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Dilated cardiomyopathy mutations in δ-sarcoglycan exert a dominant-negative effect on cardiac myocyte mechanical stability

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Dilated cardiomyopathy mutations in δ-sarcoglycan exert a dominant-negative effect on cardiac myocyte mechanical stability. Am J Physiol Heart Circ Physiol 310: H1140–H1150, 2016. First published March 11, 2016; doi:10.1152/ajpheart.00521.2015.—Delta-sarcoglycan is a component of the sarcoglycan subcomplex within the dystrophin-glycoprotein complex located at the plasma membrane of muscle cells. While recessive mutations in δ-sarcoglycan cause limb girdle muscular dystrophy 2F, dominant mutations in δ-sarcoglycan have been linked to inherited dilated cardiomyopathy (DCM). The purpose of this study was to investigate functional cellular defects present in adult cardiac myocytes expressing mutant δ-sarcoglycans harboring the dominant inherited DCM mutations R71T or R97Q. This study demonstrates that DCM mutant δ-sarcoglycans can be stably expressed in adult rat cardiac myocytes and traffic similarly to wild-type δ-sarcoglycan to the plasma membrane, without perturbing assembly of the dystrophin-glycoprotein complex. However, expression of DCM mutant δ-sarcoglycan in adult rat cardiac myocytes is sufficient to alter cardiac myocyte plasma membrane stability in the presence of mechanical strain. Upon cyclical cell stretching, cardiac myocytes expressing mutant δ-sarcoglycan R97Q or R71T have increased cell-impermeant dye uptake and undergo contractures at greater frequencies than myocytes expressing normal δ-sarcoglycan. Additionally, the R71T mutation creates an ectopic N-linked glycosylation site that results in aberrant glycosylation of the extracellular domain of δ-sarcoglycan. Therefore, appropriate glycosylation of δ-sarcoglycan may also be necessary for proper δ-sarcoglycan function and overall dystrophin-glycoprotein complex function. These studies demonstrate that DCM mutations in δ-sarcoglycan can exert a dominant negative effect on dystrophin-glycoprotein complex function leading to myocardial mechanical instability that may underlie the pathogenesis of δ-sarcoglycan-associated DCM.

THE DYSTROPHIN-GLYCOPROTEIN complex (DGC) is a large complex of transmembrane and membrane-associated proteins that resides in the lateral sarcolemma of cardiac and skeletal muscle cells. The DGC contributes to an important functional linkage between the extracellular matrix (ECM) and cytoskeletal proteins in muscle cells (20, 21). The DGC attaches and interacts with the cytoskeleton through the binding of dystrophin to F-actin (62). The central transmembrane protein dystroglycan completes the link of dystrophin and the DGC to the extracellular matrix and requires the functional glycosylation of α-dystroglycan to serve as a high-affinity matrix receptor (38, 42, 62). The critical functional roles of the DGC in muscle are still debated, but may include regulation of cell signaling (1, 10), muscle force transmission (6, 49, 50, 55), and promotion of plasma membrane stability during muscle contraction (3, 5, 19, 25, 56, 57). Contained within the DGC is a subcomplex of proteins known as the sarcoglycan complex that include sarcoglycans and sarcospan (8, 12, 36, 66). Delta-sarcoglycan (8SG) is one of six known transmembrane sarcoglycans (α, β, γ, δ, ε, and ζ) that are expressed in a tissue-specific manner to form the tetrameric sarcoglycan complex (2, 9, 17, 37, 47, 58, 63). Delta-, beta-, and gamma-sarcoglycan are highly expressed in skeletal and cardiac muscle (30), and while α-sarcoglycan is the prominent fourth member of the sarcoglycan complex in skeletal muscle, cardiac muscle expresses both α and ε-sarcoglycan. Alpha- and epsilon-sarcoglycan proteins are capable of compensating for the loss of each other within the cardiac muscle sarcoglycan complex (35).

A critical role of 8SG and the sarcoglycan complex in muscle is revealed by the fact that recessive mutations in α-, β-, γ-, and δ-sarcoglycan have been linked to limb girdle muscular dystrophy (LGMD types 2D, 2E, 2C, and 2F, respectively) (14). Genetic deletion of any of these four sarcoglycans in mice results in a dystrophic muscle phenotype (18). Mutations in ε-sarcoglycan cause movement disorders called myoclonus dystonia (26, 33, 34, 44, 51, 52, 54, 59, 61) but have not been linked to cardiomyopathy or muscular dystrophy. Recessive 8SG mutations are responsible for the cardiac and skeletal muscle phenotypes of the cardiomyopathic BIO 14.6 hamster (45, 46). In addition to progressive skeletal myopathy, patients with sarcoglycan-associated LGMD have a high prevalence of cardiomyopathy (30, 45). Recessive mutations in 8SG cause reduction or total loss of the sarcoglycan...
complex at the plasma membrane and are, thereby, thought to disrupt overall DGC function.  

In contrast to recessive mutations in SG that result in skeletal muscle dystrophy, dominantly inherited mutations in SG have been linked to familial dilated cardiomyopathy (DCM) (60). The dominantly inherited mutations in SG do not appear to cause skeletal muscle disease and, therefore, may exert specific effects in cardiac muscle or in the vasculature. Some of the initial studies on patients carrying these mutations suggested a possible haploinsufficiency mechanism of action. Mutations in SG causing truncation at lysine residue 238 (ΔK238), in some pathological samples, resulted in decrease in sarcoglycan expression at the plasma membrane (60). The ΔK238 mutation was found in the placental isoform of SG but is expressed at low levels in the developed heart (60). However, one patient identified with this mutation showed normal expression patterns of the DGC proteins but still developed DCM. Transgenic mice expressing the S151A mutation in SG that is linked to DCM in the heart show early lethality, loss of the sarcoglycan complex at the plasma membrane in cardiac myocytes, and a sequestration of nuclear proteins in cardiac muscle (27), suggesting a possible dominant-negative mode of action. However, a study of a large consanguineous family with recessive loss of function LGMD2F mutations in the SGCD gene (that encodes SG) revealed that carriers of SGCD mutations that cause LGMD2F, do not have evidence of cardiomyopathy, arguing against haploinsufficiency as a causal mechanism of action for DCM. Furthermore, a subset of the individuals carrying the LGMD2F mutation, also carried the S151A polymorphism without DCM or LGMD2F, and there was no family history of heart disease (7). Despite the controversy raised regarding the link between the S151A mutation and DCM, independent groups have found additional mutations in SG in patients with autosomal dominant DCM (32) and, more recently, polymorphisms in SG were identified as a risk factor for hypertrophic cardiomyopathy (29, 48). Therefore, significant questions remain about whether and how mutations in SG cause DCM, and whether mutations in SG can truly exert a dominant effect on DGC function that directly impacts cardiac muscle and may lead to cardiomyopathy in humans.

To directly test the hypothesis that DCM-associated mutations in SG exert a dominant effect on DGC function, we expressed wild-type SG and SG containing mutations R97Q or R71T, which have been previously linked to or implicated in the pathogenesis of DCM (7, 30, 32, 60) in adult cardiac myocytes to study their direct acute effects on the DGC and function of cardiac muscle cells. Adult rat cardiac myocytes expressing mutant SG R97Q or R71T showed no alterations in the trafficking or localization of the mutant SG to the plasma membrane. Additionally, expression of either mutant SG R97Q or R71T does not appear to alter expression or assembly of the DGC in adult cardiac myocytes. However, using a functional assay previously used to study cardiac myocytes from mice with recessive muscular dystrophy (31), the sarcolemma in cardiac myocytes expressing either mutant SG R71T or R97Q was shown to be unstable under conditions of increased mechanical strain, resulting in increased uptake of membrane-impermeable dye and cell contracture. Finally, cardiac myocytes expressing mutant SG R71T show a unique alteration of N-linked glycosylation of SG, due to creation of an ectopic consensus N-glycosylation site by the R71T mutation. Together, these support the conclusion that DCM-linked mutations in SG can have a dominant negative effect on cardiac myocyte mechanical stability similar to dystrophic cardiomyopathy, and the R71T mutation suggests a possible link between posttranslational modifications of mutant SG and the function of SG within cardiac muscle cells.

**MATERIALS AND METHODS**

**Animals.** Sprague-Dawley rats (Charles River Laboratories, strain 400) were housed in specific pathogen-free barrier facilities in the Unit for Laboratory Animal Medicine at the University of Michigan, Ann Arbor. All procedures were approved by the University of Michigan Committee for the Use and Care of Animals.

**Cardiac myocyte isolation and transduction.** Sprague-Dawley rats were anesthetized and hearts were removed. Cardiac myocytes were isolated enzymatically as previously described with minor modifications (40). A total of 20,000 cells in 200 µl of MEM with 5% FBS were plated on glass cover slips or Bioflex plates (Flex Cell International BF-3001U) that were coated with 200 µl of 50 µg/ml EHS laminin 50 (Sigma Aldrich L2020). Alternatively, 1,000,000 total cells in 1 ml MEM with 5% FBS were plated on 100-mm culture dishes coated with 1 ml of 50 µg/ml EHS laminin. Cardiac myocytes were transduced by adenovirus in culture media as described previously (40), and cells were cultured for 24–72 h to express SG.

**Myocyte immunostaining and scoring.** Immunofluorescence staining on permeabilized myocytes was performed with fixation, and cells were permeabilized with 1% Triton-X 100 in PBS as described previously (40, 41). For live-cell staining, media were aspirated, and cardiac myocytes were incubated with primary antibody suspended in MEM for 2 h at 37°C. Cardiac myocytes were washed with 5 ml MEM for 5 min three times. After final aspiration of media cardiac myocytes were fixed with 2 ml of 3% paraformaldehyde in DPBS for 10 min and then permeabilized with 1% TX-100 in PBS followed by blocking with 5% BSA in PBS and secondary antibody staining, as described previously (40, 41). Primary antibodies included: A-14 rabbit polyclonal anti-myc (Santa Cruz Biotechnology; 1:100), goat anti-human δ-sarcoglycan (R&D Systems; 1:1,000), IIH6 anti-α dystroglycan (Millipore; 1:1,000), and mouse anti-β dystroglycan (Vector Laboratories; 1:1,000). Secondary fluorophore antibodies were from Jackson Immunochemicals and applied at 1:200–1:400, and DAPI was applied at 1:10,000 from a 10 mg/ml stock solution. All microscope images were taken using an Olympus BX51 Microscope and DP70 Digital Camera System. For the live cell experiments, baseline for exposure of fluorescence images was established using isolated cardiac myocytes stained without primary antibodies, and any staining above the fluorescence in untransduced myocytes was scored as positive in scoring experiments from >5 random field counts for each experiment. Five independent experiments were scored.

**Glycosidase treatment.** Thirty micrograms of protein lysate was denatured for either endoglycosidase H (EndoH) (New England Biolabs) or peptide N-glycosidase F (PNGaseF) (New England Biolabs) treatment in the supplied denaturing buffer and incubated for 10 min at 100°C in a total reaction volume of 80 µl. Protein lysate was then incubated for 60 min at 37°C with 2 µl (1,000 units) of EndoH with 10 µl of supplied buffer G5 in a total volume of 100 µl, or protein lysate incubation was with 2 µl (1,000 units) of PNGaseF with 10 µl of supplied buffer G7 and 10 µl of supplemented supplement NP-40.

**Western blot analysis.** Protein lysates were collected in buffer containing 1% digitonin in PBS with a cocktail of protease inhibitors and cleared by brief centrifugation at 14,000 g, as described previously (31, 43). Proteins were separated using 3–15% gradient SDS-PAGE gels and analyzed by Western blot analysis, as described previously (31, 43).

**Cell-stretching assay and scoring.** Rat cardiac myocytes were plated on Bioflex plates coated with laminin and transduced with adenoaviral vectors. Cardiac myocytes were allowed to express trans-
genes for 24–48 h. Cardiac myocytes were incubated with 50 μg/ml propidium iodide in cultured media immediately prior to and during the stretch protocol. Test groups were then stretched for 24 h using a pressure protocol that impart a 20% length change across the plate at a frequency of 1 Hz using a Flexcell Fx4000 Tension Plus System. Membranes were cut from Bioflex plates, and cells were fixed and mounted for fluorescent microscopy. Cardiac myocyte staining and cell morphology [rod shaped or round (hypercontracted)] was assessed via random field counts. Five separate experiments were performed in a double-blind fashion using five random field counts each of images taken at ×200 magnification. Dye-positive cells were scored as those demonstrating propidium iodide fluorescence in the nucleus.

Statistics. Data were analyzed by ANOVA followed by post hoc Dunnett’s t-test with P < 0.05, indicating significance with n ≥ 5 separate cell preparations for each experiment.

RESULTS

DCM mutant δ-sarcoglycans can be expressed in adult cardiac myocytes at similar expression levels to wild-type δ-sarcoglycan. Adenoviral vectors containing δ-sarcoglycan expression constructs were generated using previously described techniques (41). The constructed adenoviral vectors use the cytomegalovirus (CMV) promoter to drive expression of wild-type and mutant human δSG cDNA. A myc tag was engineered on the extracellular COOH terminus to identify the exogenous protein and study its cellular trafficking and sarcolemma localization (Fig. 1A). Adult rat cardiac myocytes were isolated and transduced with adenoviral vectors expressing wild-type or mutant δSG. Cardiac myocytes were collected at 24, 48, and 72 h posttransduction. The adenoviral expressed δSG proteins became first detectable at 18–24 h posttransduction and continued to increase until expression levels stabilized between 48 and 72 h posttransduction (Fig. 1B). Protein expression after 72 h did not appear to increase (data not shown), indicating a stable level of expression had been achieved. Importantly, under these viral transduction conditions, similar levels of δSG protein expression by Western blot analysis were obtained in wild-type and R97Q and R71T mutant δSG-expressing cardiac myocytes (Fig. 1B). For wild-type δSG, and δSG mutant R97Q or R71T vectors, equivalent titers of virus were required to achieve maximal transduction efficiency of nearly 100% of adult cardiac myocytes in vitro, as detected by immunofluorescence staining using the anti-myc antibody (Fig. 1C). We tested commercial antibodies directed against the extracellular domain of δSG to compare the levels of expressed human δSG to the endogenous rat δSG. Unfortunately, we were unable to detect the endogenous rat δSG by Western blot analysis using these antibodies (data not shown), preventing direct quantification of the relative level of adeno-virus-mediated δ-sarcoglycan expression to the endogenous protein. Therefore, to address the concern that overexpression of δSG may have some effects on cell function, all of the results in these studies were compared with results from parallel experiments on cardiac myocytes expressing comparable amounts of wild-type human δSG protein by adenoviral transduction. These data indicate that R97Q and R71T mutant δSG can be expressed at levels equivalent to wild-type δSG, and the mutants appear to have normal protein stability when expressed in adult cardiac myocytes.

Adult rat cardiac myocytes expressing R97Q or R71T mutant δSG do not show DGC disruption or loss of cell viability. Genetic disruption of δSG or other sarcoglycan members in mice results in loss of expression of the entire sarcoglycan complex at the sarcolemma in muscle cells, and although dystrophin and dystroglycan are still expressed, their expression levels are significantly reduced (24, 35), indicating null mutations in δSG can disrupt DGC assembly in cardiac muscle. In contrast, overexpression of wild-type δSG, δSG mutant R97Q, or R71T, using adenoviral vectors, has no apparent effect on DGC assembly and expression (Fig. 2). Immunofluorescence analysis showed that expression of R97Q or R71T showed no change in expression and localization of several components of the DGC, including α- and β-dystroglycan or α- and β-sarcoglycan (Fig. 2C). Immunofluorescence staining...
Delta-sarcoglycan mutants R97Q and R71T properly localize to the plasma membrane. Expression of the δSG S151A mutation linked to DCM in hearts of transgenic mice reveals that δSG mutant S151A protein accumulates in the nucleus and does not localize correctly to the plasma membrane (27). Whether this defect was due to the level of overexpression or whether this represents a common mechanism or a property of other δSG mutations linked to DCM has not been studied. To investigate possible trafficking defects associated with the R97Q and R71T mutations in isolated adult rat cardiac myocytes, transduced myocytes were first analyzed for localization of the wild-type and mutant protein in fixed and permeabilized myocytes to detect the total cellular distribution of the expressed proteins. Immunofluorescent staining using an anti-myc antibody on isolated cardiac myocytes expressing δSG proteins shows no apparent difference in expression or localization of δSG mutant R97Q or R71T compared with wild-type δSG expressing cardiac myocytes (Fig. 2B) and did not appear to show any accumulation in the nucleus, as previously observed for the S151A mutation. Although immunofluorescent staining shows no obvious differences in expression or localization of mutant δSG compared with wild-type δSG, the data do not directly demonstrate that the wild-type or mutant proteins are properly integrated into the plasma membrane. To address the questions of potential trafficking defects and secretory pathway processing of these transduced proteins, we designed a live-cell labeling assay to monitor only the expressed δSG protein properly trafficked and inserted into the plasma membrane. Delta-sarcoglycan is a type II transmembrane protein that, when inserted into the membrane, presents the COOH terminus to the extracellular milieu. We took advantage of the exposure of the COOH terminus myc epitope tag engineered into the expressed human δSG proteins, to develop a live-cell staining protocol in unpermeabilized cardiac myocytes to quantify the insertion of the expressed δSG into the plasma membrane. Using live-cell anti-myc antibody staining, both δSG mutant R97Q and R71T proteins can be labeled at the membrane in unpermeabilized cells (Fig. 3A), suggesting they are properly inserted into the plasma mem-

Fig. 2. Expressing δSG using adenoviral vectors does not affect cardiac myocyte viability or cell morphology and does not alter DGC expression or localization. A: assessment of cardiac myocyte cell morphology. Expressing δSG using adenoviral vectors does not decrease overall cardiac myocyte viability and does not alter rod morphology. Data are expressed as means ± SE. B: immunostaining for δSG in cardiac myocytes following fixation and permeabilization. The red channel is myc-tagged δSG (anti-myc) and the blue channel is DAPI-stained nuclei. There is no apparent difference in gene transfer efficiency or expression levels or localization of wild-type or δSG mutant R97Q or R71T. C: immunostaining for δSG, βSG, αSG, βDG, and glycosylated δDG(IH6). Expressing δSG mutation R97Q or R71T does not alter expression or localization of membrane-bound DGC components. The δSG antibody used is human specific with minimal binding to endogenous rat δSG as seen in the untransduced panel. Scale bars are equal to 50 μm.
Delta-sarcoglycan mutant R71T is aberrantly glycosylated. The presence of N-linked glycosylation of δSG has been demonstrated in skeletal muscle (30) and in cultured CHO (28) and COS-1 cells (13). It is unclear what role δSG glycosylation plays in δSG protein and overall DGC function in muscle cells or whether δSG is equivalently glycosylated in all muscle cell types. Western blots of cardiac myocyte whole cell lysates treated with glycosidases targeting N-glycosylation demonstrated that wild-type δSG is glycosylated in isolated rat ventricular myocytes. Delta-sarcoglycan mutant R97Q protein appears to have the same glycosylation and expression pattern as wild-type δSG but oddly, δSG mutant R71T protein shows a higher-molecular-weight band, suggesting altered posttranslational modification (Fig. 4A). Incubating cell lysates with EndoH or PNGaseF for 1 h was sufficient to restore the migration of δSG mutant R71T to the same migration pattern of wild-type δSG on Western blots (Fig. 4B), indicating that the high-molecular-weight band in cardiac myocytes expressing δSG mutant R71T represents an alternately N-linked glycosylated form of δSG. The alternate glycosylation of δSG mutant R71T does not appear to be cardiac muscle cell-specific, as the upward shift in molecular weight can be observed when δSG mutant R71T is expressed in other rat muscle cell types, including rat smooth muscle cells (A7R5) and rat skeletal muscle myotubes (L6) (Fig. 5). Interestingly, the occupancy of the three putative N-glycosylation sites on wild-type δSG appears to be different in smooth muscle cells (Fig. 5), as indicated by a more prominent higher-molecular-weight band in A7R5 cells. However, the R71T mutation still results in an additional upward shift in both bands, indicating it does not affect the glycosylation on the other N-glycosylation sites in δSG. Interestingly, the detection of the expressed protein with anti-human δSG antibody, also results in a lower-molecular-weight band that is not observed with the myc antibody, suggesting a possible additional proteolytic processing of the COOH terminus of δ-sarcoglycan in cardiac myocytes. This form of posttranslational processing does not appear to be different between wild-type and δSG mutant R97Q or δSG mutant R71T.
Expression of δ-sarcoglycan mutant R97Q or R71T in adult cardiac myocytes is sufficient to cause mechanical instability of the plasma membrane following stretch. The mechanisms of how loss-of-function mutations in δSG and the DGC cause cardiomyopathy in models of limb-girdle muscular dystrophy and Duchenne muscular dystrophy have received considerable attention. We and others have shown that loss-of-dystrophin expression, cardiac specific loss of dystroglycan expression or function, or loss of sarcoglycan expression, is sufficient to cause loss of cardiac membrane integrity in vivo using vital dye uptake (4, 15, 31, 43). This loss of sarcolemma stability in dystrophic cardiac muscle can be demonstrated in isolated cardiac myocytes by subjecting them to mechanical stretching in the presence of cell-impermeant dyes (4, 31, 43). Using a modified version of these cell stretch assays in the presence of propidium iodide to irreversibly label nuclei of cells that lose sarcolemmal integrity, we assessed whether expressing δSG mutant R97Q δSG or R71T δSG exerts a dominant-negative functional effect on the DGC to cause plasma membrane instability following cell stretching (Fig. 6). Isolated cardiac myocytes were allowed to express transgenes for up to 48 h prior to cell stretching. Expressed δSG appears as early as 24 h post-transduction (Fig. 1B). However, exposing the cells to mechanical stretching at 24 h post-transduction failed to demonstrate a statistically significant effect of the δSG mutant R97Q or R71T expression on membrane stability and integrity, although modest trends toward instability existed (data not shown). In contrast, cardiac myocytes expressing δSG mutant R97Q or R71T exposed to mechanical strain at 48 h, where the expression of the mutant proteins was higher, showed a significant increase in impermeant dye uptake and increase in cell contracture, leading to rounding of cells (Fig. 6B). Representative images of each experimental group are shown. To quantify this effect on sarcolemma stability and cell contracture, wild-type, δSG mutant R97Q, and δSG mutant R71T-expressing cardiac myocytes were scored in a blinded fashion for cell morphology and dye uptake following mechanical stretching. δSG mutant R97Q and δSG mutant R71T-expressing cardiac myocytes showed a significant decrease in rod-shaped cells, and concomitant increase in round-shaped cells, compared with wild-type δSG-expressing myocytes, which had no effect compared with untransduced cells (Fig. 7, A and B). Nearly all of the round-shaped cells following stretch were propidium iodide-positive, indicating that stretch-induced sarcolemma rupture likely leads to calcium-induced cell contracture (Fig. 7C). We also quantified the cell density to determine whether stretch-induced cell detachment. Expression of δSG mutant R97Q or R71T did increase the number of attached rod-shaped cells (Fig. 7, D and E). The total number of cells appeared less affected and did not significantly affect the total

Fig. 4. DCM mutant δSG R71T expressed in cardiac myocytes undergoes alternative glycosylation. A: Western blot analysis of whole cell lysates taken from rat cardiac myocytes. Using an antibody directed against human δSG reveals additional bands in cardiac myocytes expressing DCM mutant δSG R71T. Probes using antibodies directed against δ-sarcoglycan produce multiple bands in all cell types but a molecular weight shift only in cardiac myocytes expressing δSG mutant R71T. B: Western blot analysis using anti-myc antibody of whole cell lysates subjected to glycosidase treatment. Treating with EndoH and PNGaseF shows additional bands present in cardiac myocytes expressing DCM mutant δSG R71T are alternatively glycosylated forms of δSG. Anti-myc antibodies identify only proteins expressed by the transgene.

Fig. 5. Alternative glycosylation of mutant δSG R71T occurs in other cell culture models of striated muscle, and A7R5 cells have increased overall glycosylation. Western blot analysis of whole cell lysates (anti-myc) taken from rat cardiac myocytes, smooth muscle A7R5 cells, and skeletal muscle L6 myotubes. Blotting reveals differential glycosylation of δSG mutant R71T in rat cardiac myocytes is recapitulated in skeletal muscle L6 myotubes. Additionally, A7R5 cells show molecular weight shift when δSG mutant R71T is expressed compared with WT δSG and δSG mutant R97Q. Similar volumes of lysate were loaded for all groups. RCM-rat cardiac myocytes, A7R5-rat smooth muscle cells, L6-rat skeletal muscle myotubes.

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mechanical stretching also appears to reveal a defect in cell adhesion in viable rod-shaped adult cardiomyocytes expressing δSG mutant R97Q or R71T. Overall, expression of δSG mutant R97Q or R71T is sufficient to cause a dominant-negative effect on sarcolemma stability in mechanically stressed adult cardiac muscle cells. This defect in sarcolemma stability is comparable to what has been previously observed with loss of dystrophin glycoprotein complex function in cardiomyocytes (31) and may result as a consequence of loss of an important functional interaction of the DGC with the extracellular matrix.

DISCUSSION

Recessive mutations in δSG that cause limb-girdle muscular dystrophy result in loss of δSG at the plasma membrane and number of attached rounded cells (not shown). Therefore, the mechanical stretching also appears to reveal a defect in cell adhesion in viable rod-shaped adult cardiomyocytes expressing δSG mutant R97Q or R71T. Overall, expression of δSG mutant R97Q or R71T is sufficient to cause a dominant-negative effect on sarcolemma stability in mechanically stressed adult cardiac muscle cells. This defect in sarcolemma stability is comparable to what has been previously observed with loss of dystrophin glycoprotein complex function in cardiomyocytes (31) and may result as a consequence of loss of an important functional interaction of the DGC with the extracellular matrix.
disrupt assembly and function of the rest of the DGC (24, 28). This study investigated the effects of two dominantly inherited mutations in δSG, R97Q, or R71T, in adult cardiac myocytes to provide evidence for potential mechanisms underlying their linkage to dilated cardiomyopathy in humans. These results indicate that δSG mutant R97Q and R71T can be stably expressed in cardiac muscle cells at the sarcolemma without disrupting DGC assembly, but exert a dominant-negative functional effect, causing sarcolemma instability similar to what has been observed in other models of DGC deficiency. This suggests a prominent functional role for sarcoglycans in the overall function of the DGC in stabilizing the plasma membrane, considerably more than just an accessory protein necessary for proper DGC assembly.

Complicated role of δSG in DCM pathogenesis. Previous work using transgenic mice demonstrated that cardiac specific overexpression of S151A mutation, previously linked to dominantly inherited human DCM, resulted in a lethal cardiomyopathy (27, 60). In several lines of these mice, the S151A protein abnormally trafficked to the nucleus, where it sequestered other sarcoglycans and other nuclear proteins. However, in one line of mice with high level of expression, the S151A trafficking defect was only penetrant in around 60% of the cells. Further studies of these mice have been difficult because of the lethality leading to failure to breed. The role of S151A in causing DCM in humans has been challenged by studies that found the S151A mutation in a large consanguineous family with LGMD2F, but the family members carrying this mutation did not have cardiomyopathy (7). Further, heterozygous knock-in of mutant δSG S151A in δSG-null mice restored DGC assembly and improved membrane stability, as assessed via Evans blue dye uptake (53). However, the S151A knockin shows a mild cardiac phenotype of increased heart weight normalized to body weight and run significantly less on a voluntary wheel, suggesting that mutant δSG S151A does not completely rescue the cardiac phenotype present in δSG-null mice.

We chose to focus our attention on other DCM-associated δSG mutations R97Q and R71T by using a highly efficient model system of adenovirus-mediated gene transfer to adult cardiac myocytes that we have previously used to study dominant mutations that lead to hypertrophic cardiomyopathy and other genetic forms of myopathy (39, 41). Our results indicate that the δSG mutant R97Q and R71T defects do not arise from a deficiency in δSG localization or insertion to the plasma membrane and suggest a potentially dominant-negative function of the protein at the sarcolemma, underlying a role in pathogenesis of DCM. The R97Q δSG mutant was originally suggested as a nonpathogenic variant (30). Single-stranded DNA confirmation polymorphism analysis by Tsubata et al. (60) suggested that the presence of the R97Q variant may be linked to sporadic development of DCM. PCR screening of candidate DCM-linked genes in Finnish sample populations (DCM: n = 56; control: n = 150) identified individuals carrying the R97Q mutation that did not present with classical symptoms of DCM (32), concluding that R97Q is simply a δSG polymorphism. However, the incidence of this variant in the DCM-affected population was almost twice as common compared with the control population, suggesting that genetic variation or environmental influences contribute to development of DCM in individuals with this mutation. The Finnish sample population study was also the first to identify the R71T mutation in two individuals within the same family. Both individuals had phenotypes that responded well to medication and were milder than those patients described by Tsubata et al. (60). The conflicting clinical reports illustrate the complicated role of δSG in cardiac muscle function and a lack of full understanding of the specific roles of δSG in the function of the DGC within cardiac myocytes. The presence of some δSG mutations alone may not be pathogenic without an external acute or continuous stressor, such as excess mechanical stress or hormones that result in cardiac myocyte cell signaling. The location and type of mutation on δSG likely affect interaction with proteins in the DGC related to structural and/or signaling functions. For example, there is evidence that the sarcoglycans interact directly with dystrobrevin, which is thought to have signaling roles in the DGC (65). Furthermore, the posttranslational modifications of δSG itself, especially those that may be altered by mutation, as we describe below, may be relevant to overall δSG protein function in the DGC in a similar manner to other DGC components (31, 43). The complexities of the interaction of these posttranslational modifications with other environmental or physiological stressors may markedly affect disease penetrance and pathogenesis in humans.

δSG glycosylation may modify mutant δSG sarcoglycan function. Delta-sarcoglycan protein has been shown to be glycosylated in muscle in vivo and when expressed in vitro (28, 30). Although the function of δSG glycosylation in muscle is unclear, it has been established that hypoglycosylation of dystroglycan within DGC disrupts its function as a laminin receptor and results in muscular dystrophy with associated cardiomyopathy (11, 23, 38). Surprisingly, when we expressed the δSG mutant R71T, we found an additional higher molecular weight band upon Western blot analysis with both anti-human-δSG and myc antibodies. Altering amino acid residue 71 from an arginine to a threonine creates a new consensus site for a potential N-linked glycosylation site relative to the upstream glutamine at amino acid 69. The removal of N-glycans by both EndoH and PNGaseF glycosidases converted all the glycoforms of the wild-type and δSG mutant R97Q and R71T to a similar migrating low-molecular-weight protein, showing the molecular weight shift in δSG mutant R71T is the result of ectopic glycosylation in cardiac myocytes. Oddly, it was expected that treatment with EndoH would also allow us to probe any trafficking defects since sensitivity to that particular glycosidase typically indicates a protein is retained in the endoplasmic reticulum. However, the sarcoglycan complex has previously been found localized to the plasma membrane and yet is still sensitive to treatment with EndoH (24). Interestingly, an ε-sarcoglycan mutation causing myoclonus dystonia results in an ectopic N-glycosylation site that becomes glycosylated (61). Epsilon sarcoglycan can localize to the plasma membrane in mouse brain and still retains sensitivity to EndoH treatment (22). This abnormal post-translational processing of δSG mutant R71T does not appear to be unique to cardiac muscle cells, as the R71T mutation resulted in an apparent higher molecular weight when expressed in skeletal muscle myotubes and with slightly less occupancy in smooth muscle cells. The model system developed here could provide a unique opportunity to dissect the role of the individual glycosylation sites on sarcoglycans in the function of the DGC in future studies. Overall, the abnormal ectopic glycosylation of δSG
may contribute to the functional effects of the R71T mutation in cardiac muscle cells, although the specific role of this ectopic glycosylation remains to be tested because it is possible the R71T mutation itself has an effect on function similar to the R97Q mutation. Regardless, the location of the ectopic glyco-
sylation and the R71T and R97Q mutations suggest that the COOH-terminal extracellular domains of δ-sarcoglycan may directly contribute to overall DGC function as an extracellular matrix receptor. This conclusion is consistent with previous observations in humans that truncation of the COOH terminus in γ-sarcoglycan can cause limb girdle muscular dystrophy and cardiomyopathy without causing loss of sarcoglycan complex expression (16).

R97Q and R71 mutant δSG directly impair sarcolemma stability. Previous studies have shown that loss of DGC function, through loss of αDG glycosylation or dystrophin deficiency, is sufficient to cause plasma membrane instability in mouse cardiac myocytes upon mechanical strain (31, 64). Our results clearly show the potential for dominant-negative effects of δSG sarcoglycan mutations on sarcolemma stability without dramatic alterations in DGC expression or localization. Interest-

ingly, compared with our previous studies in mouse models, untransduced rat cardiac myocytes are overall much less sensi-
tive to mechanical stretching than mouse cells and could withstand much longer mechanical stretching experiments (31). However, when cardiac myocytes expressing δSG mutant R97Q or R71T are submitted to mechanical strain for 24 h, there is a marked increase in membrane permeability (in-
creased dye uptake) and that likely leads to calcium-induced cell contracture (loss of rod-shaped morphology). The cells undergoing contracture do not appear to be cells that have just died during the experiment. While most of the rod-
and round-shaped myocytes are quiescent, many of the round-
shaped cells show spontaneous beating behavior (not shown) and remain attached to the matrix substrate. Furthermore, cells that die during the cardiac myocyte isolation and exhibit a rounded morphology prior to any experiments typically do not adhere well and are easily washed off the laminin substrate. Despite differences in extracellular domain glycosylation of R97Q and R71T δSG mutant proteins, the overall magnitude of dye uptake and cell morphology alterations following cardiac myocyte stretching were similar. Because of the relatively close proximity of these mutations within the extracellular domain of δSG, it is likely that either of these mutations, or the aberrant glycosylation of R71T, similarly affects some critical interaction of this region of the δSG protein with either another member of the DGC, the plasma membrane itself, or an extracellular substrate. Overall, these results indicate DCM-
linked δSG mutations exert a dominant-negative effect on cardiac myocyte sarcolemma stability during mechanical stress, consistent with a dominant-negative functional effect on the overall function of the DGC that normally protects the cardiac muscle cell membrane against mechanical damage. On the basis of previous work showing that muscle cell damage is an early feature of dystrophic cardiomyopathy leading to path-
ological dilated phenotype and fibrosis in dystroglycanopathy model mice (31, 43) and cardiac dysfunction in dystrophin-
deficient mice in vivo (64), we propose that this cellular phenotype of sarcolemma instability plays a causal role in the development of DCM. Because the DCM mutant δSG has dominant-negative effects without disrupting DGC expression, the data support the idea that sarcoglycans may function more than just as an accessory protein needed for assembly of the DGC and suggest that they may actually play an important prominent direct role in maintaining sarcolemma stability in striated muscle.

Study limitations. The results here indicate a contribution of mutant δSG to a dominant-negative dystrophic phenotype in isolated cardiac myocytes, but it should be noted that it comes with a few caveats. Cultured cardiac myocytes, while a good model for precise acute gene manipulation, are clearly isolated from their in vivo environment, so effects of extracellular interactions beyond the basal lamina and intracellular signaling are not addressed. Future studies could address this by using a knock-in approach similar to Rutschow et al. (53). Addition-
ally, quantification of the amount of expressed δSG protein present at the plasma membrane was not fully possible, given some of the antibody limitations noted. We were also unsuccessful at detecting known interacting partners of the DGC, such as βSG and βDG, following coimmunoprecipitation experiments, likely due the relatively low numbers of cells and low concentration of membrane proteins in these experiments, so definitive proof that the expressed proteins are fully assem-
dled into the DGC is lacking. However, the live cell labeling shown in Fig. 3A does show that the mutant appears to insert correctly in the plasma membrane at levels comparable to WT cells, without disrupting the assembly of the DGC (Fig. 2). We also show by immunostaining with a relatively human-specific δSG antibody that the adenovirus-expressed human proteins δSG do not appear to be retained as potentially misfolded proteins in the secretory pathway (Fig. 2). Perinuclear staining as evidence of secretory pathway retention was quantified and was 5% or less of all cells and not statistically different in cells expressing wild-type or mutant δSG proteins (wild-type: 4.6 ± 0.3% of cells; R97Q: 4.5 ± 0.4%; R71T: 5.1 ± 3%; means ± SE; n = 5 preparations), supporting the conclusion that over-
expressed wild-type and mutant δSG proteins traffic to the plasma membrane with similar efficiency. Although we would like to better visualize and quantify the consequences of sarcolemma damage induced by these mutants, the elastic membrane used for stretch experiments is considerably thicker than typical microscope slide coverslips, and stretching using this technique produces significant motion artifacts. Although the current system has significant advantages of testing and quantifying effects of mechanical strain on larger populations of genetically modified adult cardiac myocytes, the current platform has some significant limitations for using high-
resolution microscopy for identifying specific sites of cellular damage, high temporal resolution studies of calcium handling, and transitioning the cells to systems designed for measuring other myocyte functions, such as cell shortening. Therefore, our future work will focus on developing and adapting the stretch system with custom chambers to take advantage of these other imaging modalities and functional measures imme-
diately before and after a bout of mechanical stretching.

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