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Disruption of the Dystrophin-Glycoprotein Complex in the Cardiomyopathic Hamster*

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Cardiomyopathies are a diverse group of primary cardiac diseases, most of which have a poorly understood etiology. One type of hereditary cardiomyopathy is caused by defects in the dystrophin gene in Duchenne and Becker muscular dystrophy patients. Our laboratory has identified a complex of dystrophin-associated proteins in skeletal and cardiac muscle which span the sarcolemma, linking the subsarcolemmal cytoskeleton to the extracellular matrix. The absence of dystrophin in Duchenne muscular dystrophy patients leads to the loss of dystrophin-associated proteins in both skeletal and cardiac muscle, suggesting that a primary loss of one or more dystrophin-associated proteins might lead to other forms of cardiomyopathy. Here we report the specific deficiency of the 50-kDa dystrophin-associated glycoprotein in cardiac and skeletal muscles of the BIO 14.6 strain of cardiomyopathic hamsters, which experience both autosomal recessive cardiomyopathy and myopathy. Other dystrophin-associated proteins are well preserved in myopathic hamster skeletal muscle, but the link between dystrophin and dystroglycan is disrupted. All dystrophin-associated proteins are decreased in abundance in the cardiomyopathic hamster heart, perhaps explaining why the cardiomyopathy is more severe than the myopathy. Thus, the disruption of the dystrophin-glycoprotein complex may play a role in skeletal and cardiac myocyte necrosis of the cardiomyopathic hamster.

Cardiomyopathies develop in patients suffering from Duchenne muscular dystrophy and in many patients having other forms of muscular dystrophies (1, 2). Moreover, cardiomyopathy is the primary manifestation in some patients having Becker muscular dystrophy (3, 4). Duchenne muscular dystrophy, Becker muscular dystrophy, and muscle pathology of the *mdx* mouse result from mutations in the gene encoding dystrophin, a membrane cytoskeletal protein (5–7). In normal skeletal (8–12) and cardiac (13, 14) muscles, dystrophin is complexed with a 59-kDa intracellular dystrophin-associated protein (59-DAP),[1] a 25-kDa sarcolemmal dystrophin-associated protein, three sarcolemmal dystrophin-associated glycoproteins of 35, 43, and 50 kDa (35-DAG, 43-DAG, and 50-DAG, respectively), and a 156-kDa extracellular dystrophin-associated glycoprotein (156-DAG or 156-kDa dystroglycan). Dystroglycan binds to laminin with high affinity, indicating that at least one function of the dystrophin-glycoprotein complex is to link the subsarcolemmal cytoskeleton to the extracellular matrix (15). Due to the absence of dystrophin, all dystrophin-associated proteins are greatly reduced in skeletal muscle membranes from Duchenne muscular dystrophy patients (9, 15, 16) and *mdx* mice (12) and in cardiac membranes from at least some Duchenne muscular dystrophy patients (data not shown).

The BIO 14.6 hamster is a widely studied animal model of autosomal recessive cardiomyopathy which also experiences a muscular dystrophy (17–19). Although cardiomyopathic hamster (CMH) skeletal muscles exhibit classical signs of myopathy (central nucleation, wide variation in fiber diameter, and necrosis), the affected animals remain ambulatory and appear not to die of respiratory muscle weakness (20). Rather, BIO 14.6 hamsters experience a hypertrophic cardiomyopathy leading to heart failure and to death within one-half to one-third of their normal life span (17). Several biochemical abnormalities in the CMH heart have been described, but the genetic defect in the BIO 14.6 strain has not been identified (21–27).

Because a deficiency of dystrophin-associated proteins is associated with skeletal muscle dysfunction and cardiomyopathy in Duchenne muscular dystrophy (16) and possibly Becker muscular dystrophy patients, we investigated the status of the dystrophin-associated proteins in the BIO 14.6 cardiomyopathic hamster. 50-DAG was specifically deficient in CMH skeletal muscle. However, dystrophin and 156-DAG were less tightly associated with the sarcolemma in CMH skeletal muscle than in normal muscle. In CMH cardiac muscle 50-DAG was undetectable, and all other dystrophin-associated proteins were decreased in abundance. Thus, the dystrophin-glycoprotein complex was disrupted in both skeletal and cardiac muscles of the cardiomyopathic hamster, suggesting that a loss of structural integrity of the dystrophin-glycoprotein complex may play a role in skeletal and cardiac myocyte necrosis of the cardiomyopathic hamster.

EXPERIMENTAL PROCEDURES

Immunofluorescence—Skeletal muscle cryosections (7 μm) were preincubated 30 min in PBS (0.9% NaCl, 50 mM sodium phosphate, pH 7.5) + 3% bovine serum albumin (BSA), incubated 1 h with primary antibody diluted in PBS + 3% BSA, washed with PBS, incubated 30 min with a biotinylated secondary antibody (Vector Laboratories) diluted 1:500 in PBS + 3% BSA, washed with PBS, incubated 30 min with fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted 1:1000 in PBS + 3% BSA, washed in PBS, and mounted under FITC-Guard (Testog). Cardiac ventricular muscle cryosections (7 μm) were processed by the same method except preincubation was in

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[1] The abbreviations used are: 59-DAP, 59-kDa dystrophin-associated protein; 156-DAG, 156-kDa dystrophin-associated glycoprotein; 50-DAG, 50-kDa dystrophin-associated glycoprotein; 43-DAG, 43-kDa dystrophin-associated glycoprotein; 35-DAG, 35-kDa dystrophin-associated glycoprotein; CMH, cardiomyopathic hamster; SCARMD, severe childhood autosomal recessive muscular dystrophy; WGA, wheat germ agglutinin; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

PBS + 1% BSA and antibodies were diluted in PBS + 0.1% BSA. All incubations were performed at 37 °C. Sections from control and cardiomyopathic hamsters were placed on the same microscopy slide to insure identical treatment, and photographs for a given antibody were processed using identical conditions for both control and cardiomyopathic sections.

Immunoblot Analysis—Skeletal and cardiac muscle homogenates were prepared as described previously (10) from age-matched F1B and BIO 14.6 hamsters. Skeletal and cardiac muscle total membranes were prepared as described previously (12) from 9-week-old F1B and BIO 14.6 hamsters. Homogenates (500 μg of protein/lane) or membranes (250 μg of protein/lane) were fractionated on 3–12% gradient SDS-polyacrylamide gels by the method of Laemmli (28) and transferred to nitrocellulose according to Towbin *et al.* (29). Immunoblot staining was performed as previously described (10).

WGA-Sepharose Chromatography—KCl-washed microsomes were prepared as described previously (10) from 9-week-old F1B and BIO 14.6 hamsters. Microsomes (25 mg) were solubilized in 10 ml of 1% digitonin, 0.5 M NaCl, 0.5 M sucrose, and protease inhibitors as previously described (8). Solubilized microsomes (7.5 ml) and 1 ml of WGA-Sepharose were incubated overnight at 4 °C with mixing.

Animals and Antibodies—Male F1B control and BIO 14.6 cardiomyopathic hamsters were obtained from Bio Breeders, Fitchburg, MA. Affinity-purified rabbit antibodies against the C terminus of dystrophin or affinity-purified sheep antibodies against 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG were produced as previously described (11, 12, 30). Anti-50-DAG antibodies were affinity-purified against the entire protein (antibody 1) or against a 50-DAG peptide (antibody 2) as described (16). Monoclonal antibody McB2 against the α_2 subunit of the rat brain Na^+/K^+-ATPase (31) was the kind gift of Dr. Kathleen Sweadner. Monoclonal antibody IIID5 against the dihydropyridine receptor (32), monoclonal antibody IIH6 against dystroglycan (11), monoclonal antibody IID8 against the cardiac Ca^{2+}-ATPase (33), and an affinity-purified rabbit antibody against the C terminus of the skeletal muscle ryanodine receptor (34) were produced and characterized as previously described. Peroxidase-conjugated WGA was from Sigma.

RESULTS AND DISCUSSION

Immunofluorescence analysis demonstrated that dystrophin and all dystrophin-associated proteins were localized at the cell periphery of normal hamster skeletal muscle (Fig. 1), consistent with their localization in rabbit, mouse, and human skeletal muscle (9, 11, 12, 16). However, 50-DAG was undetectable in cardiomyopathic hamster (CMH) skeletal muscle using two distinct affinity-purified antibodies (Fig. 1, *50-DAG Ab 1* and *Ab 2*). 50-DAG was also undetected by this method in cardiomyopathic hamsters ranging from 2 to 24 weeks of age (data not shown). Sarcolemmal immunostaining intensity for dystrophin was indistinguishable between control and CMH skeletal muscle (Fig. 1, *DYS*), although hematoxylin and eosin staining of CMH skeletal muscle cryosections revealed muscle fibers of various sizes with rounded contours and central nucleation characteristic of myopathy (Fig. 1, *H&E*). 156-DAG, 59-DAP, and 43-DAG were present at apparently equal levels in normal and CMH muscle, whereas 35-DAG appeared slightly decreased in abundance at the CMH sarcolemma.

Dystrophin and all dystrophin-associated proteins were clearly localized to the sarcolemma in normal hamster cardiac muscle (Fig. 2). In addition, staining of small processes leading inward from the sarcolemma was consistent with the presence of these proteins in hamster cardiac T-tubules as has been described for rabbit cardiac muscle (14). Immunohistochemical analysis in the presence of secondary antibody alone (Fig. 2, *2° Ab*) illustrated the higher background staining observed in CMH heart. 50-DAG was not detected above background by immunofluorescence in CMH cardiac muscle from hamsters ranging from 6 to 24 weeks of age (Fig. 2 and data not shown). Dystrophin and 59-DAP were each present at slightly reduced levels in normal and CMH cardiac sarcolemma, but 156-kDa dystroglycan (156-DAG), 43-kDa dystroglycan (43-DAG), and 35-DAG were decreased in CMH cardiac sarcolemma relative to that of normal hamsters. The deficiency of multiple dystrophin-associated proteins, including dystroglycan, which binds to laminin in the extracellular matrix (15), may explain why the cardiomyopathic hamsters experience more severe cardiac symptoms than skeletal muscle symptoms. Alternatively, CMH skeletal muscle may posses a mechanism of compensating for disruption of the dystrophin-glycoprotein complex that does not exist in cardiac muscle. Interestingly, dystrophin-associated proteins are preserved in cardiac muscle of *mdx* mice (Ref. 13 and data not shown), which experience no cardiac abnormalities (35).

To more accurately compare the abundance of dystrophin and dystrophin-associated proteins in normal and cardiomyopathic hamsters, immunoblot analysis was performed on skeletal and cardiac muscle homogenates (Fig. 3). Control experiments were performed to demonstrate that any changes in

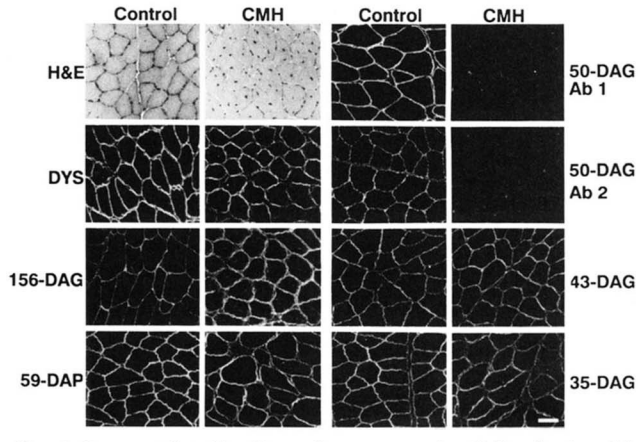

FIG. 1. **Immunolocalization of components of the dystrophin-glycoprotein complex in skeletal muscle from normal and cardiomyopathic hamsters.** Transverse skeletal muscle cryosections from 6-week-old F1B (*Control*) or BIO 14.6 cardiomyopathic (*CMH*) hamsters were stained with hematoxylin and eosin (*H&E*). Additional cryosections were labeled by indirect immunofluorescence with affinity-purified antibodies against dystrophin (*DYS*) or against 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG. Anti-50-DAG antibodies were affinity-purified against the entire protein (*Ab 1*) or against a 50-DAG peptide (*Ab 2*). Indistinguishable results were observed using skeletal muscle obtained from 6–24-week-old hamsters and, using anti-50-DAG antibodies, in 2-week-old hamsters (data not shown). *Bar*, 50 μm.

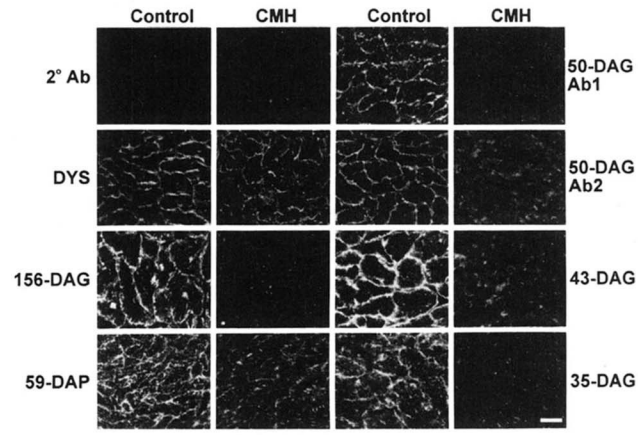

FIG. 2. **Immunolocalization of components of the dystrophin-glycoprotein complex in cardiac muscle from normal and cardiomyopathic hamsters.** Cardiac ventricle cryosections from 19-week-old F1B (*Control*) or BIO 14.6 cardiomyopathic (*CMH*) hamsters were labeled by indirect immunofluorescence with secondary antibody and streptavidin-fluorescein alone (*2° Ab*) or with affinity-purified antibodies against dystrophin (*DYS*) or against 156-DAG, 59-DAP, 50-DAG, 43-DAG, and 35-DAG. Anti-50-DAG antibodies were affinity-purified against the entire protein (*Ab 1*) or against a 50-DAG peptide (*Ab 2*). Indistinguishable results were observed using cardiac muscle obtained from 6–24-week-old hamsters (data not shown). Labels at *left* refer to the *two leftmost panels* on each *row*, and *labels* at *right* refer to the *two rightmost panels*. *Bar*, 20 μm.

protein levels in cardiomyopathic hamsters were not due to general effects in necrotic tissue. The pattern of lectin binding using wheat germ agglutinin (Fig. 3), concanavalin A (data not shown), and jacalin (data not shown) to CMH skeletal and cardiac muscle homogenates was unaffected. Additionally, integral membrane proteins involved in membrane transport or excitation-contraction coupling (specifically, the Na/K-ATPase and dihydropyridine receptor in skeletal muscle (Fig. 3*A*) and the Ca^{2+}-ATPase and ryanodine receptor in heart (Fig. 3*B*)) were present at comparable levels in both control and CMH homogenates. These results indicate that the majority of integral membrane proteins and glycoproteins are unaffected in CMH skeletal muscle and heart.

50-DAG was undetectable on immunoblots of CMH skeletal muscle homogenates (Fig. 3*A*). Dystrophin was only slightly reduced in abundance in CMH skeletal muscle, whereas 156-DAG was present at equal levels in control and CMH skeletal muscle. In heart (Fig. 3*B*), as in skeletal muscle, 50-DAG was undetected in cardiomyopathic hamsters, and dystrophin was well preserved on immunoblots of CMH cardiac homogenates. However, 156-DAG was greatly reduced in CMH heart relative to normal heart, which is consistent with immunohistochemical data (Fig. 2).

To investigate the abundance of 50-DAG, 156-DAG, and dystrophin in membrane-enriched preparations, immunoblot analysis was performed on skeletal (Fig. 4*A*) and cardiac (Fig. 4*B*) muscle total microsomes. No differences in immunostaining intensity between normal and CMH microsomes were detected for the Na/K-ATPase or dihydropyridine receptor in skeletal muscle or the Ca^{2+}-ATPase in heart (data not shown). 50-DAG was deficient in both skeletal and cardiac muscle membranes from the cardiomyopathic hamster (Fig. 4, *A* and *B*). In cardiac membranes (Fig. 3*B*) as in cardiac homogenates, dystrophin and 156-DAG were somewhat reduced in abundance. This finding is consistent with a recent report that dystrophin is reduced up to 50% in CMH ventricle (36). In CMH skeletal muscle membranes, dystrophin was somewhat reduced and 156-DAG was greatly reduced in abundance (Fig. 4*A*). Thus, although dystrophin and 156-DAG are expressed at normal levels in total CMH skeletal muscle (Figs. 1 and 3), these proteins appear to be less tightly associated with the sarcolemma in CMH skeletal muscle than in normal skeletal muscle as evidenced by their loss during membrane purification.

To assay the functional integrity of the dystrophin-glycoprotein complex, digitonin-solubilized skeletal muscle membranes from normal and cardiomyopathic hamsters were incubated

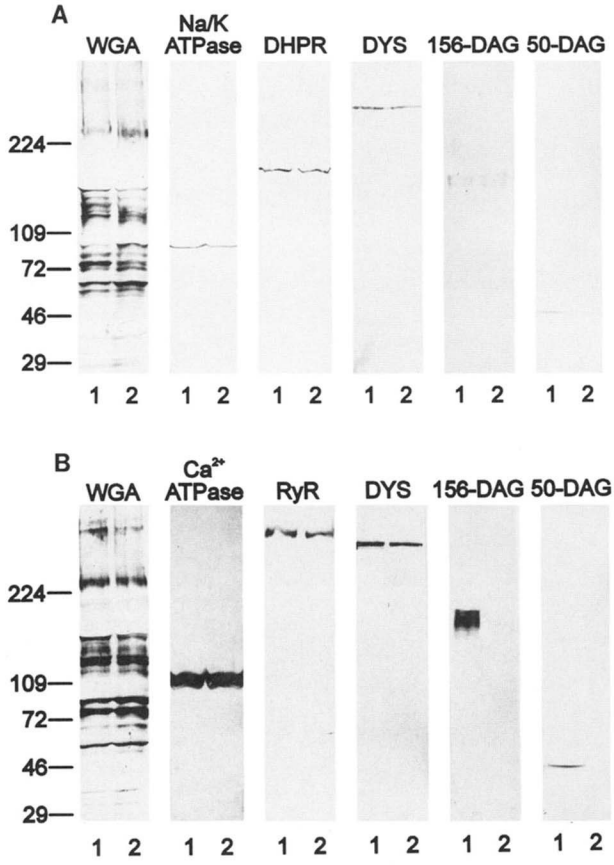

FIG. 3. **Immunoblot analysis of components of the dystrophin-glycoprotein complex in normal and cardiomyopathic hamster skeletal and cardiac muscle homogenates.** *A*, identical immunoblots of skeletal muscle homogenates from F1B control (*lanes 1*) or BIO 14.6 cardiomyopathic (*lanes 2*) hamsters were stained with peroxidase-conjugated WGA, with monoclonal antibodies against the α_2 subunit of the Na/K-ATPase or the dihydropyridine receptor (*DHPR*), with an affinity-purified antibody against dystrophin (*DYS*), with a monoclonal antibody against 156-DAG, or with an affinity-purified antibody against 50-DAG. *B*, identical immunoblots of cardiac homogenates from F1B (*lanes 1*) or BIO 14.6 (*lanes 2*) hamsters were stained with peroxidase-conjugated WGA, with a monoclonal antibody against the cardiac Ca^{2+}-ATPase, with an affinity-purified antibody against the skeletal muscle ryanodine receptor (*RyR*), which cross-reacts with the cardiac isoform, and with antibodies against dystrophin (*DYS*), 156-DAG, and 50-DAG as described in *A*. Homogenates were prepared from age-matched 10- or 24-week-old F1B and BIO 14.6 hamsters. Results at either age were indistinguishable; therefore, a representative blot is shown. Molecular weight standards ($M_r \times 10^{-3}$) are indicated.

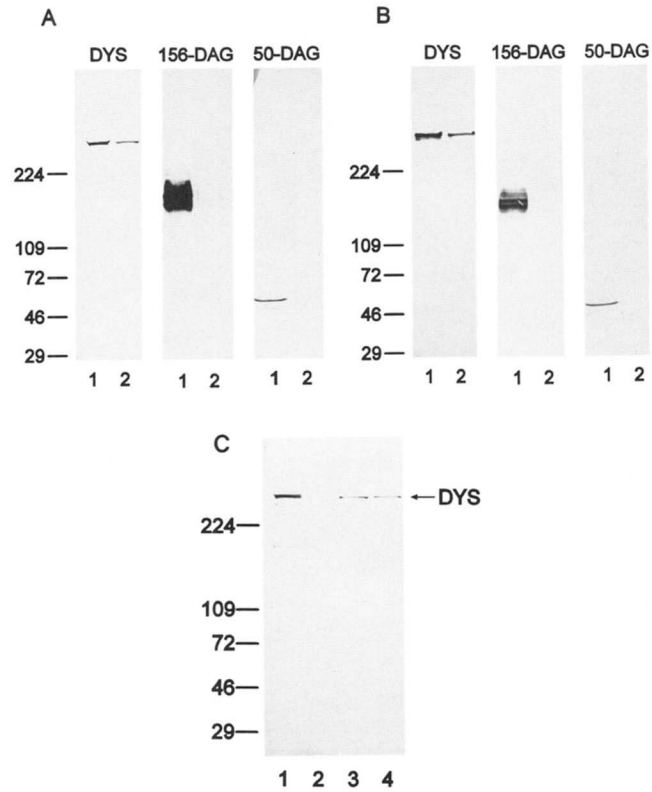

FIG. 4. **Evidence for disruption of the dystrophin-glycoprotein complex in cardiomyopathic hamster skeletal and cardiac muscle membranes.** *A* and *B*, identical immunoblots of skeletal muscle (*A*) or cardiac (*B*) membranes from F1B control (*lanes 1*) or BIO 14.6 cardiomyopathic (*lanes 2*) hamsters were stained with an affinity-purified antibody against dystrophin (*DYS*), a monoclonal antibody against 156-DAG, or an affinity-purified antibody against 50-DAG. *C*, immunoblots of equal volumes of total solubilized control (*lanes 1* and *2*) or cardiomyopathic (*lanes 3* and *4*) hamster skeletal muscle membranes (*lanes 1* and *3*) and supernatants following incubation of solubilized membranes with WGA-Sepharose (*lanes 2* and *4*) were stained with an affinity-purified antibody against dystrophin. Molecular weight standards ($M_r \times 10^{-3}$) are indicated.

with WGA-Sepharose. In normal hamsters, dystrophin was retained on WGA-Sepharose due to its association with membrane glycoproteins (Fig. 4*C*). However, dystrophin from CMH skeletal muscle was not retained on WGA-Sepharose, indicating that the dystrophin-glycoprotein complex-mediated link between membrane glycoproteins and the subsarcolemmal cytoskeleton is disrupted in cardiomyopathic hamsters. Thus, we hypothesize that dystrophin and 156-kDa dystroglycan are preserved at the CMH skeletal muscle sarcolemma (Figs. 1 and 3*A*) due to their interactions with actin and laminin, respectively, but that their association via the dystrophin-glycoprotein complex is nearly completely disrupted in the cardiomyopathic hamster (Fig. 4, *A* and *C*).

This work demonstrates that 50-DAG is deficient in skeletal and cardiac muscles of the BIO 14.6 cardiomyopathic hamster. A specific deficiency of 50-DAG in skeletal muscle of patients having severe childhood autosomal recessive muscular dystrophy (SCARMD) was also recently reported by our laboratory (16). One form of SCARMD has recently been linked to chromosome 13q12, but neither the gene nor its protein product has been identified (37). Cardiac abnormalities do develop in SCARMD patients (38), but the status of dystrophin-associated proteins, including 50-DAG, in SCARMD cardiac muscle remains to be determined. The similarity of skeletal muscle dystrophin-associated protein expression between SCARMD patients and cardiomyopathic hamsters suggests that these two conditions may share the same mechanism of pathogenesis and, perhaps, may have a defect within the same gene. Cloning and analysis of the 50-DAG gene will be required to determine if an abnormality in the gene is responsible for the CMH phenotype. If a defect in the 50-DAG gene is identified in both the BIO 14.6 hamster and patients with SCARMD, the cardiomyopathic hamster will be important as a symptomatic animal for testing potential gene therapies of SCARMD and perhaps human cardiomyopathies.

The deficiency of 50-DAG in cardiac and skeletal muscles and the decreased abundance of multiple dystrophin-associated proteins in cardiac muscle are unlikely to be nonspecific consequences of the general disease process in the cardiomyopathic hamster (*e.g.* due to activation of Ca^{2+}-dependent proteases) since many other integral membrane proteins are unaffected in CMH skeletal and cardiac muscles (Fig. 3). Additionally, 50-DAG and all dystrophin-associated proteins are found at normal levels in most forms of muscular dystrophy tested (16), indicating that dystrophin-associated proteins are not lost simply due to myocyte degeneration. Thus, if the deficiency of 50-DAG in the cardiomyopathic hamster is not due to a defect in the 50-DAG gene, 50-DAG deficiency is likely due to alteration of a 50-DAG-specific regulatory mechanism rather than a general degradation of membrane proteins. In this case, BIO 14.6 cardiomyopathic hamsters will be useful to elucidate such a mechanism.

Our results are consistent with the hypothesis that a deficiency of 50-DAG leads to dysfunction or disruption of the dystrophin-glycoprotein complex in the cardiomyopathic hamster. Breakdown of dystrophin-glycoprotein complex integrity is evidenced in CMH skeletal muscle by the ease by which 156-kDa dystroglycan is extracted from membranes (Fig. 4*A*) and by the failure of dystrophin to associate with WGA-binding glycoproteins as it does in normal muscle (Fig. 4*C*). Dystrophin-glycoprotein complex breakdown in CMH heart is demonstrated by the loss of 156-kDa dystroglycan and other dystrophin-associated proteins in cardiac muscle (Figs. 2 and 3*B*). In both skeletal and cardiac muscles, a decrease in the association between 156-kDa dystroglycan and dystrophin apparently disrupts the link between the subsarcolemmal cytoskeleton and the extracellular matrix (15). The disruption of this trans-sarcolemmal linkage may lead to a decrease in the integrity of the sarcolemma, to cellular necrosis, and eventually to myopathy and cardiomyopathy.

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