

# Animal Model

## Molecular Pathogenesis of Muscle Degeneration in the $\delta$ -Sarcoglycan-Deficient Hamster

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**The BIO14.6 hamster is an extensively used animal model of autosomal recessive cardiomyopathy and muscular dystrophy. Recently, a large deletion in the 5' end of the  $\delta$ -sarcoglycan gene was found to be the primary genetic defect in the hamster. In the present investigation, we studied the effects of the  $\delta$ -sarcoglycan deletion on transcription, expression, and function of the dystrophin-glycoprotein complex in skeletal and cardiac muscle. We demonstrated that in striated muscle the genetic defect leads to the complete deficiency of  $\delta$ -sarcoglycan and a concomitant loss of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sarcoglycan. In addition, absence of the sarcoglycan complex reduced the expression of  $\alpha$ -dystroglycan in striated muscle fibers. These findings indicated that the primary defect in the BIO14.6 hamster leads to the dissociation of the dystroglycan complex from the sarcoglycan complex and disrupted anchorage of  $\alpha$ -dystroglycan to the cell surface. Using intravenous injection of Evans blue dye as an *in vivo* tracer assay, we demonstrated that perturbation of the dystrophin-glycoprotein complex caused extensive fiber damage in skeletal and cardiac muscle of the BIO14.6 hamster. Based on our results, we propose that loss of  $\delta$ -sarcoglycan results in the impairment of sarcolemmal integrity, finally leading to muscular dystrophy and cardiomyopathy. (*Am J Pathol* 1998, 153:1623–1630)**

Dystrophin, the protein product of the Duchenne muscular dystrophy gene, is associated with a large oligomeric complex of sarcolemmal proteins and glycoproteins in striated muscle.<sup>1,2</sup> The dystrophin-glycoprotein complex (DGC), which consists of cytoskeletal elements and membrane proteins, links the F-actin cytoskeleton to the extracellular matrix.<sup>3</sup> At the cytoplasmic side of the oligo-

meric complex, dystrophin binds actin filaments along its amino terminus and spectrin-like rod domain.<sup>4</sup> With its carboxyl-terminal region, dystrophin binds to the membrane-spanning dystroglycan complex, which is composed of  $\alpha$ - and  $\beta$ -subunits.<sup>5</sup> The heterotrimeric basement membrane protein laminin 2 serves as the major extracellular ligand of  $\alpha$ -dystroglycan in striated muscle cells.<sup>5</sup> Furthermore, the sarcoglycan complex, composed of at least four single-pass transmembrane proteins,<sup>6</sup> the syntrophins,<sup>7</sup> and the recently characterized 25-kd protein sarcospan,<sup>8</sup> are closely associated with dystrophin and dystroglycan.

Several components of the DGC have been implicated in various forms of muscular dystrophy.<sup>9,10</sup> Mutations in the genes for  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan are responsible for four phenotypically similar classes of autosomal recessive limb-girdle muscular dystrophy (LGMD 2C-F).<sup>11–16</sup> Recently, a mutation in the  $\delta$ -sarcoglycan gene of the BIO14.6 cardiomyopathic hamster had been identified to cause the disease in this animal strain.<sup>17,18</sup> The BIO14.6 hamster exhibits histological features of muscular dystrophy characterized by central nucleation, wide variation in muscle fiber diameter, and necrosis.<sup>19–21</sup> In contrast to most of the reported patients with sarcoglycan deficiency, the hamsters develop cardiomyopathy leading to heart failure and death within one-half to one-third of their normal life span.<sup>22</sup>

In the present study, we have investigated the pathological manifestations of the  $\delta$ -sarcoglycan gene mutation in the cardiomyopathic hamster. We demonstrated by immunohistochemical and biochemical analysis that  $\delta$ -sarcoglycan was absent in skeletal and cardiac muscle. As a consequence of the  $\delta$ -sarcoglycan deficiency, we observed a concomitant loss of the other sarcoglycans and reduced expression levels of  $\alpha$ -dystroglycan in

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Supported by a grant from the Deutsche Forschungsgemeinschaft (Str 498/1–1 to V. Straub) and by the Muscular Dystrophy Association (to K.P. Campbell) and the L'Association Française contre les Myopathies (to K.P. Campbell). K.P. Campbell is an Investigator of the Howard Hughes Medical Institute.

Accepted for publication August 5, 1998.

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skeletal and cardiac muscle. These findings indicate that the primary defect in the BIO14.6 hamster leads to the dissociation of the dystroglycan complex from the sarcoglycan complex. Using an *in vivo* tracer assay, we demonstrated that in skeletal and cardiac muscle fibers the  $\delta$ -sarcoglycan mutation impairs sarcolemmal integrity in the cardiomyopathic hamster.

## Materials and Methods

### Animals

Male F1B control and BIO14.6 cardiomyopathic hamsters were obtained from BioBreeders (Fitchburg, MA). A colony of  $\delta$ -sarcoglycan-deficient hamsters was established at the University of Iowa by breeding female Golden Syrian hamsters (Charles River Laboratories, Wilmington, MA) with male BIO14.6 cardiomyopathic hamsters. The F1 generation females were bred with male BIO14.6 cardiomyopathic hamsters to produce the F2 generation. The offspring from the F2 generation were biopsied to identify  $\delta$ -sarcoglycan deficiency. Animals homozygous for the  $\delta$ -sarcoglycan mutation were used for further breeding. All animals were kept in the animal care unit of the University of Iowa College of Medicine according to animal care guidelines. We studied a total of 30 animals ranging from 2 to 56 weeks of age. For our studies, the F1B strain controls were age and sex matched with the cardiomyopathic BIO14.6 strain animals.

### Antibodies

Antibodies against the following components of the DGC were used: dystrophin,  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan,  $\alpha$ - and  $\beta$ -dystroglycan, and the laminin  $\alpha$ 2 chain. Monoclonal antibodies VIA4<sub>2</sub> against dystrophin,<sup>23</sup> IIH6 against  $\alpha$ -dystroglycan,<sup>24</sup> 8D5 against  $\beta$ -dystroglycan,<sup>12</sup> and rabbit polyclonal antibodies against  $\alpha$ -sarcoglycan<sup>25</sup> and the laminin  $\alpha$ 2 chain<sup>26</sup> were described previously. Monoclonal antibodies 20A6 against  $\alpha$ -sarcoglycan, 5B1 against  $\beta$ -sarcoglycan, and 21B5 against  $\gamma$ -sarcoglycan were generated in collaboration with Louise V. B. Anderson (Newcastle General Hospital, Newcastle on Tyne, UK). An affinity-purified rabbit antibody (rabbit 208) was produced against a carboxyl-terminal GST-fusion protein of  $\gamma$ -sarcoglycan. Peptide antibodies were generated against an amino-terminal peptide (MMPQEYTHHRST-MPGAAC) of  $\delta$ -sarcoglycan (rabbit 215). Polyclonal antibodies against  $\alpha$ -dystroglycan fusion protein D were affinity purified from goat 20.<sup>27</sup>

### Immunofluorescence Analysis

The heart, femoral quadriceps, sural triceps, tongue, and diaphragm muscles were sampled in all animals. Additional skeletal muscle samples were taken from muscles that showed visible Evans blue staining. All tissues were embedded in Tissue-Tek OCT mounting media (Miles Laboratories, Elkhart, IN) and frozen in liquid nitrogen-cooled isopentane. Immunofluorescence microscopy of

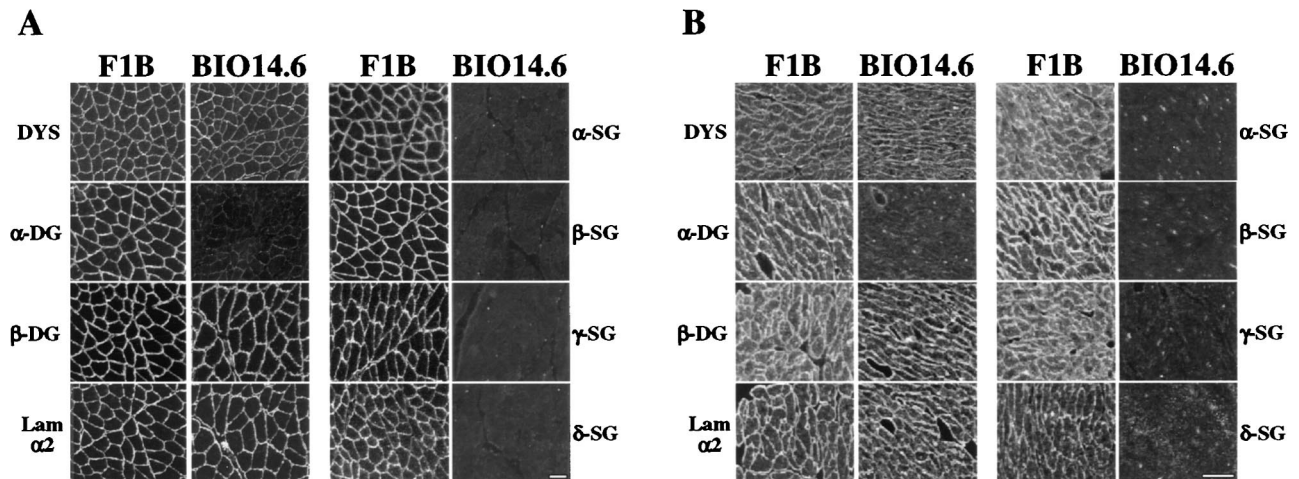
7- $\mu$ m cryosections was performed as described before.<sup>28,29</sup> Briefly, sections were blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes and then incubated with the primary antibodies for 90 minutes in 1% BSA/PBS. After washing with 1% BSA/PBS, sections were incubated with biotinylated secondary antibodies (1:500) for 30 minutes, washed with 1% BSA/PBS, and then incubated with fluorescein-isothiocyanate-conjugated streptavidin (1:1000) for 30 minutes. After a rinse with PBS, sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Histochemical examination of tissues was performed using hematoxylin and eosin (H&E) staining. Sections were observed under a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Thornwood, NY) or a MRC-600 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA).

### Northern Blot Analysis

Total RNA from control F1B and BIO14.6 skeletal and cardiac muscle was extracted using RNazol (Tel-Test, Friendswood, TX) according to manufacturer specifications, and 40  $\mu$ g of total RNA was electrophoresed on a 1.25% agarose gel containing 5% formaldehyde and transferred to Hybond N membrane (Amersham Corp., Arlington Heights, IL). RNA was cross-linked to the membrane using an ultraviolet cross-linker (Stratagene, La Jolla, CA). Membranes were then prehybridized and hybridized in 20% formamide, 4X SSPE, 5X Denhardt's, 5% SDS, 10% dextran sulfate, 40 mmol/L HCl, 0.2 mg/ml yeast RNA, and 0.4 mg/ml single-stranded DNA at 65°C with polymerase chain reaction (PCR)-labeled or random-primed cDNA. Washes were carried out at 65°C in 1X SSC/1% SDS initially and then 0.2X SSC/1% SDS. As a control for loading, human actin cDNA (Clontech Laboratories, Palo Alto, CA) was used as a probe.

### Sucrose Gradient Fractionation Analysis of Purified Dystrophin-Glycoprotein Complex

KCl-washed microsomes were prepared from age-matched control and BIO14.6 hamster skeletal muscle.<sup>30</sup> KCl-washed microsomes (450 mg) were solubilized in 100 ml of 50 mmol/L Tris/HCl, pH 7.4, 500 mmol/L NaCl containing 1% digitonin (Sigma Chemical Co., St. Louis, MO). The solubilized proteins were circulated overnight on a 10-ml wheat germ agglutinin-agarose column (Vector Laboratories). The columns were washed with 100 ml of 50 mmol/L Tris/HCl, pH 7.4, 500 mmol/L NaCl containing 0.1% digitonin and eluted with 0.3 mol/L *N*-acetylglucosamine in 50 mmol/L Tris/HCl, pH 7.4, 500 mmol/L NaCl containing 0.1% digitonin. The eluted solution was diluted to 100 mmol/L NaCl with 50 mmol/L Tris/HCl, pH 7.4, containing 0.1% digitonin and applied to a DEAE-cellulose (3-ml) column and washed with 50 ml of 50 mmol/L Tris/HCl, pH 7.4, 100 mmol/L NaCl containing 0.1% digitonin. The column was eluted with a gradient of 100 mmol/L to 300 mmol/L NaCl buffer containing 50 mmol/L Tris/HCl, pH 7.4, 0.1% digitonin. The fractions



**Figure 1.** Skeletal (femoral quadriceps) muscle (A) and cardiac muscle (B) cryosections of control (F1B) and BIO14.6 hamsters were stained with antibodies against dystrophin (DYS),  $\alpha$ -dystroglycan ( $\alpha$ -DG),  $\beta$ -dystroglycan ( $\beta$ -DG), laminin  $\alpha 2$  chain (LAM- $\alpha 2$ ), and  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan (SG). All proteins are expressed at the plasma membrane of skeletal and cardiac fibers in control animals. The  $\delta$ -sarcoglycan mutation in the BIO14.6 hamster leads to the absence of  $\delta$ -sarcoglycan from the sarcolemma and to a concomitant reduction of the other sarcoglycans and  $\alpha$ -dystroglycan. Bar, 50  $\mu$ m (A) and 20  $\mu$ m (B).

containing DGC were pooled and concentrated to 800  $\mu$ l. The samples were applied to a 5% to 30% sucrose gradient and centrifuged with a Beckman VTi65.1 vertical rotor at 200,000  $\times g$  for 90 minutes at 4°C. The gradients were fractionated into 1-ml fractions. KCl-washed microsomes from skeletal and cardiac muscle and gradient fractions were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)<sup>31</sup> on 3% to 15% linear gradient gels and transferred to nitrocellulose membranes.<sup>32</sup> Immunoblot staining was performed as previously described.<sup>30</sup>

### *Evans Blue Dye Injection and Microscopic Evaluation*

Evans blue (Sigma) was dissolved in PBS (10 mg/ml) and sterilized by passage through membrane filters with a pore size of 0.2  $\mu$ m. Hamsters were anesthetized with pentobarbital (0.75 mg/10 g of body weight) via intraperitoneal injection, and 0.25  $\mu$ l/10 g of body weight of the dye solution was injected intravenously through the front limb vein. Animals younger than 4 weeks were injected intraperitoneally. Animals were killed 48 hours after injection by asphyxiation with gaseous carbon dioxide. During the time period between injection and asphyxiation, animals were kept in standard laboratory cages. All hamsters were skinned and inspected for dye uptake in the skeletal muscles, indicated by blue coloration. Muscle sections for microscopic Evans blue dye (EBD) detection were incubated in ice-cold acetone at -20°C for 10 minutes and examined as described above.

## **Results**

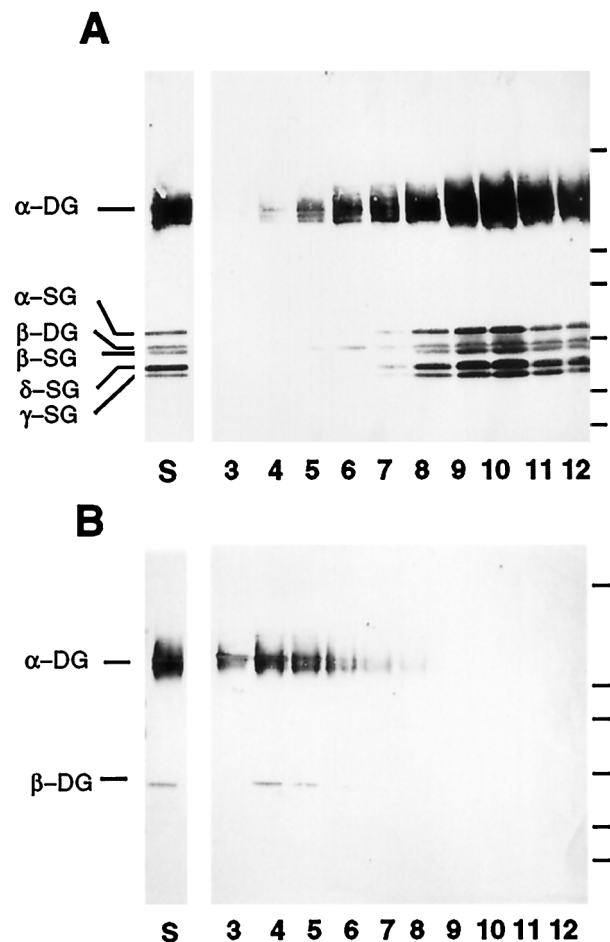
### *Immunohistochemistry*

To study the effect of the  $\delta$ -sarcoglycan mutation on the expression and localization of the sarcoglycans in skel-

etal and cardiac muscle, we performed immunofluorescence analysis on cryosections of normal and BIO14.6 hamsters. In control animals  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan were homogeneously expressed at the muscle fiber plasma membrane (Figure 1, A and B). In contrast, the  $\delta$ -sarcoglycan protein was completely deficient at the sarcolemma of BIO14.6 skeletal muscle fibers and cardiomyocytes (Figure 1, A and B). In addition, there was a severe concomitant reduction of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sarcoglycan (Figure 1, A and B). We also asked whether the  $\delta$ -sarcoglycan mutation altered expression of other DGC proteins. Immunofluorescence analysis revealed that dystrophin,  $\beta$ -dystroglycan, and the laminin  $\alpha 2$  chain were expressed at comparable levels in the BIO14.6 hamster and in control muscles (Figure 1, A and B). In contrast, expression of  $\alpha$ -dystroglycan was reduced at the fiber surface in both skeletal and cardiac muscle tissues (Figure 1, A and B). However, the degree of reduction showed variation even among littermates. In some cases,  $\alpha$ -dystroglycan was almost completely deficient at the plasma membrane; in other cases, expression levels were similar to those in control animals. We found no correlation between the degree of  $\alpha$ -dystroglycan expression and the age of the BIO14.6 hamsters.

### *Dissociation of the Dystroglycan Complex from the Sarcoglycans in the $\delta$ -Sarcoglycan-Deficient Hamster*

To confirm the reduction of all sarcoglycans at the sarcolemma in BIO14.6 muscle, immunoblot analysis was performed on sarcolemma-enriched preparations from control and BIO14.6 skeletal muscle. All four sarcoglycans were greatly reduced or absent in skeletal muscle membranes whereas dystrophin showed a slight reduction in the BIO14.6 hamster compared with control animals (data not shown). In all membrane preparations of the BIO14.6 hamster,  $\alpha$ -dystroglycan was greatly re-



**Figure 2.** Sucrose gradient fractions of purified DGC from control and BIO14.6 hamsters were resolved by 3% to 15% SDS-PAGE and transferred to nitrocellulose. The first lane of the blots (S) is an aliquot of the glycoprotein complex enriched from sWGA and DEAE columns that is concentrated and applied to a sucrose gradient. The other lanes are fractions 3 to 12 of the sucrose gradient fractionation. Control (A) and BIO14.6 (B) blots were stained with monoclonal antibodies against  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sarcoglycan ( $\alpha$ -SG,  $\beta$ -SG, and  $\gamma$ -SG) and a rabbit amino-terminal peptide antibody to  $\delta$ -sarcoglycan ( $\delta$ -SG). Additional staining was performed with monoclonal anti-dystroglycan antibodies ( $\alpha$ -DG and  $\beta$ -DG). In both A and B, the molecular weight markers are shown on the right. The molecular weights from top to bottom are 208, 107, 68, 46, and 28 kd. The entire sarcoglycan complex was absent in the muscle preparation of the cardiomyopathic hamster (B). In addition, the dystroglycans, which are reduced in the BIO14.6 muscle preparation, shifted to an earlier fraction of the sucrose gradient in diseased animals (B).

duced. Analysis of muscle extracts suggested that  $\alpha$ -dystroglycan was synthesized and fully glycosylated, but it was not associated with membranes (data not shown).

To examine the status of the dystroglycan complex in muscle lacking  $\delta$ -sarcoglycan, a glycoprotein preparation enriched in DGC components was prepared from F1B and BIO14.6 skeletal muscle using chromatography and sucrose gradient fractionation. Analysis of sucrose gradient fractions was performed for the sarcoglycans and the dystroglycans (Figure 2). The control hamster sucrose gradient profile demonstrated that the sarcoglycans and  $\alpha$ - and  $\beta$ -dystroglycan co-sedimented with peak fractions 8 to 12 (Figure 2A). In contrast, no sarcoglycan staining could be detected in BIO14.6 skeletal

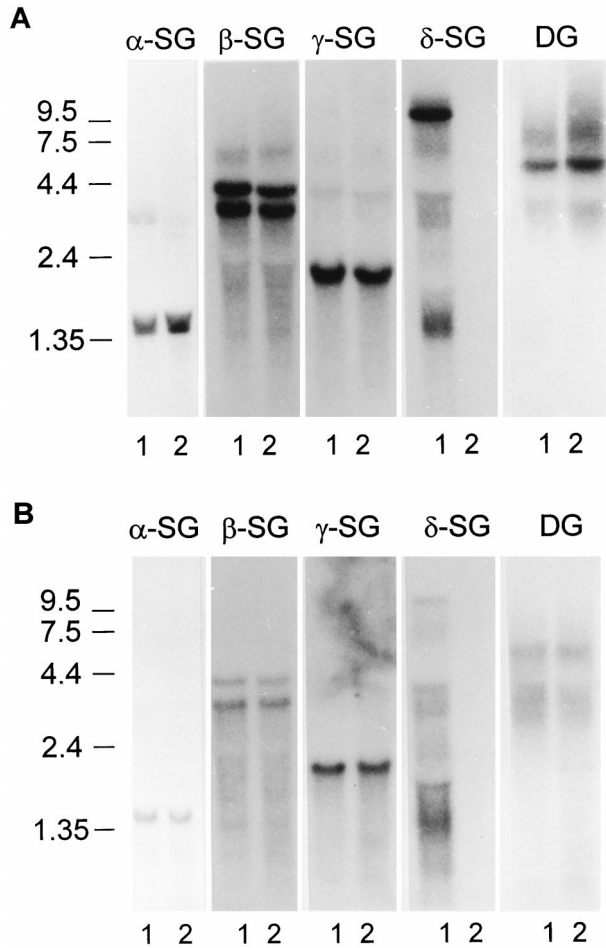
muscle fractions in agreement with immunofluorescence and immunoblot analysis (Figure 2B). Interestingly, the dystroglycan complex sedimented in two different peak fractions (4 to 6 and 8 to 12) in control hamster, whereas the BIO14.6 dystroglycan complex sedimented only in the earlier fractions 3 to 6 (Figure 2, A and B).

### *Transcript Levels of the Sarcoglycans and Dystroglycans in Striated Muscle of Normal and Cardiomyopathic Hamsters*

To determine whether the loss of the entire sarcoglycan complex and the reduction of  $\alpha$ -dystroglycan from the plasma membrane reflected transcriptional regulation, we performed RNA analysis of skeletal and cardiac tissues. For this, we used a full length reverse transcription PCR product containing the entire coding sequence of the  $\delta$ -sarcoglycan gene. A major transcript of 9.5 kb, three transcripts between 3 and 4 kb, and several transcripts around 1.35 kb were detected in skeletal muscle (Figure 3A) and cardiac tissue (Figure 3B) of the control hamster. All of these transcripts were dramatically reduced in the cardiomyopathic hamster even after overexposure of the autoradiograms. In parallel, we analyzed the expression of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sarcoglycan in control and cardiomyopathic skeletal and cardiac muscle and detected identical message levels and sizes in each case (Figure 3, A and B). Because we had demonstrated reduced expression of  $\alpha$ -dystroglycan in skeletal and cardiac muscle of diseased hamsters by immunohistochemical and biochemical analysis, we also investigated mRNA levels of dystroglycan. The dystroglycan transcript in skeletal muscle of the BIO14.6 hamster showed equal to higher levels compared with control animals (Figure 3A). In cardiac muscle, we detected identical message levels for  $\alpha$ -dystroglycan in the control and BIO14.6 strains (Figure 3B). These results confirm and extend those of previous reports.<sup>18,33</sup>

### *Sarcolemmal Damage in $\delta$ -Sarcoglycan-Deficient Muscle*

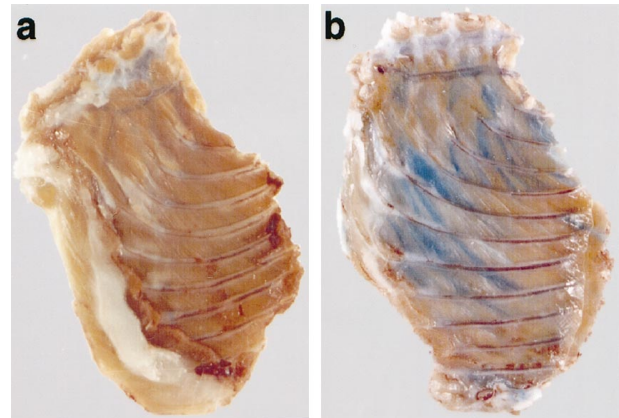
The DGC is believed to play an important role in linking the cytoskeleton to the extracellular matrix, thereby maintaining the structural integrity of the plasma membrane.<sup>34-36</sup> Here, we tested whether the mutation of the  $\delta$ -sarcoglycan gene leads to plasma membrane disruptions in skeletal muscle fibers of BIO14.6 hamsters. Membrane permeability was assessed by intravenous injection of EBD, a normally membrane-impermeant molecule. No obvious tracer uptake into skeletal muscles of F1B control hamsters at any age was detected by macroscopic inspection. Up to approximately 3 weeks of age, we also found no dye uptake into skeletal muscles of BIO14.6 animals. In contrast, intravenously administered EBD always stained skeletal muscles in BIO14.6 hamsters of 6 weeks or older (Figure 4). The extent of EBD accumulation varied among muscles. Areas of blue staining appeared mainly within the proximal limb muscles



**Figure 3.** Forty micrograms of total skeletal (A) or cardiac (B) RNA from control (lanes 1) and BIO14.6 (lanes 2) was electrophoresed on a 1.25% agarose gel containing 5% formaldehyde. Blots were hybridized with  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan (SG) and  $\alpha$ -dystroglycan random primed cDNA probes. Although  $\alpha$ -,  $\beta$ -, and  $\gamma$ -sarcoglycan transcripts appeared equal in amount between control and BIO14.6 tissues,  $\delta$ -sarcoglycan transcripts were dramatically reduced in BIO14.6 tissue. As a control for loading, the  $\delta$ -sarcoglycan blot was stripped and reprobed with actin cDNA. Molecular weights in kilobases are indicated on the left.

and the muscles of the pelvic and the shoulder girdle. Furthermore, the diaphragm and intercostal muscles always showed partial incorporation of the dye into muscle fibers if diseased animals were older than 4 weeks of age. The intercostals often showed clusters of blue fibers that ran throughout the entire length of the muscle (Figure 4). We found significant variation in the amount and distribution of blue fibers between the BIO14.6 hamsters. The uptake of the tracer into certain skeletal muscles could not be correlated with the age of the animals and showed variability even between littermates.

Skeletal muscles that macroscopically showed dye uptake always revealed red EBD autofluorescence by fluorescence microscopy analysis. In most of the cases, the intracellular staining was diffusely distributed across the cytoplasm, although sometimes the fiber periphery was more strongly stained than the center (Figure 5). Intensity of the dye signal also showed differences between fibers (Figure 5). An interesting finding was the arrangement of EBD-positive fibers into groups (Figure 5). Grouping of

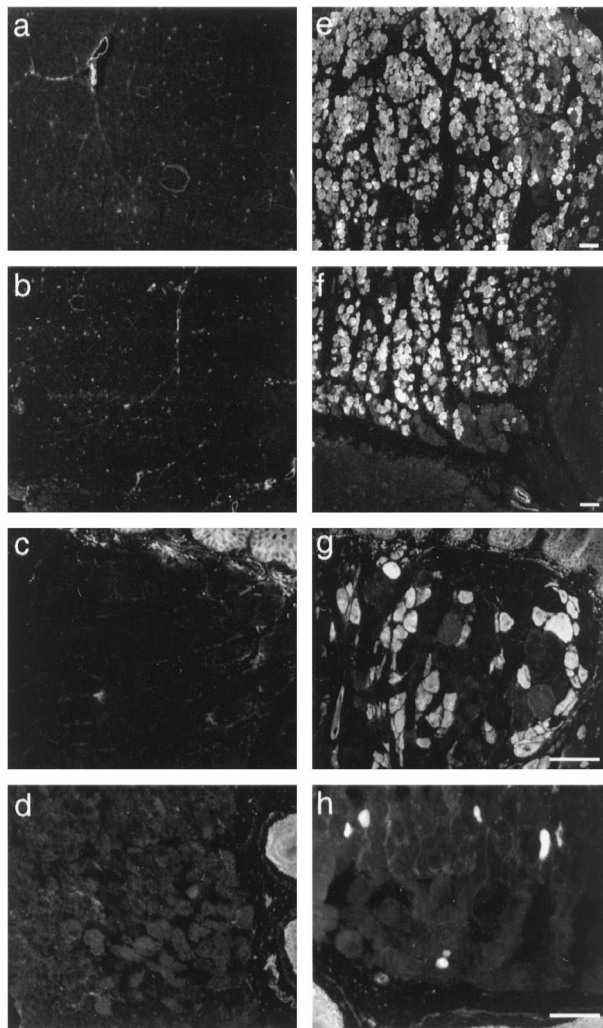


**Figure 4.** Macroscopic evaluation of Evans blue staining in intercostal muscles after intravenous dye injection into a 4-month-old control (a) and a 4-month-old BIO14.6 hamster (b). The tissue samples were fixed in an 8% formaldehyde solution 48 hours after the dye injection. In control animals (a), we never found dye uptake into fibers of intercostal muscles or other skeletal muscles. On the other hand, we always observed EBD-positive fibers within regions of intercostal muscles in cardiomyopathic animals (b). The location and extent of plasma membrane damage varied from animal to animal but was a consistent finding in the BIO14.6 strain. Besides dye uptake in intercostal muscles, we also found discoloration within the proximal limb muscles, muscles of the pelvic and the shoulder girdle, and muscles of the back and the abdominal wall. Furthermore, the diaphragm always showed partial incorporation of the dye into muscle fibers.

EBD-positive fibers was observed in all investigated muscle types (Figure 5). On longitudinal sections we could demonstrate that dye uptake was not confined to a small part of the fiber but appeared over long segments of the entire muscle cell. In addition to the muscles of the limbs and the trunk, we also looked at the muscle tissue of the tongue for EBD uptake, as tongue lesions have been reported to occur in 100% of the BIO14.6 hamsters.<sup>37</sup> In the F1B control strain, EBD-positive fibers in the tongue were not observed (Figure 5). In contrast, all BIO14.6 animals older than 4 weeks of age showed partial EBD-positive fibers (Figure 5). Furthermore, we looked at EBD uptake into skeletal muscle fibers of the esophagus in 4-month-old F1B and BIO14.6 animals. Again, we found no dye uptake into muscle fibers of control animals, whereas in diseased animals, EBD-positive skeletal muscle cells were frequently detected (Figure 5). Dye-positive fibers often showed characteristic features of degeneration and necrosis in H&E staining.

The ability of skeletal muscle to regenerate after injury is well established. The most effective regeneration occurs when the basal lamina and endomysium remain intact and new fibers can be formed within the original scaffolding shaped by the basal lamina and surrounding tissues.<sup>38,39</sup> To test whether the basement membrane of EBD-positive muscle fibers was disrupted, we stained sections with antibodies against the laminin  $\alpha 2$  chain. EBD-positive fibers showed a complete basal lamina, suggestive of normal regenerative potential (data not shown).

After intravenous EBD injection, dye uptake was not readily visible in cardiac muscle by macroscopic inspection, but microscopic analysis revealed EBD in cardiomyocytes of BIO14.6 hamsters 4 weeks or older (Figure 6). Dye uptake into cardiac muscle cells did not occur in

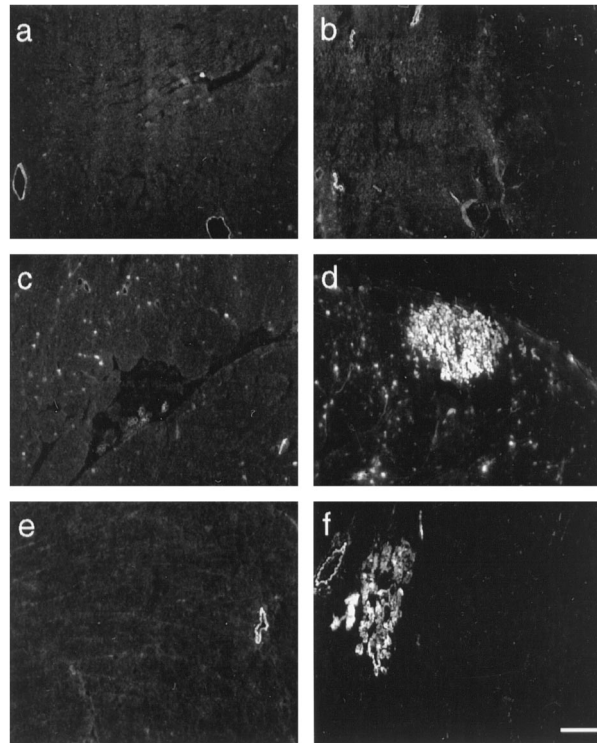


**Figure 5.** Evans blue staining on 7- $\mu$ m cryosections from 4-month-old control (a–d) and BIO14.6 (e–h) injected hamsters. The tracer assay was used to assess fiber damage in the femoral quadriceps muscle (a and e), the diaphragm (b and f), the tongue (c and g), and the skeletal muscle layer of the esophagus (d and h). Skeletal muscle fibers of control animals did not show dye uptake in any of the investigated tissues. In contrast, diseased hamsters of 4 weeks or older repeatedly showed EBD-positive fibers in all of the tissues. EBD-positive fibers frequently displayed a grouped arrangement (e and f). The epithelial layers of the tongue and the esophagus showed nonspecific fluorescent staining. Bars, 100  $\mu$ m.

diseased animals younger than 4 weeks or in control animals of any age (Figure 6). Most frequently, EBD-positive fibers were detected in small clusters in the ventricular walls (Figure 6). Affected cells were homogeneously stained with the tracer and showed signs of degeneration and necrosis by H&E staining.

### Discussion

In the present study, we have investigated the pathological manifestations of the  $\delta$ -sarcoglycan mutation in cardiac and skeletal muscle tissues of the BIO14.6 cardiomyopathic hamster. We demonstrated that loss of  $\delta$ -sarcoglycan from muscle fiber plasma membranes led to reduced expression of several other components of



**Figure 6.** Evans blue staining on 7- $\mu$ m cryosections of control (a, c, and e) and BIO14.6 (b, d, and f) cardiac muscle. Injected animals were 14 days (a and b), 5 weeks (c and d), and 1 year (e and f) old. EBD-positive cardiomyocytes were detected in diseased hamsters of 4 weeks or older (d and f). We never found EBD in cardiac muscle fibers of cardiomyopathic animals younger than 4 weeks (b). The EBD-positive fibers in cross sections may appear singly but were most often detected in small groups (d and f). Bars, 100  $\mu$ m.

the DGC, including other sarcoglycans and  $\alpha$ -dystroglycan. Our findings confirmed the tight association between sarcoglycans and suggest a close relationship between the sarcoglycan and dystroglycan complexes. Although abnormal expression of sarcoglycans and  $\alpha$ -dystroglycan in the BIO14.6 hamster had been previously shown,<sup>18,33,40,41</sup> this is the first time that specific antibodies against all sarcoglycans and the dystroglycans have been used for analysis in the animal. The affinity-purified antibody against  $\alpha$ -dystroglycan allowed us for the first time to demonstrate reduction of this peripheral glycoprotein by immunohistochemical analysis in a subset of animals. Interestingly, the reduction of  $\alpha$ -dystroglycan in BIO14.6 skeletal muscle tissue was not a consistent immunohistochemical finding and even age- and sex-matched animals revealed different expression levels, ranging from normal to severely reduced. This observation may explain the results of a previous report in which a reduction of  $\alpha$ -dystroglycan by immunofluorescence analysis was not demonstrated.<sup>40</sup> The observation may also account for the considerable variation of disease course in the cardiomyopathic animals, which can be slowly progressive and, in other cases, rapidly leading to death within 80 days.<sup>42,43</sup> Similar findings have been reported in families with sarcoglycan-deficient LGMD, where patients with the same mutation displayed both mild and severe forms of muscular dystrophy, implicating

other loci or environmental factors as mediators of the dystrophic process.<sup>44</sup> In the membrane preparations of the BIO14.6 hamster, on the other hand, we always found a reduction of  $\alpha$ -dystroglycan. This may be explained by the fact that the anchorage of  $\alpha$ -dystroglycan to the sarcolemma is impaired by sarcoglycan deficiency and that during the microsome preparation  $\alpha$ -dystroglycan may easily detach from the membrane. Our findings from Northern blot analysis of skeletal and cardiac muscle samples indicated that effects of the deletion in the  $\delta$ -sarcoglycan gene on other DGC components are post-transcriptional.

### *Functional Impairment of Plasma Membrane Integrity in Striated Muscle Tissue of the $\delta$ -Sarcoglycan-Deficient BIO14.6 Hamster*

It has recently been shown that loss of sarcolemmal integrity is a characteristic feature of dystrophin-deficient muscular dystrophies.<sup>35,45,46</sup> Due to the phenotypic similarity between Duchenne and Becker muscular dystrophy and the sarcoglycan-deficient LGMDs, we investigated the status of sarcolemmal damage in the BIO14.6 hamster by using intravenously injected EBD as an *in vivo* tracer for membrane damage. The consistent finding of EBD uptake into skeletal muscles of the cardiomyopathic hamster emphasized the role membrane lesions play in the pathogenesis of this disease model. This was further underlined by focal groups of EBD-positive fibers in cardiac muscle of the BIO14.6 hamster. The intravenous injection of EBD into BIO14.6 hamsters also revealed membrane damage in the skeletal muscle layer of the esophagus. Although only the proximal one-third of the esophagus is surrounded by skeletal muscle fibers in humans, fiber damage in this region could cause the swallowing problems reported for patients with muscular dystrophy.<sup>47,48</sup> Another interesting result we obtained by EBD injection were membrane lesions in the tongue of the BIO14.6 hamster. There are several lines of evidence that sarcolemmal damage leads to the release of growth factors into the surrounding tissue.<sup>49,50</sup> These growth factors may play an important role in the pathogenic process of fibrosis and fiber hypertrophy in dystrophic muscle tissue. They may not just be responsible for hypertrophy of the calves, as reported in patients with sarcoglycan-deficient LGMD,<sup>51</sup> but could also explain macroglossia in some of the patients with muscular dystrophy.<sup>47</sup> A common feature between the dystrophin-deficient *mdx* mouse and the sarcoglycan-deficient hamster were clusters of EBD-positive fibers in skeletal and cardiac muscle.<sup>46</sup> Moreover, both animal models reveal a reduction of  $\alpha$ -dystroglycan at the sarcolemma. Structural and functional alterations of this molecule may play an important role in the pathogenesis of muscular dystrophies associated with the DGC. Our biochemical findings gave additional evidence that the  $\delta$ -sarcoglycan mutation in the BIO14.6 hamster effects the stability of dystroglycan at the muscle fiber plasma membrane. Although we are at present unable to offer a precise description of the pathogenic pathway that accounts for muscle fiber necrosis in

muscular dystrophies, we have demonstrated that loss of plasma membrane integrity in skeletal muscle fibers might play a primary role in the course of these progressive muscle diseases. Efforts to fully understand the functional role of the sarcoglycans will involve the identification of their intracellular and extracellular binding partners.

### *Acknowledgments*

We gratefully acknowledge Rachele H. Crosbie and Connie S. Lebakken for their comments on this manuscript and the helpful discussion.

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