystrophic hamster may be localized to one of the genes coding for 50DAG, A3b, or 35DAG, which are the components of the sarcoglycan complex, and that the loss of the entire complex may result from the absence of only one of its components.

Materials and Methods

Muscles

Skeletal and cardiac muscles of dystrophic hamsters aged 6 to 12 months introduced from the BIO14.6 to the NSJ strain (NSJ-my/my) and control hamsters (NSJ-+/+) were examined.

Antibodies

Monoclonal anti-dystrophin antibody (DY2,[22] Novocastra Inc., Newcastle, UK), monoclonal anti-35DAG antibody (MA4–2[15]), and four polyclonal antibodies, anti-50DAG (PA2[7]), anti-43DAG (PA3a[7,13]), anti-A3b (PA3b[20]), and anti-laminin antibody (E-Y Inc., San Mateo, CA), were used.

Immunohistochemistry

Sections were prepared as described previously.[23] In brief, after incubation with the primary antibodies (anti-dystrophin, anti-50DAG, anti-43DAG, anti-A3b, anti-35DAG, and anti-laminin), the sections were washed with phosphate-buffered saline. They were then incubated with fluorescein isothiocyanate-labeled goat $F(ab')_2$ anti-rabbit IgG (Caltag Inc., South San Francisco, CA) in the cases of anti-50DAG, anti-43DAG, and anti-laminin; with fluorescein isothiocyanate-labeled affinity-purified antibody to mouse immunoglobulin G (IgG; Kpl Inc., Gaithersburg, MD) in the cases of anti-dystrophin and anti-35DAG; and with dichlorotriazinyl aminofluorescein-labeled goat $F(ab')_2$ anti-guinea pig IgG (Chemicon Inc., Temecula, CA) in the case of anti-A3b. After a second washing, the sections were mounted, examined, and photographed.

Immunoblot Analysis

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional PAGE were carried out as described previously.[19] Immunochemical staining of proteins was performed as described previously.[19] In brief, after incubation with the primary antibodies (anti-dystrophin, anti-50DAG, anti-43DAG, anti-A3b, and anti-35DAG) and washing, horseradish peroxidase-labeled anti-mouse IgG (Kpl Inc., Gaithersburg, MD) and horseradish peroxidase-labeled anti-rabbit IgG (Cappel Inc., West Chester, PA) were used in the cases of anti-dystrophin and anti-35DAG or anti-50DAG and anti-43DAG, respectively. Biotin-labeled anti-guinea pig IgG (Vector Laboratories, Burlingame, CA) was used in the case of anti-A3b. Finally, the protein on the membrane was visualized with 0.05% diaminobenzidine tetrahydrochloride and 0.035% hydrogen peroxide in 100 mmol/L Tris-HCl.

Before the membrane was incubated with anti-A3b, however, an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) was used to prevent endogenous nonspecific staining.

Results

Skeletal Muscle

As shown in Figure 1, anti-A3b (D) homogeneously stained the sarcolemma of control muscle as did anti-dystrophin (A), anti-43DAG (B), anti-50DAG (C), and anti-35DAG (E). On the other hand, anti-A3b (I), anti-50DAG (H), and anti-35DAG (J) hardly stained the

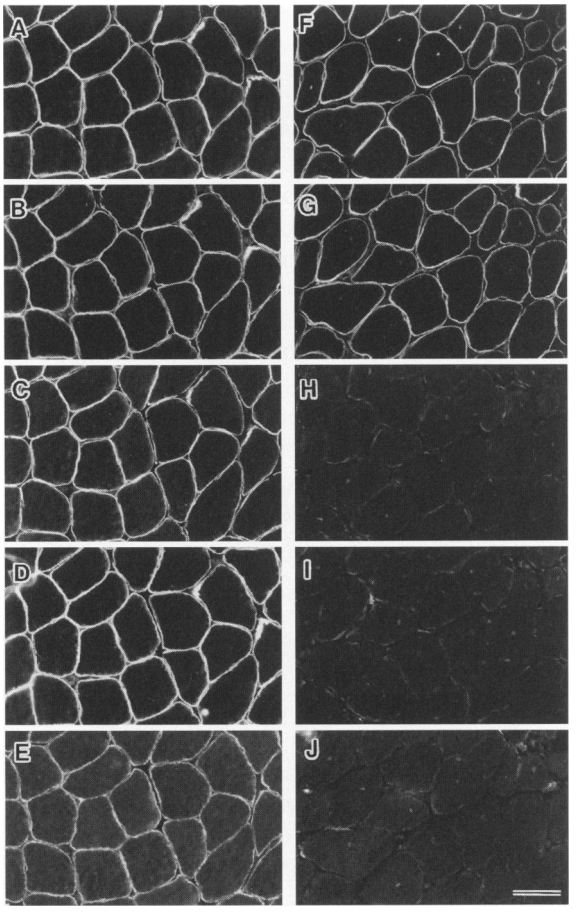

Figure 1. *Immunohistochemistry of serial sections of control* (A *to* E) *and dystrophic* (F *to* J) *skeletal muscles stained with anti-dystrophin* (A *and* F), *anti-43DAG* (B *and* G), *anti-50DAG* (C *and* H), *anti-A3b* (D *and* I), *and anti-35DAG* (E *and* J). *Control skeletal muscles were positively stained with all antibodies* (A *to* E). *Although dystrophic skeletal muscles were stained with an intensity almost equal to that observed in control muscle with anti-dystrophin* (F) *and anti-43DAG* (G), *they were hardly stained with anti-50DAG* (H), *anti-A3b* (I), *and anti-35DAG* (J). *Bar = 50 μ.*

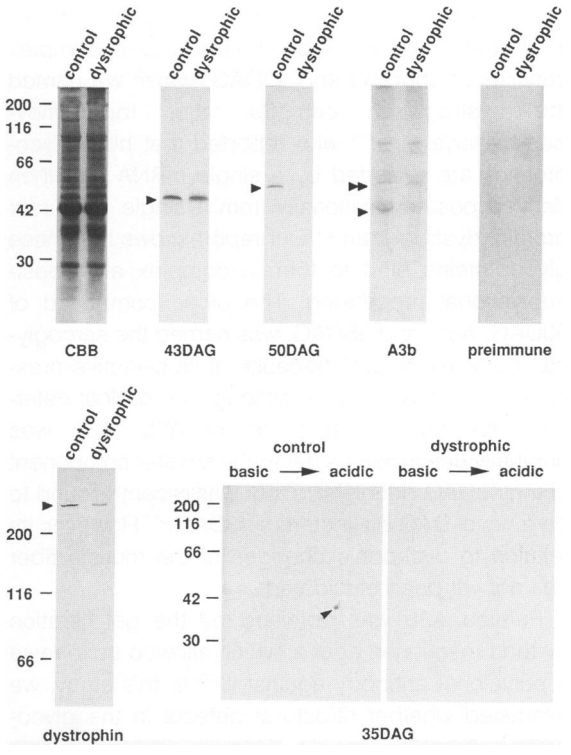

Figure 2. *Immunoblot analyses of control and dystrophic skeletal muscles stained with Coomassie brilliant blue, anti-43DAG, anti-50DAG, anti-A3b, preimmune serum of anti-A3b, anti-dystrophin, and anti-35DAG. The samples of control and dystrophic skeletal muscle extracts were placed on the left and right sides, respectively, of each blot. Because it was difficult to detect the 35DAG band with one-dimensional SDS-PAGE, two-dimensional PAGE analysis was used in the case of anti-35DAG.*[15] *Single arrowhead indicates respective bands or spot. A double arrowhead indicates the band of unknown origin. The positions of molecular markers (kd) are indicated on the left. Coomassie brilliant blue and six antibodies used are indicated at the bottom. A gel containing 6% polyacrylamide was used for the samples stained with anti-dystrophin, and gels containing 10% polyacrylamide were used for the samples stained with Coomassie brilliant blue, anti-50DAG, anti-43DAG, anti-A3b, preimmune serum of A3b, and anti-35DAG. For details see text.*

sarcolemma of dystrophic muscle, whereas anti-dystrophin (F) and anti-43DAG (G) stained it with an intensity similar to that observed in control muscle. Neither control nor dystrophic muscle was stained by the preimmune serum of anti-A3b (data not shown).

When the SDS extract of control muscle separated by SDS-PAGE was stained with anti-A3b (Figure 2), two characteristic bands were detected in addition to the bands assumed to be the endogenous biotin or biotin-binding protein bands that were stained by the preimmune serum. One was an approximately 53-kd band (double arrowhead) and the other was an approximately 42-kd band (single arrowhead). We assume that the 42-kd band corresponds to A3b, as the molecular mass of A3b is similar to that of 43DAG.[8] Mobility of this band might have been influenced to some extent by the presence of actin, as A3b migrates in close proximity to actin, which is one of the most abundant proteins in muscle. The quantity of the sample from dystrophic muscle is almost the same as that of the sample from control muscle, judging by the intensity of Coomassie brilliant blue staining. In dystrophic muscle, anti-A3b stained the 53-kd band but not the 42-kd band, corresponding to A3b. As we reported previously,[18] the bands were stained with slightly less intensity with anti-dystrophin and anti-43DAG in dystrophic muscle compared with those in control muscle. However, the band corresponding to 50DAG and the spot corresponding to 35DAG, like the band corresponding to A3b, were not found in dystrophic muscle. The immunohistochemistry and immunoblot analysis demonstrated a selective defect of the sarcoglycan complex.

Cardiac Muscle

As shown in Figure 3, anti-A3b (D) as well as the other antibodies stained the membranes, including T tubules of control muscle, as found in a previous study with anti-dystrophin and other anti-DAPs.[17,24] Anti-A3b (I), anti-50DAG (H), and anti-35DAG (J) hardly stained the membranes of dystrophic muscle, whereas anti-dystrophin (F) and anti-43DAG (G) clearly stained them. The preimmune serum of anti-A3b did not stain the membranes of control and dystrophic muscles (data not shown). The immunohistochemical findings in cardiac muscle, therefore, were similar to those in skeletal muscle, although the background staining in cardiac muscle was fairly strong.

The results of immunoblot analysis were similar to those for the skeletal muscle (Figure 4). The intensity of the band stained with anti-A3b in cardiac muscle was somewhat greater than that in skeletal muscle.

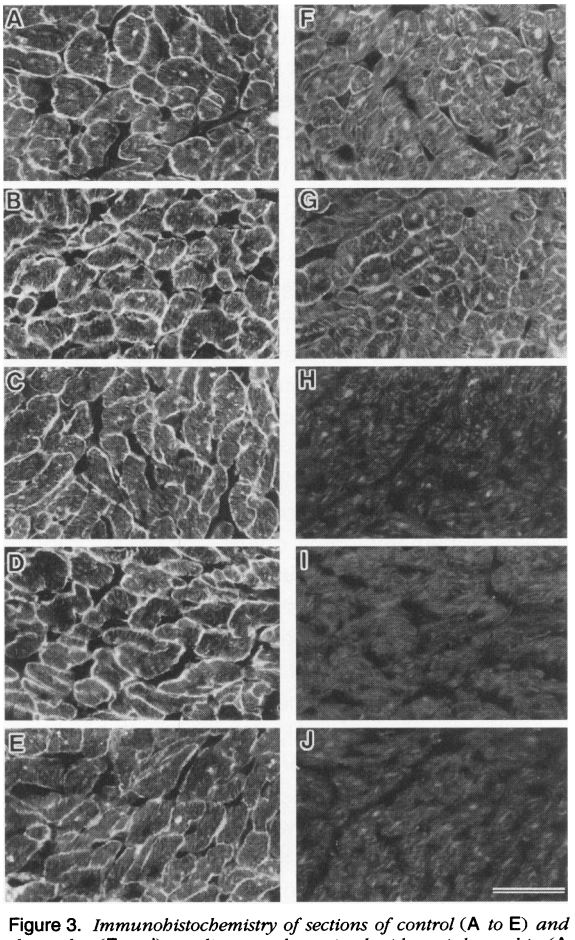

Figure 3. *Immunohistochemistry of sections of control* (**A** *to* **E**) *and dystrophic* (**F** *to* **J**) *cardiac muscles stained with anti-dystrophin* (**A** *and* **F**), *anti-43DAG* (**B** *and* **G**), *anti-50DAG* (**C** *and* **H**), *anti-A3b* (**D** *and* **I**), *and anti-35DAG* (**E** *and* **J**). *For details see text. Bar = 50 μ.*

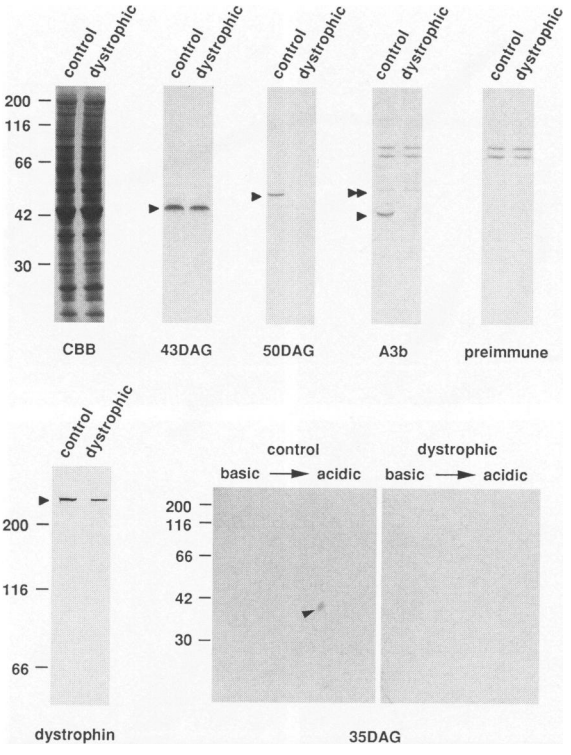

Figure 4. *Immunoblot analyses of control and dystrophic cardiac muscles stained with Coomassie brilliant blue, anti-43DAG, anti-50DAG, anti-A3b, preimmune serum of anti-A3b, anti-dystrophin, and anti-35DAG. The placement of samples is the same as that in Figure 2.*

Laminin Staining

Anti-laminin clearly stained the sarcolemma of skeletal muscle (Figure 5A, C) and the membranes of cardiac muscle (B, D) in control (A, B) and dystrophic (C, D) hamsters. There was little difference in the results of immunohistochemistry between control and dystrophic muscles.

Discussion

We have shown (Figure 6) that the dystrophin-associated glycoprotein complex binds to the cysteine-rich domain and the first half of the carboxyl-terminal domain of dystrophin[5] and that these domains bind to 43DAG among the components of glycoprotein complex,[25] and we have reported that 43DAG binds to 156DAG.[20] As 43DAG has a transmembranous domain in its internal structure and 156DAG binds to laminin, a component of the basement membrane,[21] it is very plausible that the complex composed of 43DAG and 156DAG, namely, the dystroglycan complex, serves as a link between dystrophin, a subsarcolemmal cytoskeletal protein, and the basement membrane. The sarcoglycan complex,

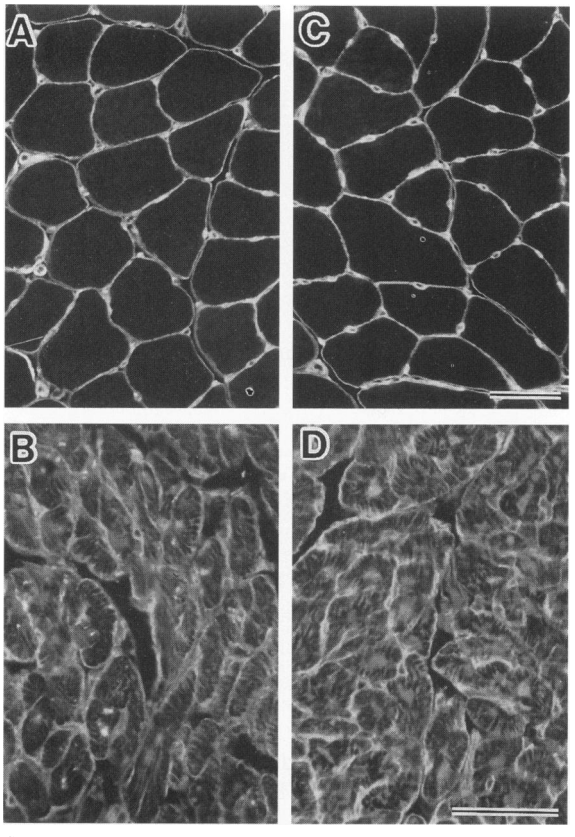

Figure 5. *Immunohistochemistry of sections of control skeletal* (A) *and cardiac* (B) *muscles and dystrophic skeletal* (C) *and cardiac* (D) *muscles stained with anti-laminin. Anti-laminin stained the sarcolemma of control and dystrophic muscles. Bar = 50 μ.*

on the other hand, does not appear to have a similar function, because none of the components of the sarcoglycan complex bind to either dystrophin or laminin. Therefore, it is likely that the dystroglycan and sarcoglycan complexes have different functional roles. This is further supported by the difference in tissue distribution of these complexes.[15,19]

We recently identified A3b as a novel DAP having molecular mass of 43 kd, but being distinct from 43DAG, and showed that A3b is a component of the sarcoglycan complex that, together with the dystroglycan complex, comprises the glycoprotein complex (Figure 6).[20] We reported previously[18] that in dystrophic hamster skeletal muscle the levels of 50DAG and 35DAG are greatly reduced whereas 43DAG is present at almost normal levels. These results indicate that some components of a protein group, now known as the sarcoglycan complex, are defective in this muscle, although at that time we were unable to prove this definitively. The introduction of octyl β-D-glucoside into the purified dystrophin-DAP complex enabled us to separate A3b from 43DAG and raise a polyclonal antibody against A3b.[20]

In the present study, we reinvestigated the skeletal muscle as well as the cardiac muscle of control and dystrophic hamsters with various antibodies, including anti-A3b. The results clearly showed that the sarcoglycan complex is greatly reduced or lost in the striated muscles of the dystrophic hamster, whereas dystrophin, 43DAG, and laminin are well preserved. The dystroglycan complex is also assumed to be preserved, although we did not test for 156DAG, which has already been shown to be preserved in the dystrophic hamster skeletal muscle.[17] Therefore, the linkage via 43DAG and 156DAG between dystrophin and laminin may remain intact,[26] even though the sarcoglycan complex is greatly reduced. With the deficiency of the sarcoglycan complex, muscles might degenerate despite the presence of the dystroglycan complex. In other words, merely the presence of the linkage between the subsarcolemmal cytoskeleton and the basement membrane is insufficient to prevent striated muscles from degenerating. Most likely, the sarcoglycan complex plays an essential role in maintaining normal muscle structure and function.

We have found that all three components of the sarcoglycan complex are lost in the dystrophic hamster muscle. However, it is not likely that the disease is due to a simultaneous defect in these three genes; the disease may be caused by a defect in any one of these three genes. When the protein product of the affected gene is abnormal, the sarcoglycan complex might not be formed and thus not be detected by immunochemical methods. Another possibility, which appears to be less likely, is that a defect in an undetermined gene, the protein product of which serves as a *cis* regulatory element that simultaneously regulates the genes of these three proteins, is the cause of the disease. In any case, additional studies are required to clarify these questions.

To accept the dystrophic hamster as an animal model for the SCARMD patient, it is necessary to identify some pathological changes in the dystrophic hamster muscle that are similar to those in SCARMD muscle. SCARMD was first reported to result from a deficiency of 50DAG.[16] Later, it was found that the deficiency of 50DAG is not always the primary cause of all cases of SCARMD. The genetic defect that leads to a 50DAG deficiency in Algerian families is localized to chromosome 13q[27] whereas the causative gene is not present in 13q for a Duchenne-like muscular dystrophy in Brazilian families with a 50DAG deficiency.[28] Therefore, it is likely that the genetic abnormalities among SCARMD patients are heterogeneous as postulated by Passos-Bueno et al.[28] We have found that in SCARMD muscle all components of the sarcoglycan complex is greatly reduced or lost.[29] Therefore,

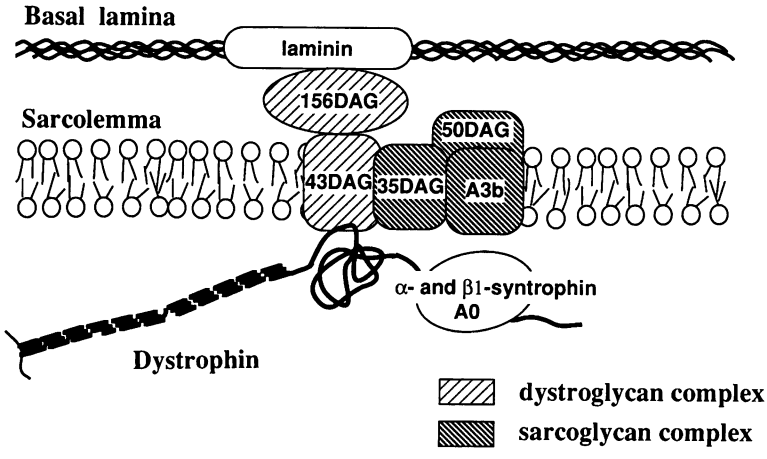

Figure 6. *Molecular architecture of dystrophin and dystrophin-associated proteins. (Modified from Suzuki et al[25])*

whatever the primary cause of SCARMD, the development of the disease may be due to the lack of the sarcoglycan complex. We can thus consider the dystrophic hamster to be a good model for SCARMD, although phenotypical expression of degenerative change is masked in the dystrophic hamster as it is in the case of *mdx* mice even though the *mdx* mice are widely used as the animal model for DMD patient.

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